Effects of Egg White Derived Peptides on Metabolic Syndrome Complications: Hypertension, Inflammation, and Insulin resistance

by

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ABSTRACT

Metabolic syndrome (MetS), a cluster of several abnormalities of hypertension, inflammation, glucose intolerance and dyslipidemia, enhances a person's risk for cardiovascular disease and type 2 diabetes. The number of people with MetS is increasing largely worldwide. The multi-faceted nature of MetS makes patients take several medications to target different aspects of this disease. In addition to significant side effects associated with synthetic drugs and possible drugs interactions, adherence to life-long therapies is usually poor. Therefore, there is increasing interest in developing functional foods or natural health products as an alternative for the prevention and management of the complications of this disease. Bioactive peptides may potentially alleviate several complications of MetS namely hypertension and insulin resistance, the key features of the disease. Several peptides with antioxidant and antihypertensive activity were previously identified from egg white protein ovotransferrin hydrolysate.

Egg white contains several proteins including ovotransferrin, therefore, we hypothesized that egg white hydrolysate would have beneficial effects on hypertension, oxidative stress, and inflammation. Moreover, due to the link between hypertension and insulin resistance, egg white hydrolysate may positively affect glucose tolerance and insulin resistance. The main purpose of the current research was to investigate the potential of egg white-derived peptides on hypertension, inflammation, glucose intolerance, and insulin resistance. The specific objectives of this thesis were to 1) evaluate the anti-inflammatory and antioxidant activities of ovotransferrin-derived antioxidant peptides on endothelial cells and their gastrointestinal stability, 2) test the *in vivo* antihypertensive effect of egg white hydrolysate (EWH) prepared with thermolysin and pepsin with *in vitro* ACE-inhibitory effect using spontaneously hypertensive rats (SHRs), 3) explore the possible effects of the EWH on adipocyte differentiation

and insulin signaling *in vitro*, 4) evaluate the potential effects of EWH on glucose and insulin tolerance in insulin resistant rats, and 5) identify bioactive peptides in EWH responsible for the observed adipogenic differentiating effects in adipocytes.

We assessed the anti-inflammatory and antioxidant capacity of sixteen antioxidant peptides-previously identified from the egg protein ovotransferrin by *in vitro* oxygen radical absorbance capacity (ORAC) method-using human umbilical vein endothelial cells (HUVECs). Surprisingly, none of the peptides showed anti-inflammatory effects in endothelial cells. While several digested peptides significantly reduced expression of pro-inflammatory adhesion molecules (ICAM-1 and VCAM-1) in response to tumor necrosis factor-α (TNF-α) stimulation, only GWNI reduced TNF-α activated superoxide generation (71.0 ± 12.9%) after GID when tested with Dihydroethidium (DHE) assay. Mass spectrometer analysis identified two new peptides, GWN and GW, in the GWNI digest; however, only GW reduced TNF-α induced VCAM-1 expression (64.3 ± 20.6%) significantly compared to the TNF-α treated cells. Our study suggested that the *in vitro* ORAC test lacked biological relevance in assessing bioactive peptides.

In vivo antihypertensive activity was studied in SHRs using the telemetric method. The effect of oral administration of EWH at low dose (250 mg/kg BW) and high dose (1000 mg/kg BW) over a period of 12 days was studied on blood pressure (BP). BP showed a significant reduction in the EWH high dose group compared to untreated controls. BP reduction was associated with enhanced *ex vivo* vasodilation, reduced oxidative/nitrosative stress, reduced angiotensin converting enzyme and angiotensin II type 1 receptor expression, while enhanced angiotensin II type 2 receptor expression. Circulating level of angiotensin II was unaffected. Thus, EWH exerted antihypertensive effects in SHRs through multiple mechanisms of vascular relaxation and RAS modulation.

Egg white ovotransferrin derived bioactive peptides have shown beneficial effects against hypertension, oxidative stress and inflammation *in vitro* and *in vivo*. We also observed that EWH exerted antihypertensive effects in SHRs. Given the interplay among hypertension, inflammation, oxidative stress and metabolic syndrome, we aimed to test the effects of EWH on differentiation, insulin signaling and inflammatory responses in 3T3-F442A pre-adipocytes. Our study suggested that EWH could promote adipocyte differentiation as shown by increased lipid accumulation, increased release of adiponectin and upregulation of peroxisome proliferator associated receptor gamma (PPARγ) and CCAAT/ enhancer binding protein alpha (C/EBP-α). In addition to enhanced insulin effects on the upregulation of protein kinase B/Akt phosphorylation, EWH treatment increased extracellular signal regulated kinase 1/2 (ERK1/2) phosphorylation to a level similar to that of insulin, indicating insulin sensitizing and mimetic properties. EWH further attenuated cytokine-induced inflammatory marker; cyclooxygenase-2 (COX-2) was reduced by 48.8%, possibly through the AP-1 pathway by down regulating c-Jun phosphorylation in adipocytes. Given the critical role of adipose in the pathogenesis of insulin resistance and MetS, EWH may have potential applications in the prevention and management of MetS and its complications.

Next, we evaluated the effectiveness of EWH on glucose and insulin tolerance in diet-induced insulin resistant rats. Supplementing a high-fat diet (HFD) with 4% EWH (equivalent to 1.2 g/Kg BW) improved glucose tolerance, muscle and adipose tissue insulin sensitivity, and inflammatory profile in insulin resistant rats. We also observed some evidence for the enhanced adipocyte differentiation or reduced hepatic glucose production in EWH-treated rats, which needs further experiments.

Since EWH is a combination of multiple peptides, we identified the bioactive peptides in EWH with beneficial effects on adipocyte differentiation in 3T3-F442A adipocytes. Our data on the purification and characterization of peptides from EWH with PPARγ stimulatory activity in 3T3-F442A cells identified 42 potent peptides from the major egg white proteins ovalbumin and ovotransferrin. We successfully validated the stimulatory effects of several novel peptide sequences including WEKAFKDED, QAMPFRVTEQE, and VFKGL. ERYPIL was another potent peptide with stimulatory effects on PPARγ expression. For the first time, adipogenic differentiating peptides have been characterized from the hydrolysate of a food-derived protein.

Thus, the present study confirmed the effectiveness of EWH on several complications of MetS, including hypertension, inflammation, oxidative stress, glucose tolerance and insulin resistance *in vitro* and *in vivo*. We could also identify the bioactive peptides in EWH with beneficial effects on pre-adipocyte differentiation. Findings from this study support the potential of egg protein-derived peptides for uses in the prevention and management of MetS complications.

PREFACE

This thesis contains original work done by Mrs. Forough Jahandideh and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta. The concept of this thesis originated from my supervisor Dr. Jianping Wu and the research was funded by the grants from Alberta Livestock and Meat Agency (ALMA) and the Natural Sciences and Engineering Research Council of Canada (NSERC) discovery grant. The use of human umbilical endothelial cells in chapter 3 of this thesis was approved by the University of Alberta Ethics Committee (Protocol # 00000944) and the investigation also conformed to the principles outlined in the Declaration of Helsinki and also Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All subjects provided informed consent before inclusion in this study. The experimental protocols for animal studies in chapters 4 and 6 of the thesis were approved by the Animal Care and Use Committee at the University of Alberta (Protocol # 611/09/10D and 1472) in accordance with the guidelines issued by the Canada Council on Animal Care.

The thesis consisted of eight chapters: Chapter 1 provides a general introduction to the context and the objectives of the thesis; Chapter 2 is a literature review on several topics relevant to this thesis (a version of chapter 2 will be submitted to the *Critical Reviews in Food Science and Nutrition* as a review article); Chapter 3 has been published as "Antioxidant Peptides Identified from Ovotransferrin by the ORAC Method Did Not Show Anti-inflammatory and Antioxidant Activities in Endothelial Cells" in the *Journal of Agricultural and Food Chemistry*; Chapter 4 has been published as "Egg white protein hydrolysate reduces blood pressure, improves vascular relaxation and modifies aortic angiotensin II receptors expression in spontaneously hypertensive

rats" in the *Journal of Functional Foods*; Chapter 5 has been published as "Egg white hydrolysate shows insulin mimetic and sensitizing effects in 3T3-F442A pre-adipocytes" in the *PLOS ONE* journal; Chapter 6 entitled " Egg white hydrolysate enhances glucose tolerance and insulin sensitivity in diet induced insulin resistant rats" will be submitted to the "*Molecular and Nutritional Food Research*" and Chapter 7 entitled "Purification and characterization of bioactive peptides in egg white hydrolysate with beneficial effects on adipogenic differentiation" has been submitted to the "*Food Chemistry*" journal; the last Chapter 8 gives the discussion on chapters of the thesis and concluding remarks with further discussion for future research direction.

Dr. Jianping Wu greatly contributed to the experimental design, data interpretation, thesis preparation and edits of all chapters. Dr. Sandra T. Davidge greatly contributed to the experimental design, data interpretation, and edits of chapters 3, 4, 5 and 6. Dr. Spencer Proctor helped with the experimental design and data interpretation of chapter 6. Dr. Catherine Chan greatly contributed to the experimental design and data interpretation of the animal study in chapter 6. I was responsible for literature search relevant for the above studies, designing and performing laboratory experiments, data collection and analysis, and manuscript preparation and edits. Dr. Subhadeep Chakrabarti provided substantial guidance and assistance for the cell culture studies and contributed to chapters 3 and 5 manuscript edits. Dr. Jude S. Morton provided technical support in vessel function studies and data interpretation. Mrs. Sareh Panahi and Mrs. Nicole Coursen have provided technical assistance in animal studies including animal surgery, husbandry, vessel dissection and mounting, and tissue collection upon termination. Miss Qi Yi Li prepared the egg white hydrolysate for studies and Mrs. Jing Zheng performed the mass spectrometry of peptide fractions in Chapter 7.

DEDICATION

This thesis is dedicated to my beloved parents,

Ahmad Jahandideh and Zahra Salehian Dehkordi

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My last, but not least, appreciation goes to my beloved husband, Reza, whose love, patience and support are always with me. You have always been a source of unwavering passion and encouragement in my life.

Contents

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Abbreviations used

ACE 2: Angiotensin converting enzyme 2

ACE: Angiotensin I converting enzyme

ACN: Acetonitrile

Akt: Protein kinase B

AMPK: AMP-activated protein kinase

Ang I: Angiotensin I

Ang II: Angiotensin II

ANOVA: analysis of variance

APoE: Apolipoprotein E

AT1R: angiotensin II type 1 receptor

AT2R: Angiotensin II type 2 receptor

ATP: Adenosine tri-phosphate

AUC: Area under the curve

BP: Blood pressure

bpm: Beats per minute

BW: Body weight

C/EBP-α: CCAAT/ enhancer binding protein alpha

CAA: Cellular antioxidant assay

CCK: Cholecystokinin

CCK-1R: CCK type 1 receptor

cGMP: cyclic guanosine-monophosphate

COX-2: Cyclooxygenase -2

CVDs: Cardiovascular diseases

DBP: Diastolic blood pressure

DHE: Dihydroethidium

DPP-IV: Dipeptidyl peptidase IV

DTT: Dithiothreitol

ECGS: Endothelial cell growth supplement

ELISA: Enzyme-linked immunosorbent assay

eNOS: Endothelial nitric oxide synthase

ERK1/2: Extracellular signal-regulated kinase 1/2

ET-1: Endothelin

EWH: Egg white hydrolysate

FBS: Fetal bovine serum

GID: Gastro-intestinal digestion

GIP: Glucose-dependent insulinotropic polypeptide

GLP-1: Glucagon-like peptide 1

GLUT: Glucose transporter

GRB2: Growth factor receptor-bound protein 2

HFD: High-fat diet

HPLC: High-performance liquid chromatography

HR: Heart rate

HUVEC: Human umbilical vein endothelial cells

iAUC: Incremental area under the curve

ICAM-1: Intercellular adhesion molecule 1

IL: Interleukin

iNOS: Inducible nitric oxide synthase

IR: Insulin resistance

IRS-1: Insulin receptor substrate-1

ITT: Insulin tolerance test

IκB: Inhibitor kappaB

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

LDL: Low density lipoprotein

L-Name: N-nitro-L-arginine methyl ester

MAP: Mean arterial pressure

MAPK: Mitogen activated protein kinase

MCh: Methacholine

MCP-1: Monocyte chemotactic protein-1

MDA: Malondialdehydes

MEK: Extracellular-signal-regulated protein kinase kinase

MetS: Metabolic syndrome

MMP: metalloproteinases

mRNA: Messenger RNA

NADPH: nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor kappa B

NO: Nitric oxide

NOS: Nitric oxide synthase

NSAIDs: Non-steroidal anti-inflammatory drugs

OGTT: Oral glucose tolerance tets

ORAC: Oxygen radical absorbance capacity

PBS: Phosphate-buffered saline

PDK: Phosphoinositide-dependent kinase

PE: Phenylephrine

PEPT1: Peptide transporter-1

PGI2: Prostacyclin

PI3K: Phosphatidylinositol 3-kinase

PIP2: Phosphatidylinositol 4,5-bisphosphate

PIP3: Phosphatidylinositol 3,4,5-trisphosphate

PPARγ: Peroxisome proliferator associated receptor gamma

RAS: Renin angiotensin system

ROS: Reactive oxygen species

SBP: Systolic blood pressure

SD: Sprague Dawley

SHR: Spontaneously hypertensive rats

SNP: Sodium nitroprusside

SOD: Superoxide dismutase

SOS: Son of sevenless

TFA: Trifluoroacetic acid

TNF: Tumor necrosis factor

TZD: Thiazolidinedione

VCAM-1: Vascular adhesion molecule-1

VSMC: Vascular smooth muscle cell

List of Amino acids

A (Ala): Alanine

C (Cys): Cysteine

D (Asp): Aspartic acid

E (Glu): Glutamic acid

F (Phe): Phenylalanine

G (Gly): Glycine

H (His): Histidine

I (Ile): Isoleucine

K (Lys): Lysine

L (Leu): Leucine

M (Met): Methionine

N (Asn): Asparagine

P (Pro): Proline

Q (Glu): Glutamine

R (Arg): Arginine

S (Ser): Serine

T (Thr): Threonine

V (Val): Valine

W (Trp): Tryptophan

Y (Tyr): Tyrosine

Chapter 1: General Introduction and Thesis Objectives

1. General Introduction

Metabolic syndrome (MetS), the major public health challenge worldwide, is considered as a cluster of abnormalities including hypertension, insulin resistance, hyperlipidemia, glucose intolerance, and abdominal obesity is the major public health challenge worldwide (Wong 2007). Individuals with MetS are essentially at twice the risk for cardiovascular disease (CVD) and about five times the risk for type 2 diabetes compared with those without the syndrome (Grundy 2008). Worldwide prevalence of MetS ranges from less than 10% to as much as 84%, depending on the region, environment, composition (sex, age, race, and ethnicity) of the studied population, as well as the definition used for the syndrome (Kaur 2014). The significant association of MetS, the concomitant presence of at least three of the five clinical features of hypertension, high fasting glucose and triglyceride, low high density lipoprotein cholesterol, and abdominal obesity, with CVD and other chronic diseases with respect to the large suffering population highlights the importance of this disease in public health. The number of people with MetS is increasing largely worldwide as a consequence of unhealthy diets high in fats and refined sugars, excessive energy intake, and sedentary lifestyle (Ott and Schmieder 2009).

Hypertension, one of the major CVD risk factors affecting 1 billion people worldwide with 9 million annual deaths (WHO 2013), is also closely associated with MetS. It has been reported that up to one-third of hypertensive patients are afflicted with MetS disorders (Schillaci, Pirro et al. 2004). Renin angiotensin system (RAS) well known for its significant role in the regulation of blood pressure also plays a key role for the onset and development of other metabolic disorders including insulin resistance defined as the impaired insulin stimulated glucose uptake by cells (Marcus, Shefer et al. 2013). Indeed, insulin resistance is considered as the key causative factor

for the pathogenesis of MetS (Angelico, Del Ben et al. 2005). In the pathogenic condition of insulin resistance, higher concentrations of insulin are required in order to maintain normal glucose levels (Eckel, Grundy et al. 2005). Chronic hyperinsulinemia negatively affects insulin sensitive cells and damages β-cells, exacerbating MetS complications, and finally leading to type 2 diabetes. The interplay between pancreas and many other organs and tissues maintains glucose homeostasis. Indeed, insulin secretion, tissue's sensitivity to insulin, and glucose uptake by cells play a major role in regulating blood glucose .

The multi-faceted nature of MetS makes patients take several medications to specifically target different aspects of this disease. In addition to the significant side effects associated with synthetic drugs and the possible dugs interactions, adherence to life-long therapies is usually poor. Therefore, development of functional foods and/or nutraceuticals containing naturally-derived compounds has gained a great deal of scientific interest as safer alternatives to synthetic drugs in recent decades.

Based on the definition of Health Canada, a functional food is similar in appearance to a conventional food and is consumed as part of a usual diet whereas a nutraceutical is an isolated or purified product from foods generally sold in medicinal forms not usually associated with food. Both functional foods and nutraceuticals need to demonstrate physiological benefits and/or reduce the risk of chronic disease (Health Canada 2013). The global growth of functional foods is estimated between $20 billion and $30 billion in the USA (Price Waterhouse Coopers 2011), $8.5 billion in Japan (Agriculture and Agri-Food Canada 2016), and $11 billion in Canada (Agriculture and Agri-Food Canada 2011).

Food-derived bioactive peptides have great potential for the development of functional foods and/or nutraceuticals for the prevention and management of many chronic diseases (Erdmann,

Cheung et al. 2008, Udenigwe and Aluko 2012, Chakrabarti, Jahandideh et al. 2014). Indeed, many of these bioactive peptides are multifunctional, having several modes of action, and therefore, exert more than one physiologically beneficial activity (Meisel 2004, Erdmann, Cheung et al. 2008).

Hen egg as an excellent source of protein, vitamins, and minerals, is a major source for the production of bioactive peptides. These peptides have shown various physiological properties including antimicrobial, immunomodulatory, anti-cancer, and anti-hypertensive properties highlighting the importance of hen egg proteins in human health, and disease prevention and treatment (Mine 2007, Liu, Oey et al. 2017). Water (75%), proteins (12%), lipids (12%), and carbohydrates and minerals (1%) are the main components of eggs. Majority of proteins are found in the egg yolk and egg white (albumen), while, a small proportion is found in the eggshell and shell membrane. The egg white makes up about 60% of the total egg weight with water and protein being the major constituents. The major proteins of egg white include ovalbumin followed by ovotransferrin and ovomucoid (Mine and Kovacs-Nolan 2006). Our lab previously identified several peptides with *in vitro* antioxidant activity from ovotransferrin hydrolysate (Shen, Chahal et al. 2010). However, the antioxidant and anti-inflammatory effects of these peptides in more physiological conditions, as well as their stability against gastro-intestinal digestion (GID) are not known.

Furthermore, our lab previously identified three novel peptides IRW, IQW, and LKP from ovotransferrin hydrolyzed with thermolysin and pepsin with anti-hypertensive effects in SHRs (Majumder, Chakrabarti et al. 2013, Majumder, Chakrabarti et al. 2015). IRW being the most potent peptide among the three, exerted its effects on reducing blood pressure through several mechanisms including RAS modulation, reducing oxidative stress and vascular inflammation,

and improving vasorelaxation in SHRs (Huang, Chakrabarti et al. 2010, Majumder, Liang et al. 2015).

2. Hypotheses and objectives

Oxidative stress, defined as an imbalance between the formation of reactive oxygen species (ROS) and the body's antioxidant system is proposed to be the common underlying pathological contributor to various degenerated diseases (Gutowski and Kowalczyk 2013). Oxidative stress and inflammatory responses are also interrelated where activation of pro-inflammatory transcriptional factors by ROS increases the production of various cytokines and chemokines, leading to the generation and propagation of inflammatory responses which in turn generate more ROS leading to a vicious cycle that has detrimental effects on cells (Haddad 2002). Indeed, increased oxidative stress and vascular inflammation are key mediators of endothelial dysfunction and further dysregulation of blood pressure. Consumption of dietary antioxidant compounds has been suggested to exert beneficial effects on oxidative stress and hypertension (Kizhakekuttu and Widlansky 2010, Baradaran, Nasri et al. 2014). Ovotransferrin hydrolysate was previously shown to contain several bioactive peptides with antioxidant activities as determined by oxygen radical absorption capacity (ORAC) method. Since the efficacy of these peptides was not tested in more physiological conditions, we first aimed to measure their potential benefits on inflammation and oxidative stress in endothelial cells.

We hypothesized that ovotransferrin-derived peptides identified previously as antioxidants by ORAC method exert antioxidant and anti-inflammatory effects in human umbilical vein endothelial cells (HUVECs) (Chapter 3). The specific objectives of this study were to:

1. Assess the stability of ovotransferrin-derived peptides under simulated GID conditions.

2. Evaluate the anti-inflammatory effects of these peptides before and after GID in endothelial cells.
3. Measure the antioxidant effects of peptides with anti-inflammatory properties in endothelial cells.
4. Identify the peptide sequence in the peptide digest with antioxidant and anti-inflammatory effects in endothelial cells.

To achieve these specific objectives, HUVECs, a widely used model for studying the vascular endothelium (Narumiya, Zhang et al. 2001) were used for the *in vitro* cell culture experiments.

However, we did not observe any significant effect of peptides with *in vitro* antioxidant activity on oxidative stress and inflammation in endothelial cells, therefore, we diverted our approach on investigating the physiological benefits of whole egg white hydrolysate (EWH) as another potential source for the production of bioactive peptides.

The rationale for the selection of EWH was as follows. Individual egg white proteins including ovalbumin (Matoba, Usui et al. 1999, Miguel and Aleixandre 2006, Abeyrathne, Lee et al. 2014), ovotransferrin (Shen, Chahal et al. 2010, Majumder, Chakrabarti et al. 2013, Majumder, Liang et al. 2015), and lysozyme (Memarpoor-Yazdi, Asoodeh et al. 2012) have been reported to exert beneficial effects on oxidative stress, inflammation, and blood pressure *in vitro* and *in vivo*. Using the whole EWH rather than individual proteins or bioactive peptides not only has the economic advantages but also theoretically may have additional physiological benefits due to the emergence of novel peptides with diverse physiological effects as well as the potential synergistic effects among peptides (Liu, Oey et al. 2017). Moreover, the link between hypertension specifically RAS impairments and insulin resistance--as key features of MetS--has been reported in the literature (Yusuf, Gerstein et al. 2001, Braga and Leiter 2009) (discussed in

Chapter 2). Developing novel therapies that can target the common pathologies to hypertension and insulin resistance is of immense importance. Adipose tissue as an insulin sensitive tissue with a central role in lipid and glucose metabolism has a critical role in maintaining energy homeostasis and metabolism (Ruan and Lodish 2003). Under MetS, adipose tissue becomes dysfunctional with perturbed insulin signaling associated with insulin resistance and chronic inflammation (Pradhan, Manson et al. 2001, Kadowaki, Hara et al. 2003, Winkler, Kiss et al. 2003, Coppack, Mohamed-Ali et al. 2005, Harwood 2012). As such, there is significant interest in developing compounds to improve insulin signaling in adipocytes (de Souza, Eckhardt et al. 2001, Khan, Murtaza et al. 2012).

Since egg white contains peptides with antioxidant, anti-inflammatory and antihypertensive properties, and considering the interplay among hypertension, inflammation, oxidative stress and insulin resistance, it is plausible that EWH would show additional benefits on other complications of the MetS namely adipose tissue dysfunction and insulin resistance. Therefore, we hypothesized that EWH reduces several complications of MetS namely hypertension, inflammation and insulin resistance.

The specific objectives of this study were to:

1. Evaluate the *in vivo* antihypertensive activity of EWH in spontaneously hypertensive rats (SHRs), the suitable animal model of hypertension.
2. Explore the potential benefits of EWH on adipogenic differentiation and insulin signaling in pre-adipocytes (*in vitro*).
3. Test the effects of EWH on glucose tolerance and insulin resistance in an insulin resistant rat model.

4. To purify and identify peptide sequences from the EWH using step-wise chromatographic methods and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

To achieve these specific objectives, spontaneously hypertensive rats (SHRs), an animal model of essential hypertension (Miguel, Lopez-Fandino et al. 2005, Majumder, Panahi et al. 2013) used in previous studies in our lab, and Sprague Dawley (SD) rats fed a high fat-diet as an animal model for insulin resistance (Hashemi, Yang et al. 2015, Yang, Hashemi et al. 2015) were used for *in vivo* studies.

Brief descriptions of each experimental chapter are given below.

Chapter 2 provides a literature review on several topics relevant to this thesis including the pathogenesis of hypertension and MetS, the interplay between RAS, adipose tissue dysfunction, and insulin resistance as well as the underlying mechanism of action of different food-derived bioactive peptides on glucose homeostasis and insulin sensitivity.

Chapter 3 evaluated the *in vitro* anti-inflammatory and antioxidant effects of 16 previously identified peptides from ovotransferrin hydrolysate with ORAC activity, and their gastro-intestinal digests in tumor necrosis factor α (TNF-α) induced HUVEC monolayers. The outcome of this study revealed that these peptides did not show any anti-inflammatory and antioxidant activities in endothelial cells. After simulated GID, however, several peptide digests significantly reduced the expression of TNF-α-induced pro-inflammatory intercellular cell adhesion molecule-1 (ICAM-1) by 65.7 ± 10.4% and vascular cell adhesion molecule-1 (VCAM-1) by 53.5 ± 9.6% to 61.0 ± 14.5%, but only Gly-Trp-Asn-Ile (GWNI) digest reduced TNF-α-activated superoxide generation by 71.0 ± 12.9% when tested with dihydroethidium (DHE) assay. Mass spectrometer analysis identified two new peptides, Gly-Trp-Asn (GWN) and Gly-Trp (GW), in the GWNI

digest; however, only GW reduced TNF-α-induced VCAM-1 expression (64.3 ± 20.6%) significantly compared to the TNF-α treated cells. Both GW and GWN showed antioxidant effects in vascular smooth muscle cells. Our study concluded that ORAC lacked biological relevance in assessing bioactive peptides.

The data from this Chapter have been published (J. Agric. Food Chem., 2016, 64 (1), 113–119).

Chapter 4 tested the antihypertensive effect of EWH *in vivo* through oral administration in adult male SHRs. The results showed that feeding SHRs with EWH at 1000 mg/kg BW for 12 days significantly reduced mean arterial pressure at day 9 and day 12 of the treatment compared to control group. The reduction of blood pressure was associated with enhanced *ex vivo* vasodilation in mesenteric arteries as determined through wire-myograph studies, reduced vascular oxidative/nitrosative stress (in aortic sections), reduced vascular angiotensin converting enzyme and angiotensin II type 1 receptor expression, while enhanced angiotensin II type 2 receptor expression in the aorta. Indeed, EWH exerted its antihypertensive effects in SHRs through multiple mechanisms of vascular relaxation and local RAS modulation.

The data from this Chapter have been published (J Funct Foods, 2016, 27, 667-673).

Chapter 5 explored the *in vitro* effects of EWH on adipogenic differentiation in 3T3-F442A pre-adipocytes. Our results indicated that EWH could promote adipocyte differentiation by increasing lipid accumulation, release of adiponectin, and upregulation of peroxisome proliferator associated receptor gamma (PPARγ) and CCAAT/ enhancer binding protein alpha (C/EBP-α). In addition to the enhanced insulin effects on the upregulation of protein kinase B/Akt phosphorylation, EWH treatment increased extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation to a level similar to that of insulin, indicating insulin sensitizing and mimetic properties of the EWH respectively. EWH further attenuated cytokine-induced

inflammatory marker; cyclooxygenase -2 (COX-2) by 48.78%, possibly through the AP-1 pathway by down regulating c-Jun phosphorylation in adipocytes. Considering the critical role of adipose tissue in the pathogenesis of insulin resistance and MetS, EWH could be effective in alleviating the complications of the MetS especially the insulin resistance.

The data from this Chapter have been published (PLoS One. 2017 Oct 3;12(10): e0185653).

Chapter 6 further investigated the *in vivo* effects of EWH on glucose tolerance and insulin sensitivity in high fat-induced insulin resistant Sprague Dawley rats. The results showed that supplementing a HFD with 4% EWH changed the body composition towards less fat mass and enhanced lean mass % of BW. Rats receiving 4% EWH showed improved glucose tolerance with no changes in the postprandial insulin compared to the HFD treated group. The improved glucose tolerance, therefore, could be as a result of enhanced tissue insulin sensitivity in EWH treated rats compared to HFD treated rats. Interestingly, both the overall insulin sensitivity measured by the insulin tolerance test, and tissue insulin sensitivity (in muscle and adipose tissue) were enhanced in EWH treated rats. The systemic inflammation was reduced in EWH treated rats as shown by reduced plasmatic pro-inflammatory cytokines IL-1α, and IL-1β and chemokine MCP-1. Finally, we observed an enhanced PPARγ2 expression in epididymal adipose tissue which can be an indication of enhanced adipogenic differentiation in this fat depot consistent with our previous *in vitro* cell culture experiment.

Chapter 7 aimed to characterize EWH through cell culture experiments. Identification of bioactive peptides in EWH is important for the future structure-function experiments as well as mechanistic studies. Indeed, EWH is a complex compound containing a wide range of bioactive peptides. Since we observed the positive effects of EWH on both preadipocyte differentiation *in vitro* and *in vivo*, along with the positive effects on enhanced glucose tolerance and insulin

sensitivity *in vivo*, the responsible peptides with PPARγ stimulatory activity from the EWH were purified and characterized *in vitro* on 3T3-F442A pre-adipocytes. 42 peptides were identified from EWH by employing several techniques including ultrafiltration, C18 cartridge purification, cation exchange, and reverse-phase chromatography, based on the structural characteristics including molecular weight, net positive charge and hydrophobicity. The fractions exhibiting strong PPARγ expression were sequenced by LC-MS/MS. We successfully validated the stimulatory effects of several novel peptide sequences including WEKAFKDED, QAMPFRVTEQE, and VFKGL from the major egg white proteins ovalbumin and ovotransferrin on PPARγ expression in pre-adipocytes.

The data from this Chapter have been submitted to the Food Chemistry.

Findings from this thesis suggest the potential of EWH as a novel compound for the development of functional foods with potential applications in the prevention and management of hypertension, inflammation, glucose tolerance, and insulin resistance in the more complicated conditions of MetS. The identified bioactive peptides in EWH may be responsible for the EWH positive effects on adipose tissue and also have potential benefits on ameliorating complications of the MetS.

Chapter 2: Introduction and Literature Review[1]

2. Introduction

Metabolic syndrome (MetS), the presence of at least 3 of the 5 clinical features such as raised blood pressure, dyslipidemia (raised triglycerides and lowered high-density lipoprotein cholesterol), raised fasting glucose, and central obesity is a major risk factor for cardiovascular disease and type 2 diabetes (Alberti, Eckel et al. 2009). The prevalence of MetS is high and increasing rapidly worldwide. Individuals with MetS are essentially at twice the risk for developing cardiovascular disease (CVD) over the next 5 to 10 years and about five times the risk for type 2 diabetes compared with those without the syndrome (Grundy 2008). The high prevalence of MetS and its significant association with chronic diseases including CVD has emerged this disease as both a public health and clinical problem (Alberti, Eckel et al. 2009).

Hypertension is a classical feature of the MetS. It has been reported that up to one-third of hypertensive patients are afflicted with MetS disorders (Schillaci, Pirro et al. 2004). Blood pressure levels are strongly associated with visceral obesity and insulin resistance (Ferrannini, Natali et al. 1997, Yanai, Tomono et al. 2008) the main pathophysiologic features of MetS (Angelico, Del Ben et al. 2005).

Indeed, renin angiotensin system (RAS) well known for its significant role in the regulation of blood pressure also plays a key role for the onset and development of insulin resistance (Marcus, Shefer et al. 2013). Chronic hyperinsulinemia exacerbates MetS complications and finally leads to the development of type 2 diabetes.

[1] This chapter will be submitted to the *Critical Reviews in Food Science and Nutrition* journal.

The multi-faceted nature of the MetS usually forces patients take several medications life-long which are associated with several side effects. Therefore, there is increasing interest in safer alternatives to synthetic drugs through developing naturally-derived compounds with inferred fewer side effects. Food proteins containing bioactive peptides have great potential for the development of functional foods and/or nutraceuticals for the prevention and management of many chronic diseases (Erdmann, Cheung et al. 2008, Udenigwe and Aluko 2012, Chakrabarti, Jahandideh et al. 2014).

While the potential benefits of bioactive peptides on hypertension (Majumder and Wu 2015), hyperlipidemia (Udenigwe and Rouvinen-Watt 2015), inflammation and oxidative stress (Chakrabarti, Jahandideh et al. 2014) have been extensively reviewed in literature, reports on the physiological benefits of peptides on glucose homeostasis and insulin sensitivity are a small number. Moreover, the potential effects of bioactive peptides on multiple targets rather than one specific activity should also be taken into considerations especially when inflammation and oxidative stress are known to underlie many chronic diseases like hypertension and insulin resistance.

Therefore, here in this chapter, we have first discussed the pathophysiology of hypertension with emphasis on the RAS. Since there has been an association between RAS overactivity and insulin resistance, the key component of the MetS, we further discussed MetS with emphasis on glucose intolerance and insulin resistance. The role of adipose tissue dysfunction in the onset and development of insulin resistance has been further highlighted and finally, protein hydrolysates and bioactive peptides with benefits on glucose homeostasis with their mechanism of action have been thoroughly reviewed.

2.1. Hypertension

Hypertension, one of the major constituents of the MetS is defined as the persistent elevation of systolic/diastolic blood pressure over 140/90 mmHg. Hypertension is a major risk factor for developing cardiovascular diseases and is a growing health problem worldwide. Globally, 51% of stroke deaths are attributable to high systolic blood pressure (WHO 2009), whereas, treating hypertension has been associated with about 40% reduction in the risk of stroke (Afridi, Canny et al. 2003). Although various pharmaceutical drugs are available for the treatment of hypertension, management of hypertension often requires lifelong adherence to the medication and is associated with significant adverse side-effects (Khanna, Lefkowitz et al. 2008). Moreover, some cases of hypertension are not adequately controlled by commonly used pharmaceutical agents especially when other comorbidities like obesity and chronic kidney disease are present (Viera 2012). Thus, there has been a growing interest in using alternative options, such as adoption of a healthy lifestyle with restricted energy and dietary sodium intake, engagement in physical activities and stopping smoking, for prevention and management of hypertension (Moser and Franklin 2007). Increasing consumer knowledge about the link between diet and health has also increased the awareness and demand for functional foods to avoid the increasing cost and the undesirable side effects of synthetic drugs (Saleh, Zhang et al. 2016). Therefore, foods as the other potential alternatives for controlling the rise in blood pressure have gained more attention in recent decades. The antihypertensive properties of several food sources including grain, vegetables and fruits, dairy products, fish, meat, egg, legumes, nuts, and seeds have been studied extensively (Erdmann, Cheung et al. 2008, Shahidi and Zhong 2008).

As mentioned earlier hypertension and vascular disorders are central to MetS, however, the underlying mechanisms for the development of hypertension in MetS are complicated and have

not been defined completely yet. Visceral obesity, insulin resistance, sympathetic overactivity, oxidative stress, endothelial dysfunction, activated renin-angiotensin system (RAS), and inflammation have been suggested as the potential mechanisms (Mendizabal, Llorens et al. 2013). A number of these underlying pathologies such as hyperactivity of the RAS, inflammation, oxidative stress, and endothelial dysfunction as the contributors to the onset and long-term persistence of hypertension (Giachini, Callera et al. 2008, Schulz, Gori et al. 2011) discussed in the following sections.

2.1.1. Systemic Renin Angiotensin System (RAS)

Blood pressure is controlled by a number of different interacting biochemical pathways. RAS, identified as a hormone-based pathway, plays an important role in regulating arterial pressure. Low blood pressure is one of the stimulants of RAS; where it triggers the secretion of enzyme renin from kidneys to the blood stream. Renin hydrolyzes angiotensinogen from liver to angiotensin I (Ang I). Angiotensin converting enzyme (ACE), is both generated by and resident in the lungs, the endothelial cells of the vasculature, and cell membranes of a number of organs in the body (Perazella and Setaro 2003), cleaves the inactive Ang I to the potent vasoconstrictor octapeptide, angiotensin II (Ang II) (Lavoie and Sigmund 2003). Ang II increases blood pressure by various mechanisms, including constricting resistance vessels, stimulating aldosterone synthesis, cardiac hypertrophic growth and remodeling, and enhancing sympathetic outflow from the brain (Oparil, Zaman et al. 2003, Michel 2004). Ang II binds to specific cell surface receptors angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptors. Most of the known harmful effects of Ang II are related to AT1 receptor activation. AT2 receptors, present in both endothelial and vascular smooth muscle cells (VSMCs), when activated, antagonize the effects

of the AT1 receptors by mediating vasodilation, releasing NO, and inhibiting vascular smooth muscle growth (Stankevicius, Kevelaitis et al. 2003, Wassmann and Nickenig 2006).

The cardiovascular risk factors such as hypercholesterolemia and diabetes mellitus are closely related to overexpression and increased activation of AT1 receptors in the vascular wall (Wassmann and Nickenig 2006, Liu, Gao et al. 2008). Ang II facilitates the oxidation and uptake of low density lipoprotein (LDL) via AT1 receptor (Keidar, Kaplan et al. 1995, Singh and Mehta 2001). The oxidized LDL further up-regulates AT1 receptor expression (Li, Saldeen et al. 2000) showing the interaction between hyperlipidemia and RAS in atherogenesis. The enhanced AT1 receptor expression has been shown in the tissues of hypercholesterolemic animals and humans (Chen, Li et al. 2006). The association between RAS and insulin resistance and diabetes has been discussed in section 3.

2.1.2. Endothelial dysfunction and inflammation

Structural and functional abnormalities in the vasculature, including endothelial dysfunction, increased oxidative stress, and vascular remodeling also contribute to the pathogenesis of hypertension. The endothelium, a monolayer of cells that lines the lumen of blood vessels, is the key regulator of vascular homeostasis. The healthy endothelium responds to physical and chemical signals by producing a wide range of factors that regulate vascular tone, cellular adhesion, smooth muscle cell proliferation, and vessel wall inflammation (Deanfield, Halcox et al. 2007). Endothelial cells secrete various vasoactive molecules that relax or constrict the vessel. Nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin are the vasodilators while endothelin I, thromboxane and Ang II (from conversion of Ang I to Ang II at the endothelial surface) are the vasoconstrictor compounds derived from endothelium (Stankevicius, Kevelaitis et al. 2003, Deanfield, Halcox et al. 2007). NO, the first identified

endothelium-derived vasodilator plays a key role in maintaining the vascular wall in an inert state in normal vascular physiology by inhibition of inflammation, cellular proliferation, and thrombosis (Schulz, Gori et al. 2011). NO is generated from L-arginine by the action of endothelial NO synthase (eNOS) in the presence of tetrahydrobiopterin. NO activates soluble guanylate cyclase in vascular smooth muscle cells generating cyclic GMP (cGMP) and eventually leading to cGMP-mediated vasodilatation through the removal of cytosolic Ca^{2+} from cell (Stankevicius, Kevelaitis et al. 2003, Forstermann and Munzel 2006). Endothelial dysfunction is generally referred to as an impairment of endothelium-dependent vasorelaxation caused by a loss of NO bioavailability or production (Deanfield, Halcox et al. 2007). Endothelial dysfunction is involved in the pathophysiology of MetS and cardiovascular diseases such as atherosclerosis, hypertension and heart failure (Cai and Harrison 2000, Albarran, Calvo et al. 2009).

The vascular endothelium also plays a key role by acting as a gate-keeper for the extravasation of leukocytes that is a hallmark of inflammation. The excessive and uncontrolled inflammatory changes often lead to the development of vascular disease, atherosclerosis, and its complications. The vascular inflammatory response involves a complex interaction between inflammatory cells (neutrophils, lymphocytes, monocytes, and macrophages), endothelial cells, vascular smooth muscle cells, and extracellular matrix (Sprague and Khalil 2009). Mediators like various pro-inflammatory cytokines (such as TNF-α, IL-1β), chemokines (such as MCP-1) and ROS (such as superoxide and peroxynitrite) are involved in both the generation and propagation of the inflammatory response. These pro-inflammatory mediators interact with specific receptors on various cell types and activate intracellular signaling pathways leading to the inflammatory changes. The nuclear factor-kappaB (NF-κB), and activator protein 1 (AP-1) are the two key

transcription factors orchestrating expression of many genes leading to the inflammatory response (Ruiz-Ortega, Lorenzo et al. 2000, Csiszar, Wang et al. 2008). When endothelial cells undergo inflammatory activation endothelial permeability and the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) increases. Enhanced adhesion molecules' expression promotes the adherence of the inflammatory cells followed by their migration across the vascular barrier, and recruitment of additional cytokines and growth factors (Pober and Sessa 2007). Endothelial cells can produce cytokines as well. Cytokines interact with mitochondria to increase the production of reactive oxygen species. Cytokine-induced activation of these pathways in endothelial cells further modifies the production/activity of vasodilatory as well as vasoconstrictive mediators attenuating the vascular relaxation (Tousoulis, Charakida et al. 2008). Few therapies have been devised to target the inflammatory component of cardiovascular and malignant diseases. The non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin with anti-thrombotic and anti-inflammatory properties are widely used to prevent and manage cardiovascular diseases (Davidge 2001, Fiorucci, Distrutti et al. 2004). However, side effects such as gastric bleeding and ulceration preclude the long-term use of NSAIDs for a large part of the population.

NF-κB and AP-1 as the key inflammatory transcription factors will be further discussed in the following sections.

2.1.2.1. NF-κB pathway

NF-κB is a redox-sensitive transcription factor with an important role in inflammatory changes in endothelial and smooth muscle cells under various pathophysiological conditions such as oxidative stress, RAS hyperactivity, hyperglycemia, and hyperinsulinemia. NF-κB describes various dimeric complexes of members of the Rel protein family, which comprises Rel (cRel),

Rel A (p65), RelB, NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) (Ghosh, May et al. 1998). NF-κB proteins are sequestered in the cytoplasm through their association with a member of the I-κB family of inhibitory proteins. Phosphorylation of I-κB by numerous pro-inflammatory stimuli such as TNF-α leads to dissociation of NF-κB complexes from the I-κB proteins, ubiquitination, and release of NF-κB proteins from the NF-κB complex. Free proteins then migrate from cytosol to the nucleus, where it binds to the promoters of target genes, leading to transcriptional activation of many pro-inflammatory genes implicated in vascular inflammation (Li and Stark 2002). Activated NF-κB induces the transcription of adhesion molecules, cytokines, chemokines, and inducible enzymes such as cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (iNOS). Chronic activation of NF-κB is generally believed to predispose arteries to atherosclerosis (Sprague and Khalil 2009).

2.1.2.2. AP-1 pathway

AP-1 is a group of transcription factors consisting of members of Jun and Fos families. The predominant forms of AP-1 in most cells are c-Jun and c-Fos heterodimers which have a high affinity for binding to the AP-1 site. AP-1 is regulated at multiple levels, such as transcription level, messenger RNA turnover, protein stability or interactions with other transcription factors as well as posttranslational modifications by upstream kinases such as Jun N-terminal kinases (Wagner 2010).Exposure of cells to pro-inflammatory cytokines modulates both the abundance and activity of AP-1 proteins (Ivorra, Kubicek et al. 2006). Activators of the mitogen-activated protein kinases (MAPKs) cascade can activate the AP-1 pathway. These activated members of MAPKs translocate to the nucleus to phosphorylate and thereby activate transcription factors that bind to the AP-1 sites in promoters (Karin, Liu et al. 1997, Balasubramanian and Eckert 2007).

The outcome of AP-1 activation depends on its composition as well as the phosphorylation state of each Fos/Jun heteropartner (Hipskind and Bilbe 1998).

2.1.3. Oxidative stress

Oxidative stress, *i.e.* the excessive and/or dysregulated generation of reactive oxygen species (ROS), is a major contributor to disease pathologies including hypertension and MetS. The term ROS encompasses a range of oxygen-containing highly reactive species including free radicals superoxide ($O2^{-\cdot}$) and hydroxyl radicals ($HO^{\cdot}$) as well as nonradical form like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen, and peroxynitrite ($ONOO^-$) (Lu, Lin et al. 2010). In low concentrations, ROS is beneficial by induction of apoptosis in damaged/aged cells, detoxification of xenobiotics by cytochrome P450 system, and killing invading microorganisms by phagocytes and as regulatory mediators in cell signaling pathways (Poli, Leonarduzzi et al. 2004, Pham-Huy, He et al. 2008). However, an excess of ROS, due to excessive production or impaired antioxidant capacities or both, is harmful and leads to oxidative stress. In pathological conditions, ROS attack nucleic acids, proteins, and unsaturated fatty acids and aggravate cellular damage (Brieger, Schiavone et al. 2012). Peroxyl radicals ($ROO\cdot$) initiate peroxidation of fatty acids. The final products of this reaction are malondialdehydes (MDA) which possess carcinogenic properties (Valko, Leibfritz et al. 2007). Proteins are another group of macromolecules affected by the ROS. Cleavage of the peptide bond, amino acid modification, and formation of cross-linked peptide aggregates during protein oxidation by ROS lead to the formation of protein derivatives possessing highly reactive carbonyl groups (ketones and aldehydes) which are involved in the complications of diabetes and many age-related diseases (Stadtman and Levine 2003).

2.1.4. Ang II, oxidative stress, and endothelial dysfunction

Excessive RAS activity, especially Ang II-AT1 receptor axis can be deleterious to the endothelium. In addition to increased vascular contractility, AT1 receptor activation by Ang II also leads to inflammatory changes through activation of redox sensitive genes, matrix metalloproteinase-2 release and ROS generation in endothelium, which are involved in the initiation and progression of endothelial dysfunction and manifested atherosclerosis (Arenas, Xu et al. 2004, Wassmann and Nickenig 2006, Kossmann, Hu et al. 2014). Figure 2- 1 shows the interplay between RAS, inflammation, oxidative stress, and endothelial dysfunction. AT1-receptor-mediated ROS production in vessels is linked to the activation of NAD(P)H oxidase, uncoupling of endothelial nitric oxide synthase and activation of xanthine oxidase, leading to enhanced superoxide production by these enzymes in vascular cells (Wassmann, Wassmann et al. 2004, Higuchi, Ohtsu et al. 2007). ROS impairs vascular relaxation by reaction with NO. The reaction between NO and superoxide produces the highly reactive peroxynitrite ($ONOO^-$) which causes nitration at the tyrosine residues of various proteins. Therefore, peroxynitrite formation both reduces NO bioavailability and hence contributes to endothelial dysfunction, and adversely affects physiological functions of many cellular proteins predisposing the endothelial cells toward inflammation and cell death (Szabo, Ischiropoulos et al. 2007).

Given the critical roles played by NO and its alterations observed under oxidative stress, regulation of ROS levels is essential for enhancing or maintaining normal endothelial functions. Therapies targeted against free radicals have been shown to be useful in minimizing vascular injury and prevention or regression of hypertension (Virdis, Neves et al. 2004, Rodrigo, Prat et al. 2008). Dietary intake of antioxidant compounds may potentially reinforce the body's oxidant status maintaining a balanced condition in terms of oxidant/antioxidant in the body (Ardalan and

Rafieian-Kopaei 2014). Therefore, there is increasing interest in food proteins and their constituent peptides as potential candidates for use as antioxidants. Indeed, numerous antioxidant peptides have been identified form different protein sources; milk (Contreras, Hernandez-Ledesma et al. 2011, Li, Jiang et al. 2013), egg (Chen, Chi et al. 2012, Nimalaratne, Bandara et al. 2015), soy (Zhang, Li et al. 2010), meat (Liu, Xing et al. 2016), fish (Ko, Lee et al. 2013, Wang, Li et al. 2013) etc. mostly *in vitro* in recent decades.

2.2. Metabolic syndrome

2.2.1. Definition

Historically, Reaven was the first to define the "syndrome X" concept in 1988 (which he later renamed to MetS) as the cluster of insulin resistance, dysglycemia, dyslipidemia and high blood pressure being the central feature in coronary heart disease and type 2 diabetes (Reaven 1988). Many international organizations and expert groups have attempted to incorporate different risk factors to define MetS since then. The World Health Organization (WHO), the European Group for the study of Insulin Resistance (EGIR), the National Cholesterol Education Program Adult Treatment Panel III (NCEP:ATPIII), the American Association of Clinical Endocrinology (AACE), the International Diabetes Federation (IDF), and the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI), have used different criteria to define MetS (Table 2- 1). The focus of WHO, AACE, and EGIR definitions is insulin resistance (or its surrogates) while is abdominal obesity in NCEP: ATPIII, and IDF (Kassi, Pervanidou et al. 2011). According to the new definition of International Diabetes Federation (IDF), for a person to be diagnosed with metabolic syndrome, one must have central obesity (determined by waist circumference measurement with ethnicity specific values for different groups) plus any two of the factors including hypertension, impaired fasting glucose, raised

triglycerides and reduced serum HDL-cholesterol or on medication for the above- mentioned abnormalities.

2.2.2. Prevalence

The high prevalence of MetS is a worldwide phenomenon and continuously increases as a consequence of unhealthy diets high in fats and refined sugars, excessive energy intake, and sedentary lifestyle (Ott and Schmieder 2009). Since different definitions have been used for MetS, the prevalence of the disease varies in different regions based on the criteria used in each definition. Furthermore, sex, age, race, diet, physical activity, and ethnicity of the population studied are other influential factors (Cameron, Shaw et al. 2004). Most reports have used the NCEP: ATPIII definition of the syndrome and in some cases this definition has been modified for differences in waist circumference between different ethnic groups. At least one-fourth of the adults in the Americas, Europe, and India have been diagnosed with MetS (Grundy 2008). The prevalence of MetS ranged from 5.6% in Chinese women to 52.8% in Polynesian men in New Zealand according to the NCEP: ATPIII criteria (Meshkani and Adeli 2009). Iranian, South Asian, African, Mexican and Arab's women had a higher prevalence of MetS compared to men while this trend was not the same in Caucasians and Hispanics (Meshkani and Adeli 2009). MetS at least doubles the risk for cardiovascular diseases in population with the disease compared to those without it. Moreover, it further raises the risk for type 2 diabetes by about 5-fold (Grundy, Cleeman et al. 2005). Additionally, MetS may increase the susceptibility of individuals to developing other abnormalities such as fatty liver, polycystic ovary syndrome, cholesterol gallstones, asthma, sleep disturbances, and some types of cancer (Grundy, Hansen et al. 2004). Therefore, this is a global challenge in prevention and management of MetS.

2.2.3. Metabolic syndrome and cardiovascular disease

There is accumulating evidence showing a strong association between MetS and cardiovascular disease (CVD) regardless of which definition used for the MetS. Based on various definitions of MetS, abdominal obesity and insulin resistance should be considered the main causes of the syndrome, both of which are also independent CVD risk factors (Meshkani and Adeli 2009). The disturbance in insulin signaling promotes cardiovascular disease through the development of abnormal glucose and lipid metabolism, hypertension, and a pro-inflammatory state. Impaired insulin signaling in vascular endothelium and other cells in the heart, kidney, and retina also alters the progression of cardiovascular disease in the MetS and affects the development of microvascular complications of diabetes mellitus. (Rask-Madsen and Kahn 2012). In the Framingham Heart Study, the MetS alone predicted 25% of all new onsets CVD (Lakka, Laaksonen et al. 2002). Hypertension, hypertriglyceridemia, and low HDL as the other components of the MetS have also been shown to be independent risk factors for CVD (Meshkani and Adeli 2009).

2.3. Glucose homeostasis

High plasma glucose level is one of the hallmarks of MetS. D-glucose is the major primary source of energy for the brain and the red blood cells and is a crucial fuel for other cell types in the body. In order to ensure normal body function, blood glucose level needs to be controlled tightly in the body by a complex network of various hormones and neuropeptides released mainly from the brain, pancreas, liver, intestine, adipose and muscle tissues. Indeed, glucose absorption from the intestine, glucose production by the liver, and uptake and metabolism by peripheral tissues determine the blood glucose concentration in the body. The pancreas

represents a key player within this network by secreting insulin and glucagon hormones (Roder, Wu et al. 2016).

Fasting glucose is around 4 mM while after a meal the blood glucose level raises reaching to about 7 mM (Saltiel and Kahn 2001). In the fasting state, the low glucose levels stimulate the secretion of glucagon, a hormone produced by the α-cells of the pancreas. Glucagon functions mainly in the liver to increase glycogenolysis, the enzymatic breakdown of glycogen to glucose, and gluconeogenesis which is the formation of glucose from glycerol, lactate and amino acids in the liver (Wang, Viscarra et al. 2015). After meal ingestion and the gastrointestinal digestion, nutrients are absorbed into the circulation via the hepatic portal vein. Therefore, liver plays a fundamental role in regulating postprandial glucose levels by controlling glucose production and storage (Grayson, Seeley et al. 2013). Ingested nutrients also stimulate the release of incretins from intestinal endocrine cells. Incretin secretion together with the rise in blood glucose stimulates insulin release from pancreatic β-cells leading to glucose clearance from the blood. Insulin is secreted at a low, basal level in the non-fed state, while after a meal, insulin concentration raises making the cells take up more glucose (Bilous 2010). Insulin secretion is biphasic: the first phase occurring early after meal ingestion suppresses hepatic gluconeogenesis. Whereas, the second phase reaching a plateau within 1–2h after the meal, stimulates glucose uptake by skeletal muscle and adipose tissue. Insulin also indirectly regulates hepatic glucose production by inhibiting the release of glucagon (Grayson, Seeley et al. 2013). Figure 2- 2 summarizes the glucose homeostasis.

Apart from the insulin-dependent pathway for glucose uptake, insulin-independent pathways also contribute to glucose uptake by cells. AMP-activated protein kinase (AMPK) is one of these pathways involved in insulin-independent glucose uptake, enhanced insulin sensitivity, and

glucose homeostasis. Physiological, pharmacological, natural, and hormonal stimuli can activate AMPK (Coughlan, Valentine et al. 2014). Increased AMPK activity in skeletal muscle results in enhanced glucose transport rate. The physiological effects of AMPK activation have been further discussed in section 6.3.7. Figure 2- 3 depicts the insulin dependent and independent pathways for glucose uptake.

Glucose is transported into various tissues by either Na^+ dependent glucose transporters that are not regulated by insulin, or by a family of specialized transporter proteins called glucose transporters (GLUTs). GLUT1 is ubiquitously expressed and probably mediates basal glucose uptake. GLUT2 is expressed in the liver, kidney, intestine, and β-cells of the pancreas. GLUT3 together with GLUT1 is involved in non-insulin mediated uptake of glucose into the brain. GLUT4 is the only glucose transporter which is regulated by insulin and is responsible for glucose uptake in muscle and adipose tissue (Bilous 2010). Upon insulin stimulation, the translocation of the glucose transporter GLUT4 from intracellular sites to the cell surface occurs. Insulin's primary actions are on adipocytes, muscle and liver cells. Up to 75% of insulin-dependent glucose disposal occurs in skeletal muscle, whereas adipose tissue accounts for about 10-15%. Although insulin does not stimulate glucose uptake in liver, it blocks glycogenolysis and gluconeogenesis and stimulates glycogen synthesis, thus regulating glucose levels. Insulin also regulates lipid metabolism by increasing fatty acid synthesis, increasing esterification of free fatty acids (FFAs), and decreasing lipolysis (Meshkani and Adeli 2009). The levels of FFA in the circulation also play a role in regulating glucose metabolism. An increase in FFA concentrations stimulates fatty acid oxidation while inhibiting carbohydrate oxidation and glycolysis (Jeppesen, Hansen et al. 2006). Insulin also affects protein metabolism by increasing protein synthesis and

decreasing proteolysis. Insulin also controls cell growth, proliferation, survival and differentiation (Meshkani and Adeli 2009).

2.4. Pathogenesis of the metabolic syndrome

There are many unclear key aspects in the delineation of risk factors that predispose an individual to MetS. The great variation in susceptibility and age of onset in individuals with similar risk profile suggests a major interaction between genetic and environmental factors (Ordovas 2007). Although obesity and insulin resistance (IR) remain at the core of the pathophysiology of MetS, a number of other factors such as chronic stress, increases in cellular oxidative stress as well as RAS overactivity can also be involved in its pathogenesis (Kassi, Pervanidou et al. 2011). IR and obesity/adipose tissue dysfunction are the focus of the current thesis and will be discussed further in the following sections.

2.5. Insulin Resistance

IR as an essential defect in type 2 diabetes is also the defining feature of the MetS (Facchini, Hua et al. 2001, Resnick, Jones et al. 2003, Angelico, Del Ben et al. 2005). IR is a pathologic state in which target cells fail to respond to normal levels of circulating insulin (Mlinar, Marc et al. 2007). Therefore, insulin is unable to provide normal glucose and lipid homeostasis. Consequently, higher concentrations of insulin are needed in order to maintain normal glucose levels (Eckel, Grundy et al. 2005). In MetS, IR is linked predominantly to a cluster of disorders including lipid and glucose metabolism, hypertension and vascular inflammation. The pathophysiology of IR involves a complex network of signaling pathways (Figure 2- 4).

2.6. Obesity and adipose tissue dysfunction

Obesity, one of the major underlying causes of the MetS, is increasing rapidly worldwide largely due to the sedentary lifestyle and positive energy balance conditioned by environmental and genetic factors. Obesity is defined by World Health Organization (WHO) as a body mass index (BMI) $\geq$ 30 kg/m^2 (Obesity: preventing and managing the global epidemic. Report of a WHO consultation 2000). Obesity is accompanied by high plasma levels of nonesterified fatty acids (NEFA) that cause IR in skeletal muscle and overload the liver with lipid, producing fatty liver and atherogenic dyslipidemia (Grundy 2008). Excessive fat, which is particularly deposited in visceral organs, generates a chronic low-grade inflammation state that contributes to the development of various metabolic and cardiovascular diseases. Moreover, fat accumulation in the liver may stimulate hepatic cytokine production. Development of obesity is a concomitant increase in fat cell size (adipocyte hypertrophy) and number (adipocyte hyperplasia) (DeClercq, Taylor et al. 2008). Adipose tissue has a crucial role in maintaining energy homeostasis and insulin sensitivity in the body. The following sections discuss different types of adipose tissues in the body with the focus on white adipose tissue morphology and functions, the adipogenic transcriptional cascade, and the role of white adipose tissue dysfunction in the development of MetS.

2.6.1. Adipose tissues

There are two fundamentally different types of adipose tissue in mammals; the white adipose tissue (WAT) and the brown adipose tissue (BAT), which differ in several important properties. WAT is widely distributed in the body and represents the primary site of fat metabolism and storage, whereas BAT is relatively scarce and its main role is to provide body heat, which is essential for newborn babies. White adipocytes are found in WAT tissue and are on average 60-

100 μm in diameter. White adipocytes have the capacity for lipid storage. Mature white adipocytes store triglycerides in a single large lipid droplet occupying the center of the cell accounting for 85-90% of the mass of the cell. Although they have variable volume, mature white adipocytes are large cells and their size can change dramatically depending on the quantity of triglycerides they accumulate. In contrast, brown adipocytes in BAT are on average 30-40 μm in diameter and function primarily in non-shivering thermogenesis. Brown adipocytes contain many cytoplasmic lipid droplets of varying sizes and many mitochondria that release heat via oxidation of fatty acids (Harwood 2012).

The focus of this thesis will be the white adipose tissue and white adipocytes will be referred as "adipocytes" throughout the thesis.

2.6.2. White adipose tissue

WAT is the predominant type of adipose tissue commonly named "fat" in mammals (Ahima 2006). As an excellent thermal insulator, WAT plays an important role in the conservation of body temperature. It also plays a vital role in energy homeostasis by storing triglycerides, responding to nutrient, neural and hormonal stimuli and secretion of adipokines (Ahima 2006). Adipose tissue is mainly composed of adipocytes that are crucial for both energy storage and endocrine activity. Precursor cells (including pre-adipocytes), fibroblasts, vascular cells and immune cells, which constitute the stromal vascular fraction of adipose tissue, are the other cell types present in the adipose tissue (Ouchi, Parker et al. 2011, Harwood 2012). The blood vessels in adipose tissue are required for the flow of nutrients and oxygen to adipocytes, and distribution of adipokines throughout the body. Vascular cells consisting of endothelial and vascular smooth muscle cells also secrete and are responsive to the adipokines secreted by the adipose tissue. Macrophages and T cells are the other active components of the adipose tissue with major roles

in determining the immune status of the adipose tissue. Fibroblasts produce extracellular matrix components to provide mechanical support for the adipose tissue. However, excess matrix components can lead to adipose tissue dysfunction and metabolic dysregulation (Khan, Muise et al. 2009). Intercellular communication within adipose tissue is therefore vital for maintaining homeostasis in adipose tissue and throughout the body.

WAT is distributed across different depots in the body with distinct molecular and physiological properties (Figure 2- 5).

Subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) are the two most abundant depots. SAT is mainly located in deposits below the skin in the abdominal, gluteus and femoral areas, whereas VAT includes the fat within the viscera of the abdominal cavity including mesenteric, omental and retroperitoneal depots and has an association with the increased risk of insulin resistance and cardiovascular disease (Ahima 2006, Rosen and MacDougald 2006).

WAT provides capacity for the storage of triglycerides that is vital for survival. In periods of calorie abundance, free fatty acids (FFAs) are stored in liver and WAT in the form of triglycerides through their esterification to glycerol (Galic, Oakhill et al. 2010). By contrast, in times of energy shortage like during fasting, the fall in insulin stimulates glycogen breakdown and lipolysis through activation of the sympathetic nervous system and elevation of glucagon, epinephrine, and glucocorticoids (Ahima 2006). Increased glycogen breakdown maintains glucose supply to the brain and vital organs in fasting state, whereas oxidation of fatty acids in adipose tissue, skeletal muscle and liver generates ketone bodies serving as an alternate source of energy for the brain and peripheral organs (Ahima 2006, Harwood 2012). Additionally, WAT possesses an active secretory function in releasing a number of endocrine and paracrine factors commonly referred as adipokines, that play a critical role in regulating metabolism in both health

and disease (Galic, Oakhill et al. 2010). Adipokines interact with central as well as peripheral organs through communicating the nutrient status of the organism to the tissues responsible for controlling energy intake and expenditure. Hence, allowing adipocytes to regulate processes in skeletal muscle, liver, pancreas, vasculature, and brain to influence a large number of diverse metabolic processes. Carbohydrate and lipid metabolism, inflammation processes, blood pressure, energy expenditure, and feeding behavior are among the affected processes by adipokines (Galic, Oakhill et al. 2010, Harwood 2012). Leptin, the first discovered adipokine, is produced mainly by adipocytes (Ahima 2006). A rise in leptin signals satiety to the brain through acting on receptors in the central nervous system and other sites. Leptin has been found to have a profound role in the regulation of whole-body metabolism by stimulating energy expenditure and inhibiting food intake. However, leptin resistance occurring in most cases of obesity limits its biological efficacy (Saltiel and Kahn 2001). Adiponectin is an adipokine exclusively secreted by mature adipocytes and possess anti-inflammatory effects. Adiponectin also increases insulin sensitivity by inhibiting hepatic glucose production and by increasing fatty acid oxidation in both the liver and skeletal muscle as a result of increased AMP-activated protein kinase (AMPK) activity (Hajer, van Haeften et al. 2008). There is an inverse correlation between levels of adiponectin in the circulation and the risk of obesity, insulin resistance, type 2 diabetes, and cardiovascular disease (Harwood 2012). There are several other adipokines with positive correlation with adiposity and development of insulin resistance such as resistin, visfatin, retinol binding protein-4, omentin etc. (Galic, Oakhill et al. 2010). However, the expression and secretion of many of these adipokines is less adipocyte-specific (Harwood 2012).

2.6.3. Adipocyte differentiation

Normal adipocyte differentiation is fundamental for generation of insulin sensitive adipocytes with enough capacity to store fats.

Adipocytes derive from multipotent mesenchymal stem cells. These cells have the capacity to differentiate into myocytes, chondrocytes, osteocytes, and adipocytes (Rosen and MacDougald 2006). Adipogenesis has mainly been described in 2 major phases: determination and terminal differentiation. The initial phase results in the conversion of the stem cells to pre-adipocytes. The pre-adipocytes then go through terminal differentiation to obtain the characteristics of mature adipocytes by acquiring the necessary characteristics for lipid transport and synthesis, insulin sensitivity and the secretion of adipocyte-specific proteins (Rosen and MacDougald 2006). During adipocyte differentiation, chronological changes in the expression of numerous genes by the appearance of early, intermediate, and late mRNA/protein markers lead to the acquisition of the adipocyte phenotype and triglyceride accumulation (Gregoire, Smas et al. 1998). Transcription factors such as peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein (C/EBP) have been identified as key players in the complex transcriptional cascade during adipocyte differentiation. Growth and differentiation of pre-adipocytes is highly controlled by communication between individual cells or between cells and the extracellular environment. Various hormones and growth factors affect adipocyte differentiation in a positive or negative manner by transferring external growth and differentiation signals to differentiating adipocytes (Gregoire, Smas et al. 1998, Rosen and MacDougald 2006). Insulin is the key hormone enhancing adipocyte differentiation. Adipocyte differentiation especially the molecular regulation of terminal differentiation has been extensively characterized by using murine cell lines such as the 3T3-L1, F442A, and ob1771 pre-

adipocytes that under appropriate hormonal control are capable of differentiating into mature white adipocytes. Having morphological similarity to fibroblastic preadipose cells, these pre-adipocyte cell lines exhibit the characteristics of adipocytes presenting within the adipose tissue after differentiation (Cowherd, Lyle et al. 1999).

Metabolically normal adipocytes are small in size, sensitive to insulin and secrete insulin sensitizing hormones such as adiponectin. Impaired differentiation of new adipocytes under metabolic disorders, on the contrary, leads to the enlargement of existing adipocytes (hypertrophy) and spillover of fat into other insulin sensitive tissues. Hypertrophic adipocytes are inflamed, insensitive to insulin and increasingly express harmful adipokines affecting other organs in the body and eventually development of insulin resistance locally and systematically (Kadowaki, Hara et al. 2003, Coppack, Mohamed-Ali et al. 2005, Harwood 2012). Therefore, enhanced differentiation of pre-adipocytes into mature adipocytes and restoration of insulin actions either by improving insulin sensitivity or by using compounds mimicking insulin functions is a key strategy for controlling MetS and its complications.

2.6.4. The adipogenic transcriptional cascade

2.6.4.1. The peroxisome proliferator-activated receptors

The peroxisome proliferator-activated receptors (PPARs), the members of the nuclear receptor superfamily of ligand-activated transcription factors, play a key role in normal adipogenic differentiation process for development of normal adipocytes. PPARs can be activated by dietary fatty acids and their metabolic derivatives in the body, and thus serve as lipid sensors (Evans, Barish et al. 2004). In mammals, there are three members of PPARs: PPARα, PPARδ (also known as PPARβ), and PPARγ. By binding to PPAR-responsive regulatory elements, the PPARs control the expression of networks of genes involved in adipogenesis, inflammation, and lipid

and glucose homeostasis through their coordinated activities in liver, muscle and adipose tissue (Ahmadian, Suh et al. 2013). The three PPAR isoforms have distinct patterns of tissue distribution and unique functions in the regulation of energy metabolism. PPARα predominantly presents in liver and has a crucial role in fatty acid oxidation. PPARδ is abundantly expressed throughout the body but at low levels in liver but is a powerful regulator of fatty acid catabolism and energy homeostasis (Evans, Barish et al. 2004) (Figure 2- 6).

PPARγ mainly present in adipose tissue regulating adipogenesis and normal adipocyte function, which will be further described in the following section.

2.6.4.2. PPARγ; the master regulator of adipogenesis

The terminal adipocyte differentiation involves a cascade of transcriptional changes that has been extensively studied using *in vitro* cell models. In the initial stage of adipogenesis, the transient dramatic induction of two members of CCAAT/enhancer binding protein (C/EBP) family of transcription factors including C/EBPβ and C/EBPδ occurs. They both directly induce expression of PPARγ and C/EBP-α. PPARγ and C/EBPα are considered as the key regulators of adipocyte differentiation. PPARγ and C/EBPα appear to act cooperatively in adipocyte differentiation by activating the expression of each other and regulating the expressions of other adipocyte-specific genes. Despite the importance of C/EBPs in adipogenesis, these transcription factors cannot function efficiently in the absence of PPARγ (Rosen and MacDougald 2006). PPARγ is most specific to adipogenic differentiation and is induced before transcriptional activation of most adipocyte genes. PPARγ has been shown to be both necessary and sufficient for fat formation (Farmer 2006). Overexpression of PPARγ can induce adipogenesis in mouse embryonic fibroblasts lacking C/EBPα, but C/EBPα cannot rescue adipogenesis when PPARγ is not expressed, showing that PPARγ is the master regulator of adipogenesis (Rosen and MacDougald

2006). PPARγ is also necessary for the mature adipocyte function. PPARγ exists as two protein isoforms: PPARγ1 and PPARγ2, with the latter containing an additional 30 amino acids at its N-terminus (Ahmadian, Suh et al. 2013). Both isoforms promote differentiation and both are highly expressed in adipose tissues. However, PPARγ1 is found in many different tissues while expression of PPARγ2 is predominantly localized to adipocytes (Rosen and MacDougald 2006). The outcome of enhanced differentiation in pre-adipocytes is the generation of smaller adipocytes which are more insulin sensitive than larger cells and represent a new pool that can enhance the absorption of circulating FFAs and triglycerides, thereby limiting their ectopic deposition into peripheral tissues, lipotoxicity and insulin resistance (Harwood 2012).

2.6.4.3. Other functions of PPARγ

In addition to the primary role in adipocyte differentiation, PPARγ also upregulates genes involved in glucose uptake in adipose tissue and controls the expression of adipokines such as adiponectin, leptin, TNF-α and resistin that communicate with other organs to affect whole-body insulin sensitivity (Evans, Barish et al. 2004). Therefore, distinct signaling pathways and target tissues that physiologically complement each other may be involved in achieving the insulin-sensitizing effects of PPARγ (Ahmadian, Suh et al. 2013). PPARγ is also expressed at lower levels in several other tissues and has been shown to exert tissue-specific functions. The relative contributions of liver and muscle PPARγ activity to insulin sensitization has been suggested in the literature (Gavrilova, Haluzik et al. 2003, Hevener, He et al. 2003). PPARγ has been shown to induce the expression of key genes involved in glucose-stimulatd insulin secretion in pancreatic beta cells (Kim, Cha et al. 2002). The various effects of PPARγ on insulin sensitivity and glucose homeostasis in different tissues obviously illustrate the promise for multiple and potentially safe routes for therapeutic interventions (Ahmadian, Suh et al. 2013). Indeed,

thiazolidinediones (TZDs), a class of antidiabetic drugs for improving metabolic control in patients with type 2 diabetes, are activators of PPARγ. The following section further describes the positive effects of TZDs on different tissues as well as their side effects.

2.6.5. Thiazolidinediones; the insulin-sensitizing drugs

TZDs, the highly effective oral medications for type 2 diabetes, have been introduced in the treatment of type 2 diabetes since the late 1990s. TZDs are potent activators of PPARγ (Evans, Barish et al. 2004). Since PPARγ is mainly present in adipocytes, the systemic insulin sensitization effects of TZDs are believed to occur through affecting adipose tissue. One of the proposed mechanisms of TZDs effects is the enhanced lipid storage ability of adipocytes since TZDs enhance adipocyte differentiation and generate smaller adipocytes. This increases the triglyceride content of adipose tissue while reduces the free fatty acids and triglycerides in the circulation, liver, and muscle, reduces lipotoxicity in muscle and liver which eventually improves whole-body insulin sensitivity. This model involves activation of genes encoding molecules that promote a combination of lipid storage and lipogenesis (de Souza, Eckhardt et al. 2001, Evans, Barish et al. 2004, Ahmadian, Suh et al. 2013). Pharmacologic activation of PPARγ in adipose tissue also inhibits the expression of TNF-α and resistin, which both promote insulin resistance, while stimulates the production of adiponectin, which promotes fatty acid oxidation and insulin sensitivity in muscle and liver. As a result, hepatic glucose production is reduced and muscle glucose use is increased (Evans, Barish et al. 2004). However, there are several reported side effects associated with the use of TZDs including weight gain, fluid retention and bone loss and congestive heart failure. It has been postulated that enhanced adipogenic capacity in WAT is responsible for TZD associated weight gain. However, selective activation of PPARγ in adipocytes of mice is sufficient to cause whole-body insulin sensitization without an increase in

weight (Sugii, Olson et al. 2009). Troglitazone was the first TZD drug withdrawn from the market due to liver toxicity whereas, both rosiglitazone and pioglitazone gained widespread use for the treatment of type 2 diabetes. In 2010, however, due to increased risk of cardiovascular events associated with the use of rosiglitazone, the European Medicines Agency recommended suspension of this drug and the Food and Drug Administration severely restricted its use. Thus, pioglitazone is the only TZD still in use (Consoli and Formoso 2013).

2.7. Adipose tissue inflammation

As mentioned earlier, inflammation is considered as one of the potential mechanisms for pathogenesis of obesity-induced IR (de Luca and Olefsky 2008). Adipose tissue is one of the key sites of inflammatory response. A combination of cell types in the adipose tissue releases pro-inflammatory adipokines which induce IR locally and systematically (Kassi, Pervanidou et al. 2011). Obesity can lead to changes in the cellular composition of the adipose tissue, alteration of the individual cell phenotypes (Ouchi, Parker et al. 2011), and occurrence of a low-grade, chronic inflammatory response characterized by altered adipokines production and increased markers of inflammation (Neels and Olefsky 2006). Chronic production of pro-inflammatory adipokines potentially targets non-adipose tissues during obesity (Saltiel and Kahn 2001). This is due to the diffuse nature and close association of adipose tissue with other metabolically active tissues. Secretion of pro-inflammatory adipokines especially TNF-α by hypertrophic and dysfunctional adipocytes suppresses adiponectin production (Degawa-Yamauchi, Moss et al. 2005) on one hand and initiates pro-inflammatory signaling cascades on the other hand. Vascular endothelial cells amplify the pro-inflammatory state by expressing cell adhesion molecules that can bind to leukocytes. Finally, recruitment of macrophages to the inflamed adipose tissue further augments the inflammatory signal (Shoelson, Lee et al. 2006, Harwood 2012). Indeed,

adipose tissue of obese animals and human subjects is infiltrated by a large number of activated macrophages with a pro-inflammatory state (Ruan and Lodish 2003, Weisberg, McCann et al. 2003, Galic, Oakhill et al. 2010, Kassi, Pervanidou et al. 2011). Visceral adipose tissue is believed to have a more important role in the development of IR than the subcutaneous adipose tissue. Interestingly, macrophages are more abundant in visceral than the subcutaneous adipose tissue (Ouchi, Parker et al. 2011). TNF-α, interleukins (IL-6) and C-reactive protein (CRP) are considered as predictors of cardiovascular events and diabetes with the occurrence of the MetS (Pradhan, Manson et al. 2001, Winkler, Kiss et al. 2003). Suppression of insulin receptor, insulin receptor substrate-1 (IRS-1) and GLUT4 expression, inhibition of tyrosine phosphorylation of insulin receptors (Ruan and Lodish 2003), prevention of the induction of PPARγ and C/ EBPα expression and adipocyte differentiation (Cawthorn, Heyd et al. 2007) are some of the detrimental effects of TNF-α in the body.

In summary, downregulation of anti-inflammatory molecules and upregulation of pro-inflammatory factors activate endothelial cells, disturb insulin signaling, and promote a dysfunctional phenotype occur in obesity (Galic, Oakhill et al. 2010).

Normal insulin signaling occurring through a complex network of different signaling pathways crucial for maintaining glucose homeostasis and insulin sensitivity in the body has been described in the following section.

2.8. Insulin signaling cascade

The effects of insulin on cell metabolism are mediated by insulin receptor expressed on most cells especially on major metabolic organs such as liver, adipose, and skeletal muscle. The insulin receptor is a heterotetramer composed of two α-subunits localized on the outside of the cell and are attached by disulfide bonds to a transmembrane β-subunit dimer (Saltiel and Kahn

2001). Binding of insulin to insulin receptor α-subunits triggers the autophosphorylation and activation of the protein tyrosine kinase within the receptor β-subunits that lead to the recruitment and tyrosine phosphorylation of receptor substrates such as insulin receptor substrate (IRS) proteins generating binding sites for Src homology 2 (SH2) domain proteins, including phosphatidylinositol 3-kinase (PI3K), the Ras guanine nucleotide exchange factor complex known as growth factor receptor-bound protein 2/son of sevenless (GRB2/SOS), and SH2 domain containing protein tyrosine phosphatase-2 (SHP2) (Copps and White 2012). Indeed, the IRS proteins phosphorylation provides the basis for the subsequent association with downstream signaling through three different pathways mediating metabolic and mitogenic responses of insulin and glucose transporter-4 (GLUT4) translocation (Saltiel and Kahn 2001, Mlinar, Marc et al. 2007). The two major signaling cascades which mediate most metabolic and transcriptional effects of insulin in adipocytes are the PI3K and the mitogen activated protein kinase (MAPK) pathway (Laviola, Perrini et al. 2006). The PI3K pathway mediates the metabolic effects of insulin including glucose/lipid/protein metabolism and insulin-stimulated glucose uptake. Recruitment of dimeric PI3K (p85/p110) to IRS produces membrane phosphatidylinositol 3,4,5-trisphosphate (PIP3) through phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2). PIP3 regulates the activation of phosphoinositide dependent kinase 1 (PDK1) which in turn phosphorylates and activates protein kinase B (PKB/Akt) at threonine and serine residues (Copps and White 2012). Akt activation mediates the metabolic effects of insulin on glucose transport through GLUT4 translocation to the plasma membrane, glycogen and protein synthesis, lipogenesis and suppression of hepatic glucose production through different downstream targets (Meshkani and Adeli 2009). A second essential branch of the insulin signaling pathway activated independently of PI3K/Akt, is the MAPK pathway. This pathway controls the mitogenic, growth

and cell differentiation effects. Shc plays a critical role in mediating the mitogenic effects of insulin, primarily through activation of the GRB2/SOS /Ras/MAPK pathway, however, compared with PI3K, is less defined. Phosphorylation and activation of the extracellular regulated kinase 1 and 2 (ERK1/2) plays a direct role in cell proliferation and differentiation via gene transcription regulation (Laviola, Perrini et al. 2006, Boucher, Kleinridders et al. 2014). Insulin signaling cascade in adipocytes is shown in Figure 2- 7.

2.9. Renin-angiotensin system (RAS), oxidative stress and MetS

IR, hyperinsulinemia, and blood pressure have been correlated in both obese and lean hypertensive subjects (Ferrannini, Buzzigoli et al. 1987). High baseline and continuously increasing fasting insulin levels has been reported to be independent determinants for the future development of hypertension in a 4-year follow-up study in healthy adults (Park, Rhee et al. 2013). RAS is well known for its significant role in regulation of blood pressure in the body. RAS also plays a key role for the onset and development of insulin resistance (Marcus, Shefer et al. 2013). RAS components expressed in various tissues in the body are referred to as the local RAS. Up-regulation of RAS component genes including angiotensinogen, ACE, and AT1R has been reported in adipose tissues of both normal weight and obese subjects (Giacchetti, Faloia et al. 2002). Over expression of local RAS influences multiple pathophysiological pathways leading to the development of insulin resistance (Paul, Poyan Mehr et al. 2006, Olivares-Reyes, Arellano-Plancarte et al. 2009). Elevated level of Ang II decreases insulin sensitivity due to the formation of ROS (Cooper, Whaley-Connell et al. 2007). ROS production activates signaling kinases which lead to inactivation of IRS-1 (Olivares-Reyes, Arellano-Plancarte et al. 2009). Ang II also retards adipocyte differentiation; thus, dysfunctional adipocytes with more lypolytic activity and pro-inflammatory state would further propagate insulin resistance. Locally produced

Ang II can also reduce blood supply to adipose depots, reducing the clearance of released free fatty acids and enhancing local inflammation (Marcus, Shefer et al. 2013). On the other hand, insulin resistance also up-regulates RAS components (Shinozaki, Ayajiki et al. 2004, Samuelsson, Bollano et al. 2006); thus a vicious cycle forms that could explain renal and cardiovascular dysfunctions observed in diabetic individuals (Figure 2- 8).

Antihypertensive drugs, such as ACE inhibitors (e.g. ramipril, lisinopril, captopril) and AT1 receptor blockers (e.g. valsartan, losartan) have long been used to prevent cardiovascular and renal damage in diabetic patients (Kaplan 2001, Scheen 2004). In the Captopril Primary Prevention Project (CAPPP) trial, the incidence of diabetes was significantly lower ($p=0.039$) in captopril group compared to the control group (Hansson, Lindholm et al. 1999), indicating the beneficial effects of ACE-inhibitors in prevention of diabetes. ACE inhibitors also improved IR by decreasing the formation of Ang II (Henriksen 2007).

These drugs are associated with side effects, which lead to a shift toward the development of naturally-derived alternatives to synthetic drugs in recent decades. Food proteins containing bioactive peptides with a wide variety of biological activities including antioxidant, antihypertensive, antilipidemic, and antimicrobial properties among others have a great potential to be used as alternatives to synthetic drugs (Erdmann, Cheung et al. 2008, Udenigwe and Aluko 2012, Cavazos and de Mejia 2013, Chakrabarti, Jahandideh et al. 2014).

The following sections briefly describe the preparation of bioactive peptides, their identification and characterization methods, and the mechanisms by which these peptides influence glucose tolerance and insulin sensitivity *in vitro* and *in vivo*.

2.10. Food protein derived bioactive peptides

Bioactive peptides are specific protein fragments that are encrypted in proteins. These peptides can only show their biological effects once they are released from their parent proteins. The diverse physiological effects of bioactive peptides from plant or animal sources have been examined extensively in literature. There are numerous reports on the identification and characterization of bioactive peptides with antioxidant, anti-inflammatory, anti-hypertensive, anti-obesity, antimicrobial and immunomodulatory properties. The length of bioactive peptides varies; from 2 (dipeptides) to about twenty amino acid residues (Korhonen and Pihlanto 2006). In some cases, much longer peptides with biological activity has also been reported. Lunasin is the peptide derived from soy protein with 43 amino acid residues with anti-cancer and hypocholesterolemic effects (Dia and de Mejia 2013, Liu, Jia et al. 2014). Bioactive peptides may influence the major body systems such as cardiovascular, digestive, nervous, and immune systems (Singh, Vij et al. 2014). The activity is based on their inherent amino acid composition and sequence. Bioactive peptides may have a specific physiological effect or act upon different systems in the body exerting multi-functional physiological properties.

2.11. Preparation of bioactive peptides

Generally, bioactive peptides can be produced by enzymatic hydrolysis using proteolytic enzymes of the digestive system or microbial origin, microbial fermentation and food processing.

2.11.1. Enzymatic hydrolysis

The most common way to produce bioactive peptides is through enzymatic hydrolysis of proteins *in vitro* or *in vivo*. Enzymatic hydrolysis is an easy to control process which is usually conducted under mild conditions resulting in obtaining products with well-defined features

(Zambrowicz, Timmer et al. 2013). Moreover, lack of residual organic solvents and toxic chemicals in the final peptide preparations have turned enzymatic hydrolysis the most preferred process in food and pharmaceutical industries for the production of bioactive peptides. The proteolysis process changes the molecular conformation of native proteins and produces functional and bioactive peptides (Adebiyi, Adebiyi et al. 2008). Gastrointestinal enzymes such as pepsin, trypsin or pancreatin, and other proteolytic enzymes with plant, fungal or microbial origins such as bromelain, papain and ficain have been used widely in the generation of bioactive peptides (Korhonen and Pihlanto 2006). Sequential hydrolysis of proteins using combination of proteolytic enzymes is more effective for the production of smaller fragments with biological activities (Singh, Vij et al. 2014).

2.11.2. Fermentation

Fermentation as an ancient method of food preservation is an efficient way to produce bioactive peptides and food grade hydrolyzed proteins. Fermented products are generally produced by both natural and controlled fermentation involving mainly lactic acid bacteria (LAB). LAB are important in the production of fermented products because of their physiological significance as well as their technological importance in texture and flavor development (Savijoki, Ingmer et al. 2006). In fermented meat products, the pH reduction as the fermentation progresses, not only inhibits the growth of many pathogenic microorganisms but also indirectly influences the protein degradation by increasing muscle proteases' activities. Polypeptides formed by meat endogenous proteases may be further degraded by bacterial enzymes during fermentation, which provide possibilities of generating bioactive peptides *in situ* in meat products (Wu, Jahandideh et al. 2015). In fermented soy milk, proteins are degraded into simpler form like oligopeptides, di-

peptides and tri-peptides and serve as a good source of bioactive peptides (Singh, Vij et al. 2014).

2.11.3. Other novel processing methods

Alternative to common methods like enzymatic hydrolysis and fermentation for the production of bioactive peptides, novel processing methods have been studied in recent decades for this purpose. Subcritical water hydrolysis is one of these alternatives in which water maintaining its liquid state at subcritical state (between the boiling point; 100 ˚C and 0.1 MPa and the critical point; 374 ˚C and 22 MPa) is used as the reaction medium without the need for enzymes or other reagents. Due to the high dissociation constant at the subcritical state, water can act as an acid, base, or bi-catalyst (Katritzky, Allin et al. 1996). This method has been employed for recovering a wide range of high added-value molecules including recovering protein hydrolysates in form of peptides or amino acids from both animal and vegetable wastes. These recovered biomolecules have a capital importance in biotechnology research, nutraceuticals, and especially in food industry, where such products can be utilized for different applications (Shitu, Izhar et al. 2015, Marcet, Alvarez et al. 2016). The subcritical water has been employed for the hydrolysis of a number of food proteins including fish (Choi, Jang et al. 2017), whey (Espinoza and Morawicki 2012, Espinoza, Morawicki et al. 2012), and egg yolk (Marcet, Alvarez et al. 2014). Since in subcritical water hydrolysis method, water is the reaction medium the produced peptides can be utilized without prior purification and/or chemical removal. Porcine placenta-derived collagen peptides produced by this method exerted *in vitro* anti-wrinkle effects as measured by elastase inhibition (Park, Kim et al. 2015). Although these peptides could penetrate rat skin, their elastase inhibitory activity was much lower than the reported values for the peptides prepared by enzymatic method (Han, Kim et al. 2013). The incorporation of subcritical water hydrolysis to a

whey protein isolate has been reported recently. The highest obtained degree of hydrolysis was 12% when the whey protein isolate was hydrolyzed with subcritical water for 17 min at 298 °C and the highest production of total amino acids was 57.4 mg/g of whey protein isolate at 300 °C for 40 min (Espinoza, Morawicki et al. 2012). Although addition of modifiers such as sodium bicarbonate has been reported to increase the degree of hydrolysis up to 4 times, there may be potential restrictions for its industrial application (Espinoza and Morawicki 2012). Delipidated egg yolk granular protein was hydrolyzed using both trypsin and subcritical water extraction. 50% of the initial protein content was recovered in the form of soluble peptides after 360 min hydrolysis with trypsin whereas, the yield increased to 95% when subcritical water extraction was employed at significant shorter reaction time. The low molecular weight peptides (<1 KDa) in trypsin hydrolysates and subcritical water hydrolysates were 14% and 63% respectively (Marcet, Alvarez et al. 2014). The advantages of subcritical water extraction are cost-effective, shorter reaction time, lack of solvent and enzyme use, and its eco-friendly nature. However, parameters which affect the extraction efficiency of this method including reaction temperature, pressure, and time, solid to water ratio, samples particle size, pH, solute characteristics, addition of a surfactant and flow rate which needs to be optimized (Shitu, Izhar et al. 2015). The biggest issue of the subcritical water hydrolysis is its non-specificity in cleaving peptide bonds in proteins. This method also results in very limited cleavage of disulfide bonds. Therefore, reduction and alkylation of proteins either prior to or post subcritical water treatment may be required to improve reported protein sequence coverage (Powell, Bowra et al. 2016). Another potential technique is high hydrostatic pressure which is employed as a pre-treatment method to enhance enzymatic digestibility of proteins to produce peptides. High hydrostatic pressure causes substantial modifications to proteins, enhances their digestibility, and ultimately influences their

functional properties (Chawla, Patil et al. 2011). Pre-treatment of whey protein isolate with high pressure conditions before an *in vitro* gastro-intestinal digestion have yielded protein hydrolysates with enhanced antioxidant and anti-inflammatory effects in intestinal epithelial cells exposed to hydrogen peroxide (Piccolomini, Iskandar et al. 2012).

2.12. Isolation, purification and characterization of bioactive peptides

Many research efforts have attempted to develop techniques and methods for the separation, purification, and characterization of proteins and bioactive peptides in recent decades. The crude protein hydrolysate can be fractionated into different groups with similar physical and chemical properties by using different analytical methods (Issaq, Conrads et al. 2002). Ultrafiltration membranes can be used in the initial steps to separate the fraction with desired molecular weight from the crude protein hydrolysate (Singh, Vij et al. 2014). Chromatographic methods have been commonly employed for the purification of peptide-rich fractions derived from protein hydrolysates. The principle of chromatographic fractionation is based on the interaction of the proteins or peptides with the stationary and mobile phases. Size-exclusion chromatography can concentrate peptides of specified molecular weight ranges whereas, ion-exchange and reverse-phase HPLC (RP-HPLC) can be used to fractionate peptides based on their charge and hydrophobicity respectively (Issaq, Conrads et al. 2002). High-performance liquid chromatography (HPLC) is the most widely used technique for analytic and preparative separations, commonly coupled with conventional UV and fluorescence detectors (Contreras, Lopez-Exposito et al. 2008). Normally, several steps of fractionation are required before the peptide sequence can be determined. In the food industry, the use of enzymatic membrane reactors allows protein hydrolysis and the subsequent separation of peptides generated by microfiltration or chromatography techniques (Welderufael, Gibson et al. 2012). Since the

introduction of soft ionization techniques, mass spectrometry has emerged as an important tool for protein identification and characterization. Mass spectrometry with high selectivity and sensitivity for peptide identification, has allowed accurate determination of molecular mass and peptide sequences, as well as detecting post-translational modifications (such as phosphorylation and glycosylation) or chemical modifications of primary sequence, identifying protein degradation products, and understanding protein conformations (Contreras, Lopez-Exposito et al. 2008, Singh, Vij et al. 2014).

2.13. Bioactive peptides with physiological effects on glucose homeostasis

Food proteins have been reported extensively as the potential precursors for the production of bioactive peptides with myriad biological activities including reducing blood glucose and hyperglycemia. While many protein hydrolysates and peptides have been the subject of extensive research in this area, marine bioactive peptides have already been commercialized and available in the market. Nutripeptin™ a product containing cod hydrolysate and Fortidium Liquamen® a white fish (*Molva molva*) autolysate are the commercialized food supplements with postprandial blood glucose-lowering effects (Guerard, Decourcelle et al. 2010). Bioactive peptides can exert their effects on glucose homeostasis through affecting different targets in the body including gut hormone release, satiety effect, insulin secretion, insulin sensitivity, glucose uptake, fat tissue modification, and intestinal glucose absorption as discussed in next sections.

2.13.1. Peptides targeting gut hormones

The gastrointestinal tract, the body's largest endocrine organ, plays a significant role in appetite control and energy regulation by secreting different regulatory peptide hormones acting on a variety of tissues to influence physiological processes. Most of these hormones are sensitive to

gut nutrient content. Changes in circulating gut hormone levels affect short-term feelings of hunger and satiety. Gut hormones specifically target the brain to regulate appetite (Murphy and Bloom 2006). Because of their significant role in appetite control and energy regulation, gut hormones have been considered as new therapeutic approaches for obesity or Type 2 Diabetes treatment (Irwin and Flatt 2013). The major peripheral peptides known to control satiety include anorexigenic (appetite suppressing) hormones such as cholecystokinin (CCK), Glucagon-Like Peptide 1 (GLP-1), and peptide YY (PYY) which play a significant role in the regulation of energy homeostasis (Caron, Cudennec et al. 2016). CCK is mainly secreted by I-cells located in the duodenum and jejunum following the ingestion of fat or protein. CCK inhibits food intake and gastric emptying, while stimulates pancreatic secretion and gall bladder contraction (Austin and Marks 2009). GLP-1, and glucose-dependent insulinotropic polypeptide (GIP) are the incretin hormones secreted from the enteroendocrine cells (K and L cells respectively) after meal ingestion (Pais, Gribble et al. 2016). GLP-1 and GIP involve in the augmentation of postprandial insulin secretion (Drucker and Nauck 2006). Given this important physiological function, there has been major interest in the potential for incretin-based pharmaceuticals as antidiabetic drugs. Indeed, GLP-1 agonists have been suggested as effective treatments for hyperglycemia and type 2 diabetes (Edwards 2013). However, both GIP and GLP-1 are rapidly degraded after release into the circulation (half-lives <7 and 2 min, respectively) due to the action of the ubiquitous enzyme dipeptidyl peptidase IV (DPP-IV) (Irwin and Flatt 2013). This issue however has been overcome through developing long-acting enzymatic resistant incretin analogues (Murphy and Bloom 2006) or inhibition of DPP-IV which results in an increased and prolonged insulin response. DPP-IV inhibitors are a class of oral antihyperglycemic agents to treat patients with type 2 diabetes (Green, Flatt et al. 2006). The five available DPP-IV inhibitors in the market

include sitagliptin, vildagliptin, saxagliptin, linagliptin, and alogliptin, which are small molecules used orally with similar overall clinical efficacy and safety profiles in patients with type 2 diabetes (Capuano, Sportiello et al. 2013).

Recently, many studies revealed the potential of food proteins as the precursor for DPP-IV inhibitory peptides. Hydrolysate and peptides from different proteins including fish (Huang, Jao et al. 2012, Li-Chan, Hunag et al. 2012), amaranth (Velarde-Salcedo, Barrera-Pacheco et al. 2013), rice bran (Hatanaka, Inoue et al. 2012), and milk (Lacroix and Li-Chan 2013) exerted DPP-IV inhibitory activity *in vitro*. DPP-IV inhibitory peptides identified from different protein sources have been reviewed recently (Patil, Mandal et al. 2015). Peptides GPHypGPAG derived from porcine skin gelatin, GPAE, and GPGA derived from salmon skin are some examples of the potent DPP-IV inhibitory peptides with IC_{50} values less than 50 μM (Patil, Mandal et al. 2015). However, *in vivo* experiments are required to assess the activity and efficacy of the peptides/protein hydrolysates with *in vitro* DPP-IV inhibitory activity on blood glucose levels. LPQNIPPL is a Gouda cheese-derived octapeptide with a high DPP-IV inhibitory activity. Administration of this peptide to female Sprague Dawley (SD) rats improved blood glucose response after an oral glucose tolerance test compared to the control. However, plasma concentration of incretin hormones was not assessed in this study (Uenishi, Kabuki et al. 2012). The ileal administration of a zein hydrolysate attenuated the glucose induced hyperglycemia in SD rats. The beneficial effects of the zein hydrolysate on blood glucose was accompanied by the enhancement of insulin secretion and active GLP-1 concentration, as well as reduced plasma DPP-IV activity in rats (Mochida, Hira et al. 2010). Lipid-free egg yolk protein hydrolyzed with pepsin produced peptides with α-glucosidase and DPP-IV inhibitory properties. YIEAVNKVSPRAGQF, YINQMPQKSRE, YINQMPQKSREA, and VTGRFAGHPAAQ

exhibited antidiabetic effects *in vitro* in addition to their ACE inhibitory, and antioxidant properties (Zambrowicz, Pokora et al. 2015). Antarctic krill protein hydrolysate contains KVEPLP and PAL peptides with both ACE and DPP-IV inhibitory properties. These peptides may have potential benefits in the management of hypertension and hyperglycemia as the two hallmarks of MetS (Ji, Zhang et al. 2017).

2.13.2. Insulinotropic peptides

Proteins/peptides stimulating insulin production may reduce plasma glucose levels. Insulin secretion is sensitive to composition as well as the concentration of plasma amino acids (Schmid, Schultefrohlinde et al. 1992). Food proteins are believed to enhance insulin secretion (Floyd, Fajans et al. 1966, Lang, Bellisle et al. 1999, Calbet and MacLean 2002). Ingestion of milk proteins especially whey has been shown to enhance insulin secretion. The essential amino acids leucine, isoleucine, valine, lysine, and threonine showed the strongest correlation with insulin response (Nilsson, Stenberg et al. 2004). However, when a mixture of these amino acids was tested in healthy subjects in a follow up study, despite a similar glycemic response to whey ingestion, no effects on insulin and incretin secretion was observed (Nilsson, Holst et al. 2007). The enzymatic hydrolysis of whey proteins (WPH) exhibited a significant increase in glucose induced insulin secretion in a pancreatic β cell line in a dose dependent manner (Gaudel, Nongonierma et al. 2013). Further, oral administration of WPH to ob/ob mice for 8 weeks also improved blood glucose clearance, reduced hyperinsulinemia, and restored the pancreatic islet capacity to secrete insulin in response to glucose (Gaudel, Nongonierma et al. 2013). Fermented soybeans have also been reported to affect glucose homeostasis (Kwon, Jang et al. 2007, Yang, Kwon et al. 2012). Meju, the Korean fermented soy product used in the preparation of other fermented soybean-based foods has anti-diabetic properties. Kwon and co-workers (2011) found

that both isoflavonoid and peptide fractions of 60 day fermented meju potentially affected adipocyte differentiation and insulin secretion in 3T3-L1 and Min6 insulinoma cells, respectively (Kwon, Hong et al. 2011). Water extracts of 60-day fermented meju, mostly containing peptides of 15 KDa molecular weight, promoted insulin-stimulated glucose uptake, adipocyte differentiation, and PPARγ activity in 3T3-L1 adipocytes. Furthermore, this fraction enhanced glucose–stimulated insulin secretion and moderately enhanced β- cell proliferation in Min6 insulinoma cells. The specific sequence of peptides, however, was not identified in this fraction (Kwon, Hong et al. 2011).

2.13.3. Incretins mimetic peptides

Strategies based on the enhancement of endogenous GLP-1 secretion are promising for the prevention of hyperglycemia. Previous reports have documented that amino acids, particularly glutamine, stimulated GLP-1 secretion and increased plasma GLP-1 level in humans (Greenfield, Farooqi et al. 2009, Samocha-Bonet, Wong et al. 2011). Low molecular weight fraction of wheat protein (gluten) hydrolysate, rich in glutamine residues, has been reported recently to increase GLP-1 secretion followed by enhanced insulin secretion, and improve glucose tolerance in SD rats (Kato, Nakanishi et al. 2017).

Peptones are commercially available protein hydrolysates with a mixture of different length peptides as well as free amino acids (Pais, Gribble et al. 2016). Meat peptones have been reported to stimulate GLP-1 secretion in NCI-H716 cell line (a human intestinal cell model) (Reimer 2006) and murine primary colonic cultures (Diakogiannaki, Pais et al. 2013). Although the active components of meat peptone on GLP-1 secretion were not identified, Reimer showed that the commercial mixture of essential amino acids (Sigma) increased GLP-1 secretion in NCI-H716 cells (Reimer 2006). Oligopeptides including Leu-Gly-Gly and Gly-Phe along with the

non-metabolizable peptide transporter-1 (PEPT1) substrate Gly-Sar have been reported to enhance GLP-1 release in murine primary L cells. Further, mechanistic experiments revealed that PEPT1 and activation of Ca^{2+} channels are involved in the Gly-Sar-stimulated GLP-1 secretion (Diakogiannaki, Pais et al. 2013). Recently, meat, casein and vegetable-derived peptones, the L-amino acids Phe, Trp, Gln and Ala, and the dipeptide glycine-sarcosine have proven to stimulate GLP-1 secretion in murine small intestinal L-cells. The elevation of intracellular calcium in L-cells was also responsible for the enhanced GLP-1 secretion in the presence of meat peptone (Pais, Gribble et al. 2016). The ileal administration of zein hydrolysate (prepared by papain) enhanced GLP-1 secretion while attenuated the glucose induced hyperglycemia by enhancing insulin secretion in SD rats (Mochida, Hira et al. 2010). In a follow up study, the anti-hyperglycemic effect of this treatment was shown in normal and diabetic rats. The oral administration of the zein hydrolysate attenuated hyperglycemia by stimulating GLP-1 and GIP secretion in normal rats. The involvement of increased GLP-1/GIP secretion was determined using GLP-1/GIP receptor antagonists. This treatment also effectively reduced the glycemic response under OGTT in a type 2 diabetic rat model (ZG) which was accompanied by increased GLP-1 and insulin secretion (Higuchi, Hira et al. 2013). Different peptides derived from porcine skin gelatin hydrolysate showed stimulating effects on GLP-1 and CCK secretion improving glycemic control in streptozotocin-induced diabetic rats. Three peptides; ANVST, TKAVEH and KAAVT enhanced GLP-1 secretion and the sequence VAAA showed DPP-IV inhibitory properties. Both peptide groups (GLP-1 secretory and DPP-IV inhibitory peptides) had aromatic amino acid residues and were strongly involved in CCK release (Huang, Hung et al. 2014). Corn protein has also shown potential benefits on incretin secretion and improving glucose tolerance in rodents (Hira, Mochida et al. 2009, Mochida, Hira et al. 2010, Higuchi, Hira et al. 2013).

2.13.4. Satietogenic peptides

Dietary proteins are believed to induce satiety feeling and thermeogenic effects. The normal protein intake should account for ~10–15% of energy intake when in energy balance and weight stable (WHO 2000). Protein-induced satiety and significantly greater weight loss in comparison with control has been shown with high protein *ad libitum* diets, lasting from 1 to 6 days, up to 6 months (Veldhorst, Smeets et al. 2008). High protein diets are considered as meals with 20% to 30% of energy from protein when consumed in energy balance (Westerterp-Plantenga, Luscombe et al. 2006). Mechanisms explaining protein-induced satiety are not fully understood, but increases in concentrations of satiety hormones, energy expenditure, and amino acid concentration have been suggested as potential mechanisms (Veldhorst, Smeets et al. 2008).

Although high protein diets have shown satiety induced effects, the amino acid composition of ingested proteins may have a major role in this process (Mellinkoff, Frankland et al. 1956). This means that the high amounts of proteins per se may not be the key factor for the satietogenic effects while the release of bioactive fractions and specific free amino acids during protein digestion has a role in this phenomenon. The satiating effect of milk has been shown in a 6-month trial with an energy-restricted diet in which the group receiving milk supplementation showed a decreased desire to eat and hunger versus the placebo group (Gilbert, Joanisse et al. 2011). The satiating effect of whey protein has been attributed to the high concentration of branch chain amino acids, particularly l-leucine, existence of certain peptides or the release of satiety hormones (Hernández-Ledesma, García-Nebot et al. 2014). In a comparative study of casein and whey, as the major proteins of milk, on gastrointestinal hormone secretion and appetite, a greater subjective satiety after whey load was found. Moreover, a higher increase in postprandial plasma amino acid concentration, and higher levels of CCK, GLP-1 and GIP was

observed in the group received whey compared to casein (Hall, Millward et al. 2003). This clearly highlights the importance of the type of protein, and confirms that the appetite response depended on the postabsorptive increase in plasma amino acids and gut hormones. Whey protein but not the commercial whey protein hydrolysate consumed before a meal reduced food intake, and post-meal blood glucose and insulin response in a dose-dependent manner in healthy young adults (Akhavan, Luhovyy et al. 2010). The authors have attributed both insulinotropic and non-insulinotropic mechanisms for the observed effects of whey protein on blood glucose whereas, whey protein hydrolysate only showed insulinotropic effects without pronounced effects on post-meal blood glucose levels. Delayed gastric emptying has been suggested as the possible non-insulinotropic mechanism due to the fact that whey proteins but not the mixture of branched-chain amino acids have been reported to enhance CCK and GLP-1 release (Nilsson, Holst et al. 2007). This indicates that non-insulinotropic mechanisms arise from the digestion of intact proteins but not the protein hydrolysate. This product contains 40% free amino acids and short peptides of up to 10 amino acids, 27% of peptides of 10–200 amino acids, 16% of 50–200 amino acids, and 17% with more than 200 amino acids as reported by the manufacturer (Akhavan, Luhovyy et al. 2010). Indeed, neither short peptides and free amino acids nor larger protein fragments could induce gut hormone release to affect the post-meal glucose levels in healthy adults in this study.

Apart from this study, however, there are numerous reports on the effects of protein hydrolysates on gut hormone release and satietogenic effects. Pepsin hydrolysates of porcine meat enhanced CCK release from enteroendocrine cell line STC-1 and further induced satiety in SD rats in a dose dependent manner (Sufian, Hira et al. 2006). Marine-derived proteins and bioactive peptides also have strong stimulating effects on GLP-1 and CCK release exerting satiety effects.

Cudennec and co-workers initially demonstrated that blue whiting (*Micromesistius poutassou*) muscle hydrolyzed with a mixture of commercial enzymes enhanced CCK secretion in STC-1 cell line (Cudennec, Ravallec-Plé et al. 2008). This marine hydrolysate mainly consisted of short peptides in range of 1000 Da. In a follow up study by the same group, satiety inducing effects of this treatment was confirmed *in vivo*. The administration of blue whiting hydrolysate to SD rats reduced the short-term food intake correlated to an increase in the CCK and GLP-1 plasma levels. Chronic administration of this marine hydrolysate also decreased the body weight gain in these rats (Cudennec, Fouchereau-Peron et al. 2012). Administration of a protein hydrolysate from smooth hound (*Mustelus mustelus*) to wistar rats for 21 days reduced the body weight compared to the control group. Despite the reduction in body weight, no significant changes in plasma CCK levels after thirty minutes of the oral administration of this hydrolysate was observed (Bougatef, Ravallec et al. 2010). Legume proteins from different sources such as soy (Nishi, Hara et al. 2003, Nishi, Hara et al. 2003, Nakajima, Hira et al. 2010, Sufian, Hira et al. 2011), and some under-utilized beans such as Country bean and Yard long bean (Sufian, Hira et al. 2007) are other potential food compounds with beneficial effects on gut hormone release and appetite suppressive effects. Jang and co-workers reported the anti-obesity effects of an isoflavone-free peptide mixture derived from black soybean (*Rhynchosia volubilis*) in mice (Jang, Moon et al. 2008). This treatment reduced food intake, through activation of the leptin-like signaling in hypothalamus, and reduced body weight gain in mice fed a high fat diet for 13 weeks. Interestingly, identification of a hepta peptide IPPGVPY in the plasma 30 min after oral administration of 1 g black soybean peptide mixture suggests the potential role of absorbed peptides in the observed physiological effects *in vivo* (Jang, Moon et al. 2008). Arginine residue in protein structures has been reported to play a role in CCK release (Nishi, Hara et al. 2001).

Soybean β-conglycinin hydrolysate suppresses food intake and gastric emptying by direct action on rat small intestinal mucosal cells to stimulate CCK release. The fragment from 51 to 63 of the β-subunit with the sequence of VRIRLLQRFNKRS had the strongest binding activity. Intraduodenal infusion of this peptide to SD rats inhibited food intake and markedly increased portal CCK concentration. Different model peptides with arginine (R) and glycine (G) residues were constructed to further explore the structure requirements for the observed effects among which only GRGRGRG had strong binding affinity (Nishi, Hara et al. 2003).

CCK acts via two types of G-protein coupled receptors. The CCK receptor-type 1 (CCK-1R), widely expressed in different cell types of the gastrointestinal tract, is the receptor implicated in the regulation of food intake (Cudennec and Ravallec 2013). Activation of CCK-1R signaling by CCK analogs have been shown to beneficially affect calorie intake, glucose tolerance and insulin sensitivity in mice (Irwin, Frizelle et al. 2013). Developing CCK-1R activating peptides therefore, appear to be another promising approach to develop functional foods with satiety suppressing effects. The gastrin/CCK-like molecules were identified for the first time from marine byproducts such as shrimp waste (*Pandalus borealis*), cod (*Gadus morhua*) head, and head and viscera of sardine (*Sardina pilchardus*) obtained after hydrolysis or autolysis (Cancre, Ravallec et al. 1999). This kind of peptides was identified in a variety of other marine byproducts (Ravallec-Plé, Charlot et al. 2001, Slizyte, Mozuraityte et al. 2009) indicating the potential of these sources for the production of bioactive peptides with value added properties. The gastrin/CCK like peptides identified in cod muscle and shrimp (*Penaeus aztecus*) head hydrolysates were shown to bind to CCK receptors *in vitro* (Ravallec-Ple and Van Wormhoudt 2003). Soy, potato and casein hydrolysates have also been shown to directly stimulate CCK1 receptor (CCK1R) expressing cells and CCK release *in vitro* (Foltz, Ansems et al. 2008).

Identification of such peptides from food proteins can either enhance CCK-1R signaling through binding to receptor, or enhance CCK-1R expression, which opens up a new venue in the obesity/hyperglycemia treatment due to the potential therapeutic effects of CCK-1R activation on obesity, hyperglycemia and related metabolic disturbances (Irwin, Frizelle et al. 2012).

2.13.5. α -glucosidase inhibitory peptides

Carbohydrate digestion is necessary to release glucose for absorption in the blood stream and utilization by cells in the body. The enzymes α-amylase which breaks down long-chain carbohydrates, and intestinal brush border α-glucosidase required for the final step in carbohydrate breakdown to cleave absorbable monosaccharides are the two key enzymes in carbohydrate digestion (Bischoff 1994). The α-glucosidase inhibitors are a class of nonsystemic drugs that delay but do not prevent the absorption of ingested carbohydrates and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and eventually, leading to reduced postprandial blood glucose and insulin peaks (Ross, Gulve et al. 2004). Acarbose, miglitol, voglibose, and emiglitate are α -glucosidase inhibitors used for the treatment of postprandial hyperglycemia in diabetic individuals. Flatulence, abdominal cramping, vomiting, and diarrhea are the common side effects associated with the chronic use of these agents (Chaudhry, Naureen et al. 2017). Therefore, studies have been conducted to identify natural sources of α -glucosidase inhibitors. *In vitro* α -glucosidase inhibitory activity has been assessed and calculated as IC_{50} value, which is the concentration of compound required to cause a 50 % inhibition of the enzyme activity. Whey protein hydrolysates (Lacroix and Li-Chan 2013, Konrad, Anna et al. 2014), and brewers' spent grain protein hydrolysate (Connolly, Piggott et al. 2014) contain peptides with modest α–glucosidase inhibitory activity. VW and YYPL are the identified peptides with α–glucosidase inhibitory activity from the sardine muscle hydrolyzed

with alkaline protease (Matsui, Oki et al. 1999). Egg protein is the other potential source of α – glucosidase inhibitory peptides. The Alcalase hydrolysate of egg white protein has been reported to contain peptides RVPSLM and TPSPR with IC_{50} values of 23.07 and 40.02 μmol/L, respectively (Yu, Yin et al. 2011). KLPGF is the albumin derived peptide with IC_{50} values of 59.5 μmol/L and 120.0 μmol/L for α–glucosidase and α- amylase respectively (Yu, Yin et al. 2012). Although *in vitro* α –glucosidase inhibitory effect of food derived peptides has been reported in literature, it appears that α –glucosidase inhibitory activity depends on the α – glucosidase enzyme origin (Oki, Matsui et al. 1999, Konrad, Anna et al. 2014). Therefore, further *in vivo* studies are necessary for validating the effectiveness of these peptides in physiological conditions.

2.13.6. Peptides enhancing glucose uptake

Another mechanism by which bioactive peptides/single amino acids have been reported to affect plasma glucose is through enhancing muscle glucose uptake. Leucine enhances glucose uptake in soleus muscle of normal SD rats via phosphatidylinositol 3-kinase (PI3-kinase) and protein kinase C (PKC) dependent pathways (Nishitani, Matsumura et al. 2002). Isoleucine is another amino acid with reported beneficial effects on glucose uptake. Single oral administration of isoleucine to SD rats significantly reduced plasma glucose level at 30 and 60 min in an oral glucose tolerance test (OGTT). Plasma insulin levels at 30 and 60 min after glucose bolus were also lower than that of the control group which reveals that insulin secretion was not affected by isoleucine. The signaling pathway analysis in C2C12 myotubes suggested that similar to leucine, PI3K and PKC were involved in the enhancement of glucose uptake by isoleucine (Doi, Yamaoka et al. 2003). In addition to single amino acids, peptides have also been reported to stimulate glucose uptake *in vitro* and *in vivo*. Whey protein hydrolysate contains several

dipeptides including IV, LV, VL, II, LI, IL and LL, which increase glucose uptake in L6 myotubes. IL, the main dipeptide in whey protein hydrolysate, further enhanced glucose uptake and glycogen synthesis rates in isolated epitrochlearis muscles (Morifuji, Koga et al. 2009). Feeding Wistar rats with commercial whey protein (Hilmar product 8000) or whey protein hydrolysate (Hilmar product 8350) enhanced GLUT-4 translocation and glycogen synthesis in liver, heart and skeletal muscle of rats. Enhanced muscle Akt phosphorylation in rats fed with whey protein hydrolysate can be partially responsible for the observed enhanced GLUT-4 translocation to the plasma membrane (Morato, Lollo et al. 2013).

2.13.7. Peptides enhancing AMP-activated protein kinase (AMPK)

As mentioned before, activation of AMPK induces insulin-independent glucose uptake and insulin-sensitizing effects, making it an interesting therapeutic target for type 2 diabetes. AMPK is dysregulated in animals and humans with MetS or type 2 diabetes, whereas AMPK activation (physiological or pharmacological) can improve insulin sensitivity and metabolic health. AMPK is an energy-sensing serine/threonine kinase that is activated by two distinct signals: i) Ca^{2+}-dependent pathway and ii) AMP-dependent pathway (Sanders, Grondin et al. 2007). Therefore, conditions that can alter the intracellular AMP/ATP ratio and calcium concentration can affect AMPK. Numerous physiological (such as exercise, fasting, and calorie restriction), pharmacological (such as metformin and AICAR), natural (resveratrol and berberine), and hormonal (such as adiponectin, IL-6 and leptin) activators of AMPK have been reported (Coughlan, Valentine et al. 2014). Although the net effect of AMPK activation is to restore energy balance through inhibition of energy consuming processes and promoting energy-producing processes, it is also involved in glucose homeostasis process. Activation of AMPK stimulates glucose uptake in skeletal muscle, fatty acid oxidation in adipose tissue, and reduces

hepatic glucose production (Zhang, Zhou et al. 2009). Overall, AMPK activation improves insulin sensitivity and glucose homeostasis.

The fact that the capacity for AMPK-mediated glucose uptake is shown to be intact in muscle cells from patients with type 2 diabetes, while insulin-induced glucose uptake is impaired (Koistinen, Galuska et al. 2003) further highlights the importance of this pathway. Metformin, the potent antihyperglycemic drug from the biguanide family is the first line oral therapy for type 2 diabetes. The main effect of metformin is to acutely reduce hepatic glucose production, mostly through inhibiting mitochondrial respiratory-chain complex 1. The reduction in liver energy status further activates AMPK providing a generally accepted mechanism for metformin action on hepatic gluconeogenic program (Zhou, Myers et al. 2001, Viollet, Guigas et al. 2012).

Several natural compounds have been reported to increase AMPK activation *in vitro* and *in vivo*. Phytochemicals like berberine, the isoquinoline alkaloid found in certain plants, and resveratrol, a polyphenol found in red wine, are two major naturally derived components with established AMPK activation and enhanced insulin sensitivity (Coughlan, Valentine et al. 2014). Additionally, α-lipoic acid, the short chain fatty acid with similar effects on AMPK activation has been reported to improve insulin sensitivity in obese rodents (Zhang, Zhou et al. 2009). Protein hydrolysates and bioactive peptides are the new category of compounds with beneficial effects on AMPK activation. The low molecular weight (300–500 Da) fractions of soybean peptides separated by the electrodialysis with an ultrafiltration membrane have been reported to improve glucose uptake in L6 muscle cells in the presence of insulin. These charged peptides also activated AMPK in muscle cells, however, no increase in glucose uptake was observed in the absence of insulin in these cells (Roblet, Doyen et al. 2014). IAVPGEVA, IAVPTGVA and LPYP, the peptides derived from soy glycinin hydrolysate, have been reported to activate AMPK

pathway in hepatic cells. These peptides also increased glucose uptake in hepatic cells via activation of Akt (Lammi, Zanoni et al. 2015). Although this study has reported the potential of these peptides for enhanced glucose uptake via distinct Akt and AMPK pathways in hepatic cells, the use of HepG2 cells does not seem to be physiologically relevant for this purpose. Since the major effect of AMPK activation in the hepatic cells is inhibition of glucose production by liver (Coughlan, Valentine et al. 2014), the use of muscle cells or fat cells is a better option for this purpose. Treatment of L6 myotubes with dipeptide WH significantly increased phosphorylation and activation of AMPKα, Glut4 translocation to the plasma membrane, and glucose uptake into L6 myotubes. It has also been shown that activation of AMPKα occurs after transportation of WH into cells via the peptide transporter (Soga, Ohashi et al. 2014).

2.13.8. Peptides with beneficial effects on insulin sensitivity

Enhanced insulin sensitivity is another major mechanism by which bioactive peptides have been reported to specifically ameliorate IR in the context of MetS. In addition to enhanced effects on glucose uptake, administration of whey protein and leucine increased insulin sensitivity in a non-obese insulin resistant rat model. The mechanism of action of these treatments, however, has not been studied (Tong, Li et al. 2014). Fish protein is another source of biologically active proteins with beneficial effects on glucose uptake and IR. High-fat feeding has been reported to induce whole body and skeletal muscle insulin resistance in rats. Feeding cod protein to rats prevented the development of insulin resistance in these rats whereas casein and soy protein feeding did not show any effects. Higher rates of insulin-mediated muscle glucose disposal were observed in cod protein fed rats (Lavigne, Tremblay et al. 2001). The effect of proteins from other fish sources on insulin sensitivity has also been further investigated recently. Bonito, herring, mackerel, or salmon fish proteins were tested in rats fed a high fat-high sucrose diet. Rats fed salmon protein

gained less body weight as well as epididymal adipose tissue compared to casein fed group. Although none of the fish proteins were effective on improving glucose tolerance, IL-6 and TNF-α expression in visceral adipose tissue of rats reduced in all fish-protein–fed groups. Moreover, the whole-body insulin sensitivity was improved in salmon-protein fed group as measured by the hyperinsulinemic-euglycemic clamp technique (Pilon, Ruzzin et al. 2011). Since, insulin sensitivity was not assessed for the other fish protein groups, it is not known whether fish source is a crucial factor for the observed effects. Moreover, fish proteins were provided by different suppliers in this study with no information on protein purity, therefore, it is possible that the observed differences on body weight gain between different fish protein groups is due to the purity of protein diets. Black soybean peptides previously reported to activate AMPK *in vitro* (in myotubes) and *in vivo* (Jang, Moon et al. 2008). These peptides also restored insulin signaling in normal and insulin resistant HepG2 cells by stimulating Akt serine phosphorylation, forkhead transcription factor, Foxo1, and glycogen synthase kinase-3β (GSK-3β) (Jang, Ko et al. 2010). Oral administration of black soybean peptides to diabetic (db/db) mice showed antidiabetic effects partially through suppression of hepatic endoplasmic reticulum stress (Jang, Ko et al. 2010). The black soybean peptides mixture exerts its anti-diabetic effects through multiple mechanisms involving the suppression of hepatic endoplasmic reticulum stress and restoration of insulin signaling and inhibition of hepatic gluconeogenesis in various cell and animal models. The black soy peptide supplementation has been reported to have a modest effect on reducing fasting glucose and improving glucose tolerance in Korean adults with prediabetes (fasting glucose ≥ 110 mg/dL) in a double blind randomized placebo-controlled trial (Kwak, Lee et al. 2010). Daily administration of aglycin, a 37-amino-acid polypeptide isolated from soybean, at dose of 50 mg/kg/d for 4 weeks effectively controlled hyperglycemia and enhanced oral

glucose tolerance in streptozotocin/high-fat-diet-induced diabetic mice (Lu, Zeng et al. 2012). While insulin signaling was perturbed in skeletal muscle of this diabetic mice model, aglycin restored insulin signaling by maintaining insulin receptor and IRS1 expression at both mRNA and protein levels. This peptide further elevated the expression of p-IRS1, p-Akt and membrane GLUT4 protein thereby increasing glucose uptake in skeletal muscle (Lu, Zeng et al. 2012).

2.13.9. Peptides affecting adipocyte differentiation

As mentioned earlier, pre-adipocyte differentiation, a process to generate small healthy adipocytes with higher capacity for lipid storage is necessary for normal energy and glucose homeostasis. Regulation of PPARγ expression and activity is important with respect to nutrition, obesity and diabetes. Inhibition of PPARγ activity by inflammatory cytokines such as TNF-α may be involved in the pathogenesis of IR, atherosclerosis, inflammation, and cancer (Ye 2008). Insulin, on the other hand, has been shown to increase PPARγ expression (Rieusset, Andreelli et al. 1999). The beneficial effects of a soy protein peptic hydrolysate on adipocyte differentiation have been recently reported in 3T3-L1 cells (Goto, Mori et al. 2013). The soy protein hydrolysate stimulated adipocyte differentiation through the up-regulation of PPARγ expression. The expression and secretion of adiponectin and insulin sensitivity was also enhanced in 3T3-L1 cells treated with the soy protein hydrolysate (Goto, Mori et al. 2013). Interestingly, FLV, a soy-derived peptide, has been reported recently to prevent adipose inflammation and IR (Kwak, Kim et al. 2016). This peptide, transported into adipocytes through the peptide transporter PepT2, inhibited the release of inflammatory molecules (TNF-α, MCP-1, and IL-6) from both TNF-stimulated adipocytes and co-cultured adipocytes/macrophages. Furthermore, FLV enhanced insulin responsiveness and increased glucose uptake in adipocytes (Kwak, Kim et al. 2016). Milk-derived peptides, IPP and VPP also enhance adipocyte differentiation through upregulation

of PPARγ and C/EBP-α. The effect of these peptides on adipocyte differentiation and adiponectin release was similar to insulin. IPP and VPP further exerted anti-inflammatory effects by inhibiting the cytokine mediated activation of the pro-inflammatory NF-κB pathway in adipocytes (Chakrabarti and Wu 2015). Oral administration of VPP to C57BL/6J mice ameliorated diet-induced chronic inflammation in adipose tissue (Aihara, Osaka et al. 2014, Sawada, Sakamoto et al. 2015). High fat feeding resulted in the accumulation of activated monocytes and pro-inflammatory macrophages in the stromal vascular fractions of the adipose tissue, while VPP supplementation significantly reduced the pro-inflammatory status in adipose tissue (Aihara, Osaka et al. 2014). VPP administration has also been reported to improve insulin sensitivity, reduce TNF-α and IL-1β expression, and macrophage accumulation and activation in diet induced obese mice (Sawada, Sakamoto et al. 2015). Despite the well documented beneficial effects of enhanced PPARγ expression and adipocyte differentiation on adipocyte function and insulin sensitivity, inhibition of adipocyte differentiation has been deemed beneficial in some publications. DIVDKIEI, an octa-peptide derived from boiled tuna, has been reported to inhibit C/EBPs and PPARγ expression in 3T3-L1 adipocytes and is believed to exert anti-obesity properties (Kim, Kim et al. 2015). However, it should be noted that inhibition of adipocyte differentiation per se without affecting whole body energy balance is not beneficial for adipose tissue health and function. Inhibition of adipocyte differentiation could possibly result in the generation of hypertrophied adipocytes with less buffering capacity for circulating fats hence redistribution of body fat into non-adipose peripheral tissues in physiological conditions (Kim and Park 2011). This would eventually lead to development of IR in these tissues. Moreover, the hypertrophied adipocytes have the pro-inflammatory state due to the endocrine characteristics of the adipose tissue, which would result in the inactivation of insulin signaling and development of

systematic IR as discussed in detail before. Chlorella protein hydrolysate has shown favorable effects on glucose tolerance and insulin sensitivity in high fat fed mice (Noguchi, Yanagita et al. 2016). Smaller adipocytes, lower triglycerides levels in liver, and reduced serum MCP-1 as well as MCP-1 mRNA expression in adipose tissue was correlated well with enhanced glucose tolerance and insulin sensitivity in chlorella hydrolysate treated mice as compared to the control group. Considering the role of MCP-1 in development of inflammation and macrophage infiltration, less adipose tissue inflammation has been suggested as the key mechanism for the observed beneficial effects of chlorella-derived peptides in obese mice (Noguchi, Yanagita et al. 2016). In a similar vein, rice bran protein hydrolysate with beneficial effects on IR, promoted the gene expression of PPARγ in adipose tissue of high carbohydrate-high fat fed rats similar to pioglitazone. Serum adiponectin was enhanced while adipose tissue inflammatory markers were decreased in rats treated with rice protein hydrolysate (Boonloh, Kukongviriyapan et al. 2015).

2.14. Peptides against RAS and MetS (Antioxidant peptides; Anti-inflammatory peptides)

Most food-derived bioactive peptides appear to act through more than a single mechanism of action and, therefore, possess multi-functional properties. Since the pathophysiology of MetS and most of chronic diseases involve several organs and pathways in the body, identifying multi-functional bioactive peptides with diverse biological effects is of great importance. Given that detrimental effects of oxidative stress and inflammation on endothelium, adipose tissue, liver, muscle, pancreas and other vital organs in the body leads to the pathogenesis of several disorders including endothelial dysfunction, hypertension, insulin resistance, and pancreatic beta cell apoptosis, identifying peptides with potential antioxidant and anti-inflammatory effects may be of great interest for the prevention and/or treatment of MetS complications. Here, we briefly

discuss the potential benefits of peptides with anti-inflammatory and/or antioxidant activities in addition to RAS modulating effects in the context of MetS.

ACE inhibitory peptides with potential anti-hypertensive properties have been identified from both animal and plant sources (Guang and Phillips 2009, Iwaniak, Minkiewicz et al. 2014). VPP and IPP are the well-known casein derived tripeptides with ACE inhibitory, vasculo-protective, antioxidant and anti-inflammatory properties (Kim, Kim et al. 2008, Aihara, Osaka et al. 2014, Nonaka, Nakamura et al. 2014, Solieri, Rutella et al. 2015). 31 weeks intake of diets containing VPP and/or IPP including fermented milk, casein hydrolysate and synthesized VPP and IPP attenuated atherosclerosis development in apolipoprotein E–deficient mice (Nakamura, Hirota et al. 2013). Additionally, VPP and IPP diets reduced mRNA expression of inflammatory cytokines such as IL-6 and IL-1β, and oxidized low-density lipoprotein receptor in this mouse model (Nakamura, Hirota et al. 2013). Oral administration of VPP for 10 weeks to male C57BL/6J mice at a dosage of 0.3 mg /ml along with a high fat diet exerted anti-inflammatory effects on the adipose tissue of these mice (Aihara, Osaka et al. 2014). The adipose tissue of VPP administered mice showed less inflammatory characteristics compared to control mice; less activated monocytes and pro-inflammatory macrophages as well as MCP-1 and IL-6 gene expression (Aihara, Osaka et al. 2014). Furthermore, these peptides have shown beneficial adipogenic differentiation and insulin mimetic and anti-inflammatory effects in adipocytes, suggesting additional benefits of these peptides in controlling MetS complications (Chakrabarti and Wu 2015).

Egg proteins are other sources for the production of anti-hypertensive peptides (Miguel and Aleixandre 2006, Miguel, Alonso et al. 2007, Majumder, Panahi et al. 2013, Jahandideh, Majumder et al. 2014, Yu, Zhao et al. 2017). Egg white hydrolysate prepared by thermolysin and

pepsin reduced blood pressure in spontaneously hypertensive rats (SHRs). This treatment enhanced vasodilation, and improved nitric oxide bioavailability in the vasculature of rats. Interestingly, further mechanistic analysis revealed that egg white hydrolysate modulated the expression level of several components of RAS; reduced ACE and AT1R expression while enhanced AT2R expression in rat's aorta (Jahandideh, Chakrabarti et al. 2016). Due to the role of Ang II/AT1R axis in generation of ROS, enhanced NO bioavailability could be the consequence of less oxidative stress in egg white hydrolysate-treated SHRs compared to control group. IRW, IQW and LKP are the ovotransferrin derived tripeptides initially identified as ACE inhibitors (Majumder and Wu 2011) and later reported to reduce blood pressure in SHRs (Majumder, Chakrabarti et al. 2013, Majumder, Chakrabarti et al. 2015). IRW and IQW further exerted antioxidant and anti-inflammatory effects in endothelial cells (Huang, Chakrabarti et al. 2010, Majumder, Chakrabarti et al. 2013). GW is a novel di-peptide derived from ovotransferrin with antioxidant and anti-inflammatory effects in human umbilical vein endothelial cells (Jahandideh, Chakrabarti et al. 2016). Both glycine and tryptophan have been reported as important amino acid residues in exerting anti-inflammatory and antioxidant properties in literature (Park, Nan et al. 2009, Ruiz-Ramirez, Ortiz-Balderas et al. 2014). Intraperitoneal injection of tryptophan to Wistar rats subjected to acute pancreatitis significantly reduced plasma levels of TNF-α while enhanced IL-10 levels with anti-inflammatory properties (Jaworek, Leja-Szpak et al. 2003). Glycine supplementation to diets of sucrose fed rats reduced blood pressure, enhanced vascular relaxation and exerted antioxidant activities in vasculature of these rats. Enhanced biosynthesis of glutathione by glycine has been reported as the potential mechanism for its antioxidant effect in this study (Ruiz-Ramirez, Ortiz-Balderas et al. 2014). Lyzozyme is another potential precursor egg protein for generation of bioactive peptides. Alcalase hydrolysate of lysozyme with ACE-

inhibitory activity has been reported to enhance vascular relaxation and reduce inflammatory markers in Zucker diabetic fatty rats (Wang, Landheer et al. 2012). In fact, lysozyme-derived peptides attenuated renovascular damage through a mechanism involving decreased oxidative stress, inflammation and COX expression (Wang, Landheer et al. 2012). Production of multi-functional egg white hydrolysates using eight different proteolytic enzymes has been reported recently (Garces-Rimon, Lopez-Exposito et al. 2016). Egg white hydrolysates prepared by pepsin and aminopeptidase from Rhizopus oryzae revealed antioxidant and anti-inflammatory effects in macrophage RAW 264.7 cell line in addition to their *in vitro* ACE-inhibitory activity (Garces-Rimon, Lopez-Exposito et al. 2016). However, when administered to obese Zucker rats, only the pepsin egg white hydrolysate showed beneficial effects; reduced epididymal fat, improved hepatic steatosis, lowered plasma concentration of free fatty acids, and exerted antioxidant and anti-inflammatory effects compared to control group (Garces-Rimon, Gonzalez et al. 2016). FRADHPFL, RADHPFL, YAEERYPIL, YRGGLEPINF, ESIINF, RDILNQ, IVF, YQIGL, SALAM, and FSL were the identified peptides in pepsin egg white hydrolysate (Garces-Rimon, Lopez-Exposito et al. 2016).

Marine proteins contain biological peptides with various biological activities including anti-hypertensive, antioxidant, anti-microbial, immunomodulatory, and anti-cancer properties (Cheung, Ng et al. 2015). Pacific cod (Gadus macrocephalus) skin gelatin appears to be a potential source for production of multi-functional peptides when hydrolyzed by proteolytic enzymes. Hydrolysis with different enzymes yielded different peptides with antioxidant and ACE inhibitory properties. When papain was used, TCSP and TGGGNV were generated (Ngo, Ryu et al. 2011). Whereas, a longer peptide, LLMLDNDLPP, was generated when gastro-intestinal enzymes were used (Himaya, Ngo et al. 2012). ACE inhibitory and antioxidant were

identified in skate (Okamejei kenojei) gelatin hydrolysate as MVGSAPGVL and LGPLGHQ. These peptides showed radical scavenging activity and upregulated the protein and gene expression levels of antioxidant enzymes in human endothelial cells (Ngo, Ryu et al. 2014). Administration of sardine protein isolate prevented insulin resistance and reduced oxidative stress induced by a high fructose diet in rats. Rats receiving sardine protein isolate had lower plasma insulin and glucose and enhanced GLP-1 levels as well as reduced tissue thiobarbituric acid reactive species (TBARS), hydroperoxides, carbonyls, and enhanced antioxidant enzymes (Madani, Sener et al. 2015). MY is an ACE inhibitory dipeptide with anti-hypertensive effects derived from sardine muscle hydrolysate (Kawasaki, Seki et al. 2000) which also exhibits antioxidant activity by protecting endothelial cells from oxidative stress via induction of heme oxygenase-1 and ferritin (Erdmann, Grosser et al. 2006). Salmon skin derived olidopeptides exerted antioxidant and anti-inflammatory effects with concomitant reduction in fasting blood glucose and protection of β-cells from apoptosis in type 2 diabetic rats (Zhu, Peng et al. 2010).

A new low-molecular-weight salmon peptide fraction with glucoregulatory and anti-inflammatory properties has been recently identified (Chevrier, Mitchell et al. 2015). This salmon derived peptide fraction prevented glucose intolerance, dyslipidemia, and adipose tissue inflammation in $LDLR^{-/-}/ApoB^{100/100}$ mice fed a high-fat/high-sucrose diet. Mice fed with salmon peptides had lower body and liver weight, lower levels of adipose tissue cytokines and chemokines as well as greater glucose tolerance, and increased insulin signaling in liver as compared with control group (Chevrier, Mitchell et al. 2015). Reduced adipose tissue inflammation may be central to the observed enhanced glucose tolerance in these mice.

Bioactive peptides LDAVNR and MMLDF derived from *Spirulina*, the filamentous blue-green edible algae, exhibited anti-inflammatory and antioxidant effects in histamine-stimulated

endothelial cells (Vo, Ryu et al. 2013). Hemp seed meal protein hydrolysate has been reported to prevent and treat hypertension in young and adult SHRs respectively (Girgih, Alashi et al. 2014). Interestingly, this treatment exerted antioxidant activity *in vitro* and *in vivo* further highlighting the interaction of oxidative stress to other pathophysiological conditions and the beneficial role of antioxidant compounds in such conditions (Girgih, Alashi et al. 2014).

2.15. Conclusion

Development of bioactive peptides against different aspects of metabolic syndrome especially on glucose and energy homeostasis is a growing research field. The recent discoveries of pathways and target cells in the management of glucose and energy metabolism have opened up new opportunities for identification of novel bioactive peptides on enhancing adipocyte differentiation and insulin signaling, CCK receptor binding and expression, incretin stimulants to name a few. However, as yet, the evidence available on the efficacy of such bioactive peptides is rare and more research is required to validate these potential benefits *in vivo*. In addition to the initial cell experiments where potential benefits of peptides are identified, animal experiments to further assess the effectiveness of such peptides in a suitable animal model is of immense importance. Understanding the mechanism(s) of function may be challenging due to the multi-functional characteristics of many bioactive peptides where several pathways are involved in their attributed biological effects. Nevertheless, in case of protein hydrolysates containing a complex array of peptides, assigning the mechanism of action to a single peptide is impossible unless the peptide(s) responsible for that particular biological effect are purified using stepwise fractionation methods. Although food-derived peptides are generally accepted as safer alternatives to synthetic drugs with fewer side effects, this aspect needs to be carefully evaluated for each individual peptide initially in cell and animal experiments and later in clinical trials.

Bioactive peptides are usually liable to proteolytic cleavage in the gastrointestinal tract during absorption and uptake. Although cleavage of peptide bonds by the gastrointestinal proteolytic enzymes and intestinal brush border peptidases usually diminishes the bioactivity of a peptide, this cleavage may sometime generate shorter peptides with enhanced bioactivity. The pentapeptide LKPNM, derived from Bonito fish hydrolysate, is the major component of the commercialized blood pressure-lowering capsules (Vasotensin 120T^{TM} by Metagenics, Aliso Viejo, CA, USA; PeptACE^{TM} Peptides 90 by Natural Factors, Monroe, WA, USA). This peptide is converted into LKP by digestive enzymes to exert its anti-hypertensive effects (Hartmann and Meisel 2007). Peptides susceptible to digestion may require protection when administered orally using strategies like encapsulation. Several physicochemical parameters like charge, molecular weight, lipophilicity, and solubility affect the bioavailability of bioactive peptides (Darewicz and others 2011). Moreover, the mode of administration (fed or fasted phase, with or without water), the dosage form (suspension, powder, micelle, emulsion, etc.), and inter-individual variability due to age, sex, disease conditions, ethnicity are other factors greatly affect the bioavailability of peptides in clinical trials (Cicero, Fogacci et al. 2017). Therefore, pharmacokinetic data is required to determine the effective dosage and frequency of administration (Yoshikawa, 2015). Determining accurate plasmatic concentrations of peptides as a measure the bioavailability is another challenge due to the low peptide concentration, and the complex matrix of plasma.

In addition to the above-mentioned factors affecting the biological effectiveness of peptides in physiological conditions, improving food supply for widespread applications of bioactive peptides is another hurdle. The cost associated with developmental processes in combination with the low process yield and lack of large-scale production technologies are other limiting factors for commercialization process of food-derived bioactive peptides (Agyei and Danquah

2011). However, applying immobilized enzymes and continuous hydrolyzing processes instead of conventional batch methods are potential solutions to reduce the cost and enhance the feasibility of the purification process (Decker and Park 2010).

In conclusion, while the results obtained from *in vitro* and *in vivo* studies of bioactive peptides are encouraging showing their potential effectiveness against complications of MetS, studies on humans are scarce. Therefore, randomized clinical trials are necessary to confirm the effectiveness of these peptides and their safety. Moreover, developing technological processes for feasible extraction and/or purification of bioactive peptides without compromising their biological effect is another challenge to overcome.

Table 2-1. Criteria for MetS definitions in adults

Organization	Insulin resistance	Abdominal obesity	Lipids	Blood glucose	Blood pressure	Other
WHO (1998)	Type 2 diabetes/ IFG/ IGT, **plus two of the other features**	Waist-to-hip ratio > 0.9 (males), > 0.85 (females) or BMI> 30 kg/m^2	TG ≥ 150 mg/dl and/or HDL-C < 35 mg/dl (males) or < 39 mg/dl (females)	Type 2 diabetes or IFG (>100 mg/dl) or IGT	≥ 140/90 mm Hg	Albumin excretion ≥ 20 µg/min
EGIR (1999)	Insulin levels > 75th percentile of non-diabetic patients, **plus two of the other features**	Waist circumference ≥ 94 cm (males), ≥ 80 cm (females)	TG ≥ 150 mg/dl and/or HDL-C < 39 mg/dl	Fasting glucose ≥ 110 mg/dl	≥ 140/90 mm Hg	–
NCEP:ATPIII (2001)	– any three of the five features	Waist circumference ≥ 102 cm (males), ≥ 88 cm (females)	TG ≥ 150 mg/dl and/or HDL-C < 40 mg/dl (males) or < 50 mg/dl (females)	Fasting glucose * ≥ 110 mg/dl	≥ 130/85 mm Hg	–
AACE (2003)	IGT **plus two or more of the other features**	BMI ≥ 25 kg/m^2	TG ≥ 150 mg/dl and/or HDL-C < 40 mg/dl (males) or < 50 mg/dl (females)	IGT	≥ 130/85 mm Hg	–
IDF (2005)	–	Increased waist circumference# (ethnicity specific) **plus any two of the other features**	TG ≥ 150 mg/dl and/or HDL-C < 40 mg/dl (males) or < 50 mg/dl (females)	Fasting glucose ≥ 100 mg/dl	SBP ≥ 130 and DBP ≥ 85 mmHg	–
AHA/NHLBI (2004)	– any three of the five features	Waist circumference ≥ 102 cm (males), ≥ 88 cm (females)	TG ≥ 150 mg/dl and/or HDL-C < 40 mg/dl (males) or < 50 mg/dl (females)	Fasting glucose ≥ 100 mg/dl	≥ 130/85 mm Hg	–

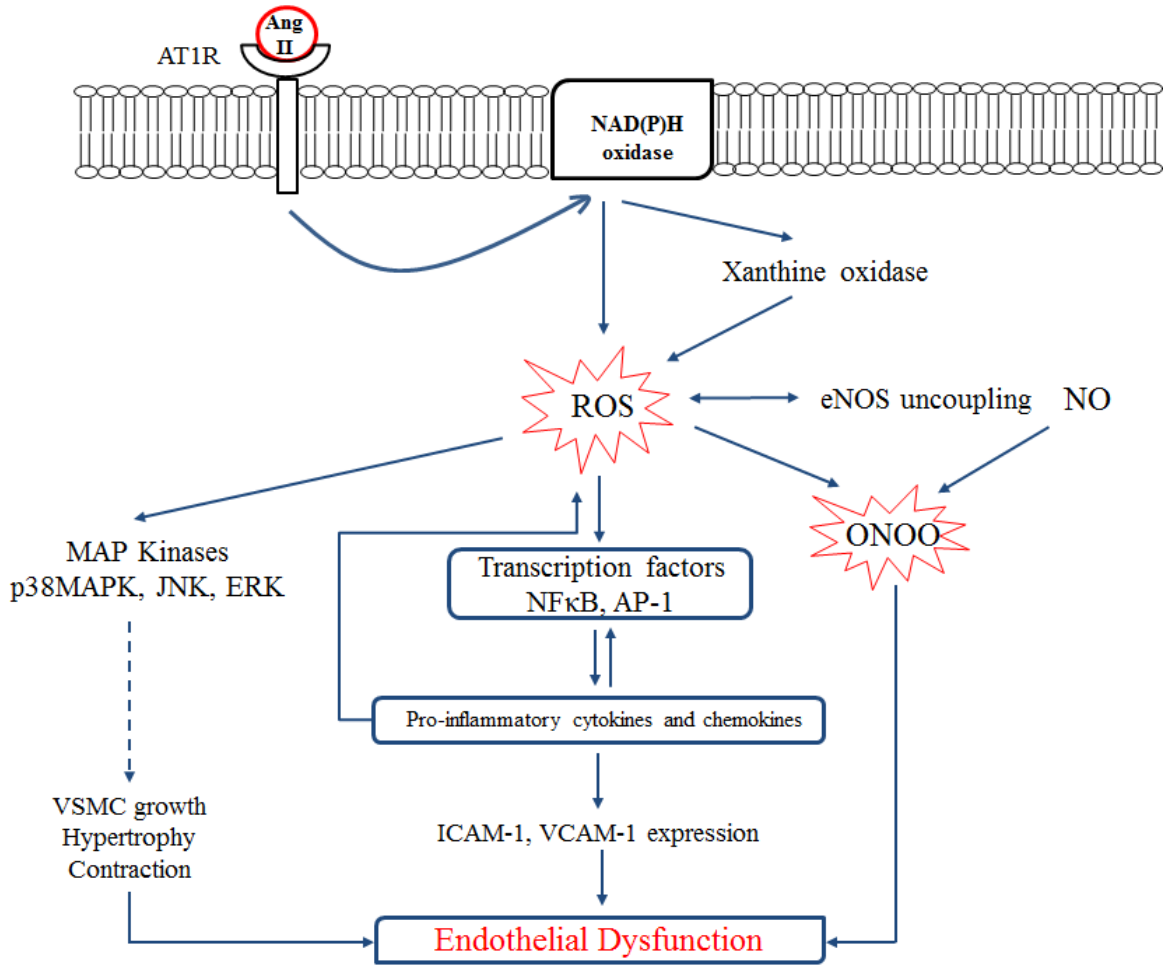


Figure 2-1. Interplay between RAS, oxidative stress, inflammation, and endothelial dysfunction. Ang II induces ROS production by activating endothelial NAD(P)H oxidase through the AT1 receptor. NAD(P)H oxidase activation further increases endothelial xanthine oxidase-mediated superoxide production and oxidative stress in the endothelium. ROS activates inflammatory transcription factors which enhance expression of adhesion molecules (ICAM-1 and VCAM-1) and/or activates MAP kinases which eventually leads to the VSMC growth and contraction and endothelial dysfunction. On the other hand, ROS induces eNOS uncoupling. NO reacts with superoxide anion resulting in peroxynitrite (ONOO−) formation which also mediates endothelial dysfunction. VSMC, vascular smooth muscle cell; ICAM-1, intracellular cells adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1. Figure adapted from (Higuchi, Ohtsu et al. 2007).

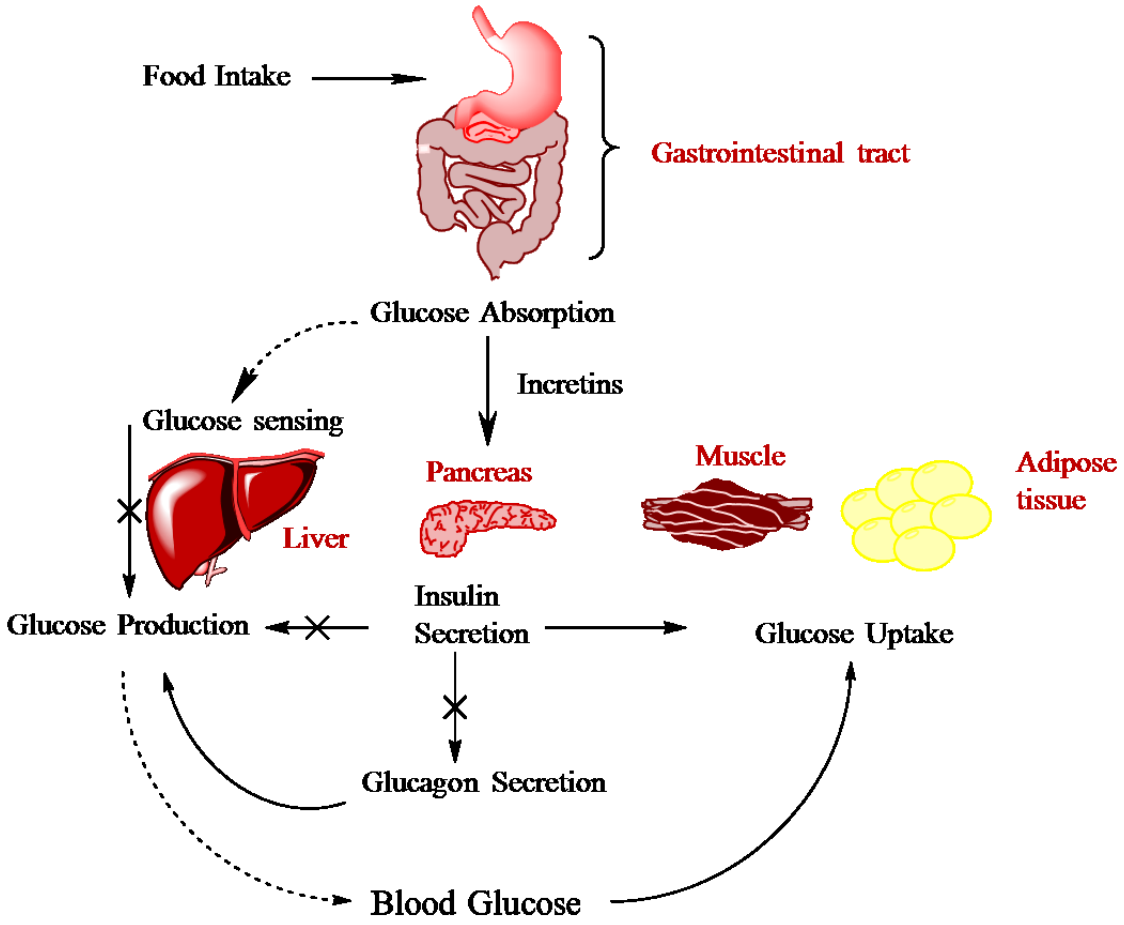


Figure 2-2. Peripheral control of glucose homeostasis. See text for details. Figure adapted from (Grayson, Seeley et al. 2013).

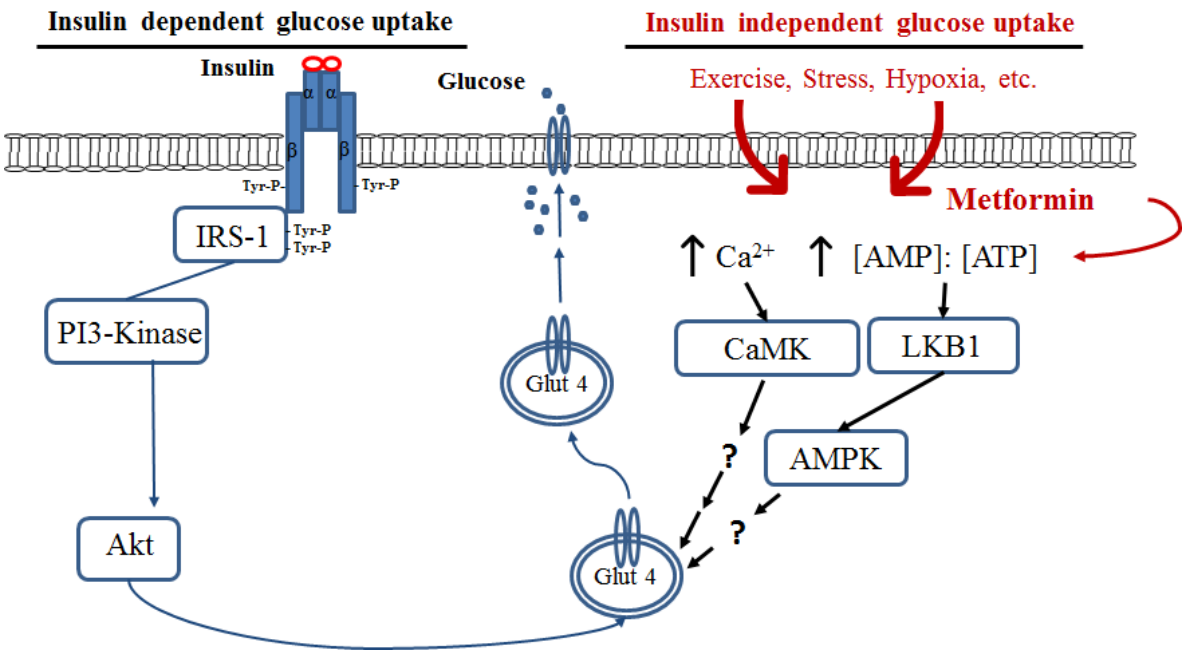


Figure 2-3. Insulin-dependent and independent glucose uptake in muscle. Glucose uptake in skeletal muscle occurs through both insulin dependent and -independent pathways under different physiological conditions. Stimuli (exercise, hypoxia, stress etc.) activate several signaling proteins (e.g., AMP-activated protein kinase (AMPK), Ca^{+2}/calmodulin-dependent protein kinase (CaMK), etc.). An increase in AMP/ATP ratio activates AMPK, while the increase in Ca^{+2} concentration activates CaMK which subsequently induce glucose transport by enhancing translocation of the glucose transporter protein 4 (GLUT4) to the cell membrane. The signaling mechanism of AMPK and glucose transport remains largely undefined. For insulin-dependent glucose uptake see text (section 3.1). Akt, protein kinase B/Akt; IRS-1, insulin receptor substrate-1; LKB1, liver kinase B1; PI3K, phosphatidylinositol 3-kinase. Figure adapted from (Jessen and Goodyear 2005, Fujii, Jessen et al. 2006, Stanford and Goodyear 2014).

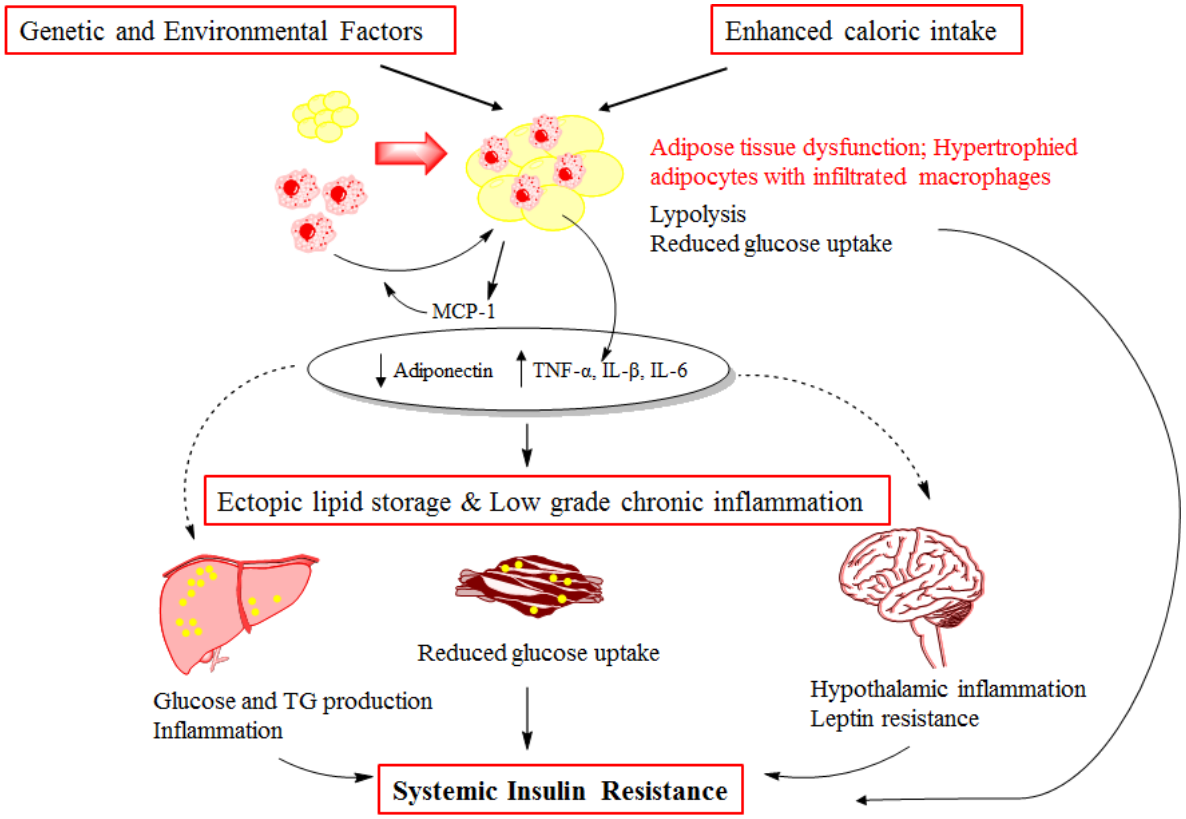


Figure 2-4. Pathophysiology of insulin resistance. Genetic, environmental and/or positive energy balance leads to weight gain and increased visceral adiposity. Enhanced visceral adiposity results in formation of hypertrophic and dysfunctional adipocytes with a pro-inflammatory nature. Adiponectin production is diminished and secretion of pro-inflammatory adipokines is increased. Recruitment of macrophages with pro-inflammatory phenotype through monocyte chemoattractant protein-1 (MCP-1), and infiltration through the adipose tissue further exacerbate the pro-inflammatory state. This low grade chronic inflammation in adipose tissue as well as other organs induces insulin resistance locally and systematically through inhibiting signaling pathways. Moreover, the loss of lipid buffering capacity and sustained levels of pro-inflammatory factors results in ectopic fat deposition in non-adipose tissues especially liver and skeletal muscle further triggering systemic insulin resistance. Figure adapted from (Van Gaal, Mertens et al. 2006, Oh and Olefsky 2016).

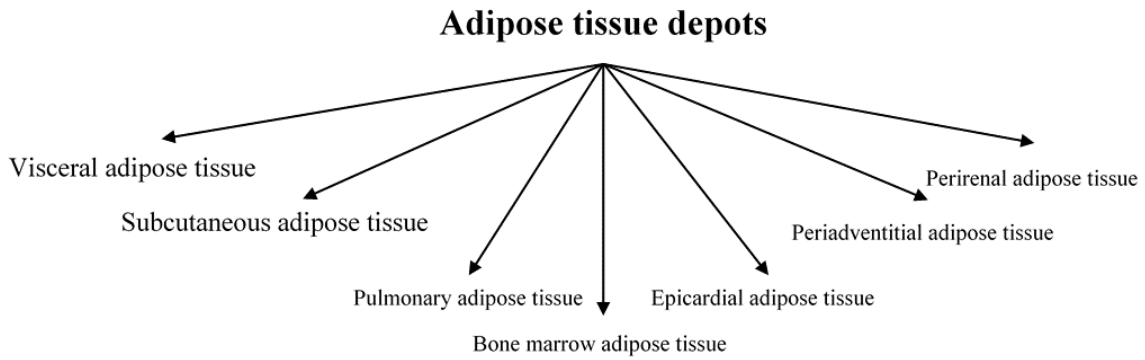


Figure 2-5. Different adipose depots. Visceral and subcutaneous depots are the major adipose depots in the body.

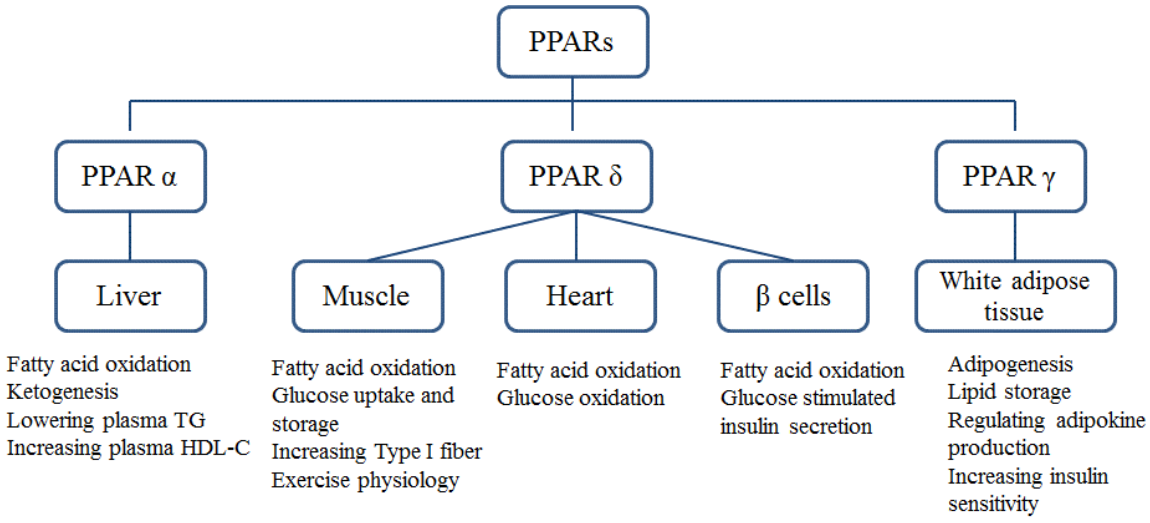


Figure 2-6. PPAR isoforms and their main physiological and/or pharmacological roles in energy metabolism. The most important PPAR isoform expressed at highest levels in each tissue is shown. Figure summarized based on (Wang 2010, Poulsen, Siersbk et al. 2012).

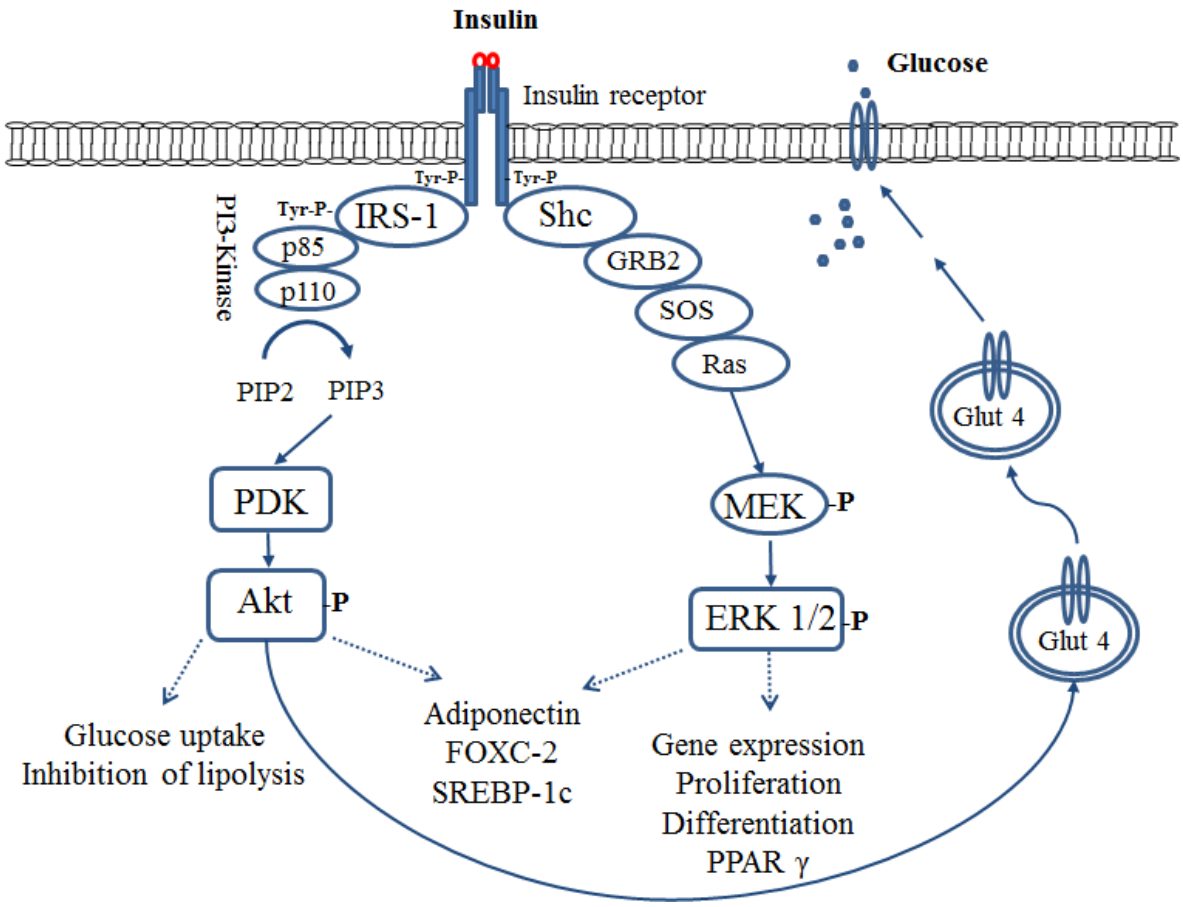


Figure 2-7. The simplified insulin signaling pathway in adipocytes. Binding of insulin to its receptor triggers insulin receptor autophosphorylation and activation of the protein kinase that phosphorylates IRS proteins on tyrosine residues. These initial tyrosine phosphorylation reactions are the basis for conveying insulin signals to a complex network that ultimately mediate the specific biological effects of this hormone. IRS protein phosphorylation generates binding sites for PI3K, GRB2/SOS, and SHP2. Recruitment of PI3K to IRS phosphorylates PIP2 to PIP3 that leads to the activation of PDK and subsequently PDK activates the serine/threonine kinase Akt/PKB. Akt activation is important for the translocation of GLUT4 to the plasma membrane and glucose uptake. On the contrary, the MAPK pathway controlling the mitogenic, growth and cell differentiation is less well understood. Shc plays a critical role in mediating the mitogenic effects of insulin, primarily through activation of the GRB2/SOS/Ras/MAPK pathway. Phosphorylation and activation of ERK1/2 plays a direct role in cell proliferation and differentiation via gene transcription regulation. ERK, extracellular signal-regulated kinase; GRB2, growth factor receptor-bound protein 2; IRS, insulin receptor substrate; MAPK, mitogen activated protein

kinase; MEK, extracellular-signal-regulated protein kinase kinase; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; SOS, son of sevenless. Figure drawn based on (Laviola, Perrini et al. 2006, Mlinar, Marc et al. 2007, Copps and White 2012).

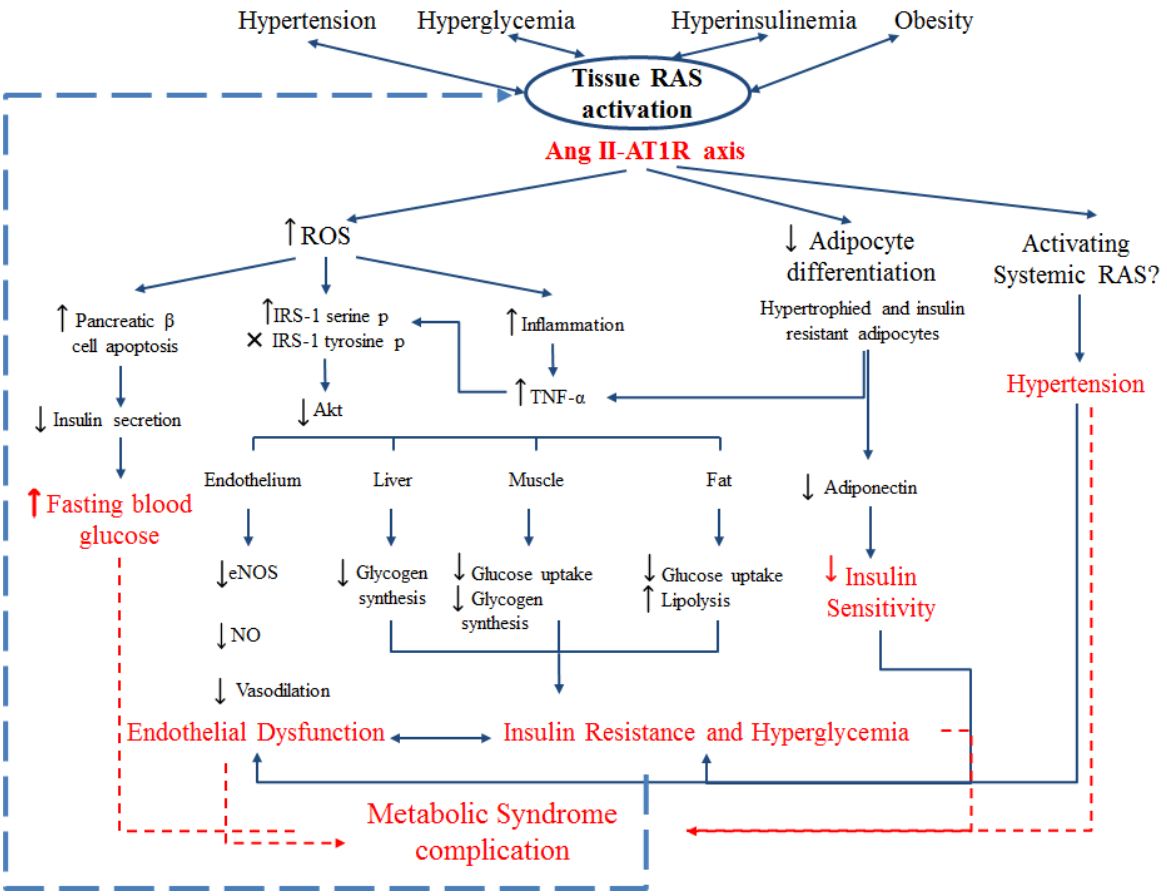


Figure 2-8. Link between the RAS, oxidative stress and metabolic syndrome. Hyperglycemia, hypertension, hyperinsulinemia and obesity enhance the expression of local RAS components especially the Ang II-AT1R axis in specific tissues. Ang II retards adipocyte differentiation leading to adipocyte dysfunction and reduced insulin sensitivity through reduced adiponectin secretion and enhanced pro-inflammatory adipokines. Ang II further, enhances reactive oxygen species (ROS) production affecting several pathways in different tissues in the body. Enhanced inflammation, disturbed insulin signaling in endothelium, liver, muscle and adipose tissue leads to endothelial dysfunction and insulin resistance. Furthermore, oxidative stress damages pancreatic β-cells that diminishes insulin secretion and enhances fasting blood glucose. Overall, local RAS overproduction contributes to the enhanced fasting blood glucose, endothelial dysfunction, systemic insulin resistance, and hyperglycemia the hallmarks of metabolic syndrome. Figure drawn based on (Kim, Montagnani et al. 2006, Putnam, Shoemaker et al. 2012).

Chapter 3: Antioxidant Peptides Identified from Ovotransferrin by the ORAC Method Did Not Show Anti-inflammatory and Antioxidant Activities in Endothelial Cells[2]

3. Introduction

Oxidative stress, defined as an imbalance between the formation of reactive oxygen species (ROS) and the body's antioxidant system, is proposed to be the common underlying pathological contributor to various degenerated diseases (Vendramini-Costa and Carvalho 2012, Gutowski and Kowalczyk 2013). Consumption of dietary antioxidant compounds has been suggested to enhance the endogenous enzymatic and non-enzymatic antioxidant systems against oxidative stress (Fang, Yang et al. 2002). A number of food-derived compounds including food proteins and their constituent peptides have been tested extensively for the potential to act as antioxidants although far fewer have been validated *in vivo* (Chakrabarti, Jahandideh et al. 2014). Chicken egg white, the excellent source of high quality proteins and bioactive peptides, has been reported as a potential source for antioxidant peptides (Davalos, Miguel et al. 2004, Chen, Chi et al. 2012, Liu, Jin et al. 2015). Ovalbumin, ovotransferrin and cystatin have been reported recently to contain several antioxidant peptides with high radical scavenging activity (Nimalaratne, Bandara et al. 2015). Antioxidant activity was mostly measured using *in vitro* chemical-based assays; these methods bear some imperfections for lacking biological relevance and not considering the complexity of the *in vivo* systems regarding bioavailability, mechanism of action and metabolism of the proposed antioxidant compounds. Therefore, the antioxidant indices determined through *in*

[2] This chapter has been published: Jahandideh F, Chakrabarti S, Davidge ST, Wu J. 2016. *Journal of Agricultural and Food Chemistry*. 64(1):113-119.

vitro chemical methods do not necessarily reflect corresponding *in vivo* efficacy in biological systems. Cell-based assays, being faster and reproducible intermediate testing methods than *in vivo* methods, are useful in assessing potential antioxidant compounds prior to more expensive *in vivo* studies (Lopez-Alarcon and Denicola 2013). Sixteen peptides were previously identified from thermolysin digest of ovotransferrin through the oxygen radical absorption capacity (ORAC)-guided fractionation in our lab (Shen, Chahal et al. 2010). It remains to be determined if the ovotransferrin-derived antioxidant peptides would also demonstrate antioxidant activity in biologically relevant systems like cells.

Oxidative stress and inflammatory responses are also interrelated. Pro-inflammatory gene expression could be induced by ROS through activation of the transcription factor nuclear factor-kappa-B (NF-κB) (Haddad 2002). Activation of pro-inflammatory transcriptional factors increases the production of various cytokines and chemokines leading to the generation and propagation of inflammatory responses (Cooper, Whaley-Connell et al. 2007, Li, Barrett et al. 2007). This process in turn generates more ROS through activation of oxidant generating enzymes and leads to a vicious cycle that has detrimental effects on cells by inducing mitochondrial dysfunction, aggravating cellular damage, cell death and finally progressing to chronic disease (Pillarisetti and Saxena 2004). Given this link, it is likely that compounds with antioxidative activity would also show anti-inflammatory properties. Due to the interplay between oxidative stress and inflammation, whether ovotransferrin derived peptides with *in vitro* antioxidant capacity would show anti-inflammatory effects is not known. Another potential issue with peptides is their stability during digestion and/or absorption; gastro-intestinal digestion (GID) may alter the structure of bioactive peptides and consequently their specific biological activities might be diminished or enhanced. Therefore, it is important to test the stability of these

peptides and determine possible changes in their activity after hydrolysis with digestive enzymes. The objectives of the present study were to 1) test if antioxidant peptides identified by ORAC could demonstrate anti-inflammatory and antioxidant activities in endothelial cells and 2) to investigate the effects of simulated GID on their stability and modulation of their biological activities.

3.1. Materials and methods

3.1.1. Reagents

Dulbecco's phosphate-buffered saline (PBS), M199 medium with phenol red, TNF-α, glutamine, pepsin (porcine gastric mucosa, 1064 units/mg protein) and pancreatin (porcine pancreas, 8 × USP specifications), porcine gelatin, heparin, and 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol) were all from Sigma Aldrich (St. Louis, MO, USA). L-glutamine, M199 medium without phenol red, and fetal bovine serum (FBS) were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). The antibiotics penicillin and streptomycin were obtained from Life Technologies (Carlsbad, CA, USA). Type 1 collagenase for cell splitting was from Worthington Biochemical Corp. (Lakewood, NJ). Triton X-100 and endothelial cell growth supplement (ECGS) were purchased from VWR International (West Chester, PA). Dihydroethidium (DHE) was bought from Molecular Probes (Eugene, OR). Mouse monoclonal primary antibody against intercellular cell adhesion molecule-1 (ICAM-1), rabbit polyclonal primary antibody against vascular cell adhesion molecule-1 (VCAM-1) were obtained from Santa Cruz Biotechnology Inc. (SantaCruz,CA, USA). The α-tubulin antibody was from Abcam (Cambridge, MA, USA). Donkey anti-mouse and goat anti-rabbit fluorochrome-conjugated secondary antibodies were from Licor Biosciences (Lincoln, NB, USA). All peptides (>95% purity with HPLC-MS/MS

confirmation) were synthesized by GenScript Corp (Piscataway, NJ). All chemicals and reagents were of analytical grade.

3.1.2. Simulated digestion of peptides

Simulated digestion of previously identified antioxidant peptides was carried out using pepsin and pancreatin as described earlier with some modifications (You and Wu 2011). The sequence of these peptides is presented in Table 1. Briefly, peptide solution (1 mM in distilled water) was acidified by 1 M HCl to pH=2 to simulate the stomach digestion phase, then pepsin (E/S: 2% w/w) was added and the solution was incubated at 37 ˚C for 2 h. Pepsin was inactivated by increasing the pH to 6.5 using 1M NaOH before addition of pancreatin (E/S: 2% w/w) to simulate the small intestine digestion at 37 ˚C for 3 hours. The hydrolysis was terminated by raising the temperature to 95 °C for 15 min, cooling to room temperature, and was kept at -80 ˚C until further experiments. Only samples prepared by this protocol are referred to as "peptide digests" throughout this chapter and the term "peptide" refers to their intact form without any digestion.

3.1.3. Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs), a model system for studying inflammation and oxidative stress in the vasculature, were used for the present study; the study protocols were approved by the University of Alberta Ethics Committee and the studies were conducted according to the principles of the Declarations of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, revised November 13, 2001, effective December 13, 2001. All subjects provided written consent to be included in this study. Cells were isolated as described earlier (Chakrabarti and Davidge 2009). Briefly, after receiving umbilical cords, blood clots were removed from the umbilical vein by flushing with PBS.

Afterwards, a type 1 collagenase containing buffer was used for separating the HUVECs from the umbilical vein. The cells were initially grown in tissue culture flasks and later plated on (for the actual experiments) to 48-well plates (80−100 K cells/well). Cells were kept in a humidified atmosphere at 37 °C with 5% CO_2/95% air in M199 medium with phenol red supplemented by 20% FBS as well as L-glutamine, penicillin−streptomycin, and 1% ECGS. Second or third passage cells were used for all of experiments.

3.1.4. Experimental protocols and treatments

On the day of the experiment, second-passage HUVEC monolayers at 80-90% confluence were quiesced for 2 h prior to the start of the experiment in a reduced serum medium containing M199 media (phenol red-free) with 1% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin (5000 U/mL). Different sets of cells were treated with ovotransferrin-derived peptides (50 μM) before/ after hydrolysis for 20 h with/ without the primary mediator of acute and chronic inflammation; tumor necrosis factor-α (TNF-α). A blank sample containing only the enzymes used for GID was used as a control for this process on cellular expression of ICAM-1 and VCAM-1. Tempol, a well-known antioxidant which metabolizes superoxide anion and many other ROS (Wilcox and Pearlman 2008) was used as the positive control in TNF-α mediated superoxide generation experiment. Cells were treated for 2 h with 20 mM of Tempol prior to TNF-α stimulation. TNF-α was used at concentrations and duration specific for different experiments (5 ng/mL, 4 h for anti-inflammatory and 10 ng/mL, 1 h for antioxidant experiments) to stimulate the cells. At the end of the specified incubation period, culture medium was removed and the cells were used either for detection of intracellular superoxide by DHE or expression of adhesion molecules by Western blot technique.

3.1.5. Western blotting

Changes in cellular levels of inflammatory molecule (ICAM-1 and VCAM-1) expression were determined by western blotting. Briefly, the cells were lysed in boiling hot Laemmli's buffer containing 50 μM dithiothreitol (DTT) as a reducing agent, and 0.2% Triton-X-100. Prepared cell lysates were run in 9% SDS-PAGE, blotted to nitrocellulose membranes and the protein bands of interest were detected with antibodies against ICAM-1, VCAM-1, and the loading control α-tubulin. Mouse monoclonal primary antibody against ICAM-1 and rabbit polyclonal primary antibody against VCAM-1were used at a concentration of 1 μg/mL. The α-tubulin antibody was used at 0.4 μg/mL. Donkey anti-mouse and goat anti-rabbit fluorochrome-conjugated secondary antibodies were used at 1:10000 dilutions. The protein bands were detected by a Licor Odyssey BioImager and quantified by densitometry using the Licor Biosciences software. Each band of ICAM-1 or VCAM-1 was normalized to its corresponding band of α-tubulin. Each gel contained the samples generated from a particular umbilical cord. Cell lysates from untreated cells (control) were loaded on every gel for comparison, and all data were expressed as % TNF-α of the same experiment.

3.1.6. Superoxide detection assay

Superoxide generation in endothelial cells was measured by DHE staining as described previously (Chakrabarti and Davidge 2009). Briefly, pre-treated HUVEC monolayers were washed once and incubated for 30 min at 37 ˚C with 10 μM DHE in the quiescing medium. Cells were then washed twice and fluorescence was visualized in a fluorescence microscope (Olympus IX81, Olympus Canada Inc., Ontario, CA). Images from three randomly chosen fields were taken for each data point. The cells in each field were counted and total fluorescence intensity

was measured to determine the mean fluorescence intensity per cell (MFI/cell). Superoxide generation was measured as fold increase in MFI/cell over the control.

3.1.7. Chromatography of peptides after digestion

Ultra-performance liquid chromatography (UPLC) was performed on a BEH300 C_{18} column (1.7 μm, 2.1 × 100 mm, Waters Inc., Milford, MA. USA) connected to an online PDA detector. The system consisted of 2 solvents: solvent A (0.05% TFA in HPLC grade water) and solvent B (0.05% TFA in HPLC grade acetonitrile) with a gradient elution in which the concentration of solvent B was increased from 0% to 50% within 15 min, and a 5-min delay was incorporated to restore the column to the initial condition (0% solvent B) for next injection. The injection volume was 10 μL; the flow rate was 0.2 mL/min and peaks were monitored at 220 nm using the photodiode array detector (Waters 2998, Waters Inc., Milford, MA. USA). Characterization of peptides with antioxidant and anti-inflammatory activities, in the hydrolysate, was carried out by liquid chromatography tandem mass spectrometry (LC/MS-MS).

3.1.8. Peptide Characterization by LC/MS-MS

Identification of peptide sequences generated after digestion of the most active sample was carried out by subjecting the sample to LC-MS/MS analysis on a q-Tof premier mass spectrometer (Waters, Milford, MA) coupled with a nanoAcquity UPLC system (Waters, Milford, MA). 5 μL of the peptides was loaded onto a nano trap column (180μm x 20mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) followed by a nano analytical column (75 μm × 150 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA). Desalting on the peptide trap was achieved by flushing trap with 1% acetonitrile, 0.1% formic acid in water at a flow rate of 10μL/min for 2 - 3 minutes. Peptides were separated with flow rate of 350 nL/min and the following gradient: starting with 1% solvent B (solvent B: acetonitrile

with 0.1% formic acid; Solvent A: 0.1% formic acid in water) and raise to 50%B in 15 min followed by 95%B at 25 min. Mass spectrometer was operated at positive mode with capillary voltage of 3.4 kV and source temperature of 100°C. Spectra were recorded over the (m/z) ranges of 200-600 in MS mode and 50-990 in MS/MS mode. Instrumental control and data analysis were performed using MassLynx software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K).

3.2. Statistical Analysis

All data presented are mean value ± SEM of four to twelve independent experiments using HUVECs isolated from different umbilical cords. One-way analysis of variance with Dunnett's or Tukey's post-test was used for the determination of statistical significance. Differences were considered significant at P value of <0.05.

3.3. Results and discussion

3.3.1. Antioxidant Peptides Identified by ORAC Did Not Show Anti-inflammatory Activity in Cells.

Our lab has previously identified 16 peptides with potential antioxidant properties (Table 1) (Shen, Chahal et al. 2010). Since oxidative stress and inflammatory responses are interrelated, we first attempted to determine if these antioxidant peptides would also show anti-inflammatory activity. When HUVECs were stimulated with TNF-α, the cellular expression of both VCAM-1 and ICAM-1 was significantly increased. This increase in protein expression was unaffected by pre-treatment with the peptides (Figure 3- 1A & B) suggesting a lack of anti-inflammatory activity of all 16 peptides in HUVECs. However, incubation of HUVECs with some but not all the peptide digests reduced VCAM-1 and ICAM-1 expression significantly (Figure 3- 1C & D).

Digestion of peptides 9, 12, 13, 14, 15 and 16 reduced the expression of VCAM-1 about 40-46% compared to the blank while ICAM-1 expression was suppressed by about 38% only by one peptide digest (Pep16). Our results indicated that digestion might cause generation of anti-inflammatory activity that could attenuate TNF-α-induced expression of inflammatory markers in endothelial cells. The chromatograms of peptides after digestion is shown in appendix 3-A. All peptide digests showed different chromatograms from their respective peptides, indicating they were all degraded by simulated gastro-intestinal digestion. For example, pep 9 (GWNI) shows the elution peak at 8.8 min while after digestion, two peaks appeared at elution time of 5.4 and 6.5 min, indicating the degradation of the pep 9 into two new peptides.

3.3.2. Peptides Did Not Affect Intracellular Basal Superoxide Levels Irrespective of Digestion.

Since all 16 antioxidant peptides identified did not show any anti-inflammatory activity in cell, we next measured their antioxidant activities further in cells. Surprisingly, none of the peptides or their digests could essentially change the basal levels of superoxide ions in endothelial cells (Figure 3- 2A & B).

Ineffectiveness of peptides on basal superoxide levels might be due to a well-functioning intracellular antioxidant system/s under resting conditions and the exogenous antioxidants might only exert their effects when this balance is altered (for example, under TNF-α stimulation). Since ORAC is a cell free method, it is not surprising that such modulation of intracellular redox status by peptides cannot be properly evaluated by this method. Therefore, in the next step, we investigated the antioxidant activity of selected peptide digests (with demonstrated anti-inflammatory activity) using a TNF-α mediated superoxide generation method in HUVECs.

Exposure of HUVECs to 10 ng/mL TNF-α for 1 h increased superoxide level significantly in these cells. Only cells treated with peptide 9 (GWNI) digest suppressed superoxide in these cells significantly ($p<0.05$) to the same level as Tempol treatment (Figure 3- 3A & B). Treatment with Tempol and peptide 9 digest reduced superoxide levels by 32% and 29% respectively, compared to the cells stimulated by TNF-α (Figure 3- 3B).

These data suggest that new peptide/s with potential antioxidant capacity in HUVECs may be generated after digestion of GWNI. These findings were further confirmed by estimation of intracellular hydrogen peroxide (H_2O_2) levels by Amplex Red Assay (Figure 3- 4).

Although several peptide digests showed anti-inflammatory activities, only one (pep 9 digest) exerted additional antioxidant effects; therefore, it is likely that these two beneficial properties are regulated by mutually independent mechanisms. Since the introduction of ORAC as a test for evaluation of antioxidant capacity of compounds in late 1990s by Cao and Prior (Cao and Prior 1999), this method has been modified over time and used extensively as the preferable method by researchers in assessing antioxidant capacity of a variety of biological samples as well as food compounds (Zheng and Wang 2001, Davalos, Miguel et al. 2004, Yida, Imam et al. 2014). Despite the extensive use of this method, accumulating evidence suggests that the antioxidants validated *in vitro* do not necessarily exhibit *in vivo* effects (Shen, Ji et al. 2007) questioning the physiological significance of these methods including ORAC for antioxidant activity. The published ORAC tables by the United States Department of Agriculture (USDA) for testing and rating variety of foods using this method for decades were consequently withdrawn in May 2012 due to the lack of correlation between ORAC values and the physiological responses. Therefore, the results of chemical-based antioxidant assays should be interpreted cautiously, and drawing any conclusions on the health benefits of proposed antioxidants should be prohibited unless

appropriately designed biologically relevant experiments are conducted (Hermans, Cos et al. 2007). The outcome of this study did not support a direct correlation between ORAC values (either high or low) and intracellular superoxide scavenging activity in HUVECs. Similar contradictory results between ORAC and cell-based assays have also been reported in the literature (Elbling, Weiss et al. 2005, Elisia, Tsopmo et al. 2011). These studies clearly indicate the limitations of chemical-based methods in evaluating antioxidant activity of compounds in environments where several factors including absorption and metabolism affect the overall activity of compounds. In fact, the real antioxidant capacity of a bioactive compound goes beyond its sole reducing or scavenging activity and adaptive responses involving activation of the antioxidant responsive signaling pathways might play a role in this regard.

3.3.3. Characterization of the Anti-Inflammatory Peptide(s) After Digestion of GWNI.

The peptide 9 (GWNI) digest, showing both anti-inflammatory and antioxidant activities, was further characterized by LC-MS/MS to identify the responsible peptides. The chromatogram of pep 9 digest is shown in Figure 3- 5 (A), where two major peaks with the retention time of 11.72 min and 12.78 min are observed. Their identities were further characterized using their corresponding MS/MS spectra as shown in Figure 3- 5 (B & C). Peptide sequences were identified by comparing the fragment peaks from MS/MS spectra with the theoretical fragment masses. As interpreted in Figure 3- 5B, b_2 ion (m/z 244.1), b_2-H_2O ion (after water loss, m/z 226.1), and a_2 ion (after loss of carbonyl, m/z 216.1) as well as y_1 and y_2 ions (m/z 133.1 and 319.1 respectively) were identified in the fragment peak at 11.72 min. Given its parent ion $[MH]^+$ m/z is 376.2, this peptide was identified as GWN. Similarly, the other peptide with fragment peak at 12.78 min was identified as GW (Figure 3- 5C), given its parent ion $[MH]^+$ m/z

of 262.1, the presence of y_1 ion (m/z 205.1), y_2-NH_3 ion (m/z 188.1; signal intensified after NH_3 loss) and b_2 ion (m/z 244.1).

Therefore, the original peptide (GWNI) was broken down into GWN and GW. After Identification of the new peptides in pep 9 digest, the synthetic forms of GW and GWN were used and tested for their anti-inflammatory and antioxidant effects in HUVECs and vascular smooth muscle cells respectively. Interestingly, only GW and not GWN significantly attenuated VCAM-1 expression in TNF-α treated cells without affecting ICAM-1 levels (Figure 3- 6A & B) while both peptides effectively reduced the basal superoxide in vascular smooth muscle cells (VSMCs).

These results suggested that while both fragments in pep 9 digest possessed antioxidant effects, the anti-inflammatory effect of pep 9 digest was largely due to the release of the active component GW from the parent peptide. The fact that GWN with an extra N at C terminal lacks anti-inflammatory properties further confirms the essential role of W and Glycine (G) or the GW sequence for the observed anti-inflammatory effects in HUVECs. Not surprisingly, these two amino acids have been reported as important amino acid residues in exerting anti-inflammatory and antioxidant properties in literature. Intraperitoneal (i.p.) injection of L-Tryptophan to Wistar rats subjected to acute pancreatitis significantly reduced plasma levels of TNF-α while enhanced the levels of the anti-inflammatory interleukin 10 (IL-10) (Jaworek, Leja-Szpak et al. 2003). Tryptophan rich peptides also reduced the inflammatory response by inhibiting the release of nitric oxide (NO) following lipopolysaccharide (LPS) stimulation in RAW264.7 cells (Park, Nan et al. 2009). Antioxidant and anti-inflammatory properties of glycine have also been reported in several studies (Fogarty, Broadfield et al. 2004, Schaumann, Kraus et al. 2013, Ruiz-Ramirez, Ortiz-Balderas et al. 2014). Further research is necessary to compare results from the cell-based

assays to *in vivo* biomarkers of antioxidants and oxidative stress to determine if these methods are indicative of biological activity before clinical trials can be undertaken for any possible health claims.

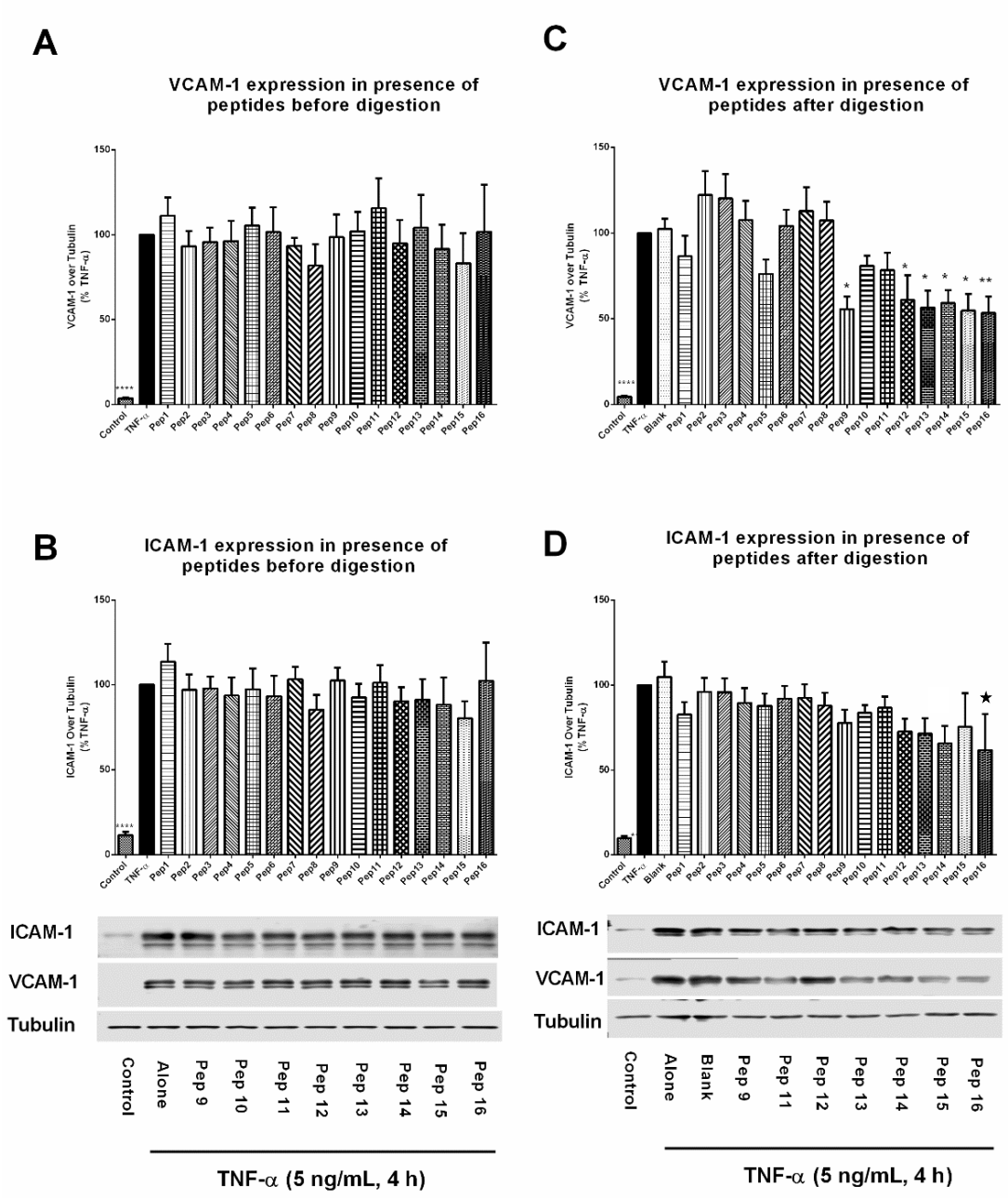


Figure 3-1. Effects of peptides on TNF-α-induced VCAM-1 (A & C) and ICAM-1 (B & D) protein expression before (A & B) and after (C & D) gastro-intestinal digestion. Confluent HUVEC monolayers were pretreated for 20 h with peptides or their digests prior to 4 h of incubation with 5 ng/mL TNF-α. VCAM-1 and ICAM-1 protein levels are expressed as % TNF-α. Bars represent mean values ± SEM, n = 6-12 separate experiments. * and ** indicate $P < 0.05$ and $P<0.01$ as compared to TNF-α respectively. The representative blots for the expression of three proteins; ICAM-1, VCAM-1 and Tubulin in TNF-α treated

HUVECs are also shown. ICAM-1 and VCAM-1 bands are normalized to their corresponding Tubulin band.

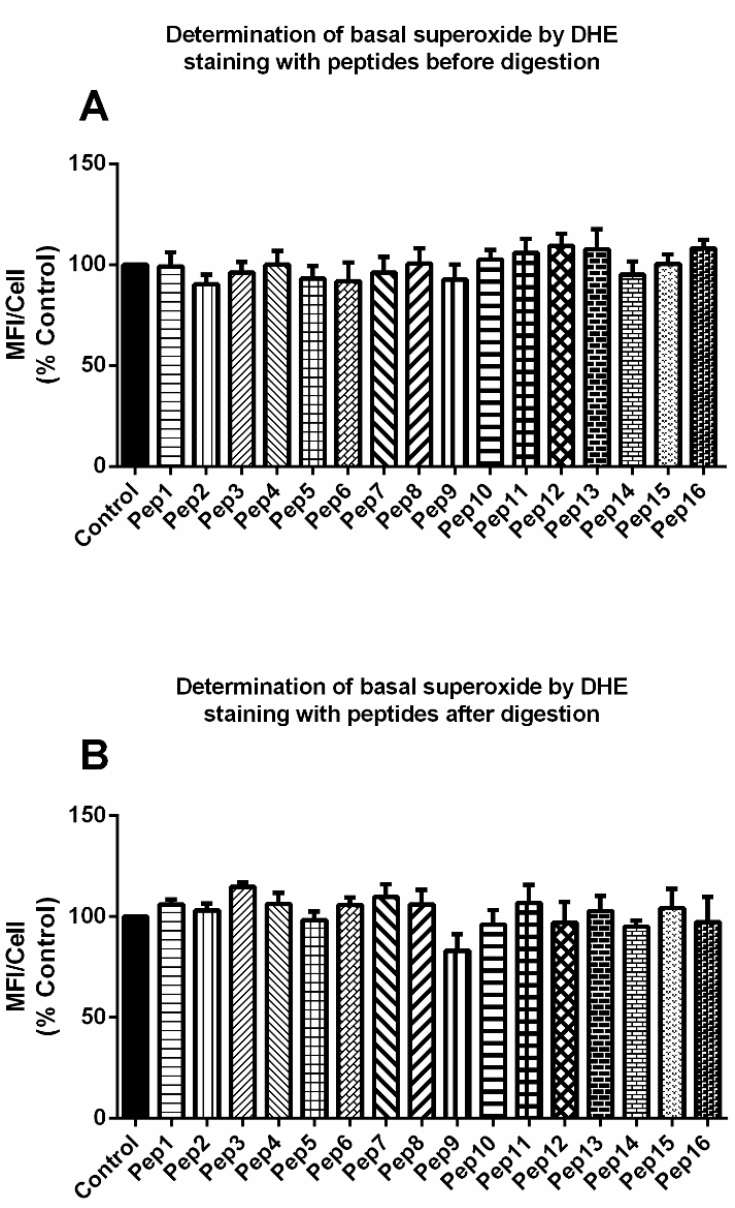


Figure 3-2. Effects of peptides before (A) and after (B) gastro-intestinal digestion on basal endothelial superoxide levels. Confluent HUVEC monolayers were pretreated for 20 h with 50 µM peptides or their digests. Data were calculated as MFI/Cell and expressed as % control. Bars represent mean values ± SEM, n = 4-6 separate experiments.

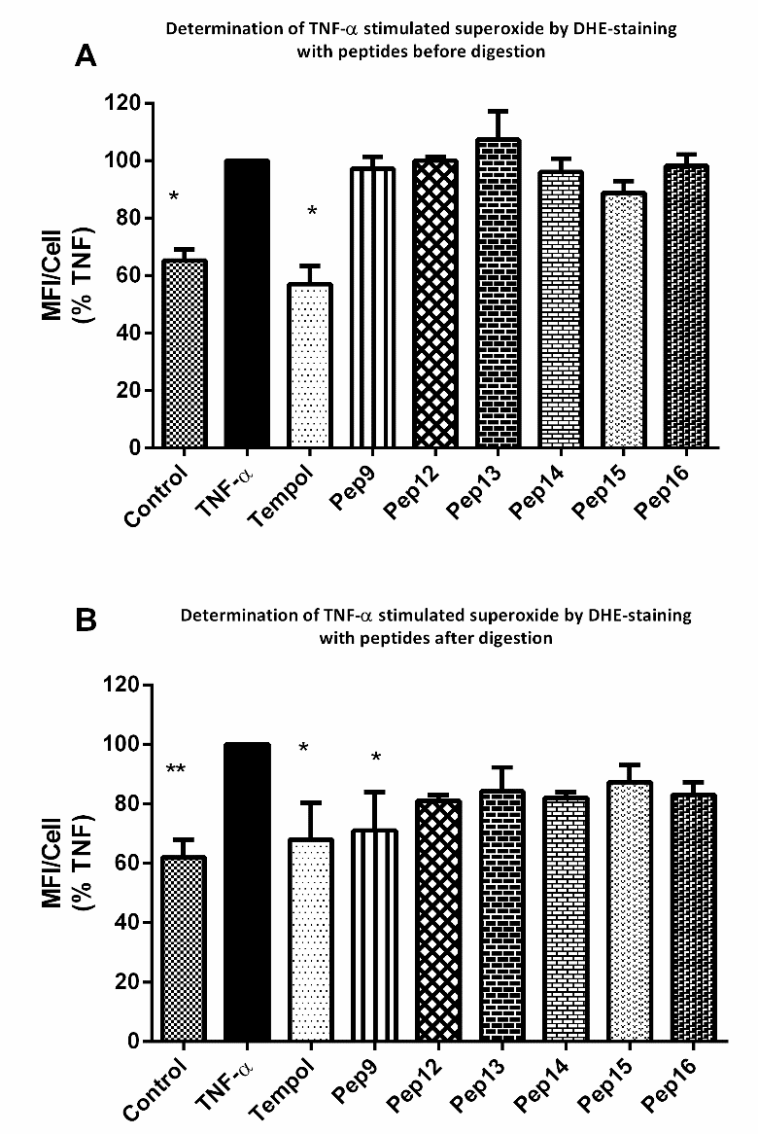


Figure 3-3. Effects of selected peptides before (A) and after (B) gastro-intestinal digestion on TNF-α induced endothelial superoxide levels. Confluent HUVEC monolayers were pretreated for 20 h with 50 μM of peptides or their digests, or 2 h with 20 mM Tempol prior to 1 h of incubation with 10 ng/mL TNF-α. Data were calculated as MFI/Cell and expressed as fold change over the TNF-α. Bars represent mean values (SEM, n = 4-7 separate experiments. * and ** indicate $P < 0.05$ and $P < 0.01$ as compared to TNF-α respectively.

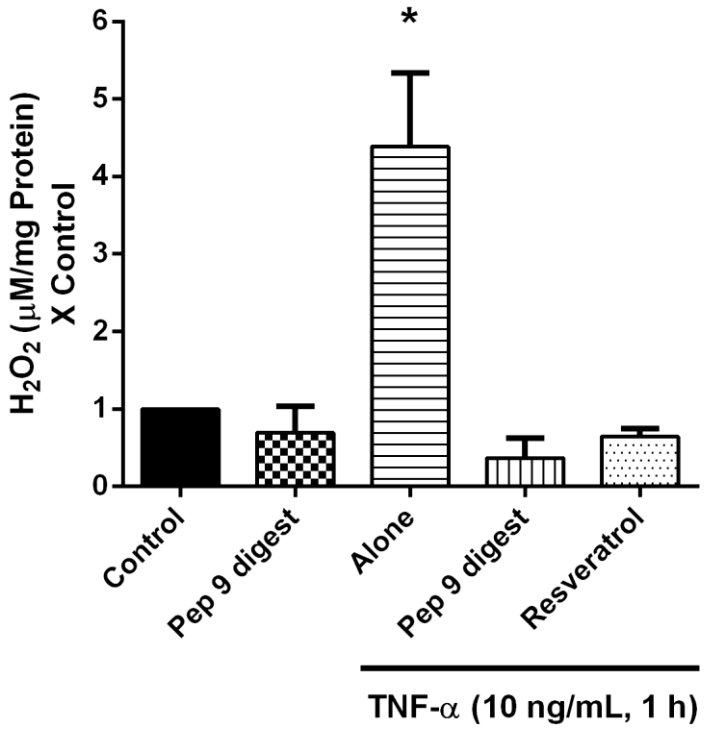
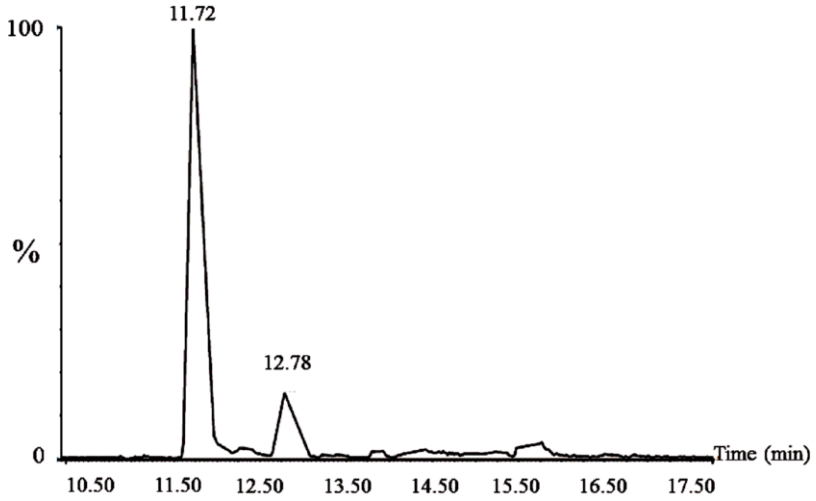
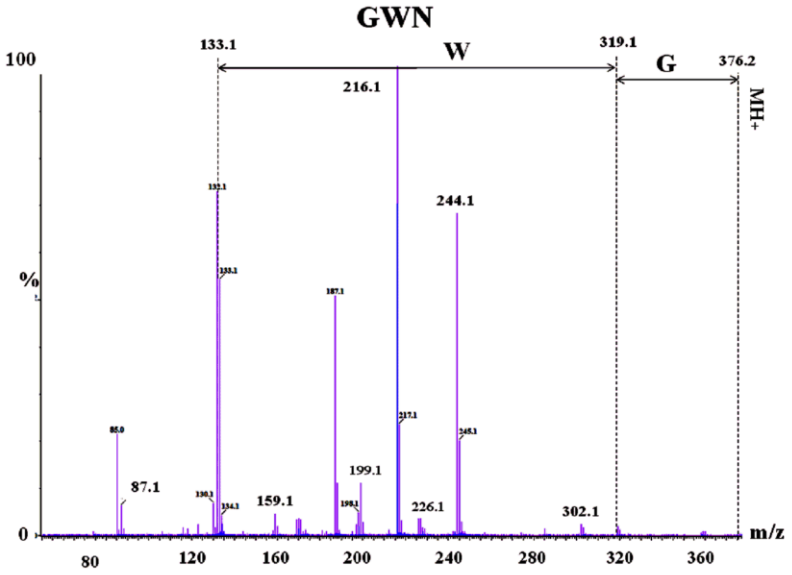


Figure 3-4. Effects of pep 9 digest on intracellular H_2O_2 production in HUVECs treated with/without TNF-α. Confluent HUVEC monolayers were pretreated for 20 h with peptide or resveratrol (as the positive control) at 50 μM concentration prior to 1 h of incubation with 10 ng/mL TNF-α. Cell lysates were prepared and H_2O_2 concentration was obtained using an H_2O_2 standard curve. The protein content in each lysate was determined using BCA method and results were normalized to their corresponding protein level. H_2O_2 levels are expressed as X control. Bars represent mean values ± SEM, n = 4 separate experiments. * indicates $P < 0.05$ as compared to the control (without TNF-α stimulation).

A


11.72
100
%
12.78
0
Time (min)
10.50
11.50
12.50
13.50
14.50
15.50
16.50
17.50

B


GWN
133.1
W
319.1
G
376.2
100
216.1
MH+
244.1
%
87.1
159.1
199.1
226.1
302.1
0
m/z
80
120
160
200
240
280
320
360

C

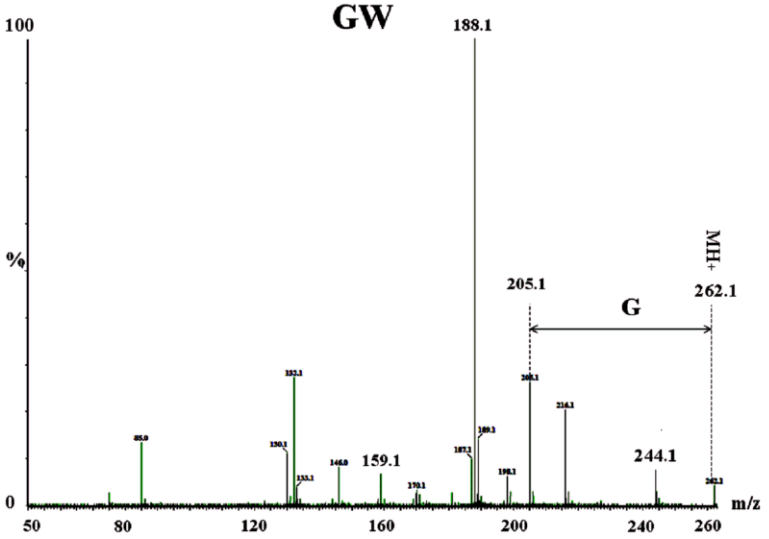

GW
100
188.1
%
MH+
205.1
262.1
G
159.1
244.1
0
m/z
50
80
120
160
200
240
260

Figure 3-5. LC-MS/MS chromatogram of the gastro-intestinal digest of peptide 9 (A). MS/MS spectrum of 376.1 Da at the retention time of 11.72 min (B), and MS/MS spectrum of 262.1 Da at the retention time of 12.78 min (C).

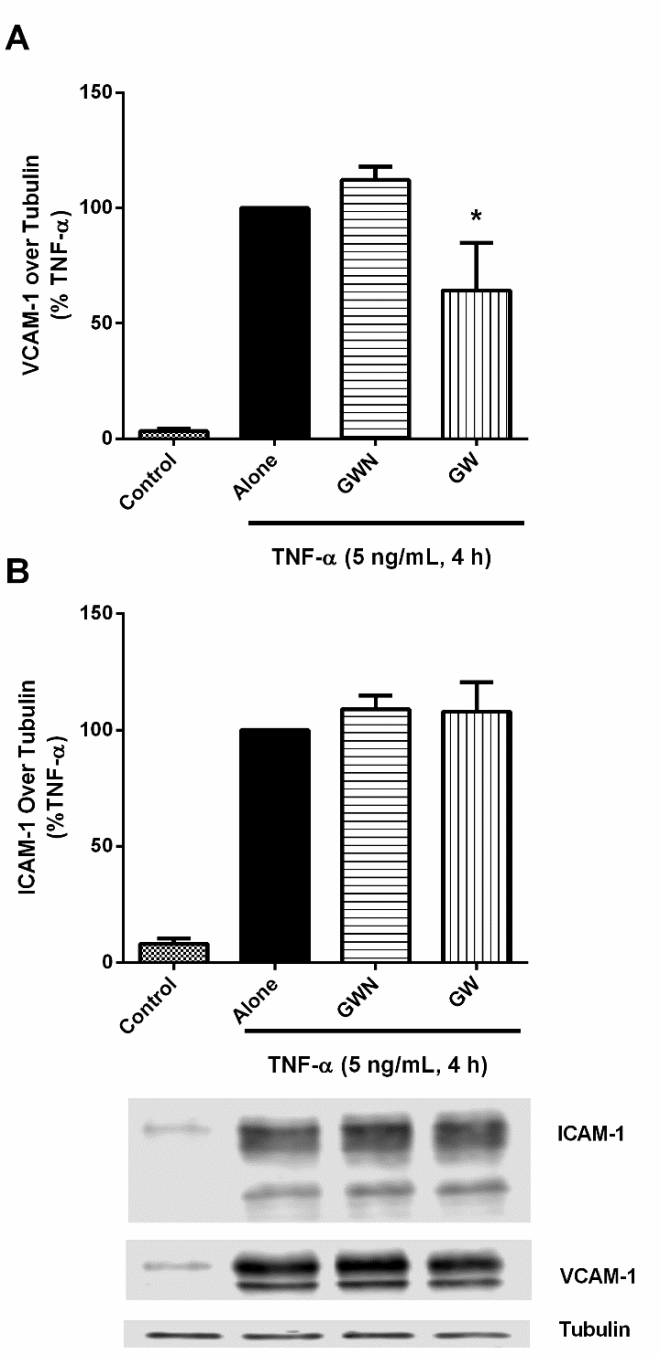


Figure 3-6. Effects of GWN and GW peptides on TNF-α-induced VCAM-1 (A) and ICAM-1 (B) protein expression. Confluent HUVEC monolayers were pretreated for 20 h with peptides prior to 4 h of incubation with 5 ng/mL TNF-α. VCAM-1 and ICAM-1 protein levels are expressed as % TNF-α. Bars represent mean values ± SEM, n = 4 separate experiments. * indicates $P < 0.05$ as compared to TNF-α.

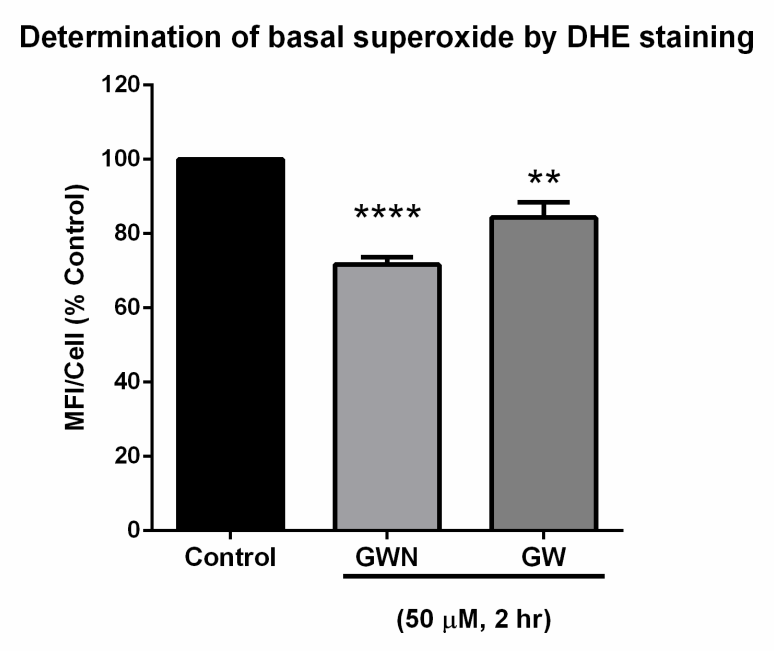
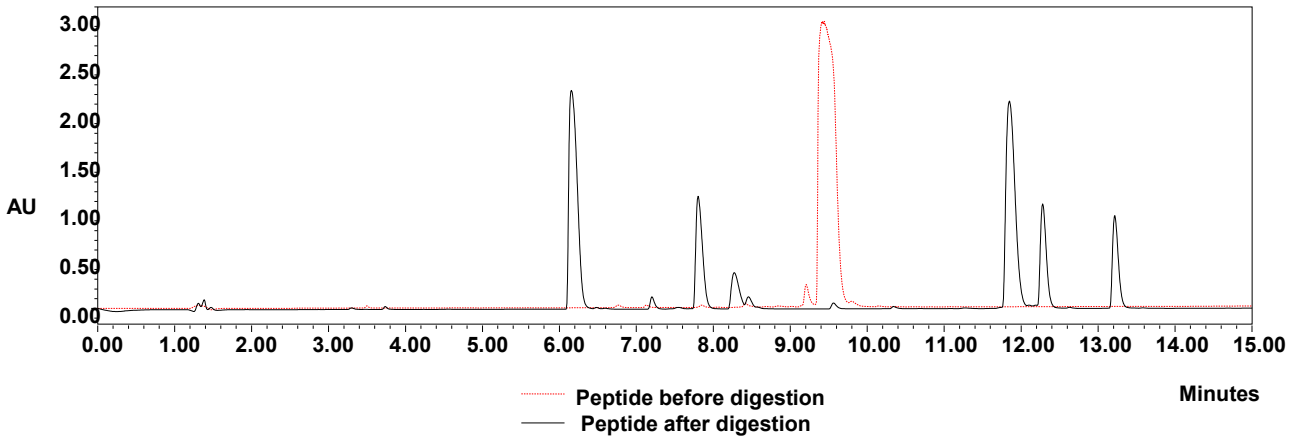


Figure 3-7. Effects of GWN and GW peptides on basal superoxide levels. Vascular smooth muscle cells were pretreated for 20 h with 50 μM of peptides. Data were calculated as MFI/Cell and expressed as % control. Bars represent mean values ± SEM, n = 3-4 separate experiments. ** and **** indicate $P < 0.01$ and 0.0001 as compared to control.

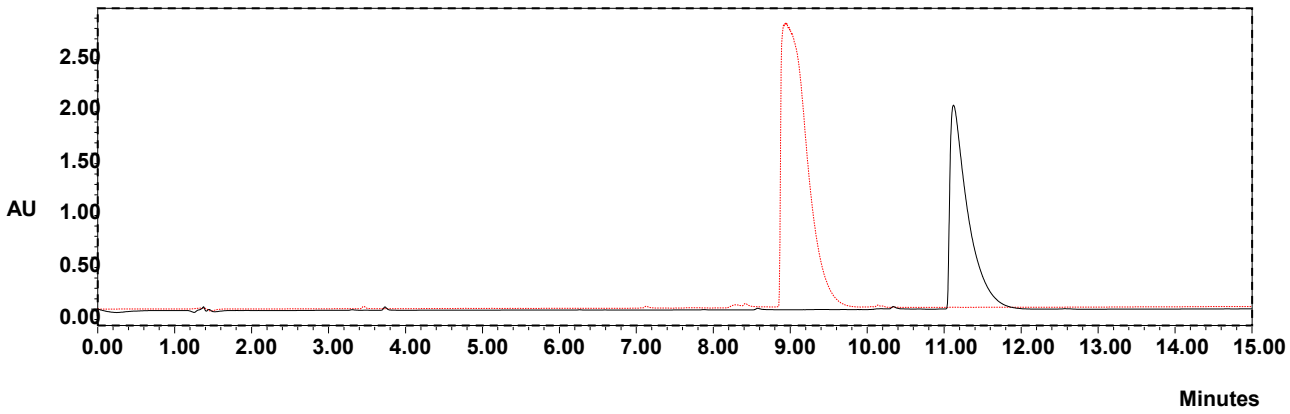
3.4. Appendix 3-A: Supplementary information

UPLC chromatograms of peptides before and after simulated gastro-intestinal digestion

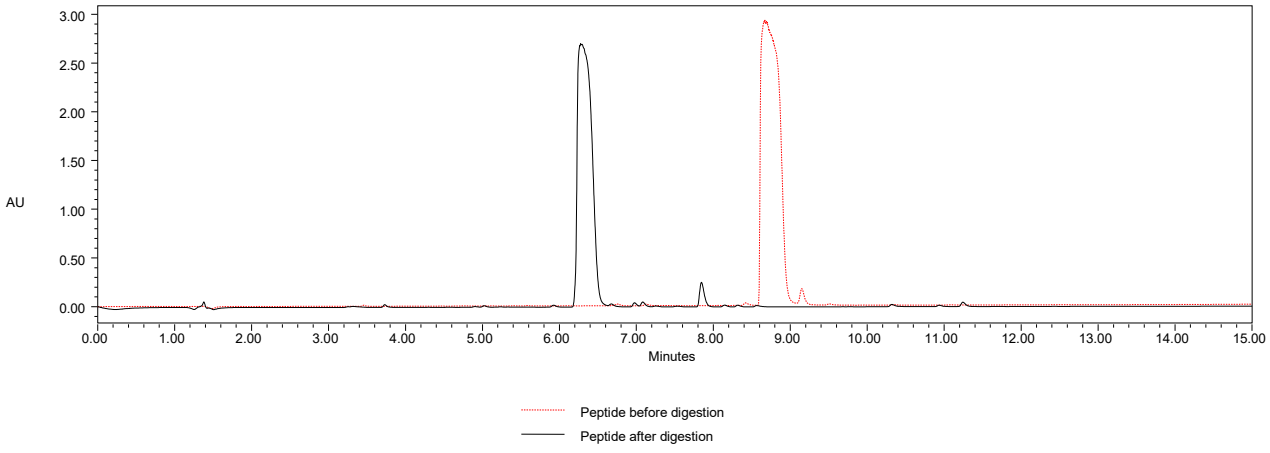
Peptide 1) AGWNIPIGT



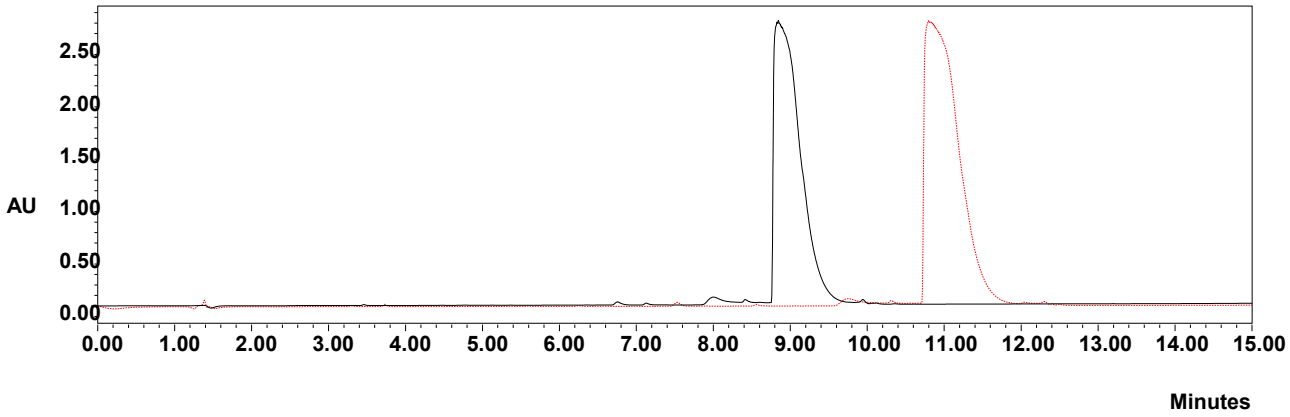
Peptide 2) AGWNIP



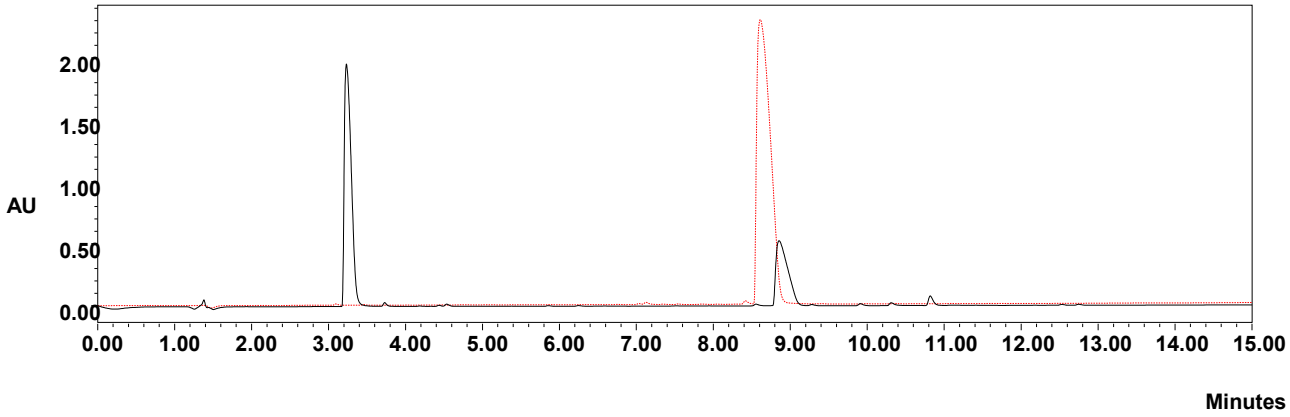
Peptide 3) AGWNI



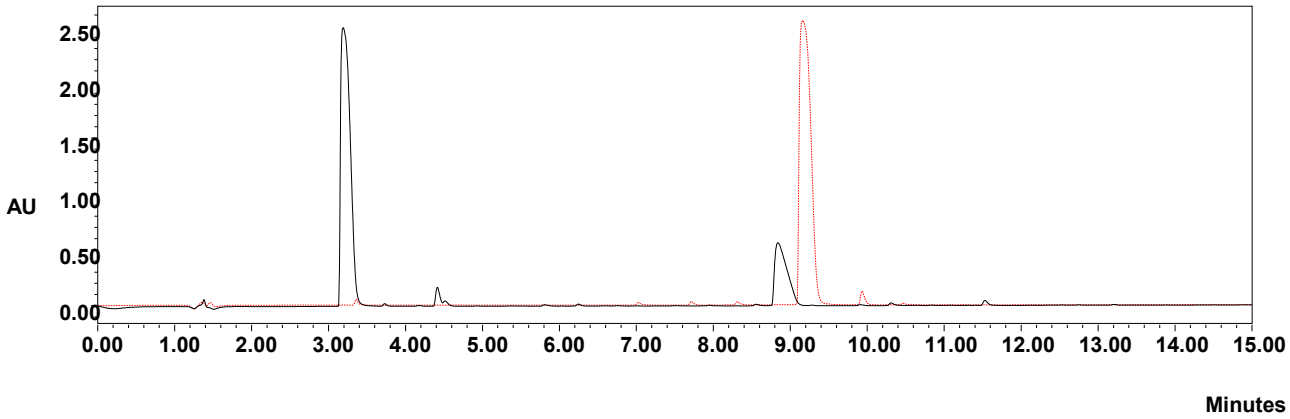
Peptide 4) WNIP



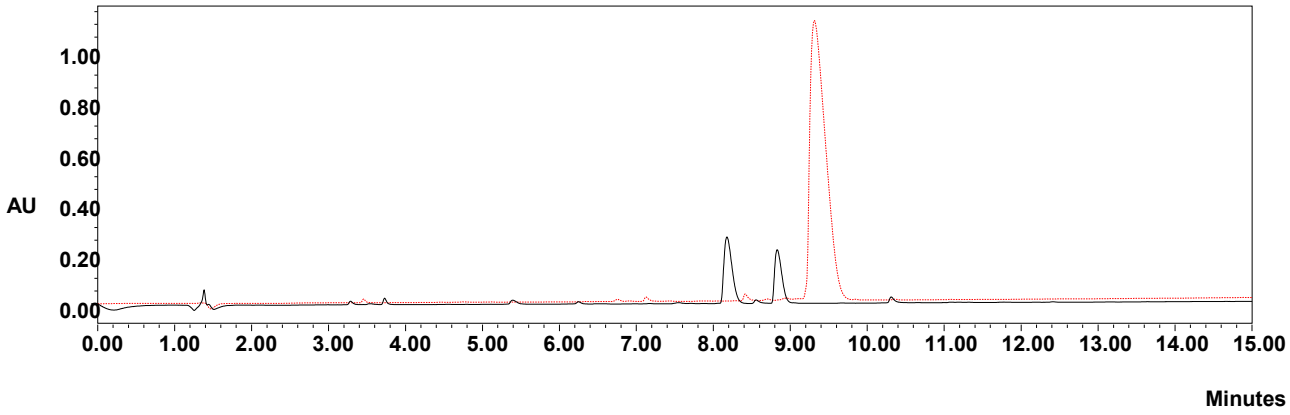
Peptide 5) LGFEY



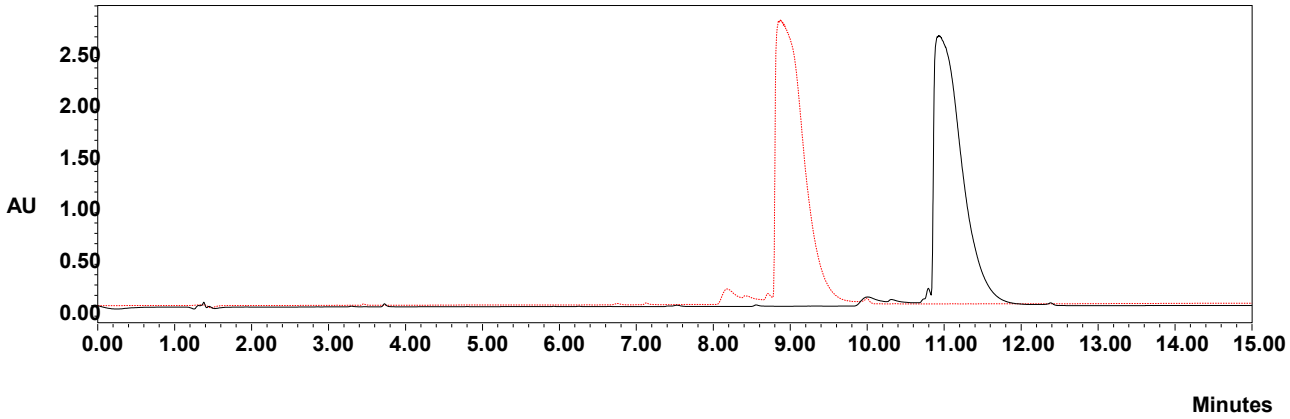
Peptide 6) LGFEYY



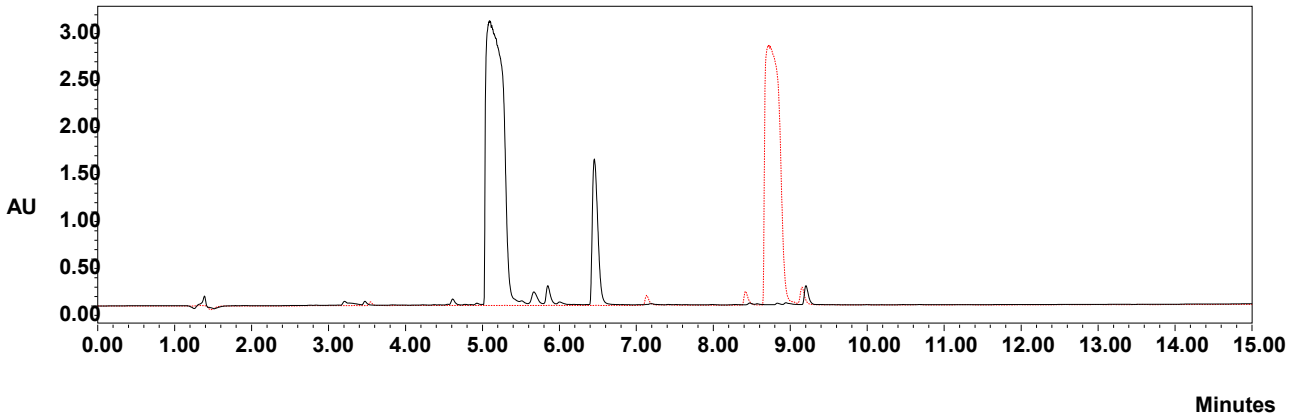
Peptide 7) VIPMGL



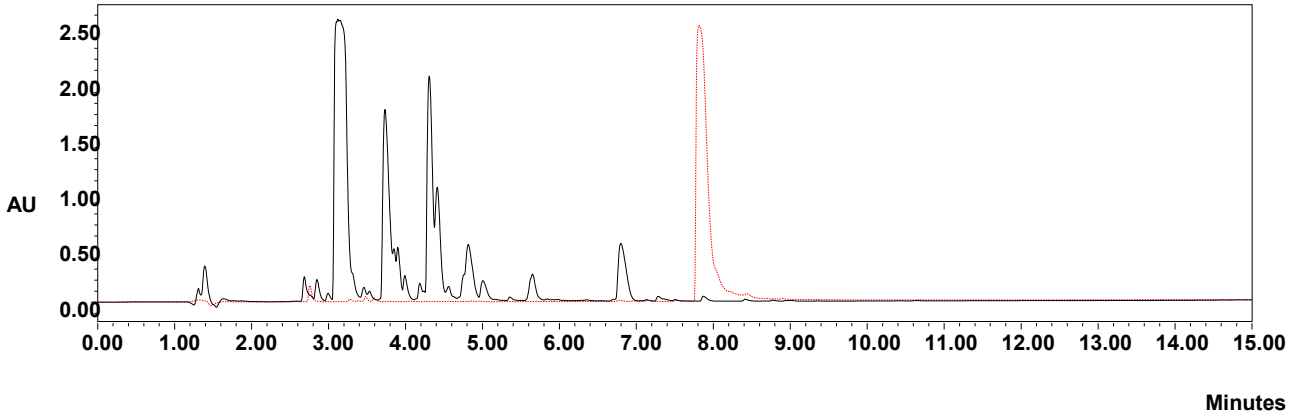
Peptide 8) GWNIP



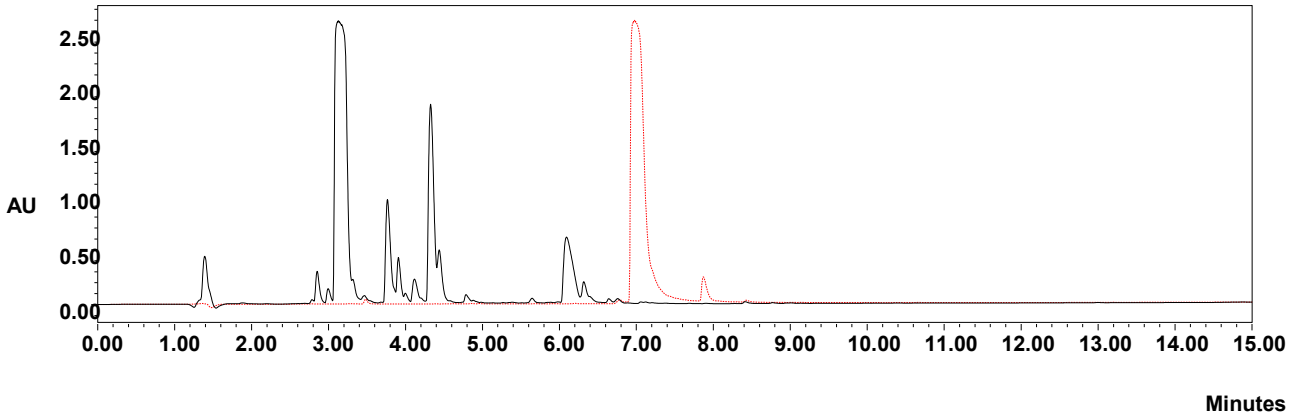
Pep 9) GWNI



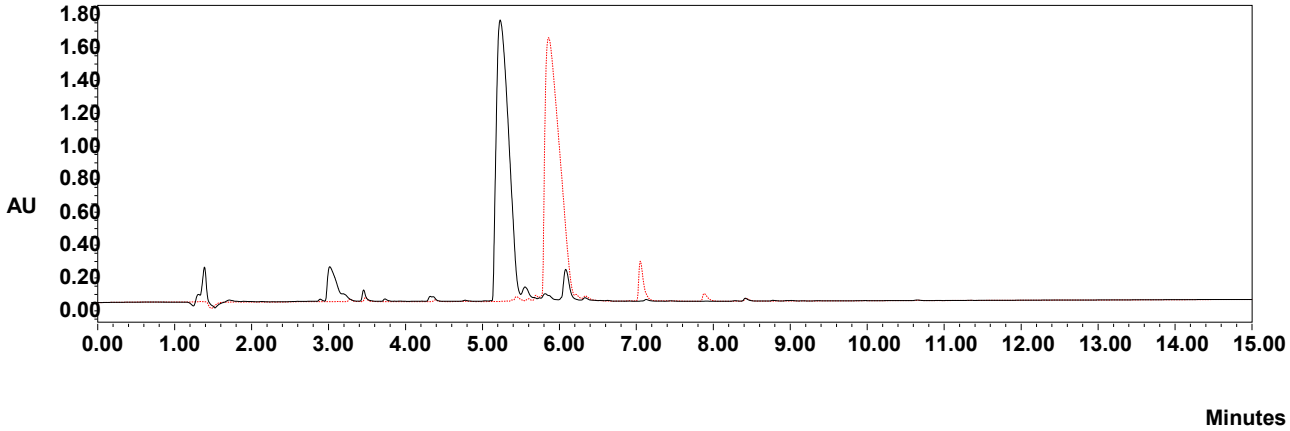
Pep10) PIAAEVYEHTEGSTTSY



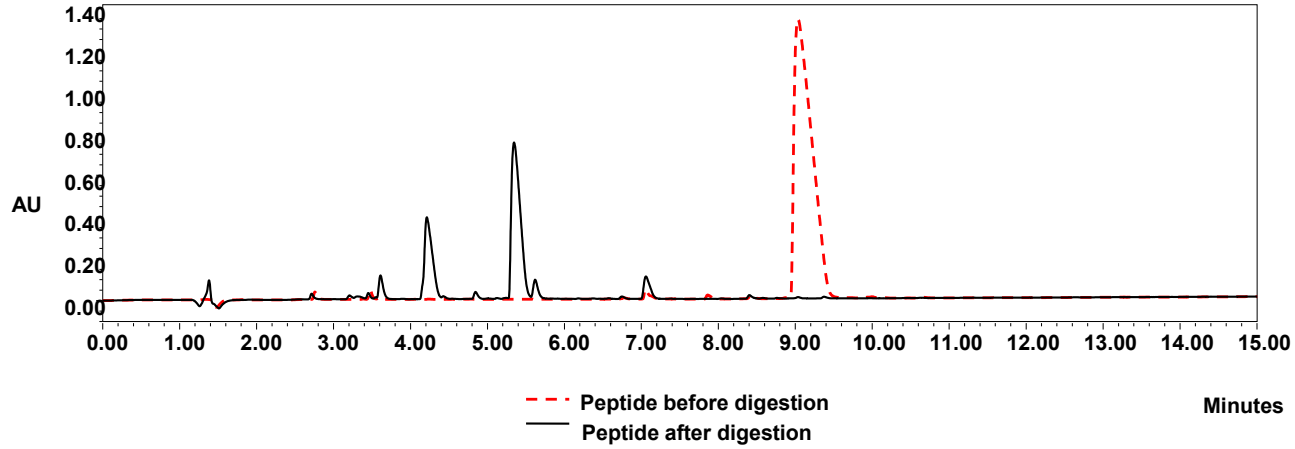
Pep11) IAAEVYEHTEGSTTSY



Pep12) LSKAQSDFG



Pep13) LVEKGDVAFI



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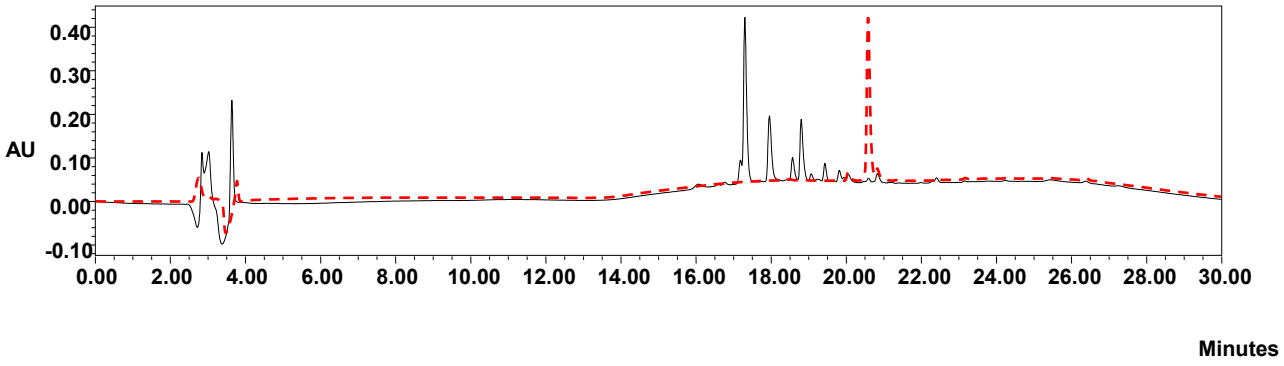

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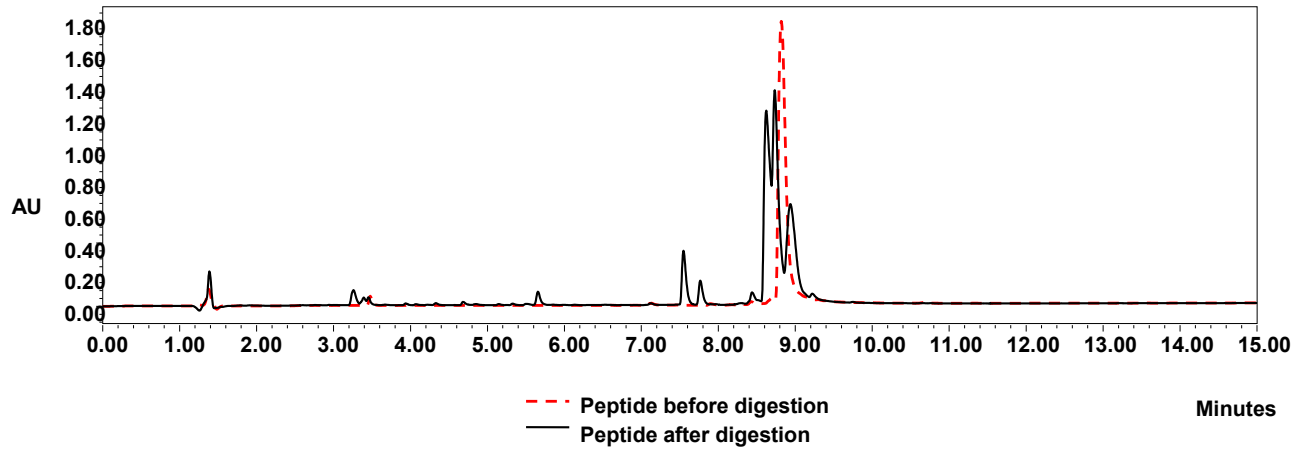

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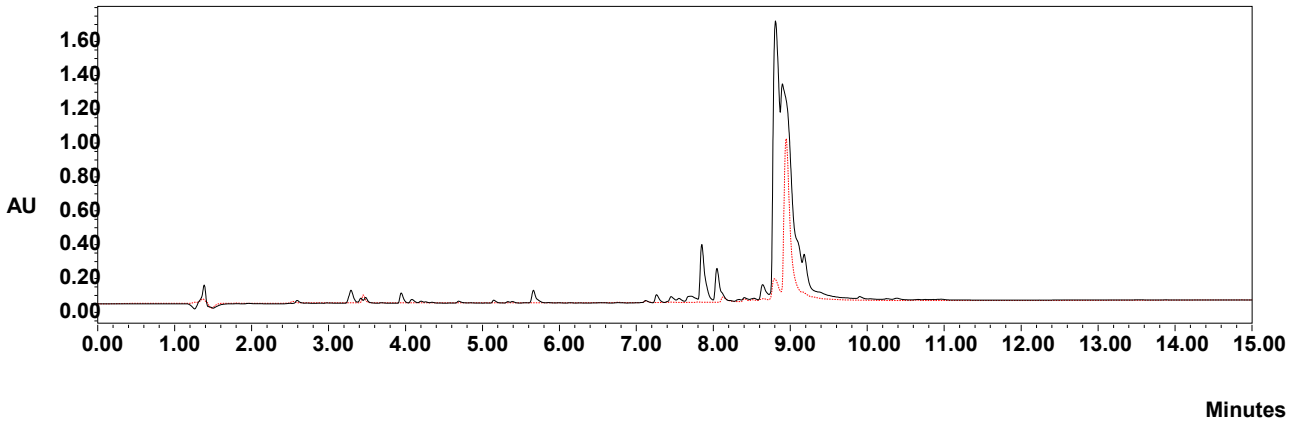


Chapter 4: Egg white protein hydrolysate reduces blood pressure, improves vascular relaxation and modifies aortic angiotensin II receptors expression in spontaneously hypertensive rats[3]

4. Introduction

Hypertension, the persistent elevation of blood pressure (BP) over 140/90 mm Hg, is associated with an increased risk of cardiovascular diseases and is a growing health problem worldwide (Chockalingam 2008, Danaei, Singh et al. 2013). A number of underlying mechanisms, including hyperactivity of the renin-angiotensin system (RAS), inflammation, oxidative stress and impaired nitric oxide generation contribute to the pathogenesis of hypertension. While a number of pharmaceutical anti-hypertensives are currently available for clinical usage, many are known to have significant side-effects in a disease that requires lifelong therapy (Khanna, Lefkowitz et al. 2008). Moreover, some cases of hypertension are not adequately controlled by commonly used pharmaceutical agents (Viera 2012). Hence, there has been a growing interest in developing novel therapies for hypertension from natural sources.

Food derived proteins and peptides are a major source of bioactive compounds with potential therapeutic applications (Saiga-Egusa, Iwai et al. 2009, Balti, Bougatef et al. 2012, Girgih, Alashi et al. 2014, Aluko 2015). Egg is an inexpensive and nutritious source of many proteins which can be used to generate novel bioactive peptides. Our previous research has identified three peptides IRW (Ile-Arg-Trp), IQW (Ile-Gln-Trp) and LKP (Leu-Lys-Pro) from ovotransferrin hydrolysate (prepared by thermolysin & pepsin) with anti-inflammatory,

[3] This chapter has been published: Jahandideh F, Chakrabarti S, Majumder K, Li Q, Panahi S, Morton JS, Davidge ST, Wu J. 2016. *Journal of functional Foods*. 27:667-673.

antioxidant and angiotensin converting enzyme (ACE) inhibitor properties (Majumder, Chakrabarti et al. 2013, Majumder, Chakrabarti et al. 2015). Indeed, these peptides showed potent BP lowering effects in spontaneously hypertensive rats (SHRs), a well-known animal model of hypertension (Dornas and Silva 2011). The isolation of specific proteins like ovotransferrin from egg white as well as using individual bioactive peptides derived from this isolated protein is expensive. Hence, there is a greater feasibility associated with using enzymatic hydrolysates of whole egg white (instead of the constituent proteins or peptides) for therapeutic applications. It is not known if egg white hydrolysate (EWH), as a source of ovotransferrin with *in vitro* ACE-inhibitory activity, would also exert antihypertensive effects *in vivo*. Given this background, we fed EWH (generated by thermolysin & pepsin) orally to SHRs to evaluate its potential as an anti-hypertensive treatment and examine the mechanisms of action focusing on various aspects of the RAS.

4.1. Materials and methods

4.1.1. Preparation of egg white hydrolysate (EWH)

Hydrolysis of egg white was carried out as described previously (Majumder and Wu 2011). Briefly, egg white slurry (5%, w/v) was first heated to 80°C for 10 min and digested with thermoase PC10F (Amano Enzyme Inc. Nagoya, Japan) 0.1% w/w at pH 8 and 65°C for 90 min. The pH was then adjusted to 2.5 for pepsin digestion. Pepsin (from porcine stomach, 10000 units/ mg, American Laboratories Inc. Omaha, NE, USA) was added at 1% w/w to the mixture at 55°C and hydrolysis was carried out for another 180 min. After heat inactivation (T= 90 °C for 15 min) of enzymes, the hydrolysate was centrifuged, and supernatant was collected and freeze dried for further experiments. The average protein content of the hydrolysate was 77.69%.

4.1.2. Ethics statement, animal model and experimental design

Rat experimental procedures were approved by the University of Alberta Animal Welfare Committee (Protocol # 611/09/10D) in accordance with the guidelines issued by the Canadian Council on Animal Care and also adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The feeding experiments were carried out using fourteen to sixteen week-old male spontaneous hypertensive rats (SHRs) weighing 290.0 ± 10.5 g obtained from Charles River (Senneville, QC, Canada). Upon arrival, rats were acclimatized for one week at the University of Alberta animal facility with a 12:12 hour cycle of light:dark in a humidity and temperature controlled (60% RH, and 23 °C) environment and maintained on standard chow and water *ad libitum*. After one-week acclimation, SHRs were chronically implanted with DSI telemetry transmitters (PA-C40; Data Sciences International, Minneapolis, MN) as described previously (Majumder, Chakrabarti et al. 2013). After one-week recovery following surgery, rats were randomly assigned into 3 groups (n=6-7): untreated (control), EWH low dose (250 mg/kg BW) and EWH high dose (1000 mg/kg BW). EWH dosages were chosen based on the previous studies done in our lab (Jahandideh, Majumder et al. 2014). Treatments were given orally once a day for 12 days after mixing with 20 mL of Ensure (Abbott Nutrition, QC, Canada). Ensure was used to enhance the palatability of the hydrolysate. The untreated group received the same volume of Ensure only (Jahandideh, Majumder et al. 2014). BP was recorded on days 0 (baseline), 3, 6, 9, and 12 for 24 h (10 sec of every 1 min) for all animals. On the morning of day 13, the animals were euthanized by exsanguination via excision of the heart under inhaled isoflurane anesthesia (isoflurane/oxygen; 1.0-2.5% mixture). Following sacrifice, blood was collected from the heart in EDTA coated tubes (BD Vacutainer, NJ, USA), and centrifuged (1,000g for 20 min at 4 °C) to obtain plasma. Tissues were removed immediately, rinsed with cold saline, weighed, flash frozen with liquid

nitrogen and stored at -80 ºC for further analysis. The mesenteric arteries were isolated immediately and used for *ex vivo* vascular function studies.

4.1.3. Data acquisition and signal processing

Chronic measurement of BP was done in a quiet room with minimal electrical interference as previously described (Jahandideh, Majumder et al. 2014). Mean arterial blood pressure (MAP) was measured while systolic blood pressure (SBP) and diastolic blood pressure (DBP) were extracted from the observed signal. Heart rate (HR) was calculated between two consecutive points and expressed in beats per minute (bpm).

4.1.4. Vascular function studies

Second order branches of the mesenteric arteries were carefully excised from SHRs and used for *ex vivo* vascular function experiments. Isolation, mounting, normalization and validating integrity of vessels was the same as described before (Jahandideh, Majumder et al. 2014). Constrictor responses of vessels were determined by assessing responses to phenylephrine (PE, 10^{-8} to 10^{-4} M, Sigma Aldrich, Oakville, Canada). In two sets of vessels, the role of nitric oxide (NO) in endothelium-dependent relaxation was assessed via studying the methacholine (MCh, 10^{-10} to 10^{-4} M, Sigma) relaxation response of vessels in the presence or absence of the nitric oxide synthase (NOS) inhibitor N-nitro-L-arginine methyl ester (L-NAME, 100 μM, Sigma). The contribution of vascular smooth muscle to relaxation was also assessed in a separate set of vessels through studying the relaxation response of vessels to sodium nitroprusside (SNP, 10^{-10} to 10^{-5} M, Sigma); which is an endothelium-independent relaxing agent. At the end of the experiment, the vessels were exposed to high potassium buffer to confirm their viability. Vessels with constriction less than 80% of their maximum constriction to PE were excluded from analysis.

4.1.5. Plasma analysis for circulating Ang II

Plasma concentrations of angiotensin II (Ang II) were quantified by ELISA kit (Ang II ELISA, Cayman Chemical, Ann Arbor, MI, USA) based on the manufacturer's instructions.

4.1.6. Immunofluroscence

Aortic specimens were embedded in Tissue-Tek O.C.T Compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) and frozen immediately in liquid nitrogen for subsequent analysis. 10 μm tissue sections were prepared, fixed in cold acetone and incubated with blocking buffer (1% BSA in phosphate-buffer saline) for 1 hour. The sections were then immunostained overnight at 4 ˚C with rabbit polyclonal antibodies for nitrotyrosine (Dilution 1:200; Chemicon, Temecula, CA, USA), ACE, ACE2, angiotensin II type 1 (AT1R) and type 2 receptor (AT2R) (Dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Incubation with a secondary antibody (Dilution 1: 500; Alexa Fluor 546 (red), Invitrogen, Burlington, ON, Canada) was performed for 30 min in the dark at room temperature. For nitrotyrosine, glass cover-slips were mounted with a Vectashield H-1200 Mounting Kit, containing nuclear stain, DAPI (Vector Laboratories, Burlington, ON, Canada) on the corresponding slides and immediately visualized under an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). Images were obtained using SlideBook imaging software (Olympus) and presented at 100x magnification. For ACE, ACE2, AT1R and AT2R, glass cover-slips were mounted with PBS and scanned by Odyssey Sa system (Licor Biosciences) with 20 μm resolution. A control image with the secondary antibody alone was used to detect any nonspecific binding. The images were then quantified by subtracting the background fluorescence in the control image, so only the fluorescence from specific immunostaining was visible.

4.2. Statistical analysis

All data presented are mean ± SEM of 4-7 experiments, as indicated in the figure legends. MCh curves were fitted using nonlinear regression, and values for area under the curve (AUC) were compared. Statistical analysis was performed using GraphPad Prism software (version 6.0). For analyses of BP data, we used a two-way analysis of variance (ANOVA) with Tukey's post-hoc test. For all other studies, a t-test was used for comparison between 2 groups while a one-way ANOVA (with Dunnett's post-hoc test) was performed for comparison involving 3 or more groups. A p-value < 0.05 was considered statistically significant.

4.3. Results and Discussion

4.3.1. EWH reduces BP in SHRs

Baseline MAP in all experimental groups was above 160 mmHg; which is an indication of hypertension in SHRs. The pathogenesis of hypertension in SHRs is similar to human essential hypertension (Okamoto and Aoki 1963). In this study, although both doses of EWH tended to reduce BP in SHRs over the experimental period compared to the untreated group, this reduction was only significant in the high dose treated group (1000 mg/kg BW) ($p<0.001$, Figure 4-1A-C). HR was not different between the groups (Figure 4-1D). Hydrolysis of egg white breaks down the protein structure generating bioactive peptides and possibly free amino acids which may be critical for the antihypertensive effects. Hydrolysis of the egg white with thermolysin and pepsin generated a complex array of peptides which could potentially contribute to the observed antihypertensive effects. The hypotensive effects of egg-derived peptides have been reported in the literature (Matoba, Usui et al. 1999, Miguel, Lopez-Fandino et al. 2005, Yu, Zhao et al. 2011, Majumder, Panahi et al. 2013, Rawendra, Aisha et al. 2013, Duan, Wu et al. 2014, Jahandideh, Majumder et al. 2014). Utilizing protein hydrolysates instead of single peptides has the

advantage of reduced cost of processing and easier incorporation into food system as a natural component. Moreover, the available peptides in the whole protein hydrolysate may have other beneficial biological activities. Since the BP was only significantly changed in high dose EWH treated animals, all the subsequent experiments were carried out on this group compared to untreated group.

4.3.2. EWH improves vascular relaxation, reduces nitrosative stress and modifies Ang II receptors expression

SHRs show impaired vasodilation, higher circulating levels of Ang II as well as increases in oxidative stress and inflammation compared to normal rats (Zicha and Kunes 1999). The effect of EWH on vascular function in SHRs was evaluated as one of the possible mechanisms for its antihypertensive effects. Since mesenteric arteries contribute significantly to the alteration of BP through affecting systemic vascular resistance (Pannirselvam, Wiehler et al. 2005), this vascular bed was studied to investigate the effects of the treatment on *ex vivo* vascular function. As evident in Figure 4-2A, vasodilation to MCh was significantly enhanced by treatment with EWH compared to the untreated group ($p<0.05$). Enhanced NO is one of the possible endothelium-dependent mechanisms of vasodilation. Incubation of mesenteric arteries with L-NAME, a NOS inhibitor, reduced vasodilation in both untreated and EWH treated animals (Figures 4-2B & 4-2C), suggesting a contribution of NO to relaxation in both groups. However, EWH further enhanced NO-dependent vasodilation compared to the untreated group as shown by a significant increase in the delta area under the curve (Δ AUC) of the MCh curves obtained for each of the two groups with and without NOS inhibition ($p<0.05$, Figure 4-2D). This increase in NO-dependent vasodilation may be due to the enhanced NO bioavailability through scavenging of free radicals or increased NO production in the vasculature. Vascular relaxation to SNP, an exogenous NO donor, was also significantly enhanced in EWH treated group compared to the

untreated group ($p<0.05$, Figure 4-3); but to a lesser extent than MCh responses. This implies the involvement of either endothelium-independent mechanisms (in addition to endothelium-dependent ones) or enhanced NO bioavailability in EWH treated compared to untreated group.

When the effect of EWH treatment on nitrosative stress in aortic sections of SHRs was further studied, we observed a significant decrease in aortic nitrotyrosine staining in EWH treated animals ($p< 0.01$, Figure 4-4). This may explain the improved NO bioavailability and vasodilation effects observed in the vascular function experiments.

Ang II, the principal component of the RAS pathway, has diverse physiological actions regulating blood pressure and salt/water balance; elevated circulating Ang II level leads to high BP in SHR. Since EWH was initially identified as an *in vitro* ACE inhibitor, plasma Ang II levels were assessed in both EWH and untreated animals. Plasma Ang II levels were not significantly different between EWH and untreated groups ($p>0.05$, Figure 4-5), indicating that EWH may not work solely as an ACE inhibitor in reducing BP in SHRs.

However, ACE expression in the EWH treated animals was significantly reduced compared to the untreated animals ($p<0.05$, Figure 4-6A). ACE2 is another contributor to Ang II plasma level; however, feeding EWH did not affect the expression level of ACE2 ($p>0.05$, Figure 4-6B).

We further assessed the expression level of Ang II receptors. Ang II acts through two main receptors, angiotensin type 1 and type 2 receptors. Binding to AT1R receptor causes vasoconstriction in vascular smooth muscle cells and other potentially harmful consequences while binding to AT2R receptor induces opposing effects (Balakumar and Jagadeesh 2014). Interestingly, EWH significantly reduced the expression of AT1R and enhanced concomitantly that of AT2R in SHRs compared to the untreated group ($p< 0.05$, Figures 4-7A & 7B).

Since the AT1R is responsible for the most known pathogenic effects of Ang II in the body, this finding offers a potentially novel way to attenuate the effect of an overactive RAS pathway. This beneficial effect was further enhanced by increased AT2R expression, contributed collectively to improved vasodilation in vascular function experiments. Therefore, although rats in the EWH and untreated groups had almost the same levels of circulating Ang II, the antihypertensive effects observed upon EWH feeding were due mainly to different expression levels of Ang II receptors. It is possible that the EWH treatment increases the breakdown of receptor protein and/or reduces the protein synthesis of the receptor. To the best of our knowledge, this is the first study reporting the effects of a food compound on modulating AT1R and AT2R expression *in vivo*.

Since BP is regulated by several organs in the body and is sex-specific, using only male SHRs and lack of normotensive control rats is one of the limitations of our study. The effects of EWH on sympathetic nervous system through binding to opioid receptors remains to be answered in future studies. It is also interesting to investigate the effects of EWH on BP post treatment to see if the effect on BP remains or not.

In conclusion, the present study demonstrated the role of EWH in reducing BP in SHRs through several mechanisms. The reduction in BP was concomitant with an increased vasodilation, reduced nitrosative stress, reduced ACE and AT1R expression, enhanced AT2R expression. While our findings are novel, further research is needed to ascertain the role of EWH on mechanisms involved in endothelial independent vasorelaxation to achieve a comprehensive understanding of underlying mechanisms. The findings from this study may establish the potential of egg derived bioactive peptides in the management of hypertension and associated complications.

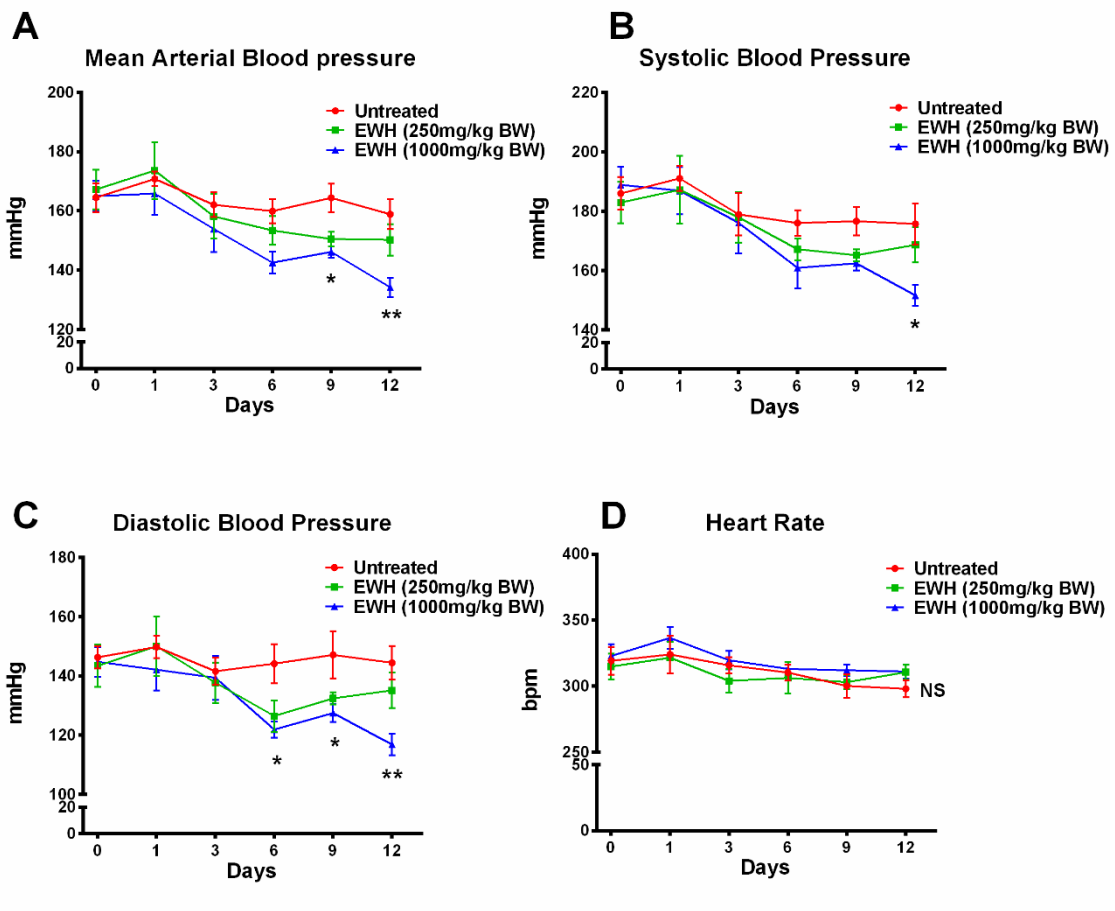


Figure 4-1. Egg white hydrolysate (EWH) reduces BP in SHRs. (A, B, and C) MAP, SBP, and DBP (mmHg) values for untreated or EWH treated (250 and 1000 mg/Kg BW) SHRs over a period of 12 days. BP values for each represent the mean BP recorded over a 24 hr period. (D) HR (bpm) of SHRs in treatment groups over 12 days. Treatment with EWH (1000 mg/Kg BW) significantly lowered MAP (A), SBP (B), and DBP (C) but not heart rate (D). Data represented as mean ± SEM from n=6–7 animals per treatment group. * indicates $p<0.05$ and ** indicates $p<0.01$ compared to untreated group. NS indicates not significant compared to the untreated group.

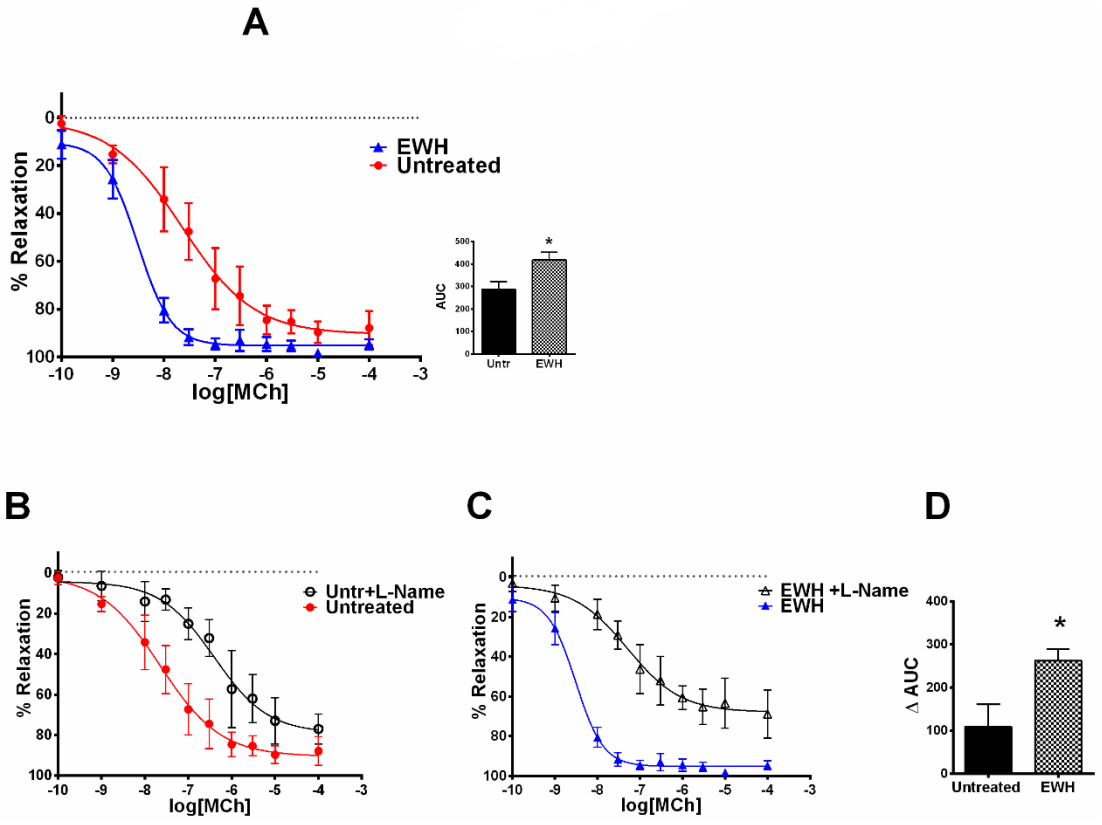


Figure 4-2. EWH treatment induces nitric oxide contribution to vasodilation in mesenteric arteries of SHRs. (A) EWH (1000 mg/ Kg BW) significantly increased vasorelaxation to MCh. Pre-incubation with L-NAME (100 µM) reduced vasorelaxation in untreated rats (B) as well as EWH treated rats (C). EWH further enhanced NO dependent vasorelaxation compared to untreated group (D). Data represented as mean ± SEM from n=4–6 animals per treatment group. * indicates $p<0.05$ compared to untreated group. AUC = Area Under the Curve.

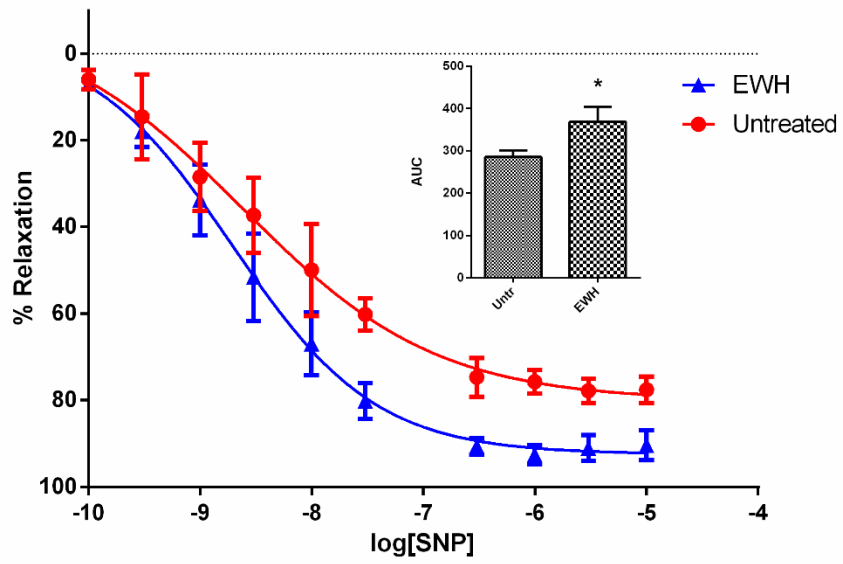


Figure 4-3. EWH treatment enhances vasodilation in the presence of exogenous nitric oxide in mesenteric arteries of SHRs. EWH (1000 mg/ Kg BW) significantly increased vasorelaxation to SNP in SHRs. Data represented as mean ± SEM from n=4–7 animals per treatment group. * indicates $p<0.05$ compared to untreated group. AUC = Area Under the Curve.

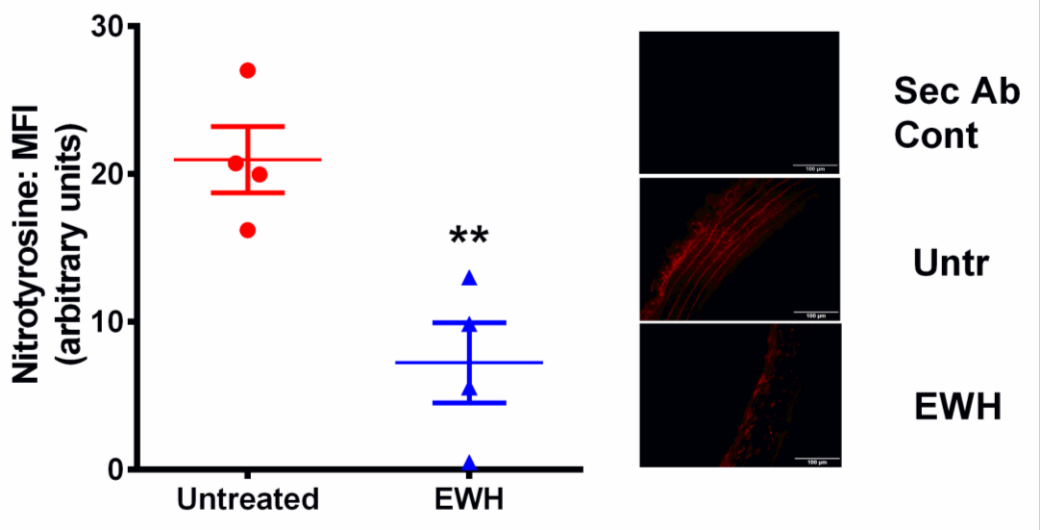


Figure 4-4. EWH treatment reduces tissue nitrotyrosine in SHRs. Immunostaining for nitrotyrosine in aortic sections of untreated and EWH (1000 mg/ Kg BW) treated SHRs. Data represented as mean ± SEM from n=4 animals per treatment group. ** indicates $p<0.01$ compared to untreated group.

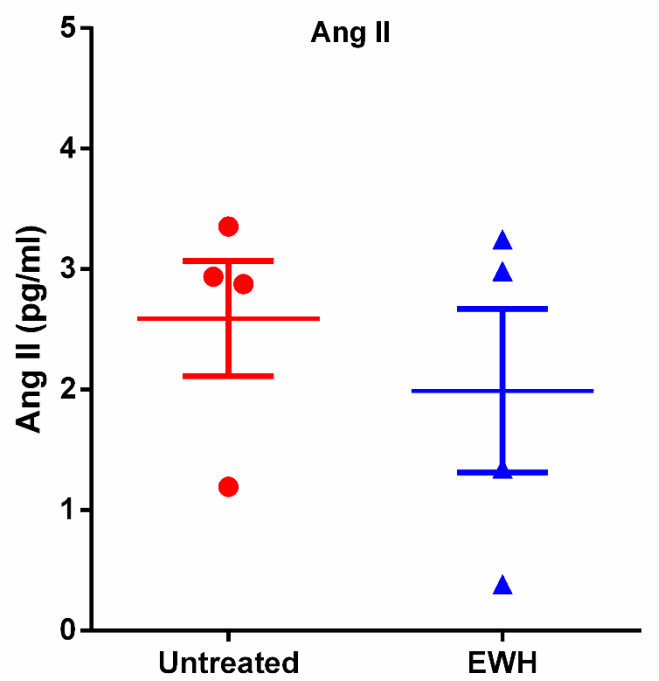


Figure 4-5. Effects of EWH on plasma circulatory Ang II. Ang II level was not affected after 12 days of treatment with EWH (1000 mg/ Kg BW). Data represented as mean ± SEM from n=4 animals per treatment group.

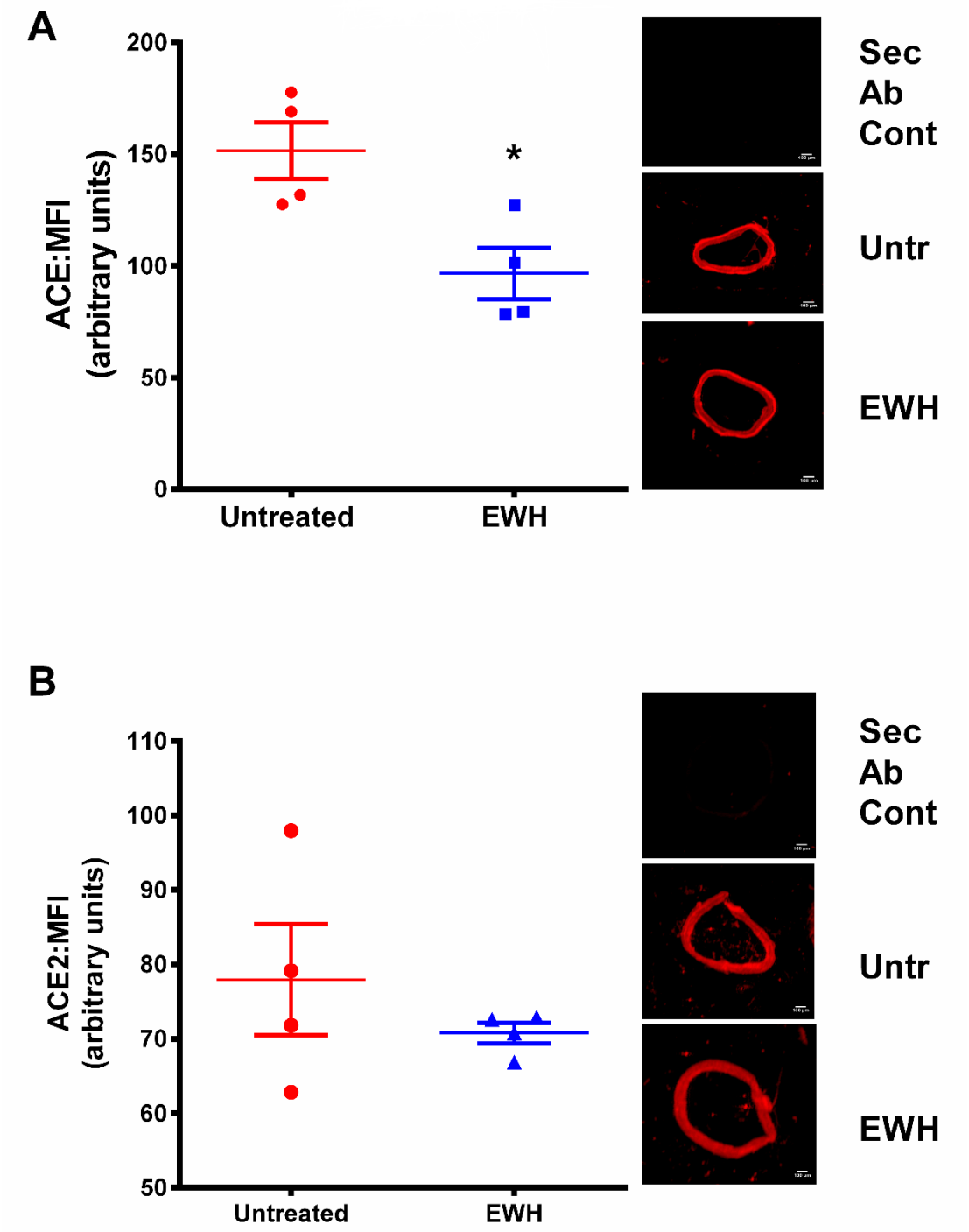


Figure 4-6. EWH reduces ACE but not ACE2 expression in aorta of SHRs. Immunostaining for ACE (A) and ACE2 (B) in aortic sections from untreated and EWH (1000 mg/ Kg BW) treated SHRs. Data represented as mean ± SEM from n=4 animals per treatment group. * indicate $p<0.05$ compared to untreated group.

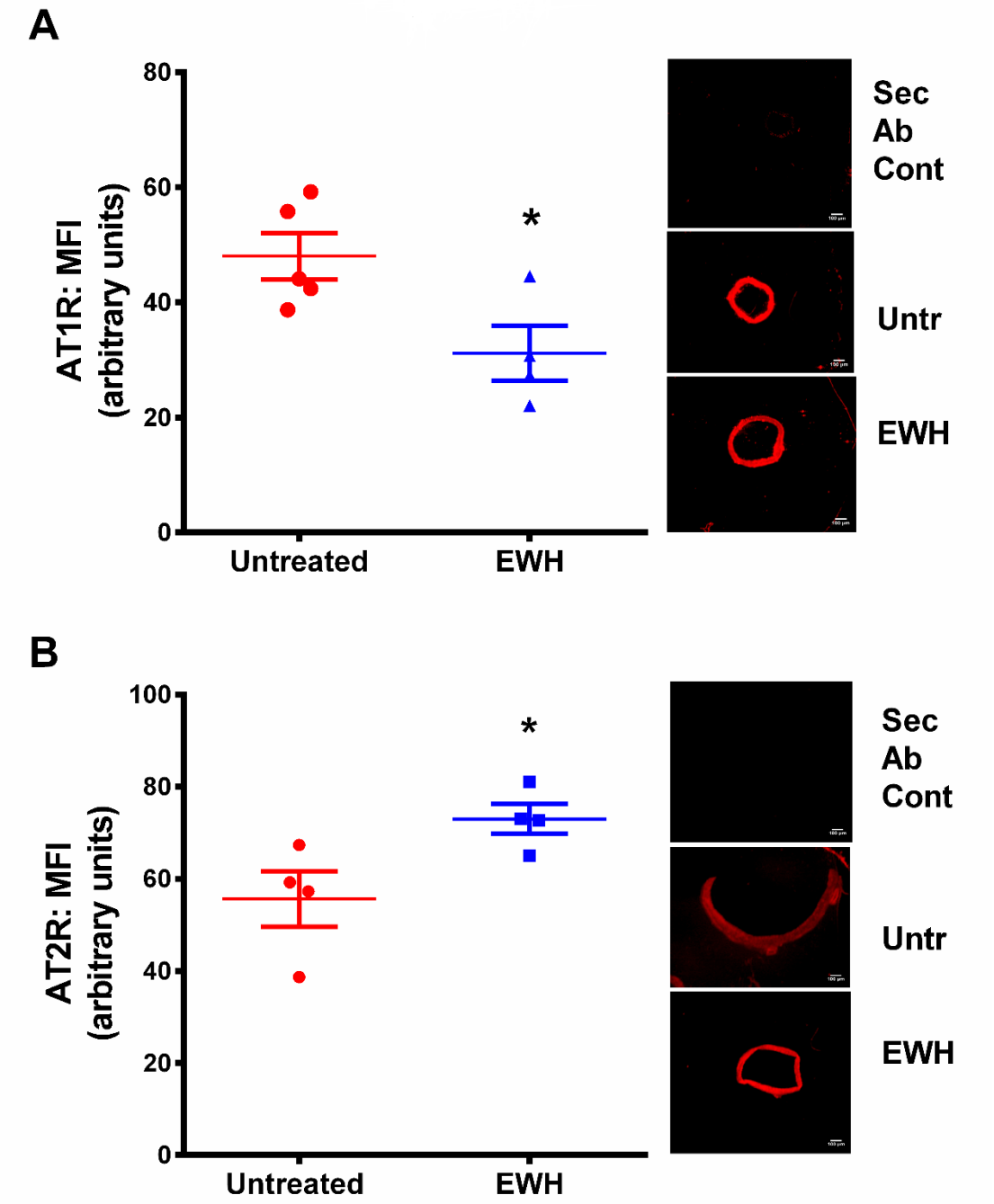


Figure 4-7. EWH reduces AT1R and increases AT2R expression in aorta of SHRs. Immunostaining for AT1R (A) and AT2R (B) in aortic sections from untreated and EWH (1000 mg/ Kg BW) treated SHRs. Data represented as mean ± SEM from n=4-5 animals per treatment group. * indicate $p<0.05$ compared to untreated group.

Chapter 5: Egg white hydrolysate shows insulin-mimetic and sensitizing effects in 3T3-F442A pre-adipocytes [4]

5. Introduction

Metabolic syndrome, a combination of several abnormalities that increase the risk for type II diabetes and atherosclerosis is global health problem of growing concern (Wong 2007, Potenza and Mechanick 2009, Salazar, Carbajal et al. 2013). It consists of atherogenic dyslipidemia (elevated triglycerides and low high-density lipoproteins), hypertension, glucose intolerance, and pro-inflammatory states (Grundy 2008).

Hypertension and insulin resistance are the key features of metabolic syndrome. Renin angiotensin system (RAS), the classical pathway for controlling blood pressure and fluid balance, has also a role in the pathogenesis of metabolic syndrome (Yvan-Charvet and Quignard-Boulange 2011). In the RAS, the enzyme angiotensin converting enzyme (ACE) plays a critical role in the formation of angiotensin II (Ang II), the primary active peptide of this system which increases blood pressure by enhancing vascular constriction. RAS blockade by ACE-inhibitors or angiotensin receptor blockers beneficially affects insulin sensitivity and prevents the development of diabetes (Yusuf, Gerstein et al. 2001, Braga and Leiter 2009). Given the role of RAS impairments in the pathogenesis of hypertension and metabolic syndrome, there is much interest in developing novel therapies that can target the common pathologies to hypertension and insulin resistance in more complicated disease conditions (Coppack, Mohamed-Ali et al. 2005, Zarich 2005, Potenza and Mechanick 2009).

[4] This chapter has been published: Jahandideh F, Chakrabarti S, Davidge ST, Wu J. PLoS One. 2017 Oct 3;12(10): e0185653).

Insulin is essential for normal metabolic functions of various tissues in the body (Havel 2002). Adipose tissue with a central role in lipid and glucose metabolism is a key target of insulin (Ruan and Lodish 2003). Insulin promotes differentiation of pre-adipocytes into mature adipocytes; a process accompanied by incorporation of lipid droplets and upregulation of immunomodulatory proteins like peroxisome proliferator associated receptor gamma (PPARγ) (Lu, Wang et al. 2006). Collectively, insulin actions on adipose tissue appear to be beneficial and anti-inflammatory in nature. Under metabolic syndrome, insulin signaling in adipose tissue is perturbed, associated with insulin resistance and chronic inflammation (Pradhan, Manson et al. 2001, Kadowaki, Hara et al. 2003, Winkler, Kiss et al. 2003, Coppack, Mohamed-Ali et al. 2005, Harwood 2012). As such, there is significant interest in developing therapeutic agents to improve insulin signaling in adipocytes, either by insulin sensitizing agents or through agents mimicking insulin actions (Strowski, Li et al. 2004, Sueta, Nakamura et al. 2012). The insulin sensitizing drugs thiazolidinediones (TZDs) enhance adipocyte differentiation. This increases lipid partitioning into adipocytes and decreases circulating, hepatic, and intramuscular triglycerides thus enhances insulin sensitivity (de Souza, Eckhardt et al. 2001).

Use of pharmacological drugs for controlling different complications of metabolic syndrome is associated with significant risk of side-effects especially when lifelong therapy is required (Khan, Murtaza et al. 2012). Not surprisingly, there is growing interest in developing naturally based products to attenuate insulin resistance as safer alternatives. Food derived products are valuable sources of novel therapeutic agents which are generally perceived as safer options compared to synthetic pharmacological drugs (Chakrabarti, Jahandideh et al. 2014). Several food proteins derived hydrolysates and peptides have undergone evaluation for therapeutic usage in metabolic disorders (Erdmann, Cheung et al. 2008, Shahidi and Zhong 2008).

Egg is a valuable source of dietary proteins. In addition to the nutritional value, egg proteins are also a source for peptides with myriad bioactive properties (Sumner, Gow et al. 2011), including ACE inhibition (Majumder and Wu 2009, Majumder, Chakrabarti et al. 2013, Majumder, Panahi et al. 2013, Jahandideh, Majumder et al. 2014, Majumder, Chakrabarti et al. 2015). Previous work from our research group has demonstrated the potential of egg white protein ovotransferrin derived bioactive peptides against hypertension, oxidative stress and inflammation *in vitro* and *in vivo* (Huang, Chakrabarti et al. 2010, Huang, Shen et al. 2012, Majumder, Chakrabarti et al. 2013). Moreover, we have recently reported the effects of egg white hydrolysate (EWH) on reducing blood pressure in hypertensive rats (Jahandideh , Chakrabarti et al. 2016). EWH significantly reduced blood pressure through modulating RAS components, reducing nitrosative stress and enhancing vascular relaxation (Jahandideh, Chakrabarti et al. 2016). While some features of metabolic syndrome such as inflammation and hypertension appear amenable to treatment with egg white protein derivatives (Huang, Chakrabarti et al. 2010, Huang, Shen et al. 2012, Majumder, Chakrabarti et al. 2013, Moon, Lee et al. 2013, Jahandideh, Chakrabarti et al. 2016), their actions on adipocyte functions have remained largely unknown.

Given the interplay among hypertension, inflammation, and metabolic syndrome, the objective of the study was to test the effect of EWH on differentiation, insulin signaling and inflammatory responses in 3T3-F442A pre-adipocytes. The findings of this study indicate insulin mimetic and insulin sensitizing as well as anti-inflammatory actions of EWH in adipocytes which may potentially prevent or alleviate the complications of metabolic syndrome.

5.1. Material and methods

5.1.1. Reagents

Pasteurized liquid egg white was purchased from Egg Processing Innovation Cooperative (Lethbridge, Alberta, Canada). Dulbecco's phosphate buffered saline (PBS), LipidTox dye and dithiothreitol (DTT) were all bought from Sigma Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were from Gibco/ Invitrogen (Carlsbad, CA, USA). The murine tumor necrosis factor alpha (TNF-α) was obtained from Peprotech (Rocky Hill, NJ, USA). Triton-X-100 was from VWR International (West Chester, PA, USA). Type 1 Collagenase used for cell splitting was from Worthington Biochemical Corporation (Lakewood, NJ, USA). Thermoase PC10F (from *Bacillus thermoproteolyticus* Var. Rokko) was purchased from Amano Enzyme Inc. (Nagoya, Japan). Pepsin (from porcine stomach, 10000 units / mg) was purchased from American Laboratories Inc. (Omaha, NE, USA).

5.1.2. Preparation of egg white hydrolysate (EWH)

Hydrolysis of egg white was carried out according to our previous method with slight modifications (Jahandideh, Chakrabarti et al. 2016). Briefly, liquid egg white was diluted with water at a ratio of 1:1 (v/v) to obtain a solution with 5% protein solid. After adjusting the pH to 8.0 with 2 M NaOH solution, and the temperature to 65 °C, thermoase (0.1%, w/w) was added and protein digestion was carried out for 90 min. The enzyme was then inactivated by adjusting pH to 2.5 for pepsin digestion. The mixture was further hydrolyzed at 55 °C by 1% pepsin for 180 min. The reaction was terminated by heating the solution at 95 °C for 15 min and the hydrolysate was centrifuged and then condensed to obtain approximately 10% solid. The hydrolysate was then spray dried and the powder was collected and stored -20 °C for further experiments. EWH was desalted with 50% acetonitrile/deionized water using Sep-Pak C18

cartridges (product #: WAT043345, Waters, Ontario, Canada) to remove salts in the hydrolysate for using in cell experiments.

5.1.3. Cell culture & differentiation

The murine pre-adipocyte cell line 3T3-F442A (Sigma Aldrich; Cat# 00070654) was used. Cell culture method is similar to our previous study (Chakrabarti and Wu 2015). The cells were obtained in passage 8, thawed and expanded in culture using DMEM supplemented with 10% FBS (heat-inactivated) and antibiotics. The cells were grown in T-25 flasks to confluence prior to sub-culture in gelatin-coated 48 well plates. All studies were performed using cells in passages 11-37.

To determine the ability of EWH to induce adipogenic differentiation, the cells (grown in 48 well plates) were incubated in standard culture medium (DMEM + 10% FBS + antibiotics) in the presence of EWH or insulin for 72 h without changing the medium. Adipogenic changes were determined by the appearance of intracellular lipid droplets (as shown by LipidTox staining), upregulation of PPARγ and CCAAT/ enhancer binding protein alpha (C/EBP-α) (determined by western blot) and release of adiponectin (measured by ELISA). Insulin (10 µg/mL) was used only as a positive control for inducing differentiation.

S961/Insulin Receptor Antagonist (cat#051-86, Phoenix pharmaceuticals Inc. USA) was used at the concentration of 200 nM to investigate the involvement of insulin receptor for the potential effects of EWH on insulin signaling.

For inflammation studies, confluent monolayers of cells were treated with/ without EWH for 48 h followed by administration of murine TNF-α (10 ng/mL, 24 hrs for COX-2, 15 minutes for cell signaling experiment).

5.1.4. Intracellular lipid staining

Intracellular lipid accumulation, a marker for adipogenic differentiation, was determined by LipidTox staining as described in our previous study (Chakrabarti and Wu 2015). Briefly, the cells were treated for 72 h with EWH or insulin (positive control), fixed and stained with LipidTox (1:250 in phosphate buffered saline) for 30 min and counter-stained with the nuclear dye Hoechst 33342 (1:10000 dilution in PBS). The cells were then visualized under an Olympus IX81 fluorescent microscope (Carson Scientific Imaging Group; Markham, Ontario, Canada). Images were obtained and analyzed using the Metamorph imaging software (Molecular Devices, Sunnyvale, CA) and presented at (200X) magnification. A control image from a group of cells without LipidTox was used to detect any nonspecific fluorescence. The images were then quantified by subtracting the background fluorescence of the control image, so only fluorescence from the lipid-specific staining was visible. The cell nuclei were stained by the DNA stain Hoechst3342. The fluorescence intensity was then measured for quantitative analysis and quantified as mean intensity per cell (MFI/cell) and expressed as % of untreated cells.

5.1.5. Adiponectin measurement

The culture media from untreated and EWH (or insulin) treated cells were centrifuged (10,000 g for 10 min at 4^{o}C) to yield cell-free supernatants which were stored at -80 ^{o}C until time of the assay. These supernatants were thawed and used in the Mouse Adiponectin DuoSet ELISA kit (R&D Systems; Minneapolis, MN, USA) following the manufacturer's instructions. Data were normalized to supernatants from the untreated cells.

5.1.6. Western blotting

Western blotting was done on 3T3-F442A cell lysates prepared at the end of experimental procedures as described in our previous studies (Chakrabarti and Davidge 2009, Chakrabarti and

Wu 2015). Protein bands for C/EBP-α (rabbit polyclonal antibody from Cell Signaling Technology, Boston, MA, USA, cat# 2295), PPARγ (rabbit polyclonal antibody from Cell Signaling Technology, cat# 2430), phospho-Akt (rabbit polyclonal antibody from Cell Signaling Technology, cat #9271), Akt (mouse monoclonal from Santa Cruz, cat#sc-81434), phospho-ERK1/2 (rabbit polyclonal antibody from Cell Signaling Technology, cat#9101), ERK1/2 (mouse monoclonal antibody from Cell Signaling Technology, cat#4696), phospho-IRS-1 (rabbit polyclonal antibody from Cell Signaling Technology, cat#3070), IRS-1 (mouse monoclonal antibody from Santa Cruz Biotechnology, Santa Cruz, CA, USA, cat# sc-8038), phospho-p65 (rabbit polyclonal antibody from Santa Cruz Biotechnology, cat# sc-3033) and p65 (mouse monoclonal antibody from Santa Cruz Biotechnology, cat# sc-8008) were normalized to α-tubulin (rabbit polyclonal antibody from Abcam, Cambridge, MA, cat# ab15246). Anti-tubulin was used at 0.4 μg/ml, while all other antibodies were used at 0.5-1 μg/ml. Goat anti-rabbit and Donkey anti-mouse conjugated secondary antibodies were purchased from Li-cor Biosciences (Lincoln, NB). The protein bands were detected by a Li-cor Odyssey BioImager and quantified by densitometry using corresponding software Odyssey v3.0 (Li-cor). Cell lysates from untreated cells were loaded on every gel and all data were expressed as % of the corresponding untreated control.

5.1.7. Statistical analysis

All data are expressed as mean±SEM (standard error of mean) of 4-8 independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) with an appropriate post-hoc test (Dunnett's test for comparison to control group; Tukey's test for multiple comparisons). For studying interactions between 2 independent variables (e.g. EWH and insulin), two-way ANOVA was used. A repeated measures test was used when applicable. The PRISM 6 statistical

software (GraphPad Software, San Diego, CA) was used for the analyses. A value of $p< 0.05$ was considered significant.

5.2. Results

5.2.1. EWH treatment induces adipogenic differentiation in 3T3-F442A cells

Adipogenic differentiation in pre-adipocytes is characterized by increases in intracellular lipid droplets and release of adiponectin (Fu, Luo et al. 2005, Christensen, Minet et al. 2009). Treating 3T3-F442A cells with EWH for 72 hrs resulted in increased intracellular lipid accumulation as determined by LipidTox staining (Figure 5- 1a). These changes were accompanied by higher levels of adiponectin released into the culture medium, further demonstrating the pro-differentiation properties of EWH (Figure 5- 1b). Interestingly, both effects induced by EWH were similar in magnitude to those of insulin, suggesting that the beneficial effects of EWH could be comparable to the physiological effects of insulin.

5.2.2. EWH upregulates markers of adipocyte differentiation in 3T3-F442A cells

In addition to lipid accumulation and adiponectin release, adipocyte differentiation is also accompanied by increased expression of a number of proteins involved in different stages of this process. For example, PPARγ, an anti-inflammatory metabolic modulator is highly expressed in differentiated adipocytes and contributes to insulin sensitizing actions (Hamm, El Jack et al. 1999). Similarly, C/EBP-α, a transcriptional regulator, is upregulated during differentiation where it co-ordinates the expression of downstream proteins involved in adipogenesis (Fu, Luo et al. 2005). Indeed, 72 hrs incubation of 3T3-F442A cells with EWH upregulated both PPARγ (Figure 5- 2a) and C/EBP-α (Figure 5- 2b), demonstrating the successful induction of adipogenic differentiation event at the molecular level. Both effects were also comparable to those induced by insulin, the physiological agonist of adipocyte differentiation.

5.2.3. EWH upregulates PPARγ expression dose-dependently in 3T3-F442A cells

The effects of different concentrations of EWH on PPARγ expression was also investigated in 3T3-F442A cells. Figure 5- 3 illustrates that EWH enhanced PPARγ expression in a dose-dependent manner. EWH at concentrations of 2.5, 5 and 10 mg/mL enhanced PPARγ expression significantly as compared to untreated cells at $P<0.01$, $P<0.001$ and $P<0.0001$ respectively.

5.2.4. EWH exerts both insulin mimetic and insulin sensitizing effects

Given the similarity in EWH responses to insulin effects, we then investigated the effect of EWH on key insulin signaling pathways in these cells. The mitogen activated protein kinase ERK1/2 is an important downstream signaling target of insulin, which is phosphorylated (and hence, activated) by insulin treatment (Eduardsen, Larsen et al. 2011). Treatment with EWH alone showed increased ERK1/2 phosphorylation in pre-adipocytes, while insulin-induced ERK1/2 activation in EWH-treated cells was comparable to that observed in EWH-free cells (Figure 5-4a), suggesting a potential insulin mimetic action of EWH.

Another major signaling target of insulin is protein kinase B (PKB)/Akt. Akt regulates many cellular processes including metabolism, proliferation, cell survival, growth and angiogenesis (Hers, Vincent et al. 2011). Akt phosphorylation is a key event involved in mediating the beneficial actions of insulin in glucose transport in adipose tissues (Kleiman, Carter et al. 2009). Interestingly, EWH alone had no effects on Akt phosphorylation; while insulin actions on Akt phosphorylation were enhanced in EWH-treated cells over and above the response seen in control cells (Figure 5- 4b). Indeed, a 2-way ANOVA showed a significant interaction between EWH and insulin effects on Akt phosphorylation indicating a novel insulin sensitizing action for EWH in these cells.

5.2.5. EWH appears to involve insulin receptor signaling in adipocytes

Next, we examined if the observed EWH actions were dependent on signaling through the insulin receptor. Insulin binding to its receptor leads to phosphorylation of an associated protein, insulin receptor substrate 1 (IRS-1), which is widely used as a marker for insulin receptor mediated functions (Velloso, Carneiro et al. 1995, Zolotnik, Figueroa et al. 2012, Zhang, Nakatsu et al. 2013). Interestingly, EWH treatment alone enhanced phosphorylation of insulin receptor β (IRβ) (Figure 5- 5a, $P<0.05$), while the protein expression level of IRβ was not affected by the treatment (Figure 5- 5b, $P>0.05$). Furthermore, EWH treatment alone also enhanced IRS-1 phosphorylation (Figure 5- 5c) significantly ($P<0.01$) compared to untreated cells indicating potential insulin mimetic actions of EWH in these cells. However, EWH did not further enhance the phosphorylation of IRβ and IRS-1 in the presence of exogenous insulin. Only insulin was able to induce significant phosphorylation of IRβ and IRS-1, which remained unaffected by concomitant presence of EWH (Figures 5- 5a and 5- 5c).

5.2.6. Insulin mimetic effect of EWH on ERK phosphorylation is mediated through insulin receptor in adipocytes

Since EWH exerted insulin mimetic effects in 3T3-F442A cells (Figure 5- 4a) and was mediated through insulin receptor (Figure 5- 5a), we aimed to further explore this possibility by using S961, an insulin receptor antagonist. As indicated in Figure 5- 6, EWH enhanced ERK phosphorylation significantly compared to untreated cells ($P<0.001$) (similar to insulin, $P<0.05$), whereas, incubating in the presence of the insulin receptor antagonist (S961) blocked the observed effects of both insulin and EWH on the levels comparable to the untreated control. This data suggests that, the insulin mimetic effects of EWH on ERK phosphorylation is potentially mediated through insulin receptor.

5.2.7. EWH modulates inflammatory response in 3T3-F442A cells

Finally, we investigated the effects of EWH on inflammatory changes in these cells. Adipocyte inflammation releases harmful cytokines which leads to the loss of protective adipokines, increases insulin resistance and contributes to the pathogenesis of metabolic syndrome (Bell 2000, Rodrigues, Vendramini et al. 2012). We used TNF-α, a pro-inflammatory cytokine involved in various inflammatory, atherosclerotic and metabolic disorders, to induce inflammation in these cells. Treatment with TNF-α for 24 hrs upregulated cyclooxygenase -2 (COX-2) levels in 3T3-F442A cells (1.64 ± 0.21), while a 48 hrs pre-treatment with EWH abolished this response (0.84 ± 0.19) (Figure 5- 7a), indicating potentially beneficial anti-inflammatory capabilities of EWH. Further examination of underlying pro-inflammatory signaling/transcriptional pathways revealed a reduction of TNF-α-mediated c-Jun phosphorylation in EWH-treated cells. TNF-α increased c-Jun phosphorylation to159.20 ± 12.17% in adipocytes, while EWH treatment restored it to the basal level (98.15 ± 15.08%) (Figure 5- 7b). This may account for the mechanisms underlying the anti-inflammatory actions of EWH.

5.3. Discussion

Adipose tissue playing a vital role in energy homeostasis, is one of the critical target organs for insulin actions (Ruan and Lodish 2003). Adipose tissue secretes a number of adipokines interacting with central and peripheral organs in the body (Havel 2002). This insulin sensitive tissue influences diverse metabolic processes including carbohydrate metabolism, lipid metabolism, inflammation, blood pressure, energy expenditure, and feeding behavior (Smorlesi, Frontini et al. 2012, Bou, Todorcevic et al. 2014). In metabolically normal conditions adipocytes are small in size, sensitive to insulin and secrete insulin sensitizing hormones such as adiponectin

(Fu, Luo et al. 2005). In metabolic disorders on the contrary, adipocytes become larger in size, inflamed, insulin resistant, and increasingly express harmful adipokines leading to adipose tissue dysfunction, insulin resistance and associated diseases (Kadowaki, Hara et al. 2003). In fact loss of insulin sensitivity in adipose tissue adversely affects glucose utilization and lipid storage leading to ectopic deposition of fat in insulin sensitive tissues which contributes towards the development of insulin resistance and pathogenesis of both type 2 diabetes and metabolic syndrome (Muller-Wieland, Kotzka et al. 1998, Coppack, Mohamed-Ali et al. 2005). Enhanced differentiation of fibroblast-like pre-adipocytes into mature adipocytes by the use of compounds mimicking insulin functions or enhancing insulin sensitivity provides a novel strategy for controlling the complications of metabolic syndrome (Salazard, Bellon et al. 2004, Strowski, Li et al. 2004, Rekovets, Sirenko et al. 2010).

PPARγ and C/EBP-α are the two key molecules involved in adipocyte differentiation and regulation of the adipogenic network (Kadowaki, Hara et al. 2002). Over expression of PPARγ can induce adipogenesis in mouse embryonic fibroblasts lacking C/EBPα, but C/EBPα cannot rescue adipogenesis when PPARγ is not expressed, showing that PPARγ is the master regulator of adipogenesis (Hamm, El Jack et al. 1999, Chinetti, Fruchart et al. 2000, Tsuchida, Yamauchi et al. 2005). Thiazolidinediones with insulin sensitizing effects promote pre-adipocytes differentiation by PPARγ activation (Spiegelman 1998, Rikimaru, Wakabayashi et al. 2012). Insulin also promotes adipogenic effects by upregulating both PPARγ and C/EBP-α in adipocytes (MacDougald, Cornelius et al. 1995, Hamm, El Jack et al. 1999). Our study revealed that EWH treatment also exerted insulin-like differentiating effects on pre-adipocytes. In accordance with observed insulin-like effects of EWH on upregulation of pre-adipocyte differentiation markers, this treatment also enhanced PPARγ and C/EBP-α to the same extent as

insulin in adipocytes. So, it is plausible to propose that the observed insulin-like properties of EWH on adipogenic response may at least be in part due to involvement of these 2 regulators in 3T3-F442A cells. Indeed, several groups have identified plant derived novel compounds that promote adipogenic effects at least partially through upregulation of PPARγ (Christensen, Minet et al. 2009, Beh, Khoo et al. 2013).

Binding of insulin to insulin receptor triggers the phosphorylation of IRβ and consequently, insulin receptor substrate (IRS) proteins providing the basis for the subsequent association with downstream signaling through different pathways mediating metabolic and mitogenic responses of insulin (Valverde, Kahn et al. 1999, Zhang, Nakatsu et al. 2013). While phosphorylation and activation of Akt is responsible for most of the known metabolic effects of insulin, ERK phosphorylation mediates mitogenic and transcriptional effects of insulin in adipocytes (Laviola, Perrini et al. 2006). When investigating the effects of EWH on insulin signaling, we also observed an insulin mimetic effect of EWH on ERK1/2 phosphorylation in these cells. In addition to the observed insulin mimetic effects on 3T3-F442A cells, EWH also exhibited insulin sensitizing effects by enhancing insulin-mediated Akt phosphorylation. Akt acts not only as a regulator of glucose transport but also involves in several other metabolic actions including glycolysis, protein synthesis, lipogenesis, glycogen synthesis, suppression of gluconeogenesis, cell survival, determination of cell size and cell-cycle progression (Whiteman, Cho et al. 2002). The fact that EWH affected both ERK and Akt phosphorylation in adipocytes indicates the potential effects of this treatment on both pathways of insulin signaling.

Moreover, since phosphorylation of IRβ and IRS-1 was significantly enhanced in EWH treated cells while no further increase was observed in the presence of exogenous insulin suggesting that EWH exerts its insulin mimetic effects through insulin receptor which was further supported by

the study of an insulin receptor antagonist (Figure 5- 6). Therefore, the insulin sensitizing effect of EWH might be via targets downstream to IRS-1 such as phosphoinositide-3 kinase (PI3-kinase) and phosphoinositide-dependent kinase 1 (PDK1).

There is an interest to assess the potential of established antihypertensive compounds for protection against insulin resistance and other complications of metabolic syndrome due to the role of RAS impairment in the pathogenesis of such diseases (Medvedev and Kumova 2007). RAS blockade has been reported to inhibit the body fat mass increase (Mathai, Naik et al. 2008, de Kloet, Krause et al. 2009), and improve insulin resistance and glucose tolerance in type-2 diabetic rodents (Lee, Song et al. 2008, Weisinger, Stanley et al. 2009). Captopril, the pharmacological ACE inhibitor, has been reported to enhance adipocyte differentiation and reduce inflammation in various tissues (Roncal, Reungjui et al. 2009). Insulin sensitizing effects of RAS blockade have also been reported in clinical studies in patients with risk factors (Pershadsingh and Kurtz 2004, Jandeleit-Dahm, Tikellis et al. 2005, Henriksen 2007) suggesting additional benefits of these drugs in a complex condition like metabolic syndrome. Interestingly, the anti-hypertensive EWH with RAS modulating properties (reducing vascular ACE and angiotenstin II type 1 receptor expression) also enhanced pre-adipocyte differentiation, and induced insulin mimetic and sensitizing effects in 3T3-F442A cells. Similarly, milk derived peptides IPP and VPP, with ACE-inhibitory and anti-inflammatory properties exerted insulin mimetic adipogenic effects by promoting the differentiating of pre-adipocytes in 3T3-F442A cells (Chakrabarti and Wu 2015). The flaxseed protein hydrolysate contains peptide fractions with anti-hypertensive (Udenigwe, Adebiyi et al. 2012) as well as anti-diabetic properties (Doyen, Udenigwe et al. 2014). Since hypertension, inflammation and insulin resistance present concomitantly in many cases of metabolic syndrome, EWH as a novel naturally based compound

with multiple benefits against hypertension, inflammation and insulin functions may serve as an effective option for the management of complications of this disease.

Adipose tissue inflammation with dysregulated adipokine secretion plays a critical role in the development of a variety of cardiometabolic disorders including metabolic syndrome, type 2 diabetes, inflammatory and vascular disorders and eventually development of coronary heart disease (Coppack, Mohamed-Ali et al. 2005). EWH upregulated the expression of anti-inflammatory molecules such as PPARγ and adiponectin, demonstrating the potential benefits of EWH on adipocyte function and metabolic syndrome. EWH also prevented the TNF-α-mediated induction of the pro-inflammatory enzyme COX-2, a molecule that contributes to the pathologic complications of metabolic syndrome (Murasaki, Omori et al. 2006, Renna, Diez et al. 2013). This anti-inflammatory effect is likely due to its interference with the AP-1 transcription factor pathway which is involved in COX-2 expression and can be modulated by the inhibition of c-Jun phosphorylation (Park, Kim et al. 2007, Zhang, Li et al. 2008, Hsu, Lee et al. 2015). TNF-α stimulates the pro-inflammatory phenotype in adipose tissues leading to the development of insulin resistance and metabolic syndrome (Ruan and Lodish 2003). Indeed, suppression of TNF-α has been suggested as a potential therapy against metabolic syndrome (Sonnenberg, Krakower et al. 2004). VPP and IPP prevented inflammatory changes in 3T3-F442A cells (Chakrabarti and Wu 2015). In another study, Sawada et al. have reported that VPP inhibited adipose inflammation *in vitro* and *in vivo* (Sawada, Sakamoto et al. 2015). VPP also enhanced insulin sensitivity in obese mice and inhibited macrophage accumulation and activation in fat tissues (Sawada, Sakamoto et al. 2015). Moreover, beta-mercaptoethanol (BME), the pharmacological redox regulator and radical scavenger, has also been reported to down-regulate the expression of inflammatory cytokines and promote adipocyte differentiation (Guo, Li et al. 2012). Our data

with EWH further supports its role as a novel regulator of adipose functions with additional anti-inflammatory benefits.

Bioactive peptides in the EWH are potentially responsible for the observed effects of EWH on adipogenic differentiation, insulin signaling and anti-inflammatory responses in adipocytes. Purification and fractionation of EWH to identify its responsible peptides with beneficial effects on adipocyte differentiation, which is underway, is essential in understanding the structure requirements of food-derived bioactive peptides with beneficial effects on adipose tissue function.

In conclusion, our study demonstrated that EWH, with RAS modulating properties promoted adipocyte differentiation through a combination of insulin mimetic and insulin sensitizing actions on 3T3-F442A cells. In addition, EWH also increased expression of the anti-inflammatory hormone adiponectin and suppressed cytokine mediated inflammatory response in these cells. Considering the fundamental role of adipose tissue dysfunction in the pathogenesis of hypertension, inflammation, insulin resistance, and metabolic syndrome, EWH may have potential benefits in the prevention and management of metabolic syndrome.

5.4. Acknowledgements

The authors would like to thank Qi Yi Li for the preparation of EWH for this study.

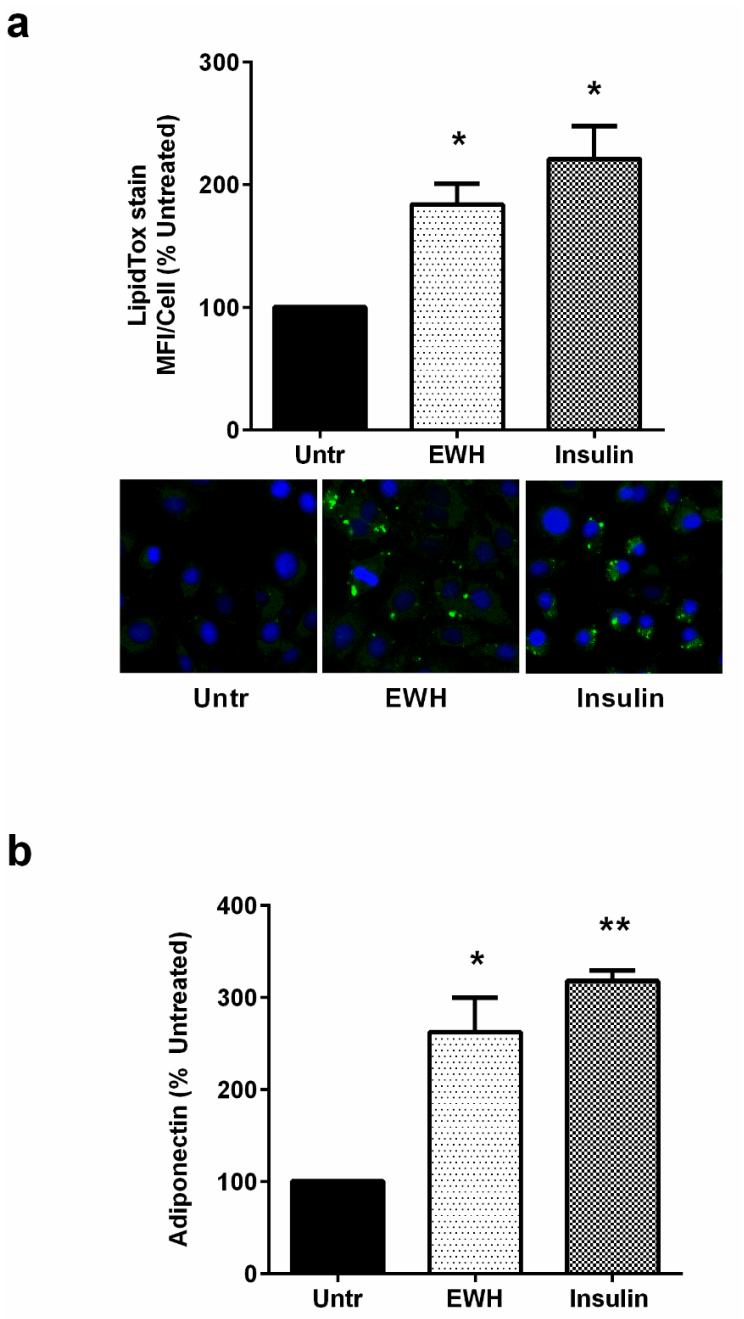


Figure 5-1. EWH treatment induces adipogenic differentiation in 3T3-F442A cells. 3T3-F442A cells were incubated with EWH (5 mg/mL) or insulin (positive control; 10 µg/mL) for 72 hrs. (a) Following incubation, the cells were fixed and stained with the neutral lipid-specific dye LipidTox (green), the nuclear stain Hoechst3342 (blue) and visualized under fluorescence microscopy. A set of representative images are shown. (b) The cell-free culture supernatants were collected and analyzed by ELISA to determine adiponectin levels. Data are presented as mean±SEM of 4-5 independent experiments. * and ** indicate $p<0.05$ and $p<0.01$ respectively compared to the untreated control (Untr).

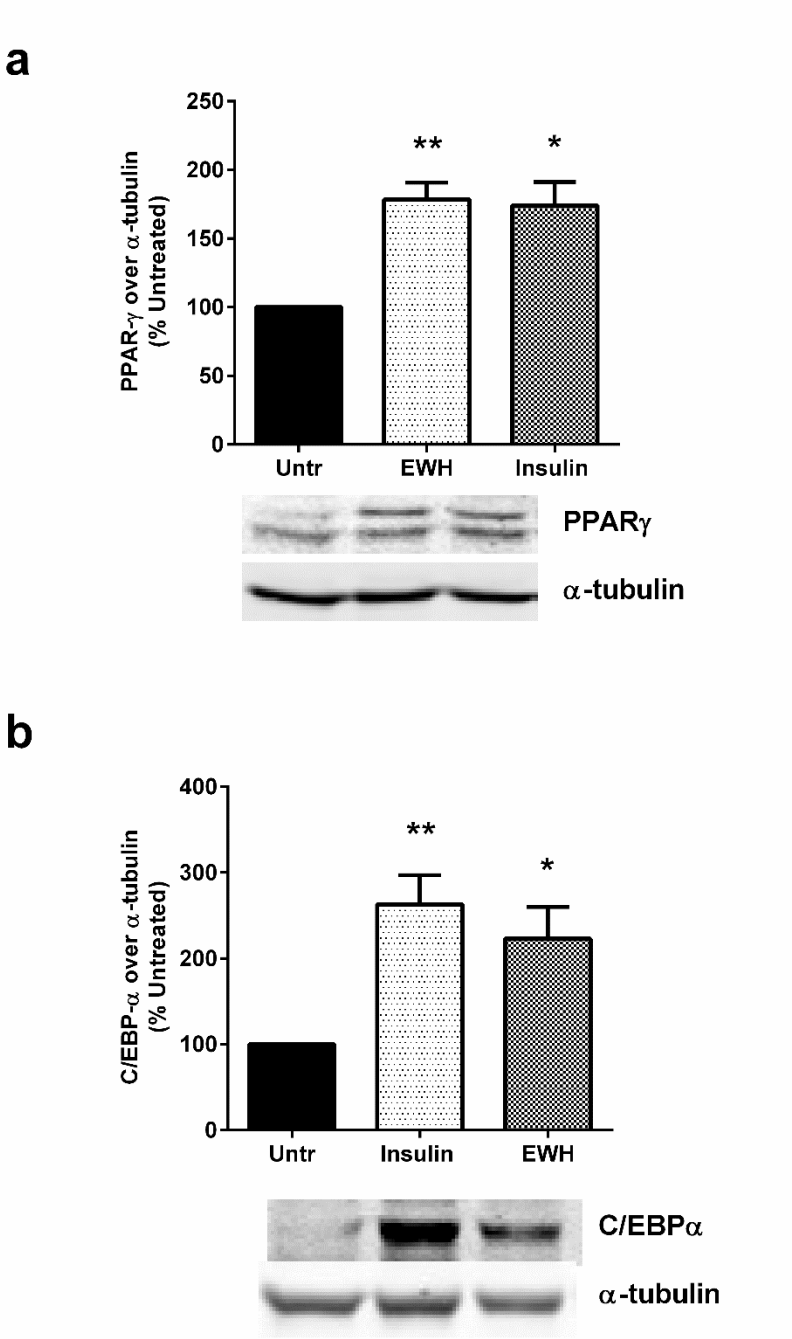


Figure 5-2. EWH upregulates markers of adipocyte differentiation. 3T3-F442A cells were incubated with EWH (5 mg/mL) or insulin (positive control; 10 µg/mL) for 72 hrs. The cells were then lysed and western blotting of the lysates was performed with antibodies against PPARγ (a), C/EBP-α (b) and α-tubulin (loading control; both a and b). A representative set of images are shown. Bands were quantified by densitometric analysis. Data are presented as mean±SEM of 4-5 independent experiments. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively, compared to the untreated control (Untr).

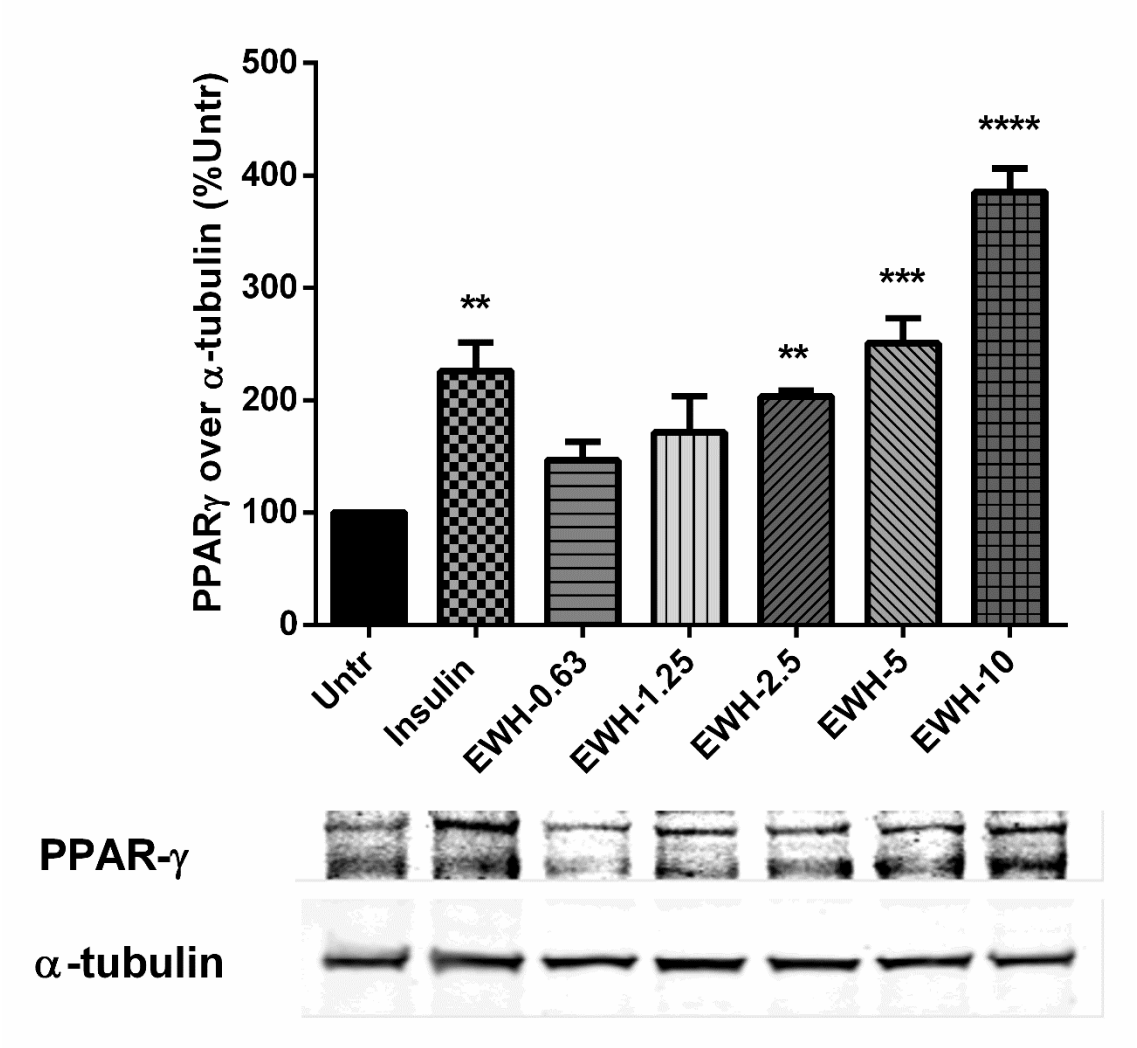


Figure 5-3. EWH upregulates PPARγ expression in a dose-dependent manner. 3T3-F442A cells were incubated with different dosages of EWH (0.63-10 mg/mL) or insulin (10 µg/mL) for 72 hrs. The cells were then lysed and western blotting of the lysates was performed with antibodies against PPARγ and α-tubulin (loading control). A representative set of images is shown. Bands were quantified by densitometric analysis. Data are presented as mean±SEM of 3-4 independent experiments. **, *** and **** indicate $p<0.01$, $p<0.001$ and $p<0.0001$ respectively, compared to the untreated control (Untr).

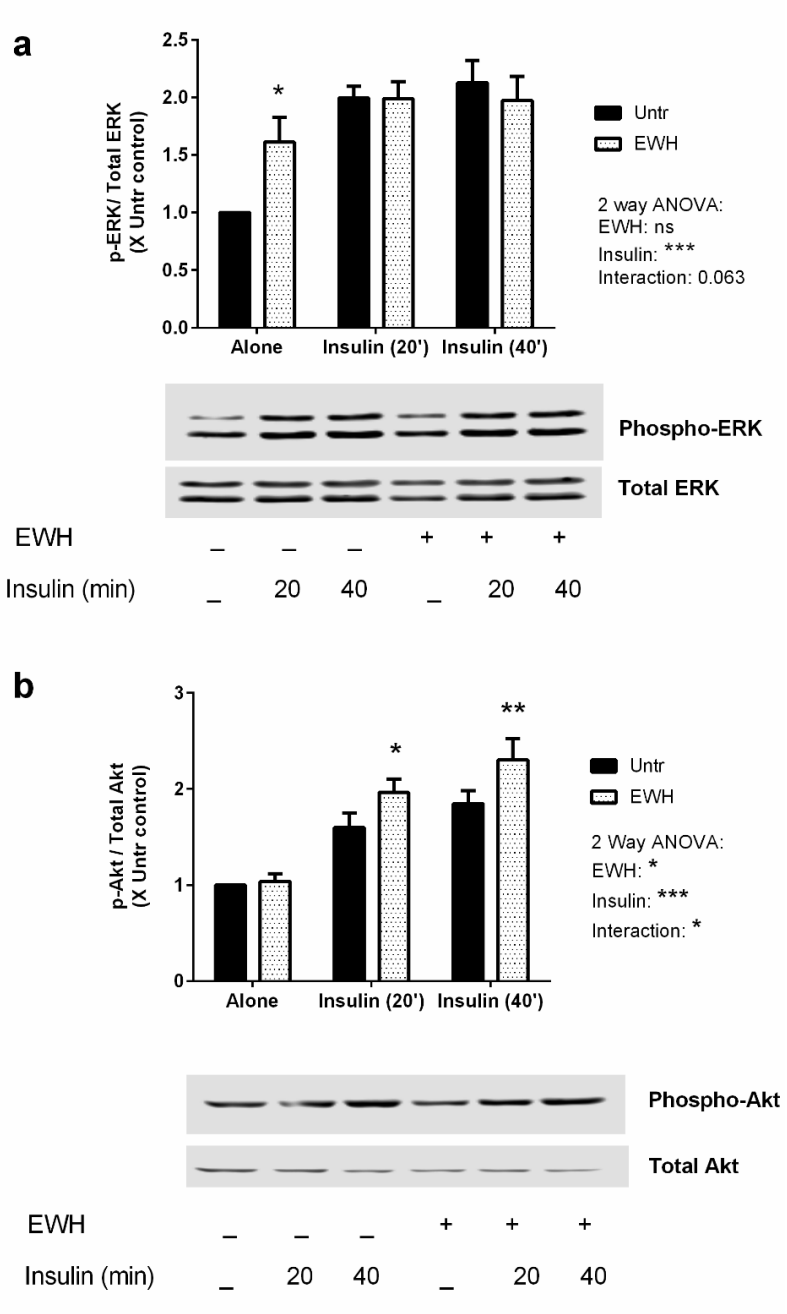


Figure 5-4. EWH differentially modulates insulin-mediated phosphorylation of ERK and Akt. 3T3-F442A cells were incubated with EWH (5 mg/mL) for 48 hrs prior to stimulation with insulin (10 µg/mL) for 20 or 40 min. The cells were then lysed and western blotting of the lysates was performed with antibodies against the total and phosphorylated forms of ERK (a) and Akt (b). Representative sets of images are shown. Bands were quantified by densitometric analysis. Data are presented as mean±SEM of 5 independent experiments. *, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively compared to untreated control by a two-way ANOVA. 'ns' indicates: not significant.

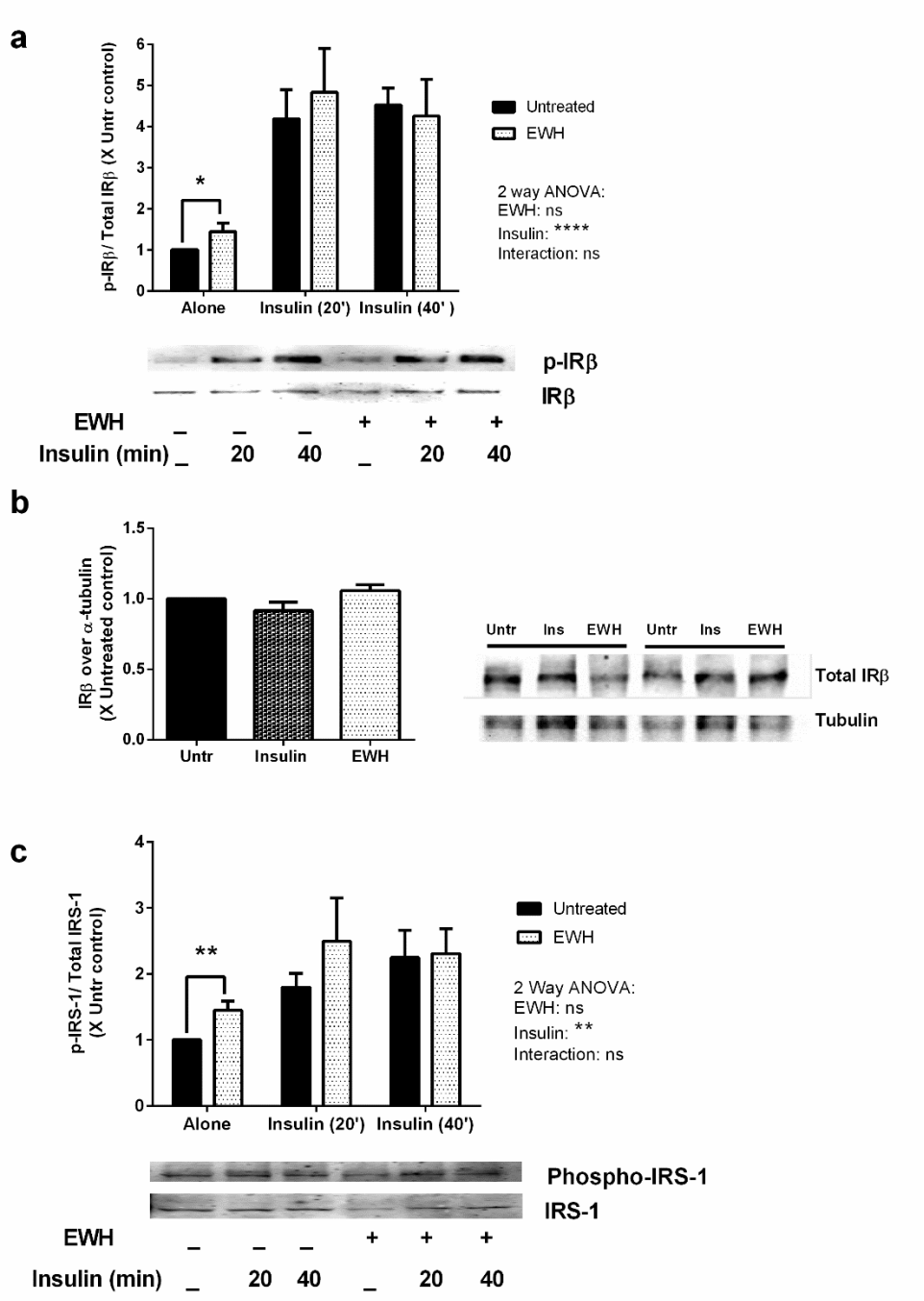


Figure 5-5. EWH actions are partly mediated through insulin receptor. 3T3-F442A cells were incubated with EWH (5 mg/mL) for 48 hrs prior to stimulation with insulin (10 µg/mL) for 20 or 40 min. The cells were then lysed and western blotting of the lysates was performed with antibodies against the: total and phosphorylated forms of IRβ (a), total IRβ and α-tubulin (loading control) (b), and total and phosphorylated forms of IRS-1 (c). Representative sets of images are shown. Bands were quantified by densitometric analysis. Data are presented as

mean±SEM of 4-7 independent experiments. *, ** and **** indicate $p<0.05$, $p<0.01$, and $p<0.0001$ respectively.

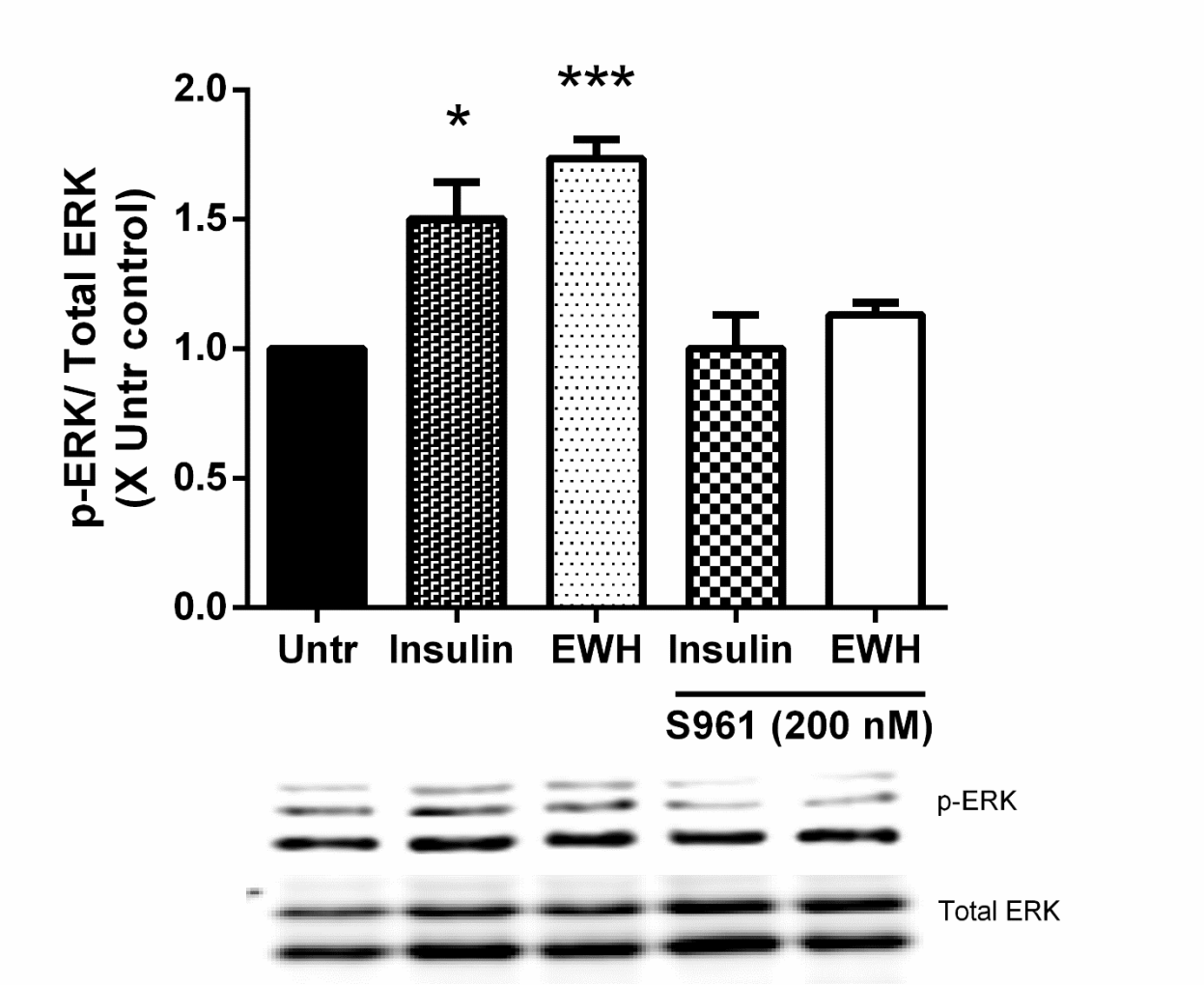


Figure 5-6. EWH mediates its insulin mimetic effects through insulin receptor. 3T3-F442A cells were incubated with EWH (5 mg/mL) or insulin (positive control; 10 µg/mL) in the presence/absence of S961 (insulin receptor antagonist; 200 nM) for 72 hrs. The cells were then lysed and western blotting of the lysates was performed with antibodies against the total and phosphorylated forms of ERK. A representative image is shown. Bands were quantified by densitometric analysis. Data are presented as mean±SEM of 3 independent experiments. *, and *** indicate $p<0.05$ and $p<0.001$ compared to untreated cells respectively.

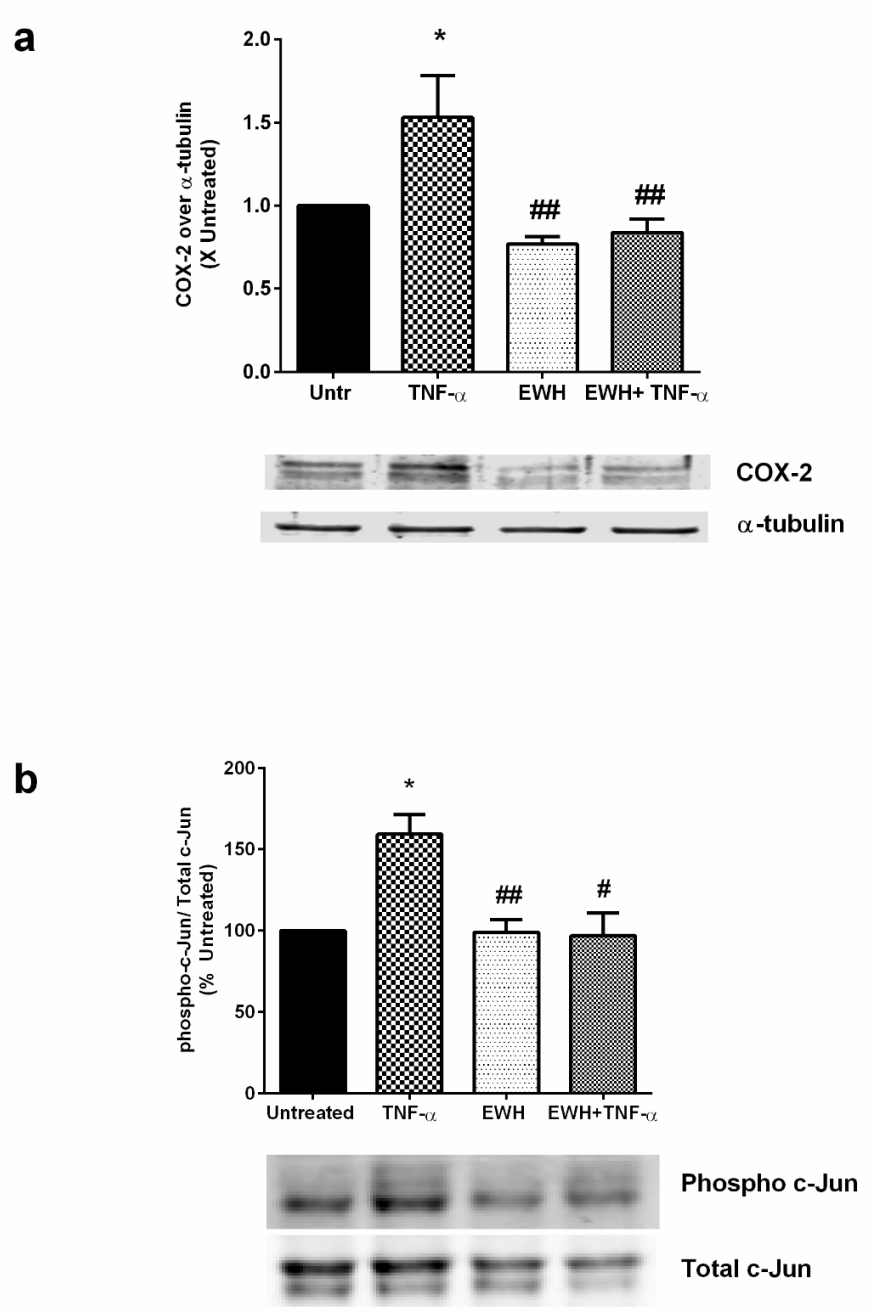


Figure 5-7. EWH exerts inhibitory effects on adipocyte inflammation. The 3T3-F442A cells were incubated with EWH (5 mg/mL) for 48 hrs prior to stimulation with TNF-α for either (a) 24 hrs (for inflammatory marker expression) or (b) 15 min (for transcription pathway experiment). The cells were then lysed and western blotting of the lysates was performed with antibodies against COX-2 and the loading control α-tubulin (a) or the total and phosphorylated forms of c-Jun (b). A representative set of images are shown. Bands from the COX-2 study were quantified by densitometric analysis. Data is mean±SEM of 4-6 independent experiments. * indicates

$p<0.05$ compared to the untreated (Untr) group, while ## indicates $p<0.01$ compared to TNF-α alone.

Chapter 6: Egg white hydrolysate enhances glucose tolerance and insulin sensitivity in diet induced insulin resistant rats

6. Introduction

Consumption of energy-dense foods, sedentary lifestyle, and genetic predisposition are the main determinants of obesity, cardiovascular diseases (CVDs), and metabolic syndrome (MetS). Indeed, MetS, the major public health challenge worldwide, is a cluster of abnormalities including hypertension, insulin resistance, hyperlipidemia, glucose intolerance, and abdominal obesity (Wong 2007) doubling the risk for CVDs and increasing the risk for type 2 diabetes 5 times compared with those without the syndrome (Grundy 2008). Lifestyle changes including dietary interventions and physical activity are the first lines of therapy for both CVDs and MetS (Pereira, Kottke et al. 2009). Although being simple, the success rate of these interventions is usually poor and patients often need to take several medications to alleviate the complications associated with hypertension, dyslipidemia, insulin resistance, and obesity. Given the serious side-effects associated with pharmacological drugs and their poor effectiveness in some cases, scientific attention has been drawn towards application of naturally derived compounds for the management of the MetS complications (Amiot, Riva et al. 2016, Lin, Xiao et al. 2016). Antioxidants, low glycaemic index carbohydrates, dietary fibers, minerals, monounsaturated and polyunsaturated fatty acids have been shown to beneficially affect MetS complications including blood pressure, plasma lipids, and insulin resistance (Abete, Goyenechea et al. 2011).

Bioactive peptides are another group of food-derived compounds encrypted within the primary sequences of food proteins and released by enzymatic digestion or food processing, play a vital role on regulation of blood pressure (Majumder and Wu 2015), reducing hyperlipidemia

(Udenigwe and Rouvinen-Watt 2015), and attenuating inflammation and oxidative stress (Chakrabarti, Jahandideh et al. 2014) as reviewed extensively in literature. Food proteins and their derived peptides have also been reported to improve glucose tolerance and insulin sensitivity. Whey protein (Morato, Lollo et al. 2013, Tong, Li et al. 2014), fish protein (Lavigne, Tremblay et al. 2001, Pilon, Ruzzin et al. 2011), and rice protein (Boonloh, Kukongviriyapan et al. 2015) or their bioactive peptides are some of the examples.

Several dipeptides including IV, LV, VL, II, LI, IL and LL were identified from a whey protein hydrolysate that could increase glucose uptake in L6 myotubes. IL, the main dipeptide in whey protein hydrolysate, further enhanced glucose uptake and glycogen synthesis rates in isolated epitrochlearis muscles (Morifuji, Koga et al. 2009). Feeding Wistar rats with a commercial whey protein hydrolysate (Hilmar product 8350) enhanced GLUT-4 translocation and glycogen synthesis in liver, heart and skeletal muscle of rats. Enhanced muscle Akt phosphorylation in rats fed with whey protein hydrolysate is partially responsible for the observed enhanced GLUT-4 translocation to the plasma membrane (Morato, Lollo et al. 2013).

Black soybean peptides (<10 KDa) have been reported to restore insulin signaling in normal and insulin resistant HepG2 cells by stimulating Akt serine phosphorylation, and glycogen synthase kinase-3β (GSK-3β) (Jang, Ko et al. 2010). Oral administration of black soybean peptides to diabetic (db/db) mice also showed antidiabetic effects (Jang, Ko et al. 2010). The black soy peptide supplementation has been reported to have a modest effect on reducing fasting glucose and improving glucose tolerance in Korean adults with prediabetes (fasting glucose ≥ 110 mg/dL) in a double blind randomized placebo-controlled trial (Kwak, Lee et al. 2010). Daily administration of aglycin, a 37-amino-acid polypeptide isolated from soybean, at dose of 50 mg/kg/d for 4 weeks effectively controlled hyperglycemia and enhanced oral glucose tolerance

in streptozotocin/high-fat-diet-induced diabetic mice (Lu, Zeng et al. 2012). While insulin signaling was perturbed in skeletal muscle of diabetic mice model, aglycin restored insulin signaling by maintaining insulin receptor and IRS1 expression at both mRNA and protein levels. This peptide further elevated the expression of p-IRS1, p-Akt and membrane GLUT4 protein thereby increasing glucose uptake in skeletal muscle (Lu, Zeng et al. 2012). One of the mechanisms by which many protein hydrolysates exert their favorable effects on glucose homeostasis and insulin sensitivity is through affecting adipose tissue by reducing adipose tissue inflammation, enhancing adipocyte differentiation and generation of smaller adipocytes, and secretion of adiponectin (Pilon, Ruzzin et al. 2011, Aihara, Osaka et al. 2014, Boonloh, Kukongviriyapan et al. 2015, Sawada, Sakamoto et al. 2015, Noguchi, Yanagita et al. 2016).

We reported the beneficial effects of egg white hydrolysate (EWH) on blood pressure reduction in spontaneously hypertensive rats (SHRs) in Chapter 4. One of the mechanisms by which EWH reduced blood pressure in SHRs was through modulating renin angiotensin system (RAS). The expression of the vascular angiotensin converting enzyme (ACE) and angiotensin type 1 (AT1R) receptor was reduced, while vascular angiotensin type 2 receptor (AT2R) expression increased; RAS is thought to link insulin resistance in animals and in subjects (Henriksen 2007, Olivares-Reyes, Arellano-Plancarte et al. 2009, Marcus, Shefer et al. 2013, Underwood and Adler 2013). The effects of valsartan (an angiotensin receptor blocker) on incidence of diabetes was studied in a randomized control trial study called the Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research (NAVIGATOR) in which a 14% reduction in the incidence of diabetes was observed in patients receiving valsartan compared to placebo group (Group, McMurray et al. 2010).

Furthermore, we previously reported the effects of EWH on adipogenic differentiation and insulin signaling in 3T3-F442A pre-adipocytes (Chapter 5), since adipogenic compound have shown potential benefits on glucose and insulin tolerance, here we aimed to examine the effects of EWH on glucose tolerance and insulin sensitivity in a rat model of MetS.

6.1. Experimental procedures

6.1.1. Animals and diets

The animal care protocols were approved by the University of Alberta Animal Welfare Committee (Protocol # 1472) in accordance with the guidelines issued by the Canadian Council on Animal Care also adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Male Sprague Dawley rats at eight weeks of age were purchased from Charles River Canada (St. Constant, QC, Canada) and housed two per cage with ad libitum access to normal chow and water for one week. Two animal trials were conducted to fulfill the purpose of the study. In both trials, after one-week of acclimatization, all rats received a high fat diet (HFD) (20% w/w) for 6 weeks to induce glucose intolerance as shown before (Hashemi, Yang et al. 2015, Yang, Hashemi et al. 2015). The HFD-fed rats were then randomly assigned to one of the following groups: HFD, HFD+1% EWH, HFD+2%EWH, and HFD+4% EWH (n=8 each) in the first trial and to HFD and HFD+4% EWH (n=8 each) in the second trial. The protein content of the EWH was 77.7%. The majority of EWH-derived peptides (more than 85%) were in the range of 1.36 to 6.51 KDa whereas, 11.21% of peptides had higher molecular weight between 6.51 to 12.38 KDa. About 3. 75% of the EWH-derived peptides had a molecular weight less than 189 Da (Appendix 6-A). Casein was used to make all diet groups isonitrogenous. Diet composition of the experimental groups is shown in Table 6- 1. Animals in both trials received treatment diets for six weeks with ad libitum access to food and

water. In the second study, half of the animals (n=4) in control (HFD) and treatment (HFD+4%EWH) groups were injected by insulin intraperitoneally (or saline in the other half) 10 minutes before euthanizing to study insulin signaling in muscle and fat.

6.1.2. Glucose and insulin tolerance tests

After 5 weeks of experimental diets (11 weeks in total on HFD), rats in both trials were performed an oral glucose tolerance test (OGTT) after an overnight fast. Fasting blood glucose was measured using a glucometer (Accu-Check Compact Plus, Roche Diagnostics, Laval, QC, Canada) and blood was collected from a tail vein for insulin determination. Then, each rat was administered orally 2g of glucose per kg of body weight and blood glucose was measured at 10, 20, 30, 60, 90 and 120 min. 50 µL of blood was taken at each time point during OGTT, centrifuged to obtain plasma, and stored at -20 ˚C for insulin measurement. For insulin tolerance test (ITT), rats were fasted for 4 hours after six weeks of treatment (12 weeks in total on HFD). Fasting blood glucose was measured and then each rat received 1.5 IU/kg BW dose of insulin via an intraperitoneal injection. Blood glucose was determined at 15, 30, 60, 90 & 120 min post injection. Homeostatic model assessment insulin resistance (HOMA-IR) was generated based on the following equation: fasting glucose (mg/dL) × fasting insulin (µU/mL)/405.

6.1.3. Body weight (BW), food intake and body composition measurement

BW was monitored weekly and food intake was measured for 24 hours 3 times a week throughout the study. Magnetic resonance imaging (MRI) technique was applied to specify lean and fat mass body composition in the second animal trial using an EchoMRI Whole Body Composition Analyzer (Echo Medical Systems LLC, Houston, TX, USA) in the last week of the treatment.

6.1.4. Tissue Collection

At the end of the study, rats were euthanized using isofluorane anesthesia after an overnight fast. A 3-5 mL blood sample was taken by cardiac puncture from all rats in both animal trials except for the insulin injected rats to obtain plasma. Muscle, liver, kidneys, and fat pads were excised, washed in phosphate buffer saline, weighted, and immediately frozen at -80°C until further analysis.

6.1.5. Plasma analysis

The blood samples were centrifuged (1,000 g for 20 min at 4°C) to obtain the plasma. Plasma was then stored at -80 °C until further analysis. Triglycerides (TG) concentration (Wako Pure Chemical Industries Ltd; cat # 992-02892/ 998-02992), total cholesterol (Wako Pure Chemical Industries Ltd.; cat # 999-02601), and non-esterified fatty acids (NEFA) (Wako Pure Chemical Industries Ltd.; cat # 999-34691/ 995-34791/ 991-34891/ 993-35191) concentrations were measured using direct colorimetric enzymatic reactions as per the manufacturer's instructions. Inflammatory cytokines in the plasma were measured using the Rat Inflammation ELISA Strip (Signosis Inc. cat # EA-1201). Samples from the OGTT were assayed for insulin using ELISA kit (Alpco Diagnostics, Salem, N.H., USA). Samples were analyzed in triplicate and performed in a single batch.

6.1.6. Tissue homogenization and protein extraction

Liver and muscle samples were ground on dry ice using mortar and pestle and then 20 mg of the tissue powder was homogenized in 200 µL extraction buffer consisting of lysis buffer (Cell Signaling Technology, cat # 9803), 1mM phenylmethanesulfonylfluoride, and protease inhibitor cocktail tablet (cat # S8820 Sigma). The homogenate was placed on ice for 15 min and

centrifuged at 14000 rpm for 10 min at 4 ˚C to collect the supernatant. Protein was extracted from the adipose tissue by a commercially available kit (invent Biotechnologies Inc. cat# AT-022) as per manufacturer's instructions. The protein content in extracts was measured using the BCA assay and aliquots of samples were stored at -80 ˚C for future western blotting.

6.1.7. Western blot

Briefly, protein extracts were separated by SDS-PAGE electrophoresis on 10-12% polyacrylamide gels, transferred to a polyvinylidene fluoride membrane and incubated with an anti-PPARγ (rabbit polyclonal from Cell Signaling Technology, cat# 2430), anti-p-Akt (rabbit polyclonal antibody from Cell Signaling Technology, cat #9271), anti-Akt (mouse monoclonal from Santa Cruz, cat # sc-81434), anti-ACE (rabbit polyclonal antibody from Santa Cruz, cat#20791), anti-ACE2 (rabbit polyclonal antibody from Abcam, cat#ab87436), anti-angiotensin II type 1 receptor (AT1R) (rabbit polyclonal antibody from Santa Cruz, cat#1173), anti-angiotensin II type 2 receptor (AT2R) (rabbit polyclonal antibody from Santa Cruz, cat#9040), and anti-β-actin (mouse monoclonal from Santa Cruz, cat# sc-47778) antibodies. PPARγ, ACE, ACE2, AT1R, and AT2R bands were normalized to β-actin as the loading control. Goat anti-rabbit and Donkey anti-mouse conjugated secondary antibodies were purchased from Li-cor Biosciences (Lincoln, NB). The protein bands were detected by a Li-cor Odyssey BioImager and quantified by densitometry using corresponding software Odyssey v3.0 (Li-cor).

6.2. Statistical analysis

All data presented are expressed as means ± SEM of 3-10 rats from each treatment group as indicated in figure legends. Statistical analysis was performed using the GraphPad Prism software, version 6.0. Data was checked for normal distribution by the Shapiro-Wilk test. For

analyses of OGTT, insulin ELISA, ITT, and insulin sensitivity in fat and muscle, we used a two-way analysis of variance (ANOVA). For all other data, one-way ANOVA, Kruskal-Wallis test, and student's t-test were used. Bonferroni and Dunn's post-hoc tests were performed to assess differences between groups were appropriate. A p value of 0.05 was considered statistically significant.

6.3. Results

6.3.1. Food intake, body and tissue weight

No significant differences was observed in the body weight (Fig 6- 1A) and food intake (Fig 6- 1B) between diet groups throughout the study ($P>0.05$). Similar food intake between groups indicates that the palatability of the diets did not affect the results. Moreover, there was no significant differences in relative liver (Fig 6- 2A) and kidneys (Fig 6- 2B) weights between control and treatment groups ($P>0.05$). However, rats fed with 4% EWH tended to have lower fat pads mass compared to the HFD treated rats (Figs 6- 2C & D) as measured by the relative retroperitoneal and epididymal fat pad weights.

6.3.2. Body composition

Since feeding rats with 4% EWH potentially reduced retroperitoneal and epididymal adipose mass, in the second trial we assessed the effect of 4% EWH on body composition (Fig 6- 3). MRI data revealed a reduction in fat mass (Fig 6- 3A, $P=0.05$) and lean mass (Fig 6- 3B, $p=0.06$) as % of final body weight between control and HFD+4% EWH treated groups.

6.3.3. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

The glucose tolerance in rats receiving HFD and different dosages of EWH is shown in Fig. 6- 4A as responses over 120 min and in Fig. 6- 4B as the glucose area under the curve (AUC). Rats

receiving 4% EWH had significantly lower glucose response at t = 30 ($P<0.05$), t = 60 ($P<0.01$), t = 90 and 120 ($P<0.05$) compared to the HFD group. The AUC during OGTT also showed a trend towards lower values in HFD+4% EWH compared to the control group ($P=0.08$). Despite a better glucose tolerance in HFD+4% EWH group, no significant difference in insulin concentrations was observed during OGTT between HFD+4% EWH and HFD groups (Fig 6- 5A & B, $P>0.05$).

Rats in EWH treated groups tended to have lower blood glucose levels during the glucose disappearance phase (0–30 min) of the insulin tolerence test but not significant ($P>0.05$). However, the difference became more evident between control and treatment groups during the recovery phase (60–120 min). Indeed, rats in HFD+4% EWH exhibited significantly lower glucose levels at t = 90 ($p<0.05$) and t = 120 min ($p<0.05$) in ITT (Fig 6- 6).

6.3.4. Fasting plasma glucose, insulin, lipids, and inflammatory markers

The metabolic profile of rats fed different diet groups has been summarized in Table 6- 2. There was no significant difference in fasting glucose and insulin levels between different groups ($P>0.05$). Rats fed with HFD+4% EWH showed lower fasting plasma TG levels as compared to HFD treated rats ($P<0.05$) while no significant difefrences were observed between groups for the NEFA and total cholesterol ($P>0.05$).

Plasma inflammatory markers were also assessed in HFD and HFD+4%EWH treated rats at the end of the study (Figure 6- 7). TNF-α and rantes levels were comparable between the two groups. EWH feeding significantly reduced the level of IL-1α ($P<0.05$), IL-β ($P<0.05$), and MCP-1 ($P<0.01$) compared to HFD treated rats.

6.3.5. Tissue insulin sensitivity as measured by p-Akt to total Akt

HFD+4% EWH treatment improved glucose and insulin tolerance in rats as shown by OGTT and ITT data. Moreover, since we observed that EWH enhanced insulin sensitivity by increasing phosphorylation of Akt (p-Akt) in our previous cell experiment study in adipocytes, we further investigated altered tissue insulin sensitivity as one of the possible mechanisms of action of EWH on enhanced glucose tolerance in HFD treated rats. Initially, in the first trial, we assessed the level of p-Akt to total Akt in liver, muscle and retroperitoneal fat pad as major insulin sensitive organs where rats were euthanized in fasted state. Fig 6- 8 A shows that p-Akt was enhanced in liver ($P=0.06$), muscle ($P<0.05$), and retroperitoneal fat pad ($P<0.05$) of rats fed with HFD+4% EWH compared to HFD treated rats. In the second trial, we studied tissue insulin sensitivity more specifically by injecting rats with insulin or saline interaperitoneally 10 min before euthanizing. Muscle, retroperitoneal, and epididymal fat pads were studied for this purpose as the major tissues responsible for insulin-dependent glucose clearance. Fig 6- 8 B shows similar results on enhanced p-Akt to Akt in muscle ($P<0.05$), retroperitoneal ($P<0.05$) and epididymal ($P=0.08$) fat pads of rats fed with HFD+4% EWH compared to HFD treated rats. Fig 6- 9 clearly shows that while insulin administration failed to enhace p-Akt in muscle and adipose tissue of the HFD-treated rats ($P>0.05$) which is an indication of insulin resistance state, feeding rats with HFD+4% EWH enhanced insulin sensitivity significantly in muscle ($P<0.05$), epididymal ($P<0.05$), and retroperitoneal fat depots ($P<0.01$).

6.3.6. Adipose tissue protein expression level of peroxisome proliferator activator receptor gamma (PPARγ)

Since EWH enhanced insulin sensitivity in adiopose tissues, we investigated the potential effects of the treatment on adipose cell differentiation by measuring PPARγ protein expression in both retroperitoneal and epididymal adipose tissues. Figure 6- 10 shows the protein expression of

PPARγ2 was significantly enhanced following EWH treatment in epididymal fat (Fig 6- 10A, $P<0.05$) compared to HFD treated rats. Although, PPARγ2 expression level was also enhanced in retroperitoneal fat, this increase was not significance (Fig 6- 10B, $P>0.05$).

6.3.7. Tissue expression of RAS components

Local RAS links to insulin resistance and type 2 dabetes (Underwood and Adler 2013). Modulation of RAS through renin and ACE inhibition, as well as AT1R antagonism enhance glucose tolerance and whole-body insulin sensitivity in insulin resistant animal models or insulin resistant humans with hypertension (Lender, Arauz-Pacheco et al. 1999, Henriksen, Jacob et al. 2001, Dietze and Henriksen 2008, Lastra, Habibi et al. 2009, Goossens 2012, Marchionne, Diamond-Stanic et al. 2012).The effects of ACE2/ANG1-7/MAS axis on insulin resistance have also been suggested recently (Giani, Mayer et al. 2009, Takeda, Yamamoto et al. 2013). RAS overexpression in skeletal muscle may impair insulin signaling and reduce glucose transporters expression restricting glucose uptake (Henriksen 2007). As one of the potential mechanisms for the observed enhanced insulin sensitivity, we measured the expression of different components of RAS namely ACE, ACE2, AT1R, and AT2R in liver and muscle of rats fed with HFD and HFD+4% EWH (Figs 6- 11 & 6- 12). However, we did not observe any significant differences in the protein expression level of studied RAS components in liver and muscle between HFD and HFD+ 4% EWH groups.

6.4. Discussion

The present study revealed that supplementing a HFD with 4% EWH: (i) changed the body composition towards less fat mass and enhanced lean mass % of BW; (ii) improved glucose tolerance with no changes in the postprandial insulin in glucose intolerant rats; (iii) enhanced overall insulin sensitivity as measured by the insulin tolerance test; (iv) reduced plasmatic pro-

inflammatory cytokines IL-1α, and IL-1β, and chemokine MCP-1; (v) enhanced insulin sensitivity in muscle and fat in both fasted and insulin stimulated states; and (vi) enhanced PPARγ expression in epididymal adipose tissue which can be an indication of enhanced adipogenic differentiation.

Diet induced and genetic models are the two major animal models used in literature to study MetS. Diet induced models are generally appreciated as closer animal models to pathogenesis of MetS in humans (Buettner, Scholmerich et al. 2007, Panchal and Brown 2011). High fat diet has been used extensively for induction of MetS symptoms in rodents (Huang, Hong et al. 2008, Gollisch, Brandauer et al. 2009, Shihabudeen, Roy et al. 2015). Prolonged high fat diet feeding is well known to induce insulin resistance and glucose intolerance (Cerf, Chapman et al. 2012, Hashemi, Yang et al. 2015, Yang, Hashemi et al. 2015), adipocyte dysfunction and inflammation (Johnson, Trasino et al. 2007, Shihabudeen, Roy et al. 2015), and dyslipidemia (Shih, Shlau et al. 2014, Jung, Cho et al. 2016) in rodents. Therefore, high fat induced insulin resistant Sprague Dawley rats were chosen as the animal model in this study. Supplementing HFD with 4% EWH changed the body composition by reducing fat mass at the end of the study. Results from the OGTT also indicated that rats fed with 4% EWH exhibited improved glucose tolerance as compared to the control group. With no changes in insulin concentrations in OGTT blood samples, 4% EWH treated rats are potentially more sensitive to insulin. An insulin tolerance test was then conducted to further explore this possibility, and the results suggest an overall increased insulin sensitivity in EWH treated rats as shown by the significant effect of the treatment ($P<0.01$) by two-way ANOVA. Furthermore, 4% EWH tended to increase glucose disappearance in the first phase (0-60 min) and potentially suppress hepatic glucose production in the second phase (60-120 min) of the insulin tolerance test. Several food proteins and

bioactive peptides have been reported to improve glucose and insulin tolerance in literature. Supplementation of rats' diet with 15% whey protein (but not with 5%) reduced insulin resistance and improved glucose uptake in insulin resistant rats (Tong, Li et al. 2014). Feeding cod protein (at 23% w/w dose) along with HFD for 4 weeks also prevented the development of insulin resistance in rats whereas casein and soy protein (at the same dose) did not show any effects. Higher rates of insulin-mediated muscle glucose disposal were observed in cod protein fed rats (Lavigne, Tremblay et al. 2001). Recently, chlorella protein hydrolysate has also shown favorable effects on glucose tolerance and insulin sensitivity at 1 g/Kg BW dosage in high fat fed mice potentially through affecting adipose tissue (Noguchi, Yanagita et al. 2016).

Based on the ITT data, to further explore whether 4% EWH treatment enhanced insulin signaling in tissues, we assessed the phosphorylation level of Akt in major insulin sensitive tissues namely liver, muscle and fat. We observed an enhanced Akt phosphorylation in fat (retroperitoneal fat depot), and muscle in 4% EWH treated rats in the fasting state. Despite the improved insulin signaling in the fasted state, we did not observe any significant differences in fasting glucose and insulin between HFD and treatment groups. Feeding rats with whey protein hydrolysate has been reported to enhance both the muscle total Akt protein (Morato, Lollo et al. 2013) which can be an indication of increased insulin signaling capacity, as well as Akt phosphorylation in skeletal muscle with no changes in fasting glucose and insulin levels (Morato, Lollo et al. 2013) similar to our data. This enhanced capacity for insulin signaling may be partially responsible for the enhanced GLUT-4 translocation to the plasma membrane in the skeletal muscle (Morato, Lollo et al. 2013). Despite the enhanced Akt phosphorylation, no change on the protein expression of total Akt (Fig 6- 9D) was observed in our study indicating that insulin signaling capacity was no affected by 4% EWH treatment. In another study, daily administration of aglycin, a 37-amino-

acid polypeptide isolated from soybean, at dose of 50 mg/kg/d for 4 weeks effectively controlled hyperglycemia and enhanced oral glucose tolerance in streptozotocin/high-fat-diet-induced diabetic mice (Lu, Zeng et al. 2012). Diabetic mice had reduced protein levels of insulin receptor, IRS1, Akt and GLUT4 in skeletal muscle compared to normal mice, while aglycin enhanced insulin receptor and IRS1 gene and protein expression significantly compared to the diabetic mice. This peptide further elevated the expression of p-IRS1, p-Akt and membrane GLUT4 protein (Lu, Zeng et al. 2012).

In Chapter 5 we showed a significant increase in insulin sensitivity through enhanced Akt phosphorylation (normalized to total Akt) in EWH treated adipocytes after stimulating cells with insulin. Our observations that EWH 1) improved glucose tolerance in OGTT, 2) enhanced overall insulin sensitivity through ITT, and 3) enhanced Akt phosphorylation in fasted state in addition to our previous data in cell study, led us to further explore tissue insulin sensitivity following insulin stimulation. Interestingly, consistent with our previous data, insulin sensitivity was enhanced significantly in epididymal ($P<0.05$) and retrperitoneal ($P<0.01$) fat depots as well as muscle ($P<0.05$) in EWH treated rats (Fig 6- 9A-C). Therefore, we concluded that enhanced insulin sensitivity in adipose tissue and muscle could be one of the possible mechanisms for the observed improved glucose tolerance in 4% EWH treated rats.

When assessing metabolic profile in rats treated with EWH, we did not observe any significant effects of the treatment on plasma lipid components except for the fasting plasma TG. Indeed, rats treated with 4% EWH exhibited lower levels of plasma TG levels. Our current data does not explain the change in plasma TG levels and this aspect may deserve further exploration in future studies. The reduced plasma TG level may be due to the reduced de novo lipid synthesis by the liver or enhanced accumulation in the tissues especially in adipose tissue as opposed to

remaining in the circulation. Insulin increases de novo lipid synthesis in hepatocytes. It has been shown that insulin regulates the sterol regulatory element binding protein-1c (SREBP1c) downstream of the Akt. SREBP1c is the dominant insulin-stimulated isoform in the liver, responsible for inducing lipogenic gene expression and promoting fatty acid synthesis (Horton, Goldstein et al. 2002). Our preliminary data indicated an increased Akt phosphorylation in the liver of 4% EWH treated rats compared to HFD treated group. Therefore, it appears that the potential effects of EWH on lipid metabolism is through downstream of Akt in liver, if any. Another possibility is the enhanced accumulation of fat in adipose tissue. Smaller adipocytes having more capacity to store lipids contribute to the reduced plasma TG and increased insulin sensitivity. Indeed, our data on enhanced insulin sensitivity in adipose tissue along with the increased PPARγ2 expression in the epididymal fat can be an indication for the enhanced adipocyte differentiation *in vivo* leading to the reduced plasma TG levels. Further experiments are necessary to better show any potential links between the observed effects of EWH on lipid metabolism and adipocyte function.

Furthermore, we observed reduced secretion of several pro-inflammatory markers (IL-1α and β and MCP-1) in the plasma of rats treated with 4% EWH which is an indication of less systemic inflammation in these rats compared to the HFD treated rats. Obesity has been linked with a low-grade, chronic inflammatory response characterized by altered adipokines production and increased markers of inflammation (Neels and Olefsky 2006). Considering the critical role of inflammation in the occurrence of insulin resistance (de Luca and Olefsky 2008), the importance of the anti-inflammatory effect of the EWH in high fat treated rats is more highlighted. IL-1β, the major pro-inflammatory cytokine, has been reported to be critically involved in inflammation-induced insulin resistance by reducing the expression of insulin signaling and glucose transporter

proteins (Bing 2015). MCP-1 is also suggested to be directly implicated in insulin resistance through attracting macrophages to adipose tissue and promoting IL-1 and TNF-α release, impairing insulin stimulated glucose uptake and insulin receptor tyrosine phosphorylation, and inhibiting adipocyte growth and differentiation (Mlinar, Marc et al. 2007). Inflammation within white adipose tissue, one of the major sites for inflammatory responses, induces insulin resistance locally and systematically due to the diffuse nature and close association of adipose tissue with other metabolically active tissues (Kassi, Pervanidou et al. 2011). Although we did not target the association of adipose tissue specifically to the less inflammatory phenotype of EWH treated rats, our data on enhanced PPARγ2 expression in the epididymal fat, and tissue insulin sensitivity, consistent with the outcomes of our previous study in cells, points to a potential effect of the treatment on adipose tissue. The effect of food proteins and their hydrolysates on reduced adipose tissue inflammation has been reported in literature. Feeding rats with fish proteins reduces visceral adipose tissue expression of IL-6 and TNF-α as compared to casein fed rats (Pilon, Ruzzin et al. 2011). Rice bran protein hydrolysate with beneficial effects on insulin resistance, promoted the gene expression of PPARγ in adipose tissue of high carbohydrate-high fat fed rats similar to pioglitazone. Adipose tissue inflammatory markers were also decreased in rats treated with rice protein hydrolysate (Boonloh, Kukongviriyapan et al. 2015). In another study, generation of smaller adipocytes, lower triglycerides in liver, reduced serum MCP-1, as well as adipose tissue mRNA expression were correlated with enhanced glucose tolerance and insulin sensitivity in chlorella hydrolysate treated mice as compared to the control group (Noguchi, Yanagita et al. 2016). Considering the role of MCP-1 in development of inflammation and macrophage infiltration, less adipose tissue inflammation was suggested as the potential mechanism for the observed beneficial effects of chlorella-derived peptides in obese

mice (Noguchi, Yanagita et al. 2016). Since whole protein hydrolysates used in the above-mentioned studies were not characterized for their bioactive peptides, it is not known which peptide(s) are responsible for the reported effects. Identification of the responsible peptides in protein hydrolysates would help better understand the structure-function relationship of these peptides. VPP, the tripeptide derived from whey protein initially identified as an ACE inhibitor with reported antihypertensive effects (Nakamura, Mizutani et al. 2009, Nakamura, Mizutani et al. 2011) and beneficial effects on adipogenic differentiation and inflammation *in vitro* (Chakrabarti and Wu 2015), also ameliorated diet-induced chronic inflammation in adipose tissue *in vivo* (Aihara, Osaka et al. 2014, Sawada, Sakamoto et al. 2015). Furthermore, VPP administration improved insulin sensitivity, reduced TNF-α and IL-1β expression, and macrophage accumulation and activation in diet induced obese mice (Sawada, Sakamoto et al. 2015). Although reduced adipose inflammation was suggested as a potent mechanism for the enhanced insulin sensitivity, the precise molecular mechanism remained to be identified.

We like to note that one more explanation for the observed less inflammatory phenotype of EWH treated rats could be due to the reduction in fat mass with pro-inflammatory characteristics. Since our data revealed that 4% EWH reduced fat mass, we cannot exclude this possibility. Future experiments on macrophage infiltration to adipose tissue, its morphology, and gene and protein expression of inflammatory markers derived from adipose tissue will help better shape the potential benefits of EWH on adipose tissue health and function.

Finally, modulation of RAS components as another potential mechanism for the observed enhanced insulin sensitivity following treatment with 4% EWH was studied in liver and muscle of rats in treatment and control groups. It appeared that this pathway in muscle and liver was not involved in the observed effects of EWH on insulin sensitivity in the current study. We did not

measure the protein expression of RAS components in adipose tissue of treatment and control rats in this study, which worth investigation especially when EWH treatment reduced AT1R expression about 50% in 3T3-F442A adipocytes (Appendix 6-B).

In conclusion, results in this study confirm the beneficial effects of chronic supplementation with 4% EWH on glucose tolerance and insulin sensitivity in diet induced insulin resistant SD rats mainly through affecting tissue insulin sensitivity.

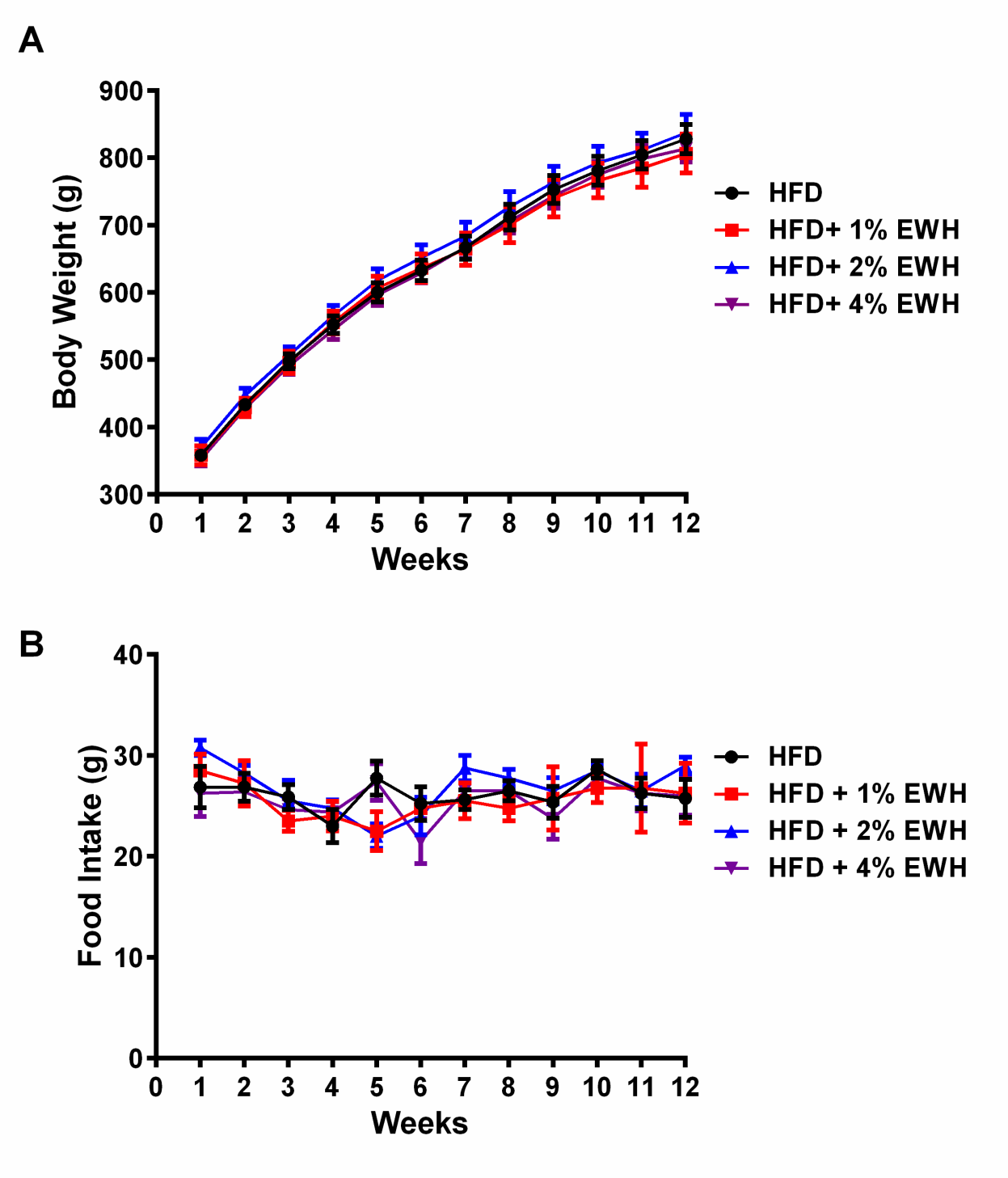


Figure 6-1. Effects of feeding EWH at different dosages on (A) body weight and (B) food intake in rats throughout the 12-week study period. There was no significant difference between groups as assessed by two-way ANOVA and Bonferroni's post-hoc comparison test ($P>0.05$). Data is shown as the Mean ± SEM for n= 7-16 rats.

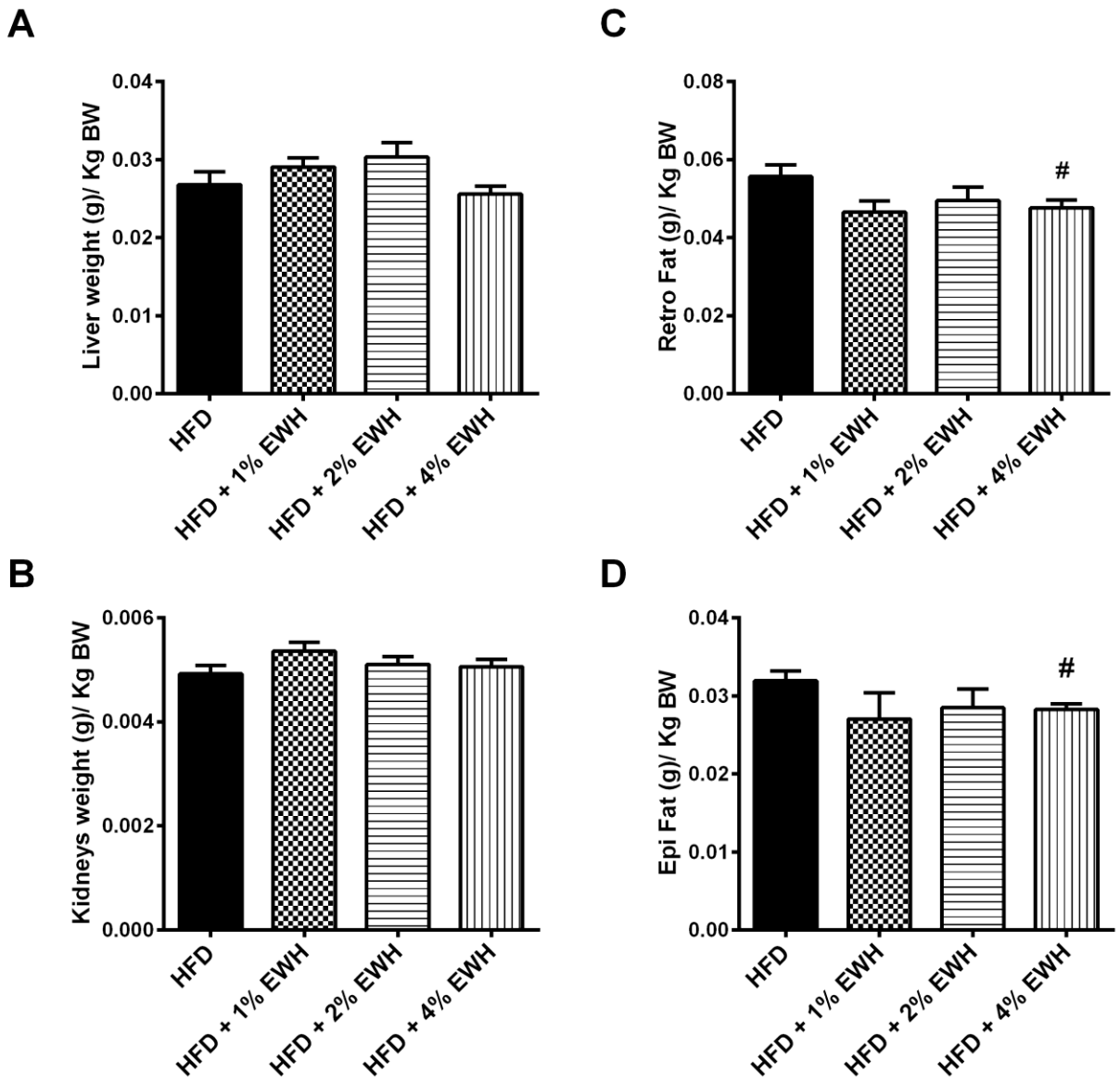


Figure 6-2. Effects of feeding EWH at different dosages on relative tissue weight in rats by the end of the study. (A) relative liver, (B) kidneys, (C) retroperitoneal fat pad, and (D) epididymal fat pad weight in different treatment groups. Data is shown as the Mean ± SEM for n= 7-16 rats. Data has been analyzed by one-way ANOVA and Bonferroni's post-hoc comparison test. # shows significant difference compared to HFD by two-tailed t-test ($P< 0.05$).

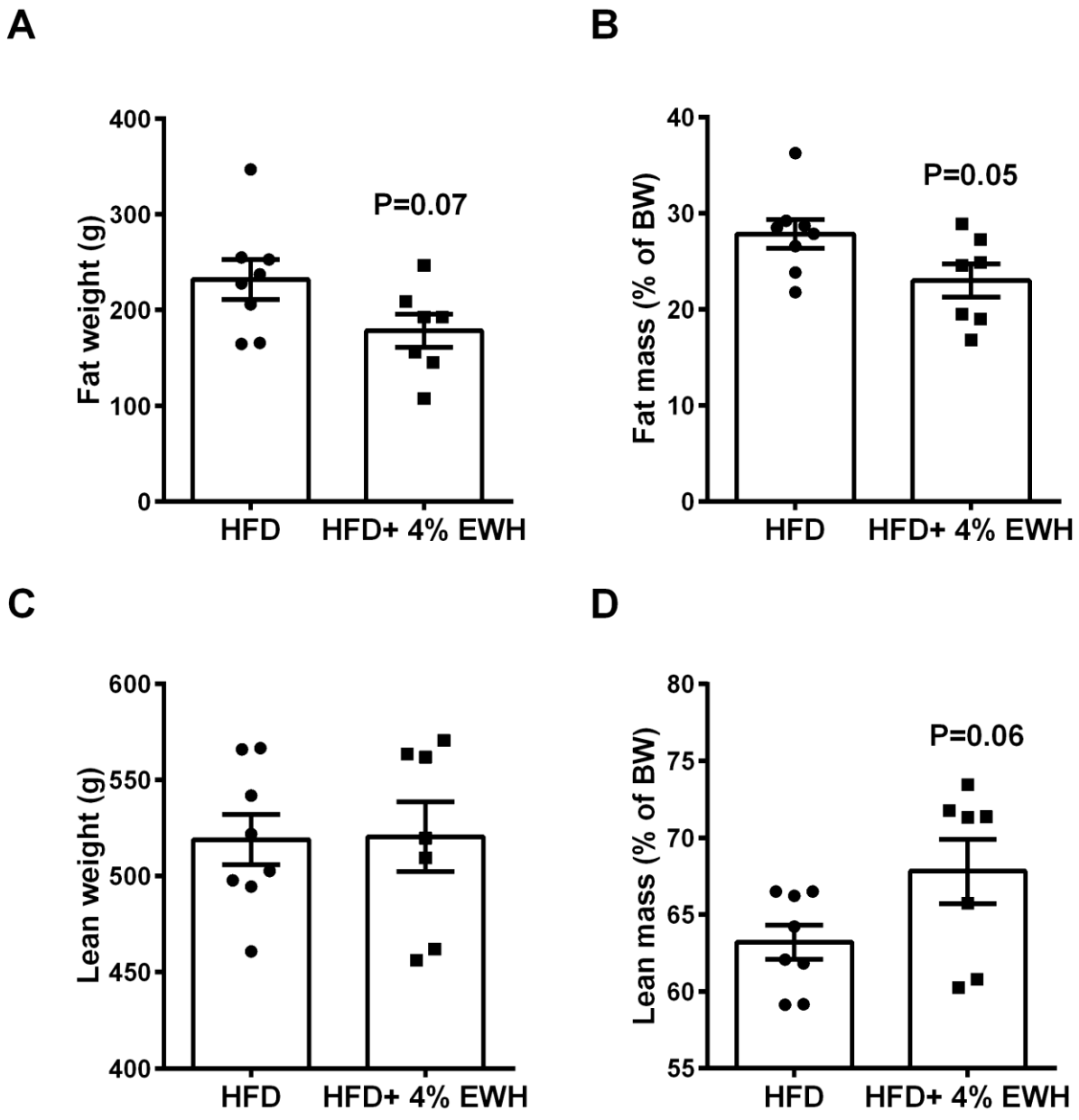


Figure 6-3. Effect of 4% EWH feeding on body composition determined by MRI. Rats fed with 4% EWH tended to have lower (A) absolute and (B) relative fat mass, (C) no change in absolute and (D) tended to have higher relative lean mass. Data is shown as Mean ± SEM for n = 7-8 rats as analyzed by two-tailed t-test.

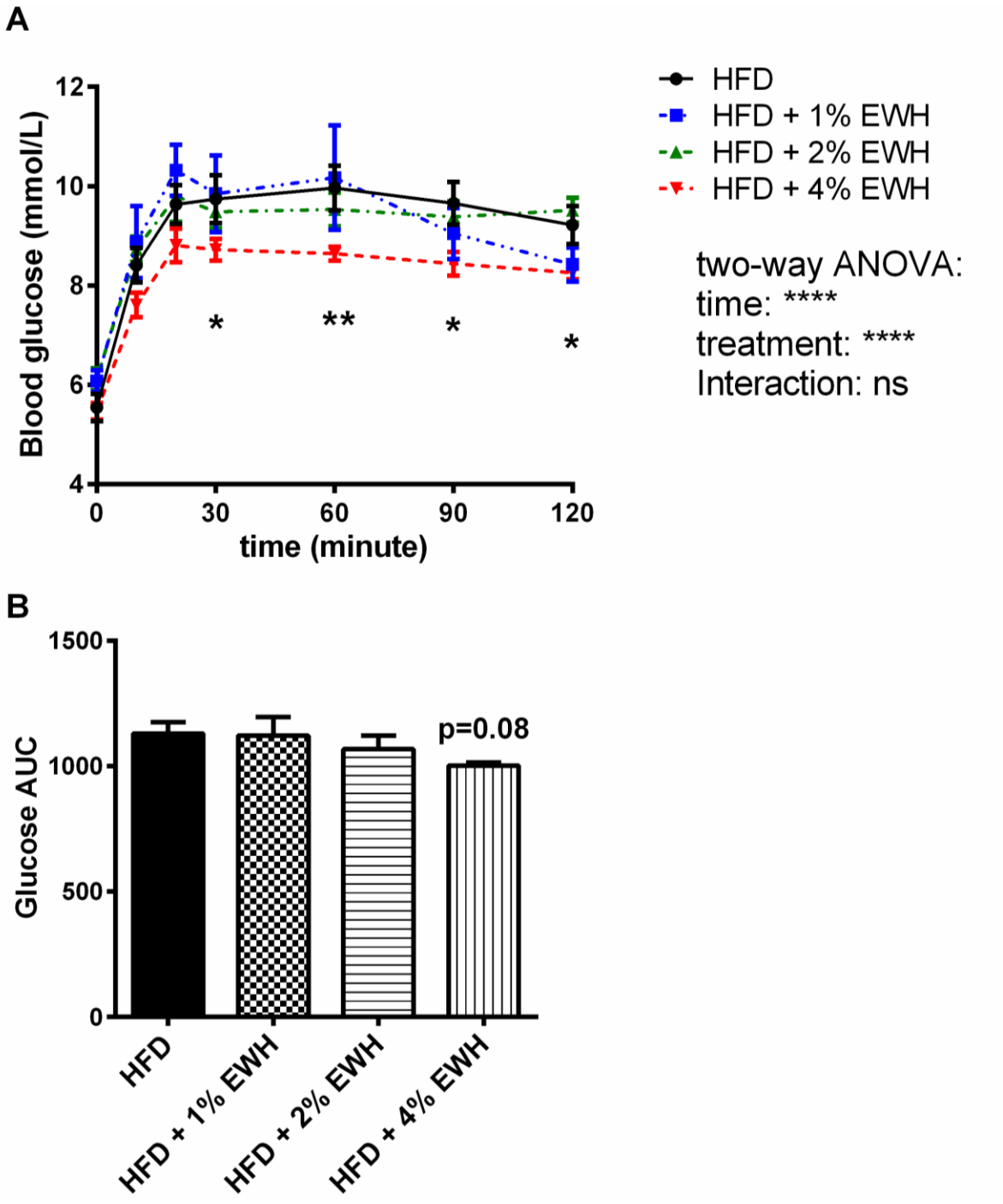


Figure 6-4. Effect of feeding different dosages of EWH on oral glucose tolerance. (A) OGTT was performed after 5 weeks of treatment after overnight fast in rats fed with a HFD (20% w/w) supplemented with 1, 2, and 4% of EWH by oral administration of 2g/kg glucose. (B) The area under the curve (AUC) was calculated for glucose during OGTT. *, **, and **** show significant difference compared to HFD by one-way ANOVA followed by Bonferroni's post-hoc comparison test at $P< 0.05$, $P<0.01$, and $P<0.0001$ respectively.

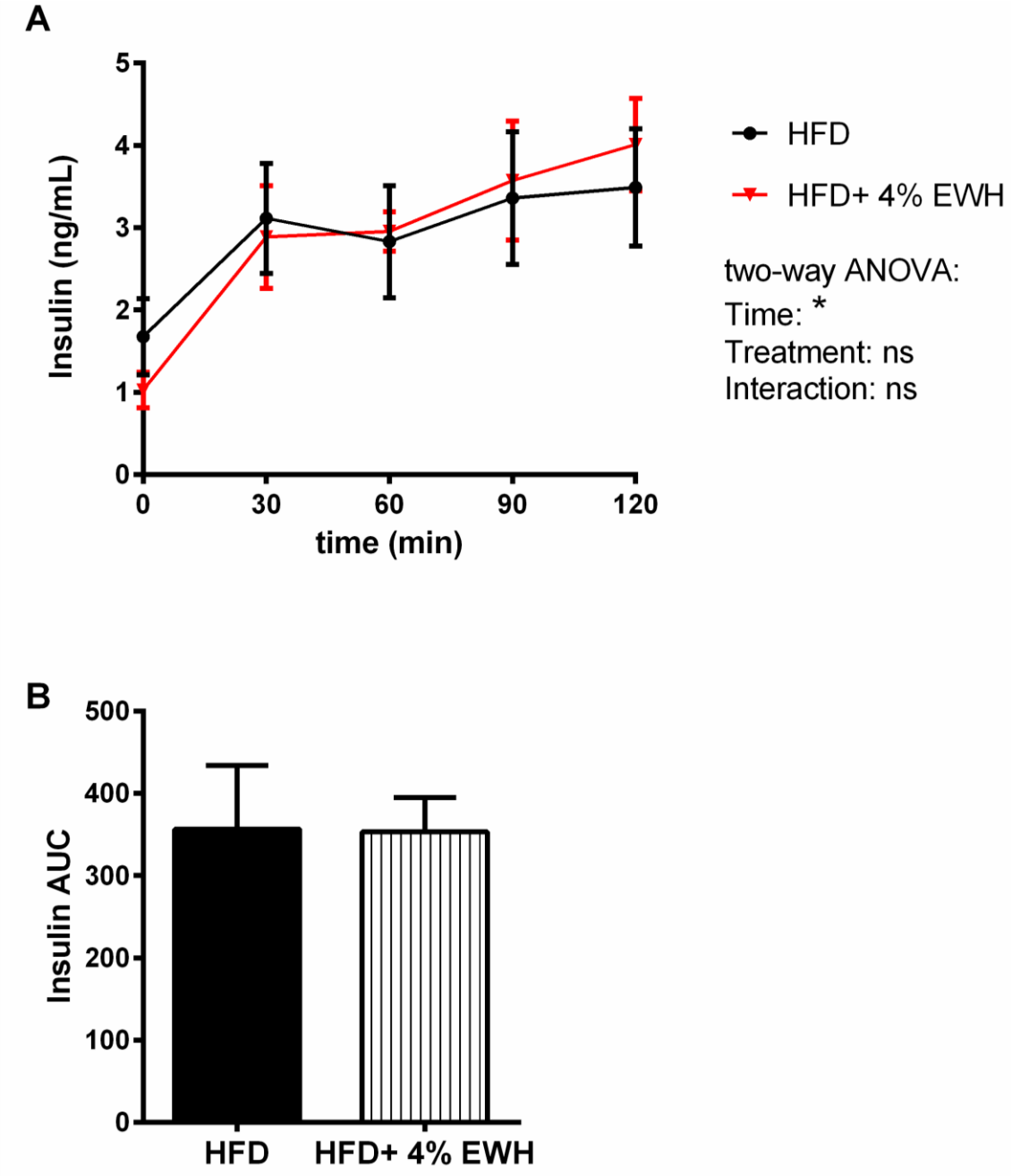


Figure 6-5. Effect of feeding 4% EWH on plasma insulin levels. (A) Plasma insulin concentrations measured in blood samples collected during the OGTT. (B) The AUC for insulin during OGTT. Data is shown as the Mean ± SEM for n= 4 rats. There were no significant differences between HFD and HFD+4% EWH on plasma insulin levels as assessed by two-way ANOVA followed by Bonferroni's post-hoc comparison test (P>0.05).

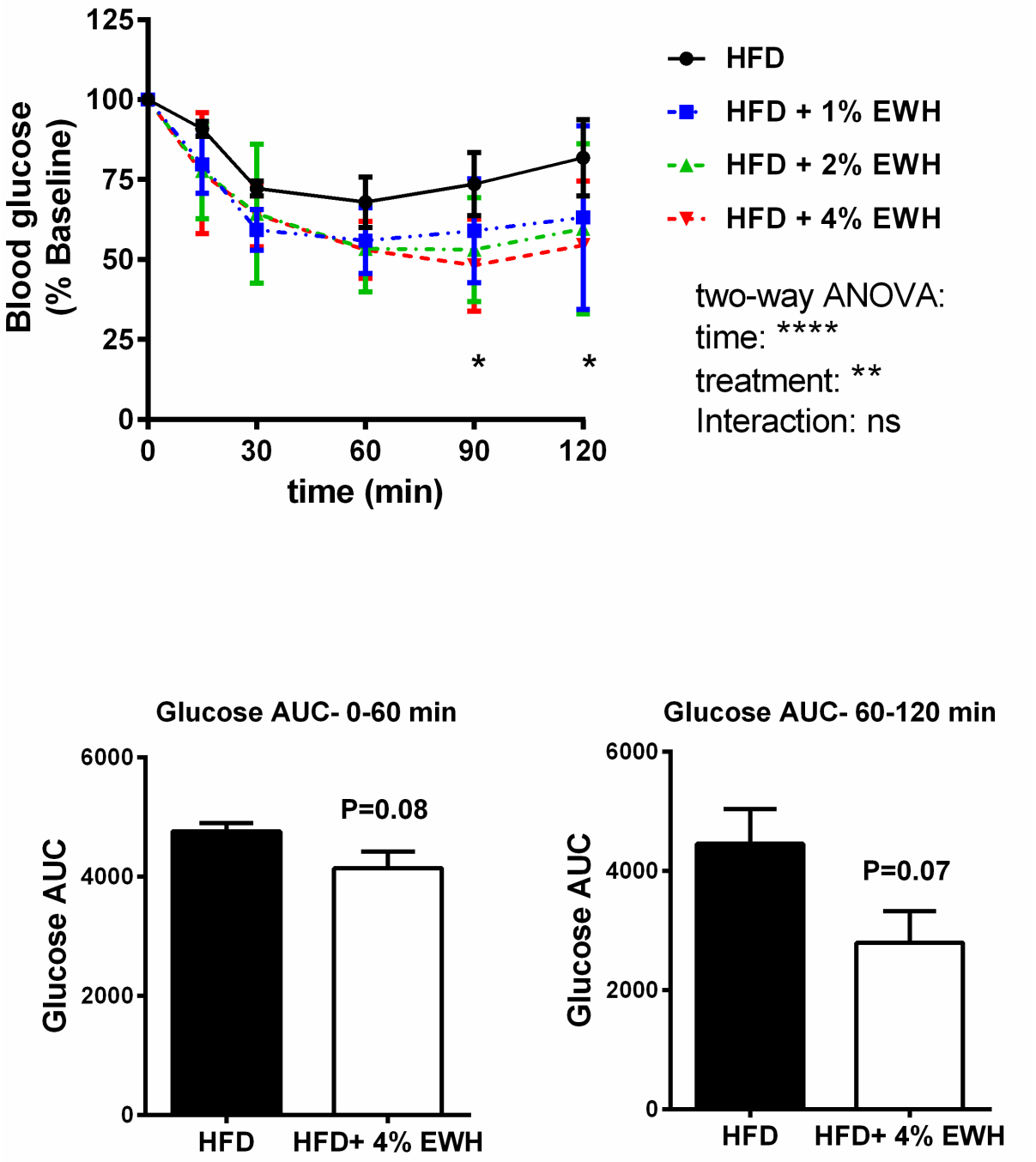


Figure 6-6. Effect of 6 weeks of feeding different dosages of EWH on insulin tolerance. ITT was performed on rats after a 4 hour fast. Blood glucose levels are shown as % of basal glucose. Data is shown as the Mean ± SEM for n= 5 rats analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. *, **, and **** shows significant difference at $P<0.05$, $P<0.01$ and $P<0.0001$ respectively. Area Under the Curve (AUC) was calculated from t= 0 to t = 60 min and t = 60 to t = 120 min for blood glucose in FHD and HFD+4% EWH and analyzed by two-tailed t-test.

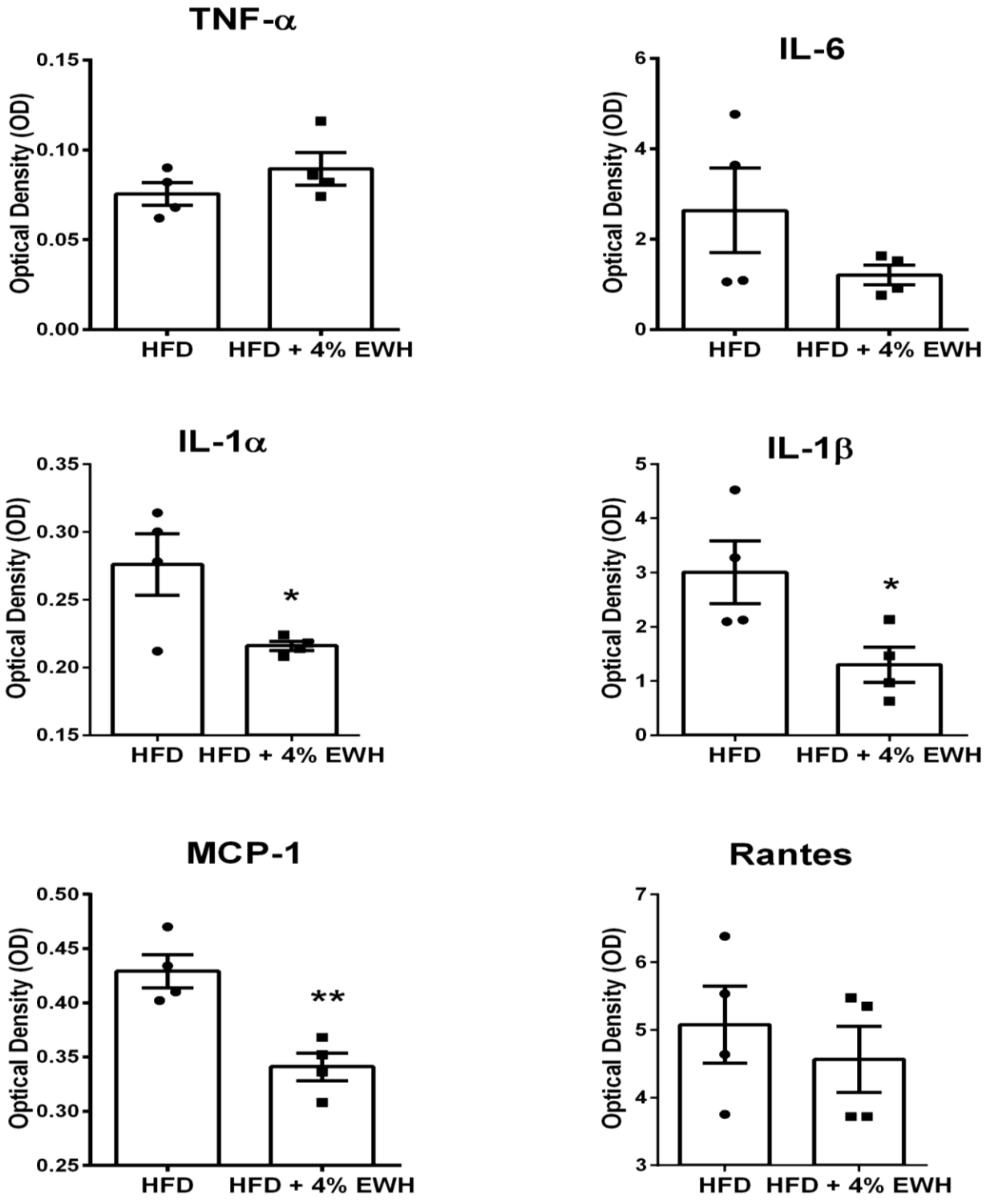


Figure 6-7. Effect of EWH feeding on plasma inflammatory markers. Data is shown as Mean ± SEM for n = 4 analyzed by two-tailed t-test. * and ** show significant difference at $P<0.05$ and $P<0.01$ respectively.

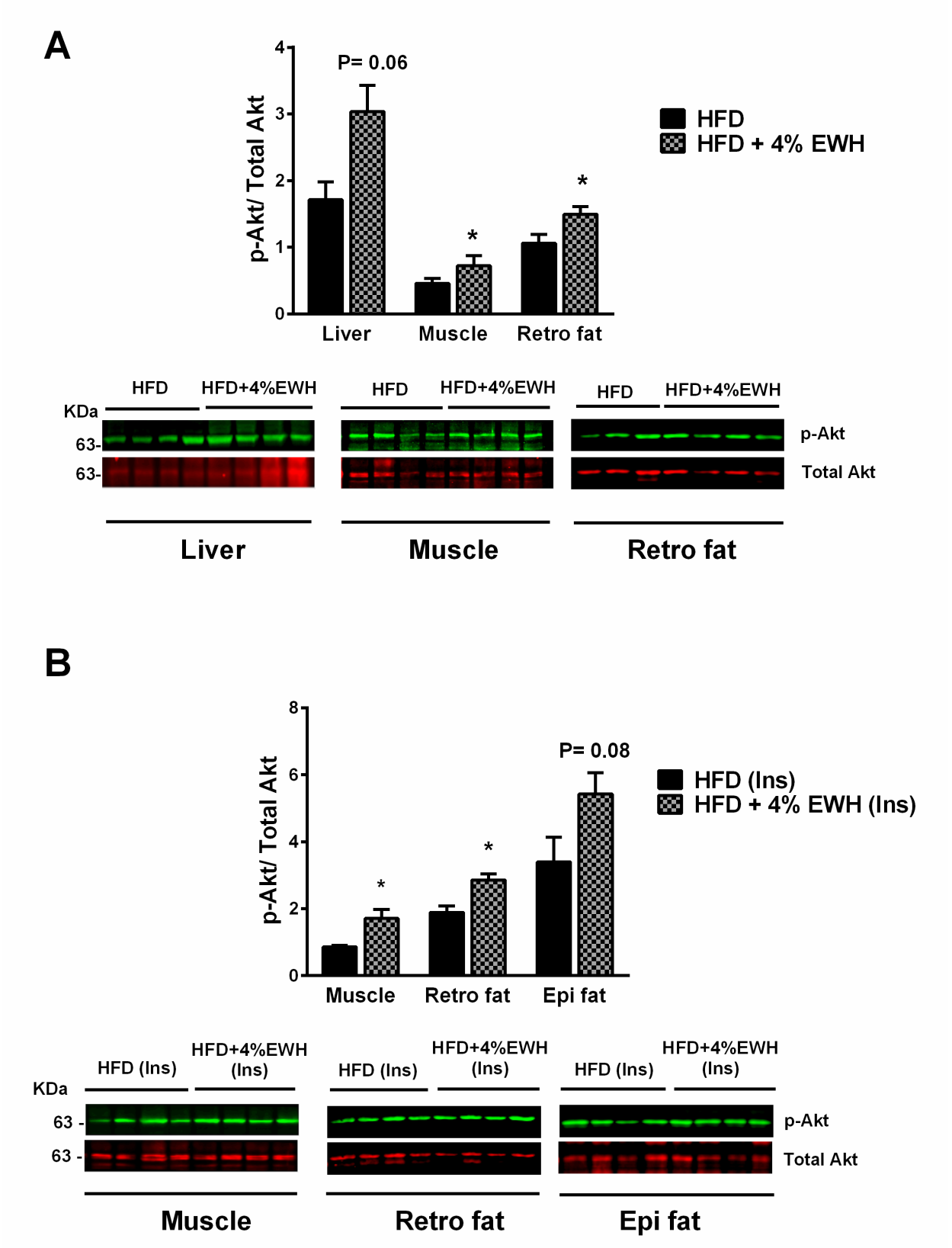
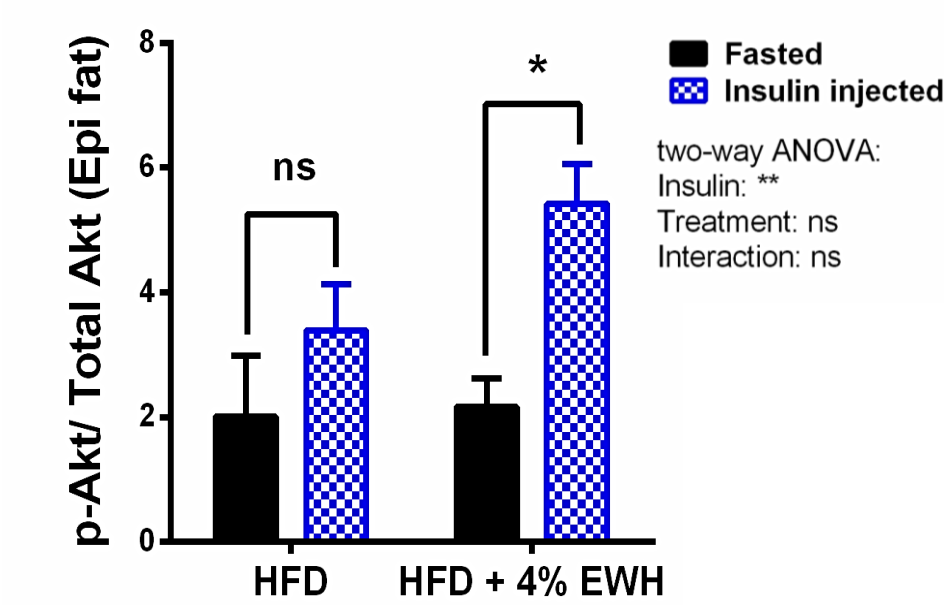
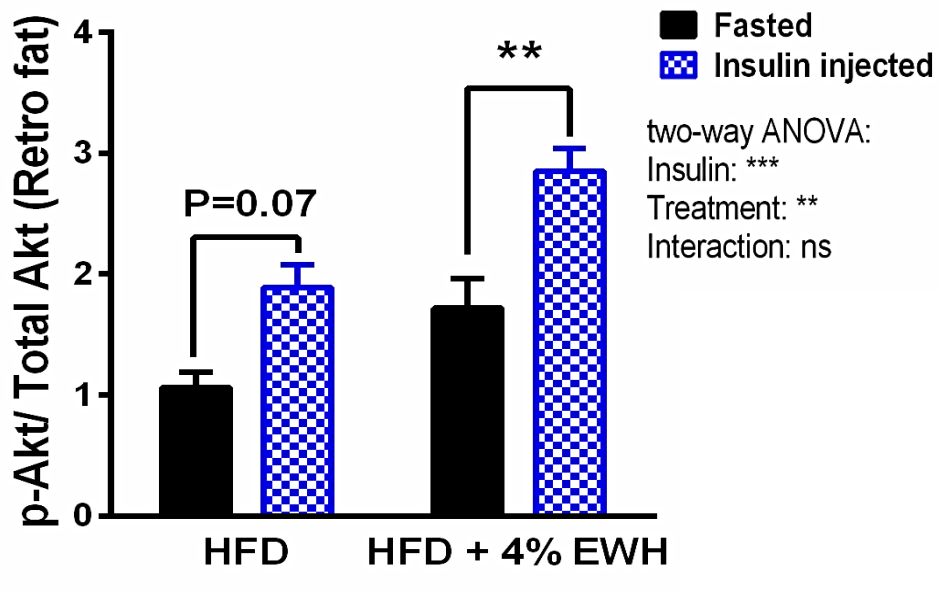

A
P= 0.06
p-Akt/ Total Akt
HFD
HFD + 4% EWH
Liver
Muscle
Retro fat
HFD
HFD+4%EWH
KDa
63-
p-Akt
Total Akt
B
P= 0.08
HFD (Ins)
HFD + 4% EWH (Ins)
Epi fat
HFD+4%EWH (Ins)
63 -

Figure 6-8. Effects of feeding 4% EWH on tissue insulin sensitivity in rats euthanized in the fasted state (A) and injected by insulin (i.p.) 10 min before euthanizing in the second animal trial. The protein band of p-Akt was normalized to total Akt as a measure of insulin sensitivity in liver, muscle, and retroperitoneal fat pad (A) and in muscle, Retroperitoneal and epididymal fat pads (B) in HFD and HFD+4%EWH treated groups. Data is shown as the Mean ± SEM for n= 4-8 rats. Data has been analyzed by two-tailed t-test. * shows significant difference at $P< 0.05$ as compared to their corresponding control (HFD group).

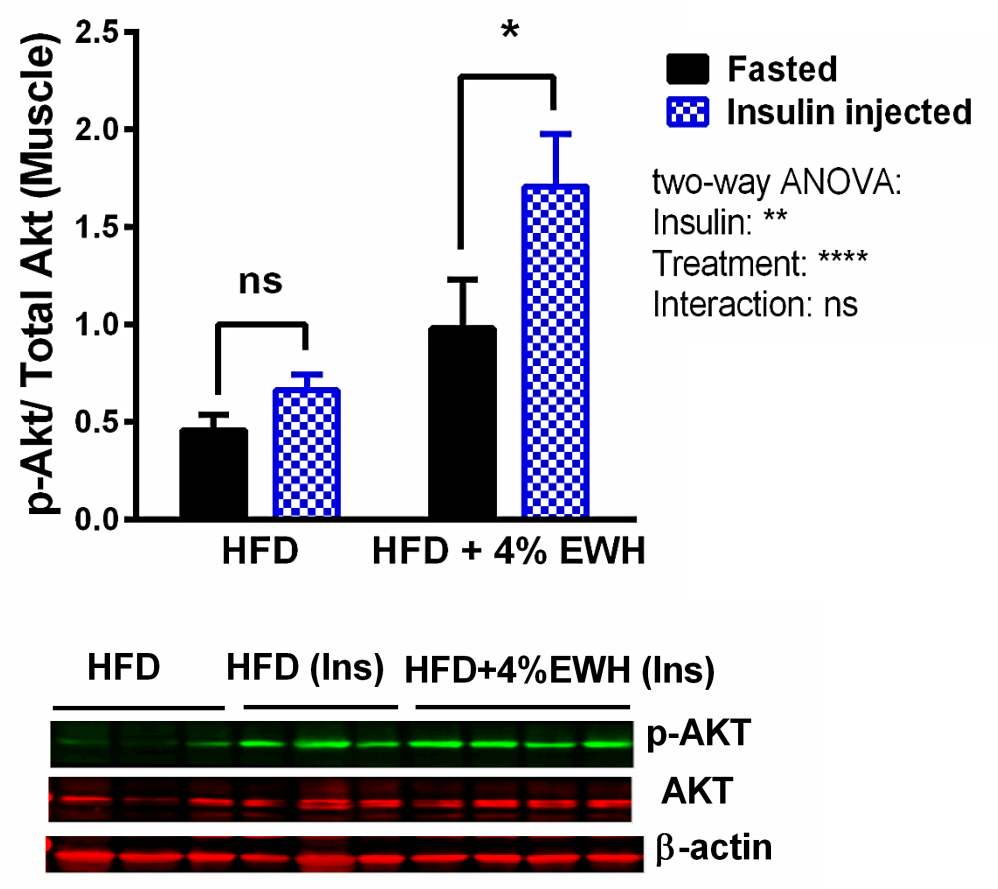
A


p-Akt/ Total Akt (Epi fat)
8
6
4
2
0
ns
*
HFD
HFD + 4% EWH
Fasted
Insulin injected
two-way ANOVA:
Insulin: **
Treatment: ns
Interaction: ns

B


p-Akt/ Total Akt (Retro fat)
4
3
2
1
0
P=0.07
**
HFD
HFD + 4% EWH
Fasted
Insulin injected
two-way ANOVA:
Insulin: ***
Treatment: **
Interaction: ns

C


p-Akt/ Total Akt (Muscle)
2.5
2.0
1.5
1.0
0.5
0.0
*
ns
Fasted
Insulin injected
two-way ANOVA:
Insulin: **
Treatment: ****
Interaction: ns
HFD
HFD + 4% EWH
HFD
HFD (Ins)
HFD+4%EWH (Ins)
p-AKT
AKT
β-actin

D

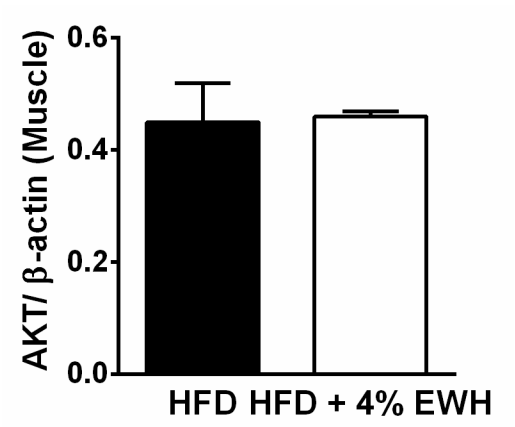

AKT/ β-actin (Muscle)
0.6
0.4
0.2
0.0
HFD
HFD + 4% EWH

Figure 6-9. Effects of feeding 4% EWH on insulin sensitivity in epididymal fat (A), retroperitoneal fat (B), and muscle (C) and the total expression of Akt in muscle (D) compared to HFD treated rats. The protein band of p-Akt was normalized to total Akt as a measure of insulin sensitivity and to β-actin (D) in HFD and HFD+4%EWH treated groups. Data is shown as the Mean ± SEM for n= 4-8 rats. Data has been analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test in A and B and a two-tailed t-test in C. *, **, ***, and **** shows significant difference at $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$ respectively.

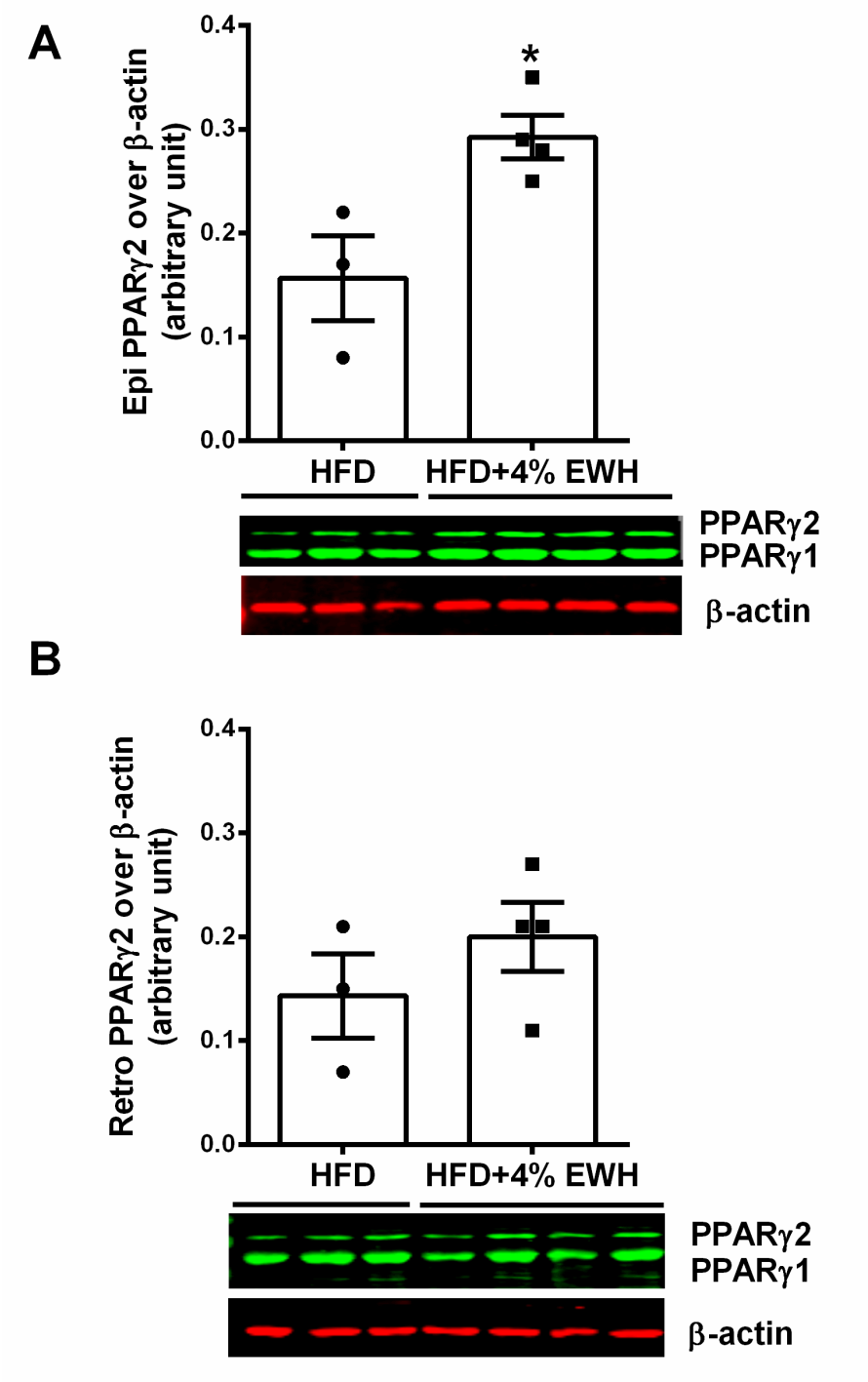


Figure 6-10. Effects of feeding 4% EWH on adipose tissue PPARγ expression. The protein band of PPARγ2 was normalized to β-actin as the loading control in (A) epididymal fat, and (B) retroperitoneal fat in HFD and HFD+4%EWH treated groups. Data is shown as the Mean ± SEM for n= 3-4 rats. Data has been analyzed by two-tailed t-test. * shows significant difference at P< 0.05.

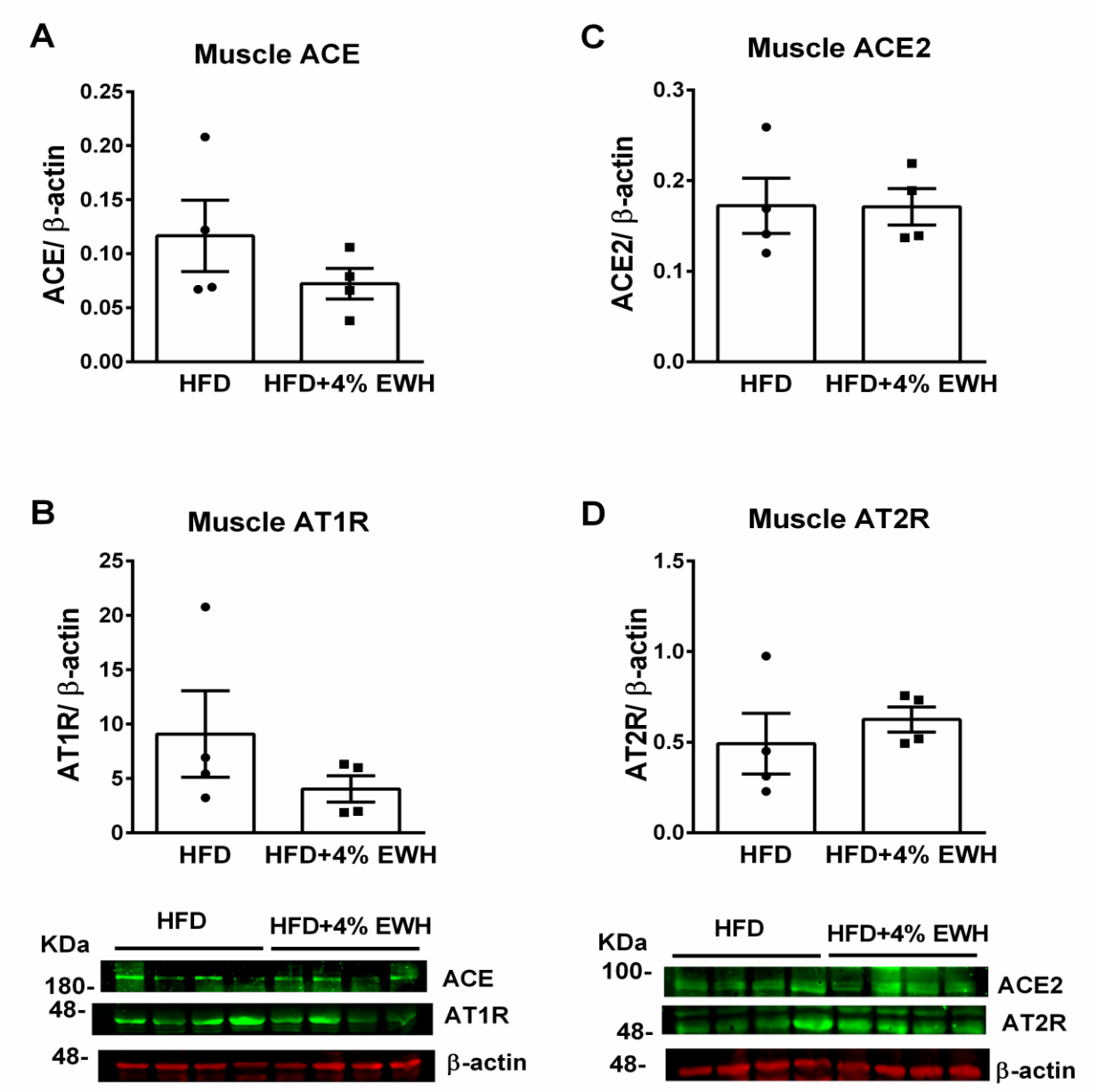


Figure 6-11. Effects of feeding 4% EWH on muscle RAS components expression. The protein band of ACE (A), AT1R (B), ACE2 (C), and AT2R (D) was normalized to β-actin as the loading control in HFD and HFD+4%EWH treated groups. Data is shown as the Mean ± SEM for n= 4 rats.

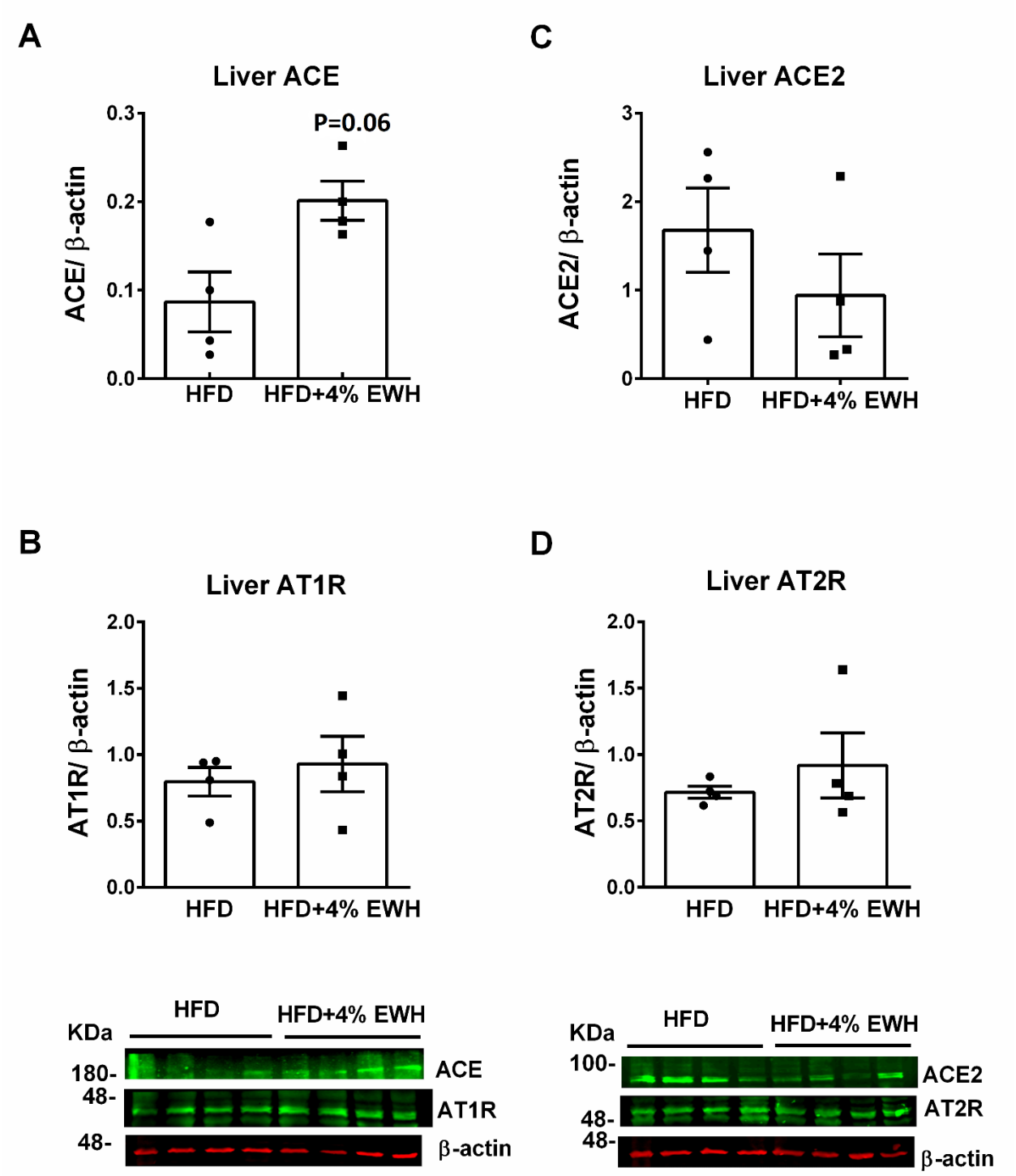
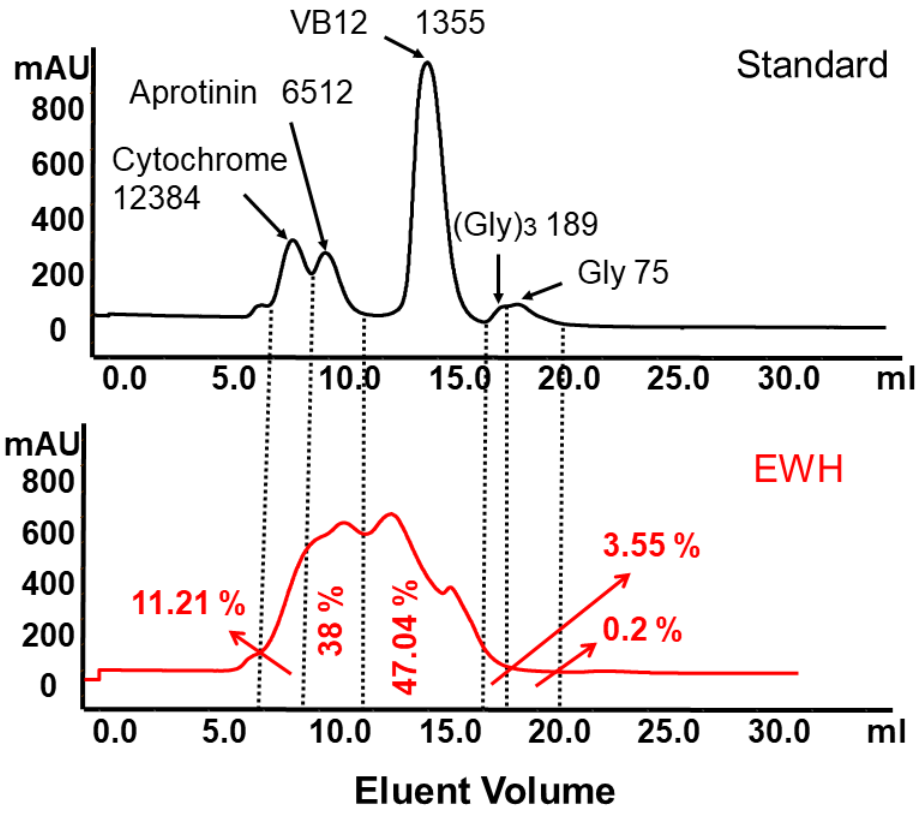


Figure 6-12. Effects of feeding 4% EWH on liver RAS component expression. The protein bands of ACE (A), AT1R (B), ACE2 (C), and AT2R (D) was normalized to β-actin as the loading control in HFD and HFD+4%EWH treated groups. Data is shown as the Mean ± SEM for n= 4 rats. Data has been analyzed by two-tailed t-test. * shows significant difference at P< 0.05.

6.5. **Appendix 6-A. Supplementary data. Size profile of EWH peptides.**



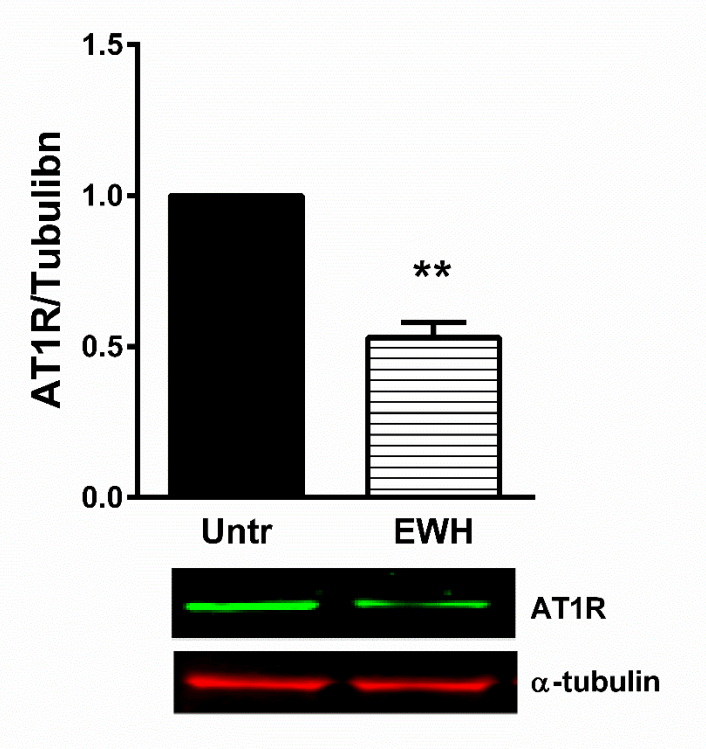
The majority of EWH-derived peptides (more than 85%) are in the range of 6.51 to 1.36 KDa. A small fraction of peptides (11.21%) with higher molecular weight between 12.38 to 6.51 KDa also present in the EWH.

Size exclusion chromatography

The molecular weight distribution of EWH peptides was determined using size-exclusion chromatography on an AKTA liquid chromatography system (GE Healthcare, Uppsala, Sweden) coupled with a Superdex Peptide 10/300GL column at room temperature. EWH was dissolved in 30% aqueous acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) and filtered through 0.22 µm filters. 100 µL of the sample was injected into the column and separated using an isocratic elution at a flow rate of 0.6 mL/min with 30% ACN containing 0.1% TFA. The absorbance of the eluent was monitored at 215 nm. Molecular

weight markers (cytochrome c, 12384 Da; aprotinin, 6512 Da; vitamin B12, 1355 Da; (glycine)3, 189 Da; and glycine, 75 Da) were run under identical conditions to obtain the standard curve.

6.6. Appendix 6-B. Supplementary data. EWH reduced AT1R expression in 3T3-F442A pre-adipocytes compared to untreated control group.



Data is shown as the Mean ± SEM for n= 4. Data has been analyzed by two-tailed t-test. ** shows significant difference at P< 0.01.

Table 6- 1. Composition of the experimental diets (g/Kg)

Ingredients (g/Kg)	Groups			
	HFD	HFD + 1% EWH	HFD + 2% EWH	HFD + 4% EWH
Casein	280	271.3	262.5	245
EWH	0	10	20	40
Sucrose	200	200	200	200
Lard	195	195	195	195
Maltodextrin	115	115	115	115
Corn starch	85	85	85	85
Cellulose	58	58	58	58
Mineral mix	43	43	43	43
Soybean oil	30	30	30	30
Vitamin mix	19	19	19	19
L-Cystine	3.5	3.5	3.5	3.5
Calcium phosphate	3.4	3.4	3.4	3.4
Choline bitartrate	3	3	3	3

Table 6- 2. Metabolic profile of rats fed high fat diet (HFD) containing different dosages of egg white hydrolysate (EWH).

Diet groups	HFD	HFD + 1% EWH	HFD + 2% EWH	HFD + 4% EWH
Fasting glucose (mg/dL)	92.54 ± 3.06	102.6 ± 3.46	101.4 ± 2.49	98.59 ± 1.85
Fasting insulin (uIU/ml)	44.78 ± 7.77	36.48 ± 13.24	34.58 ± 3.24	35.90 ± 7.17
HOMA-IR	10.75 ± 2.32	10.09 ± 3.88	9.21 ± 1.06	9.10 ± 2.74
TG (mg/dL)	98.31 ± 8.61	105.10 ± 4.19	80.59 ± 11.37	67.22 ± 8.59*
NEFA (mEq/L)	0.59 ± 0.06	0.58 ± 0.09	0.59 ± 0.06	0.62 ± 0.06
Total cholesterol (mg/dL)	80.78 ± 6.88	92.67 ± 5.10	63.16 ± 12.27	64.27 ± 10.26

HOMA-IR, homeostatic model assessment insulin resistance (fasting glucose (mg/dL) × fasting insulin (μU/mL) /405; TG, triglyceride; NEFA, non-esterified fatty acids. Data is shown as Mean ± SEM for n = 4-12 rats analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. * shows significant difference compared to HFD ($P<0.05$).

Chapter 7: Purification and Identification of Adipogenic Differentiating Peptides from Egg White Hydrolysate[5]

7. Introduction

Bioactive peptides are food protein derived fragments ranging from 2 to 50 amino acid residues with reported antioxidant, anti-inflammatory, anti-hypertensive, anti-obesity, antimicrobial and immunomodulatory properties (Moller, Scholz-Ahrens et al. 2008, Udenigwe and Aluko 2012). They can potentially impact the body's function(s) and health through action on cardiovascular, endocrine, and immune systems (Korhonen and Pihlanto 2006, Mine 2006, Singh, Vij et al. 2014). The inherent amino acid composition and sequence of the peptides determine peptide specific bioactive properties. Enzymatic hydrolysis of proteins is the most common way to produce bioactive peptides form food sources; while bioactive peptides are also reported naturally present in food commodities or prepared by fermentation (Udenigwe and Aluko 2012). An egg white hydrolysate (EWH) prepared showed anti-hypertensive effect in spontaneously hypertensive rats through renin angiotensin system (RAS) modulation; reducing vascular ACE and AT1R, and enhancing AT2R expression (Jahandideh, Chakrabarti et al. 2016). The RAS contributes to the underlying pathophysiology of insulin resistance (Olivares-Reyes, Arellano-Plancarte et al. 2009, de Kloet, Krause et al. 2010, Underwood and Adler 2013). RAS blockade, by ACE inhibitors or angiotensin receptor blockers (ARB), prevents the new onset of diabetes (Elliott and Meyer 2007, Group, McMurray et al. 2010), beneficially affects insulin sensitivity and glucose homeostasis, and prevents cardiovascular complications in diabetic patients (Braga and Leiter 2009, Frigolet, Torres et al. 2013). While activation of ACE/Ang II/ AT1R axis has

[5] This chapter has been submitted to the "*Food Chemistry*" journal.

been reported extensively to play a pivotal role in the pathogenesis of insulin resistance, AT2R activation appears to be beneficial and would contribute to the treatment of insulin resistance and the associated diseases (Ohshima, Mogi et al. 2012, Shum, Pinard et al. 2013).

Adipose tissue with a central role in lipid and glucose metabolism is a major insulin sensitive organ (Ruan and Lodish 2003). Under normal metabolic conditions insulin promotes differentiation of pre-adipocytes into mature adipocytes through upregulation of peroxisome proliferator associated receptor gamma (PPARγ) (Lu, Wang et al. 2006). Healthy adipocytes are sensitive to insulin and secrete the anti-inflammatory adipokine; adiponectin (Harwood 2012). Under abnormal conditions however, adipose tissue becomes dysfunctional with perturbed insulin signaling associated with insulin resistance and chronic inflammation (Pradhan, Manson et al. 2001, Kadowaki, Hara et al. 2003, Winkler, Kiss et al. 2003, Coppack, Mohamed-Ali et al. 2005, Harwood 2012).

RAS blockade has also shown favorable effects on adipose tissue and insulin sensitivity (Iwai and Horiuchi 2009, Jing, Mogi et al. 2013). Valsartan and olmesartan, the ARBs, have been reported to enhance adipocyte differentiation with enhanced expression of adiponectin and PPARγ, reduction of inflammatory markers, increased adipose tissue insulin stimulated glucose uptake, and improved insulin sensitivity in both diabetic rodents and human subjects (Furuhashi, Ura et al. 2004, Tomono, Iwai et al. 2008, Pscherer, Heemann et al. 2010, Goossens, Moors et al. 2012).

Interestingly, we also observed additional benefits of EWH on adipocyte differentiation, and adiponectin secretion along with insulin sensitizing and mimetic effects in 3T3-F442A pre-adipocytes (chapter 5). Moreover, feeding EWH to insulin resistant rats improved glucose and

insulin tolerance by enhancing insulin sensitivity in muscle and adipose tissue of these rats (Chapter 6).

Here, we aimed to identify the bioactive peptide(s) in EWH responsible for enhanced adipogenic differentiation. PPARγ, the master regulator of adipogenesis and the anti-inflammatory metabolic modulator which is highly expressed in differentiated adipocytes (Rosen and MacDougald 2006) was selected as the marker in our cell experiments. Fractionation and purification using chromatographic methods have been employed for this purpose and several peptides were identified in the last step. Figure 7- 1 shows the experimental design of this study. The identified peptides were further validated for the observed adipogenic differentiating effects in 3T3-F442A pre-adipocytes.

7.1. Methods

7.1.1. Preparation of egg white hydrolysate (EWH)

Hydrolysis of egg white was carried out according to our previous method with slight modifications (Jahandideh, Chakrabarti et al. 2016). Briefly, liquid egg white (purchased from the Egg Processing Innovation Cooperative, Lethbridge, Alberta, Canada) was diluted with water to obtain a solution with 5% protein solid. Thermoase (0.1%, w/w) was added to the solution after pH and temperature adjustment (pH=8, T=65 °C) and protein digestion was carried out for 90 min. The mixture was further hydrolyzed at 55 °C by 1% pepsin (pH=2.5) for 180 min. The reaction was terminated by heating the solution at 95 °C for 15 min and the hydrolysate was centrifuged and then condensed to obtain approximately 10% solid. The hydrolysate was then spray dried and the powder was collected and stored at -20 °C for further experiments.

7.1.2. Cell culture & differentiation

The murine pre-adipocyte cell line 3T3-F442A (Sigma Aldrich; Cat# 00070654) was used. Cell culture method is similar to the previously reported study (Chakrabarti and Wu 2015). The cells were obtained in passage 8, thawed and expanded in culture using DMEM supplemented with 10% FBS (heat-inactivated) and antibiotics. The cells were grown in T-25 flasks to confluence 80-90% prior to sub-culture in gelatin-coated 48 well plates. All studies were performed using cells in passages 11-18.

To determine the ability of EWH fractions/identified peptides to induce adipogenic differentiation, the cells (grown in 48 well plates) were incubated in standard culture medium (DMEM + 10% FBS + antibiotics) in the presence of insulin (at 10 μg/mL, as a positive control to induce differentiation) or the EWH fractions (at 5 mg/mL concentration)/synthetic peptides (100 μM) for 72 hr without changing the medium. Adipogenic changes were determined by measuring the upregulation of PPARγ expression using western blot technique.

7.1.3. Western blotting

Western blotting was done on 3T3-F442A cell lysates prepared at the end of experimental procedures. Protein band for PPARγ (rabbit polyclonal antibody from Cell Signaling Technology, cat# 2430) was normalized to α-tubulin (rabbit polyclonal antibody from Abcam, Cambridge, MA, cat# ab15246). Tubulin and PPARγ antibodies were used at 0.1 and 0.5 μg/ml respectively. Goat anti-rabbit conjugated secondary antibodies were purchased from Li-cor Biosciences (Lincoln, NB). The protein bands were detected by a Li-cor Odyssey BioImager and quantified by densitometry using corresponding software Odyssey v3.0 (Li-cor). Cell lysates from untreated cells were loaded on every gel and all data were expressed as X of the corresponding untreated control.

7.1.4. Ultrafiltration and C18 cartridge purification

The EWH powder was first dissolved in 0.1% trifluoroacetic acid (TFA) in water at the concentration of 40 mg/mL. After passing through an ultra-filtration (UF) membrane with 5 kDa molecular weight cutoff (Millipore, Billerica, MA) in an Amicon 121 8400 UltraFiltration Cell (Millipore Corp., Bedford, MA, USA), the permeate was fractionated using C18 cartridges. The permeate was loaded on pre-conditioned C18 Sep-Pack Vac cartridges (WAT043395) and washed 3 times with 0.1% TFA in water to remove salts. The cartridges were then washed one time with 10% acetonitrile (ACN)/water solution to collect fraction 1. The same procedure was continued with 20%, 30%, 40% and 50% ACN/water solutions to elute fractions 2, 3, 4, and 5, respectively. Each fraction was collected separately, freeze dried, and used for cell culture experiments to measure PPARγ expression in 3T3-F442A cells.

7.1.5. Cation exchange chromatography

The most active fraction from the C18 cartridge (Fraction 1) was selected for further purification by fast protein liquid chromatography (FPLC). The freeze-dried sample (Fraction 1) was dissolved in 10 mM ammonium acetate buffer at 5 mg/mL and 2 mL of the solution was loaded to the HiPreP 16/10 SP FF cation exchange column coupled with an ÄKTA explorer 10XT system (GE Healthcare, Uppsala, Sweden). After washing the column with 2 column volume (CV) of 10 mM ammonium acetate (pH 4) at a flow rate of 5 mL/min, the sample was eluted with 0.5 M ammonium carbonate (pH 8.8) by gradients up to 35% in 6 CV. The elution was monitored at 280 nm; 8 fractions were collected, freeze dried and tested for PPARγ expression in cells.

7.1.6. Purification by reverse phase liquid chromatography

Two potent fractions (F4 & F5) obtained from the previous step, were selected for the purification by reverse phase-high performance liquid chromatography (RP-HPLC) on a Waters Xbridge Prep C18 column (5 µm, 10 x 150 mm; Waters Associates, Milford, MA, USA) attached with Waters 600 HPLC system (Waters Inc., Milford, MA. USA). Instrumental control, data collection and data processing were carried out by Empower Version 2. Samples were automatically injected by Waters 2707 autosampler. Fractions were eluted with 0.1% TFA in water (solvent A) and 0.1% TFA in ACN (solvent B) using the following gradient; 10% to 20% B from 0-30 min, 20% to 50% B from 30-40 min, 50% to 60% B from 40-43 min, 60% to 10% B from 43-48 min, and 10% B from 48-55 min at a flow rate of 4 mL/min. Elutions were all monitored by Waters 2998 photodiode array at 220 nm. 10 fractions were collected from each of the 2 fractions (F4 & F5) based on major peaks in the chromatogram, concentrated using vacuum-rotary evaporator at 35 ˚C, and freeze dried for further analysis in cells. The most active fractions (F4-Fr5 & F5-Fr10) were then subjected to further analysis by liquid chromatography tandem mass spectrometry (LC-MS/ MS) to identify the peptide(s) sequences.

7.1.7. Analysis of peptide sequences by LC-MS/MS

The most active fractions were sent for sequence analysis using a Waters (Micromass) QTOF Premier (Waters Inc., Milford, MA. USA) paired with a Waters Atlantis dC18 (75 µm × 150 mm, 3 µm) UPLC column (Milford, MA). Samples were eluted using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). At a constant flow rate of 0.35 µL/mL, peptides were separated with the following gradients: starting with 6% solvent B and raised to 25%B in 23 min, to 45%B in 15 min, to 75%B in 5 min to elute all the peptides. Ionization was performed by electrospray ionization technique (ESI) with a nano-Lockspray ionization source in a positive ion mode. A MS/MS full-scan was performed for each sample

with acquisition ranges of 400–1600 in MS mode and 50-1990 in MS/MS mode. Instrumental control and data analysis were performed using MassLynx software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.). Obtained MS/MS data were analyzed by proteomic software Mascot (version 2.2; Matrix science). The sequences were searched in NCBI protein database to match parent protein. Peptide sequences were selected based on spectra intensity to be chemically synthesized (GenScript Corp., Piscataway, NJ, USA) for verification of bioactivity in cells.

7.2. Statistical analysis

All data presented are mean value ± SEM of 3−4 independent experiments. One-way analysis of variance with Dunnett's post-test was used for the determination of statistical significance. A value of $P < 0.05$ was considered significant.

7.3. Results and discussion

7.3.1. Ultrafiltration and C18 cartridge purification

The enzymatic hydrolysate prepared from egg white by thermolysin and pepsin (EWH) was found to enhance PPARγ expression *in vitro* in 3T3-F442A pre-adipocytes (Chapter 5). We employed a 5 KDa UF membrane to enrich oligopeptides in EWH before further fractionation by Sep-Pack C18 cartridges.

The initial step of EWH fractionation using C18 cartridges generated 5 fractions (Fractions 1-5). We measured PPARγ expression in adipocytes in the presence of these 5 fractions in addition to the UF derived retentate to explore the possible loss of bioactive peptides during the ultrafiltration process. Among all fractions, fraction 1 washed out with 10% acetonitrile/water (the most hydrophilic fraction between all) showed the highest PPARγ expression comparable to insulin. The enhanced PPARγ expression capacity was diminished in cells incubated with more

hydrophobic fractions (fractions 2 and 3) and was lost in the presence of fractions 4 & 5 as well as UF retentate (Figure 7- 2). This implies that smaller peptides with more hydrophilic nature in EWH were responsible for the enhanced PPARγ expression in adipocytes and ultrafiltration did not adversely affect the biological activity of bioactive peptides.

7.3.2. Purification by cation exchange and reverse phase chromatography

The cation exchange chromatography of Fraction 1, separating peptides based on their positive charge, produced 8 fractions (F1-8; Figure 7- 3A). Results from the cell study indicated that some peptide fractions obtained from this step exerted stimulatory effects on PPARγ expression while a few did not show any effect (Figure 7- 3B). F4 and F5 fractions as the most potent fractions were selected for further purification by the reverse phase liquid chromatography using Xbridge C18 RP-HPLC column which separates peptides based on hydrophobicity.

10 major peaks were identified in the chromatogram of F4 (Figure 7- 4A), and PPARγ expression was the highest in its fifth fraction (Fr5; Figure 7- 4B). Based on the elution time (22-25 min), and the gradient of buffer B (10-20% B), this fraction (Fraction 1-F4-Fr5) should be more hydrophilic compared to other fractions in Fraction 1-F4.

Further purification of F5, the second potent fraction derived from cation exchange chromatography, using RP-HPLC also yielded 10 fractions (Fr1-10) as depicted in Figure 7- 5A. Fr10 was found to be the most potent fraction between all (Figure 7- 5B). Interestingly, considering the time of elution (38-40 min) and buffer B gradient (20%-50%), this fraction (Fraction1-F5-Fr10) appears to have a more hydrophobic nature between the other fractions in Fraction 1-F5. Therefore, hydrophobicity per se was not a critical characteristic for peptides with adipogenic differentiation capacity. Instead, peptide sequence and/or amino acid residues played a more effective role with this regard.

7.3.3. Peptide identification by mass spectrometry

Two active peptide fractions, Fraction 1-F4-Fr5 and Fraction 1-F5-Fr10, were subjected to LC-MS/MS peptide sequencing analysis. Representative MS and MS/MS spectra for manual de novo sequencing of Fraction 1-F5-Fr10 is presented in Figure 7- 6. Totally, 42 peptide sequences were identified from both fractions as listed in Table 7- 1. These peptides varied in length (5-18 amino acids), amino acids composition, and polarity. Majority of identified peptides in these two fractions derived from major egg white proteins; ovalbumin and ovotransferrin (Mine 2006). Out of the 42 identified peptides, 34 peptides derived from ovalbumin, the most abundant protein in egg white, while 7 peptides identified from ovotransferrin, the second major egg white protein. One of the peptides from Fraction 1-F4-Fr5, LSARTAEVTKEQLEE, derived from ovoglycoprotein precursor. Chicken egg white is rich in glycoproteins containing 13.6% of hexoses (mannose and galactose), 13.8% of glucosamine, and 3% of sialic acid (Ketterer 1965). Due to cost and time constraints, we selected the major peptides identified in each fraction based on their mass spectra for the validation. Of the few selectively synthesized peptides from each fraction tested for PPARγ expression, all enhanced PPARγ expression while this increase was significant in QAMPFRVTEQE and VFKGL treated cells ($p<0.05$), and WEKAFKDED tended to increase PPARγ expression ($p=0.055$) in adipocytes as shown in Figure 7- 7A & B. These results clearly show the efficiency of peptide purification process in identifying peptides with adipogenic differentiating properties.

The structure-function relationship of adipogenic differentiating peptides is not known, but our results show that these peptides were rich in acidic and hydrophobic amino acid residues. specifically, Asp (E), Leu (L), and Lys (K) had the highest prevalence in the identified peptides followed by Ile (I), Phe (F), and Pro (P). Consistent with our results, Pro, Ile and Val amino acid residues present in the milk-derived tri-peptides; IPP and VPP have been reported to exert

beneficial effects on adipocyte differentiation (Chakrabarti and Wu 2015). VPP further exerted beneficial effects on adipose tissue and insulin sensitivity *in vivo* (Aihara, Osaka et al. 2014, Sawada, Sakamoto et al. 2015). A soy-derived tripeptide FLV is shown to prevent adipose inflammation and insulin resistance (Kwak, Kim et al. 2016). This peptide inhibited the release of inflammatory cytokines and enhanced insulin responsiveness and glucose uptake in adipocytes (Kwak, Kim et al. 2016). Phe, Leu, and Val as the amino acid residues of this tri-peptide seem to be important for the observed effects. Food-derived protein hydrolysates have been reported to improve glucose tolerance and insulin sensitivity *in vivo*, where improved adipose tissue function through enhanced adipocyte differentiation and reduced inflammation is one of the potential mechanisms (Boonloh, Kukongviriyapan et al. 2015, Noguchi, Yanagita et al. 2016). Although the active components were not identified in these protein hydrolysates, bioactive peptides may play a significant role in the reported effects. Indeed, identification of responsible bioactive peptides in such protein hydrolysates would help the structure-function studies. Since the EWH (with beneficial effects on adipogenic differentiation; Chapter 5) also improved glucose tolerance and insulin sensitivity in insulin resistant rats (Chapter 6), the identified peptides form EWH in the current study may also have additional benefits on adipose tissue function and insulin sensitivity *in vivo*.

7.4. Conclusion

In this study, for the first time, we reported several novel peptide sequences from major egg white proteins ovalbumin and ovotransferrin exhibiting stimulatory effect on PPARγ expression. As of now there is limited report on the structure-function relationship of adipogenic differentiating peptides which challenges the efficient and strategic preparation, purification, and identification of these peptides. Our findings suggested that Asp, Leu, and Lys residues in

sequence might contribute to the enhanced PPARγ expression in pre-adipocytes. Future research efforts are required to explore the potential benefits of these peptides on adipose tissue, and insulin sensitivity *in vivo*. Considering the pivotal role of adipose tissue dysfunction in insulin resistance complications, identification of bioactive peptides with beneficial effects on adipocytes for the development of natural-based products is of immense importance.

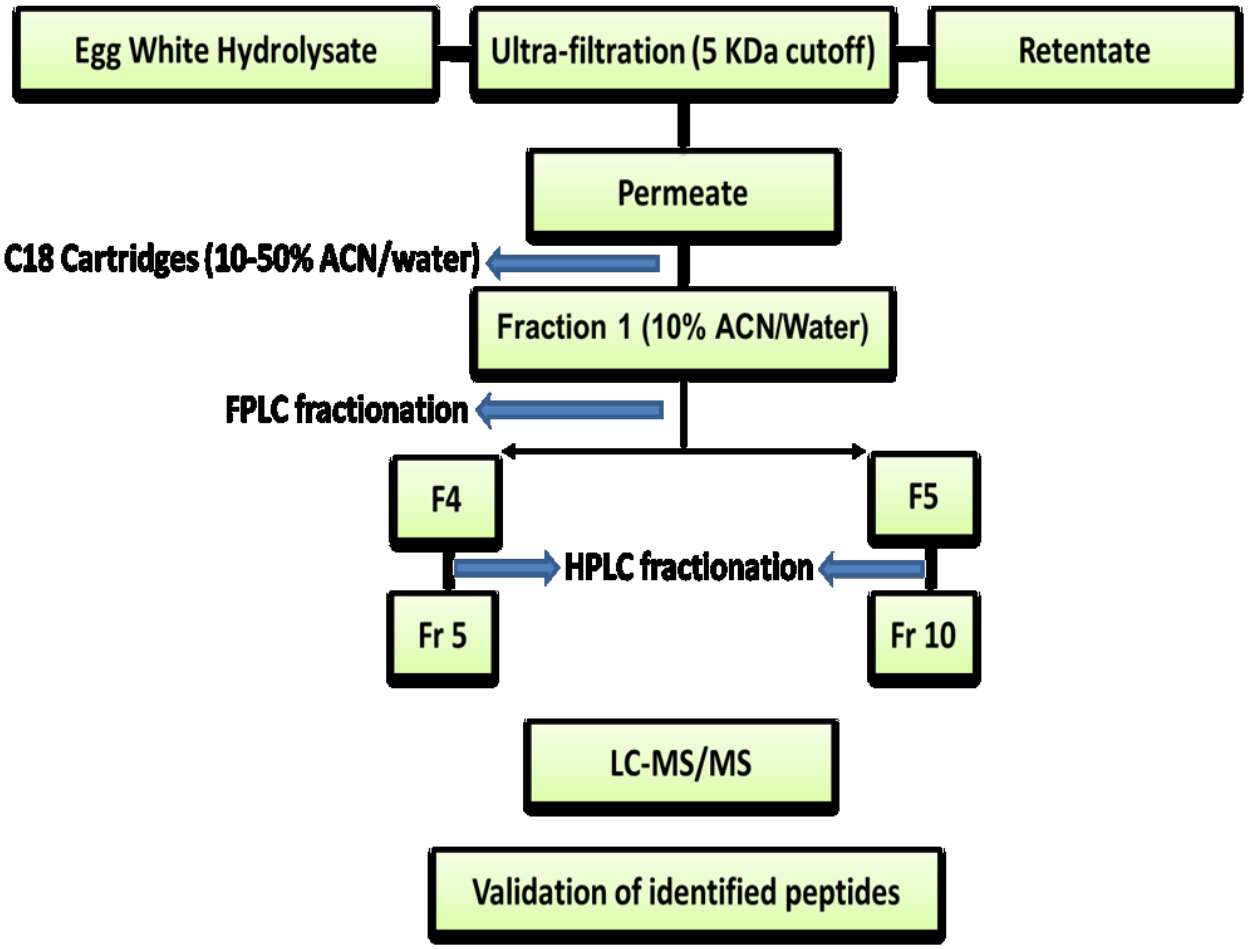


Figure 7-1. Schematic diagram for egg white hydrolysate (EWH) fractionation protocol.

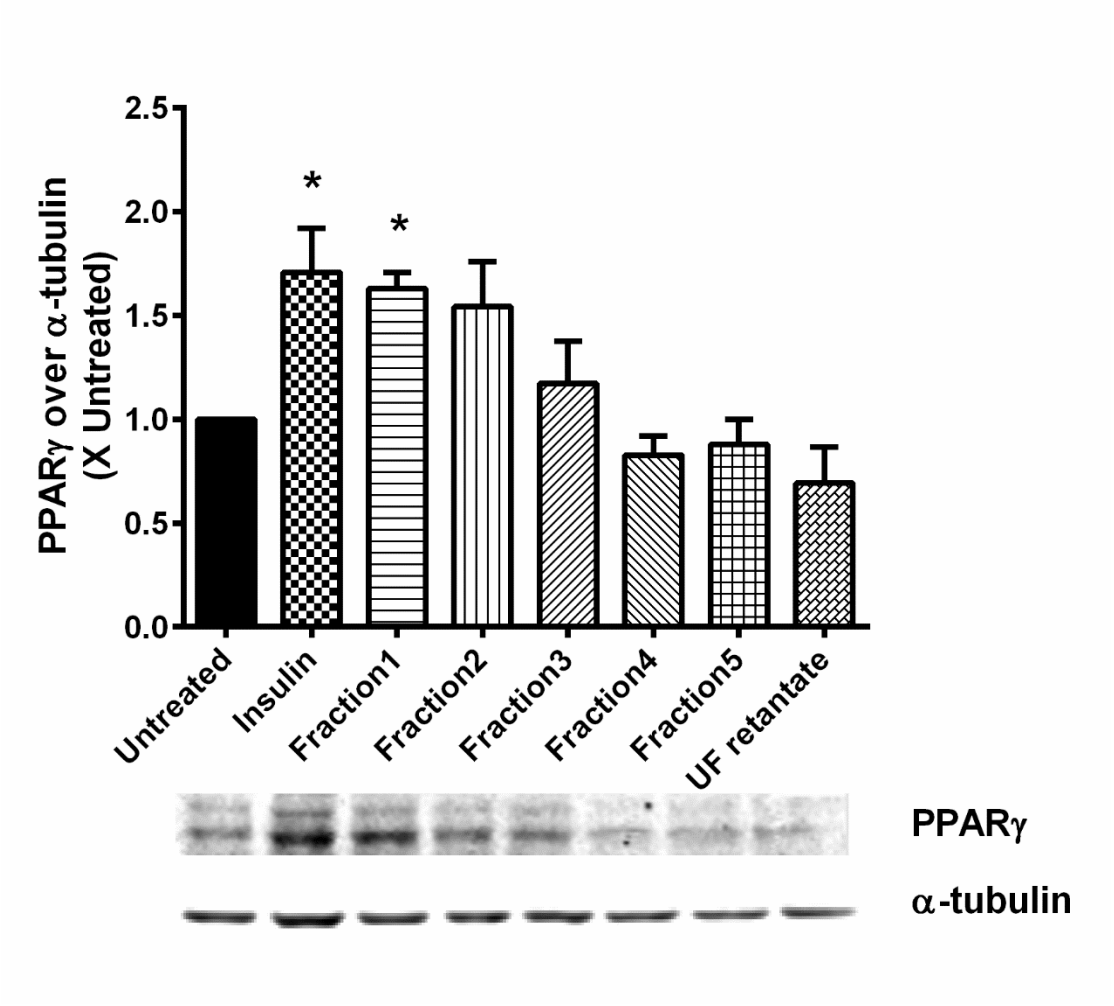


Figure 7-1. Step I fractionation by Sep-Pack C18 cartridges. EWH was first passed through a 5 KDa UF membrane and the permeate and retentate were collected. The permeate was fractionated using 10-50% ACN/water solutions by Sep-Pack C18 cartridges to obtain 5 fractions (Fractions 1-5). These fractions along with the retentate collected from the UF membrane was used in 3T3-F442A adipocytes. Fraction 1 was the most potent fraction in increasing PPARγ expression comparable to insulin. Data is shown as Mean ± SEM for 3 individual experiments. Data was analyzed by one-way ANOVA and * means significant at $P<0.05$ compared to untreated group.

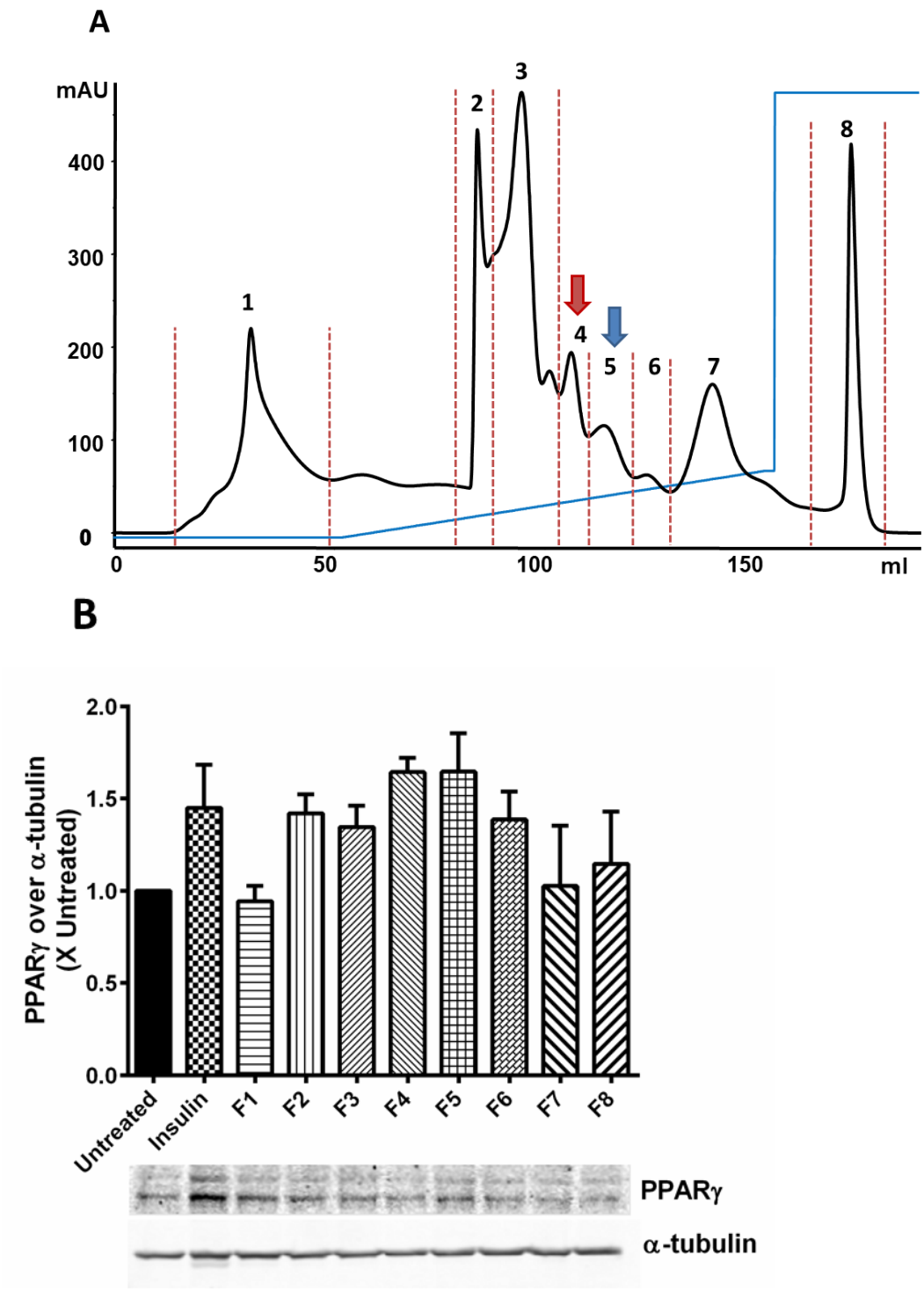


Figure 7-2. Step II fractionation by cation exchange chromatography. (A) Cation exchange chromatography of Fraction 1 generated 8 fractions (F1-F8). (B) Individual fractions were tested in 3T3-F442A adipocytes for PPARγ expression. Fractions 4 & 5 (F4 & F5) indicated by colored

block arrows were the most active ones and chosen for next step of fractionation. Data is shown as Mean ± SEM for 3 individual experiments.

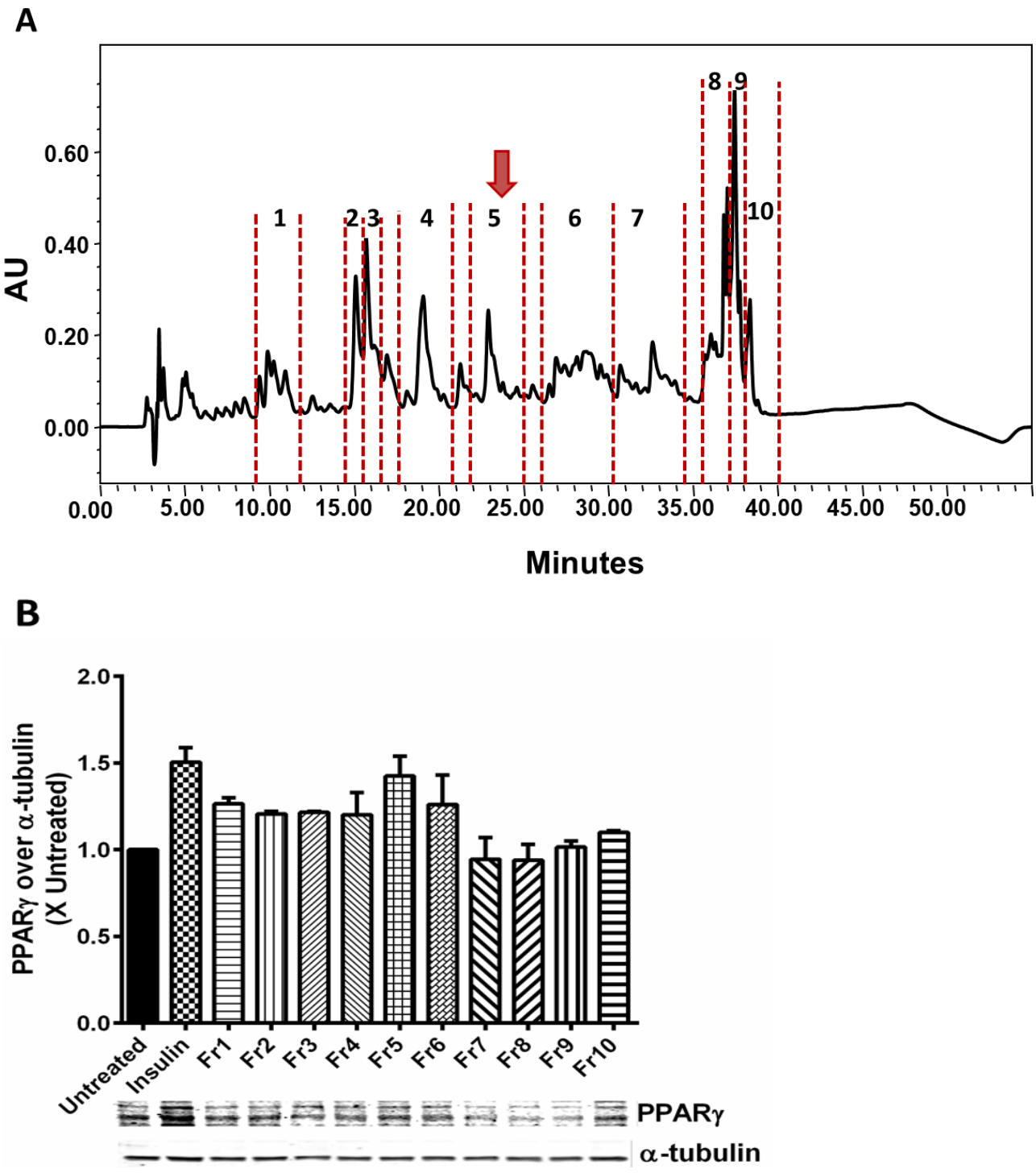


Figure 7-3. Step III fractionation of F4 by reverse phase high performance liquid chromatography (RP-HPLC). (A) RP-HPLC chromatography of F4 yielded 10 major fractions (Fr1-Fr10). (B) Individual fractions were tested in 3T3-F442A adipocytes for PPARγ expression. Fraction 5 (Fr5) indicated by red block arrow was the most active one and chosen for MS/MS analysis. Data is shown as Mean ± SEM for 3 individual experiments.

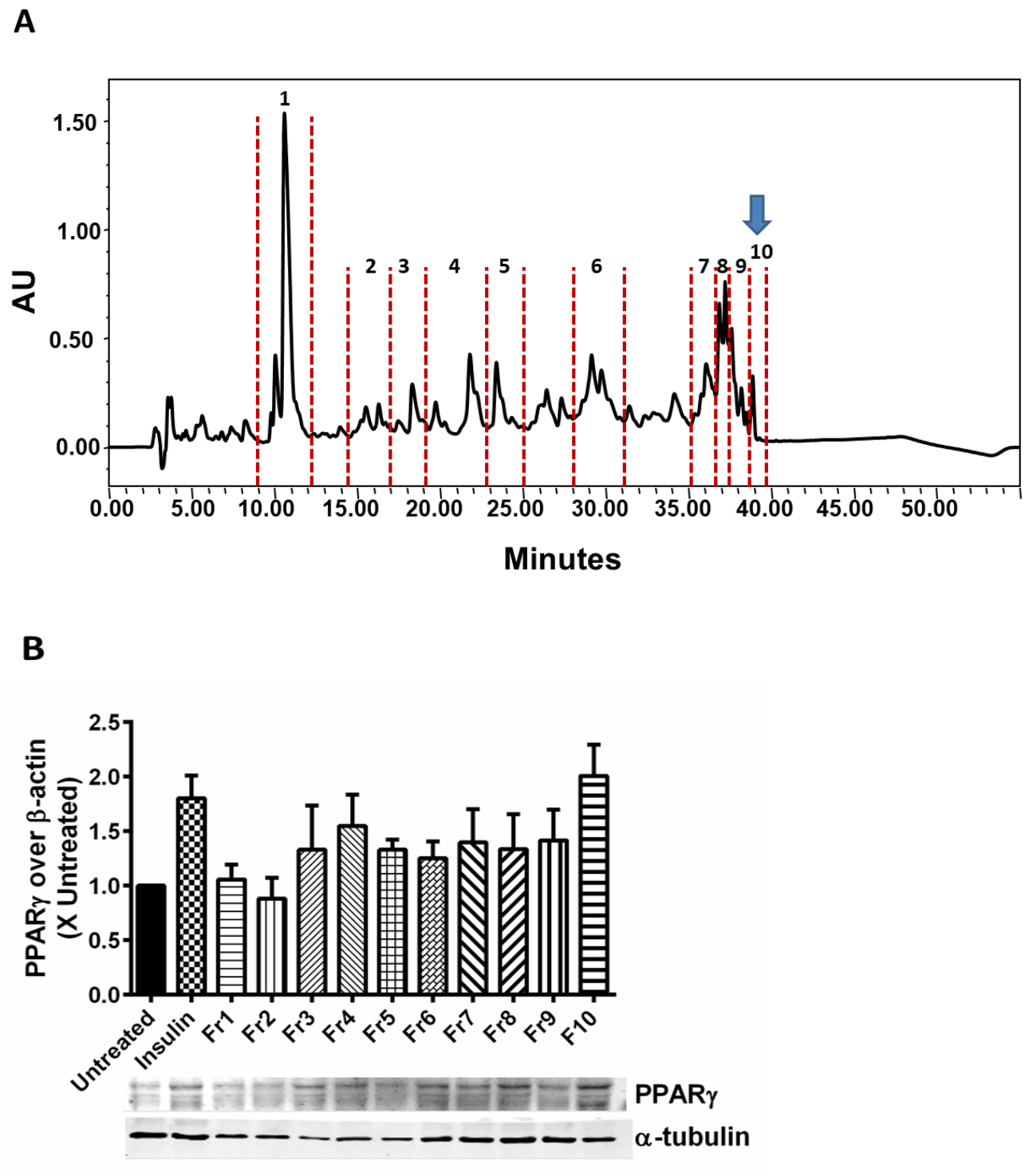
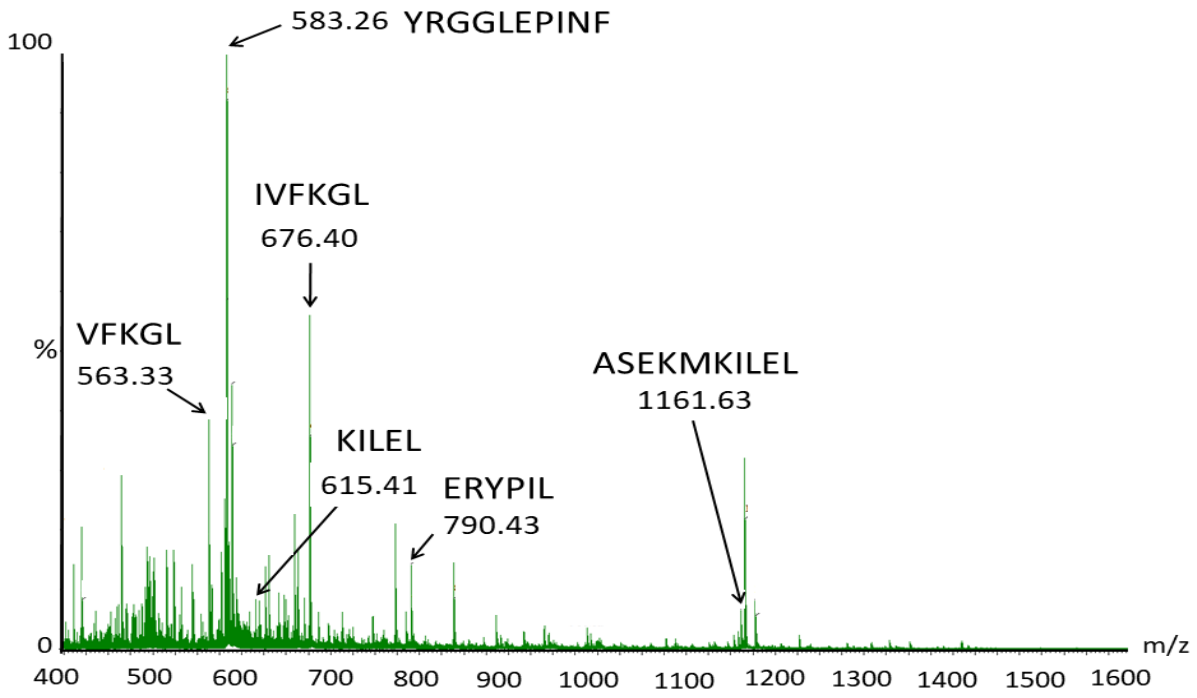


Figure 7-4. Step III fractionation of F5 by reverse phase high performance liquid chromatography (RP-HPLC). (A) RP-HPLC chromatography of F5 yielded 10 major fractions (Fr1-Fr10). (B) Individual fractions were tested in 3T3-F442A adipocytes for PPARγ expression.

Fraction 10 (Fr10) indicated by blue block arrow was the most active one and chosen for MS/MS analysis. Data is shown as Mean ± SEM for 3 individual experiments.

A


583.26 YRGGLEPINF
100
IVFKGL
676.40
VFKGL
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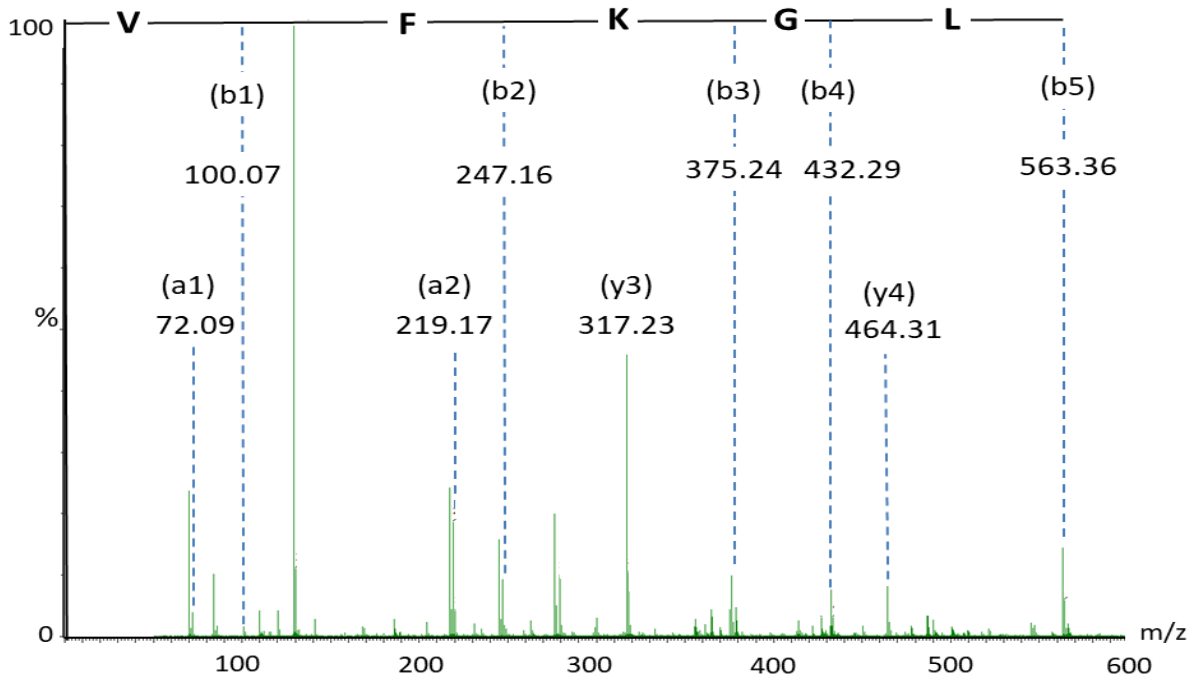
B


100
V F K G L
(b1)
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72.09
(a2)
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(y3)
317.23
(y4)
464.31
%
0
m/z
100 200 300 400 500 600

Figure 7-5. MS spectrum of Fraction 1-F5-Fr10 from egg white hydrolysate (A) and de novo sequencing of one representative peptide VFKGL (m/z 563.33) using MS-MS spectrum (B).

Table 7-1. Peptide sequences identified in fractions with PPARγ stimulatory activity from egg white hydrolysate by LC-MS/MS.

Fraction	Peptide sequence	Observed m/z	Calculated mass	Length (amino acids)	Parent Protein
F4-Fr5	ILNQITKPND	578.33	1154.63	10	Ovalbumin
F4-Fr5	WEKAFKDED	584.27	1166.52	9	Ovalbumin
F4-Fr5	WEKAFKDEDTQA	734.34	1466.67	12	Ovalbumin
F4-Fr5	**M**PFRVTEQESKPVQ(oxi)	564.62	1690.83	14	Ovalbumin
F4-Fr5	QA**M**PFRVTEQE(oxi)	676.31	1350.62	11	Ovalbumin
F4-Fr5	EKAFKDEDTQAMP	755.36	1508.68	13	Ovalbumin
F4-Fr5	ILQMCS	694.38	693.32	6	Ovotransferrin
F4-Fr5	SDILQMCS	896.43	895.38	8	Ovotransferrin
F4-Fr5	ERYDDES	913.4	1165.56	7	Ovotransferrin
F4-Fr5	LSKAQSDFGVD	583.8	1165.56	11	Ovotransferrin
F4-Fr5	DLTKCLFKVREGT	755.36	1508.8	13	Ovotransferrin
F4-Fr5	LSARTAEVTKEQLEE	852.44	1702.87	15	Ovoglycoprotein precursor
F5-Fr10	VFKGL	563.33	562.34	5	Ovalbumin
F5-Fr10	SIINF	593.34	592.32	5	Ovalbumin
F5-Fr10	KILEL	615.41	614.4	5	Ovalbumin
F5-Fr10	IVFKGL	676.41	675.43	6	Ovalbumin
F5-Fr10	ESSINF	722.38	721.36	6	Ovalbumin

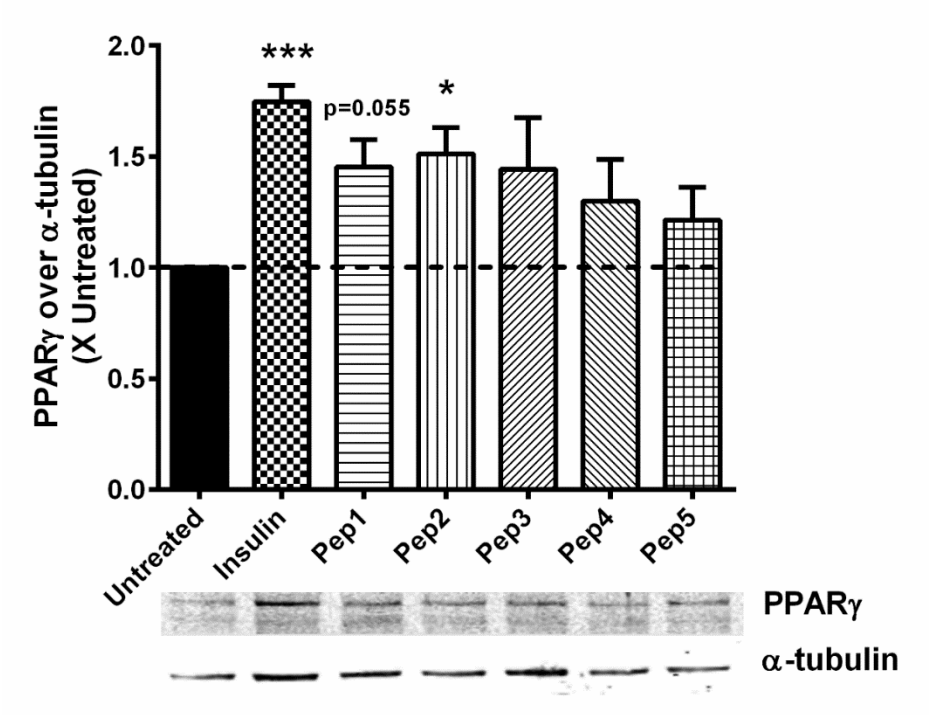
Continued.

Fraction	Peptide sequence	Observed m/z	Calculated mass	Length (amino acids)	Parent Protein
F5-Fr10	LEPINF	732.4	731.38	6	Ovalbumin
F5-Fr10	AIVFKGL	747.48	746.46	7	Ovalbumin
F5-Fr10	RELINSW	459.26	916.47	7	Ovalbumin
F5-Fr10	VNAIVFKGL	480.81	959.58	9	Ovalbumin
F5-Fr10	ARELINSW	494.77	987.51	8	Ovalbumin
F5-Fr10	FRADHPFL	501.77	1001.5	8	Ovalbumin
F5-Fr10	SEK**M**KILEL(oxi)	553.81	1105.6	9	Ovalbumin
F5-Fr10	EFRADHPFL	566.32	1130.55	9	Ovalbumin
F5-Fr10	YAEERYPIL	1153.57	1152.58	9	Ovalbumin
F5-Fr10	ASEKMKILEL	1161.63	1160.64	10	Ovalbumin
F5-Fr10	YRGGLEPINF	583.28	1164.59	10	Ovalbumin
F5-Fr10	LYRGGLEPINFQ	703.88	1405.73	12	Ovalbumin
F5-Fr10	RVTEQESKPVQMMY	575.95	1724.82	14	Ovalbumin
F5-Fr10	MPFRVTEQESKPVQ**MM**(oxi)	657.3	1968.91	16	Ovalbumin
F5-Fr10	GLEPINF	789.42	788.4	7	Ovalbumin
F5-Fr10	ERYPIL	790.44	789.43	6	Ovalbumin
F5-Fr10	TREMPFS**M**(oxi)	507.73	1013.43	8	Ovalbumin

Continued.

Fraction	Peptide sequence	Observed m/z	Calculated mass	Length (amino acids)	Parent Protein
F5-Fr10	PAEKMKILEL	586.35	1170.66	10	Ovalbumin
F5-Fr10	SAI**M**LKRVP(oxi)	515.78	1029.6	9	Ovotransferrin
F5-Fr10	NKIRDLL	436.23	870.52	7	Ovotransferrin

A



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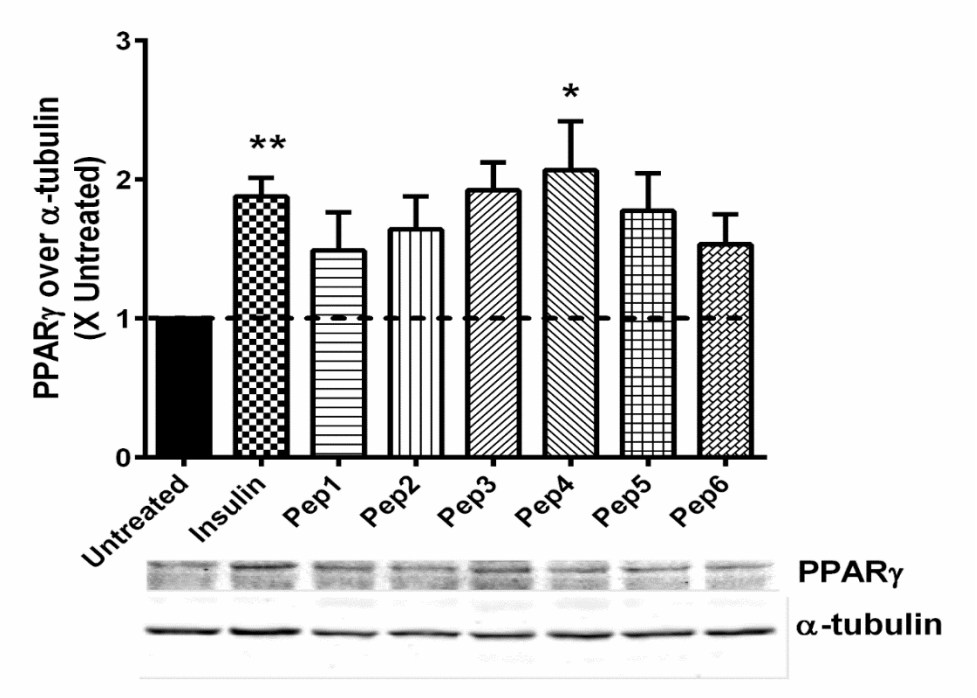

Figure 7-6. Validation of adipogenic differentiating effect of selected peptides identified from egg white hydrolysate fractions. (A) Synthetic peptides WEKAFKDED (pep 1), QAMPFRVTEQE (pep 2), WEKAFKDEDTQA (pep 3), ILQMCS (pep 4), and ERYDDES (pep

5) identified from the Fraction1-F4-Fr5 of the EWH and (B) Synthetic peptides ASEKMKILEL (pep 1), KILEL (pep 2), ERYPIL (pep 3), VFKGL (pep 4), IVFKGL (pep 5), and YRGGLEPINF (pep 6) identified from the Fraction1-F5-Fr10 of the EWH were tested for PPARγ expression in 3T3-F442A adipocytes. Data is shown as Mean ± SEM for 3 individual experiments. Data was analyzed by one-way ANOVA. *, **, and *** mean significant differences at $P<0.05$, $P<0.01$, and $P<0.001$ compared to untreated cells respectively.

Chapter 8: General discussion and future directions

8. Key findings of the present research

The main purpose of the current research was to investigate the efficacy of egg white derived peptides on several complications of metabolic syndrome (MetS) namely hypertension, inflammation, glucose intolerance, and insulin resistance. A schematic illustration of our main findings on the effects of egg white hydrolysate (EWH) and its suggested working mechanisms studied through *in vitro*, *ex vivo*, and *in vivo* conditions is presented in Figure 8- 1.

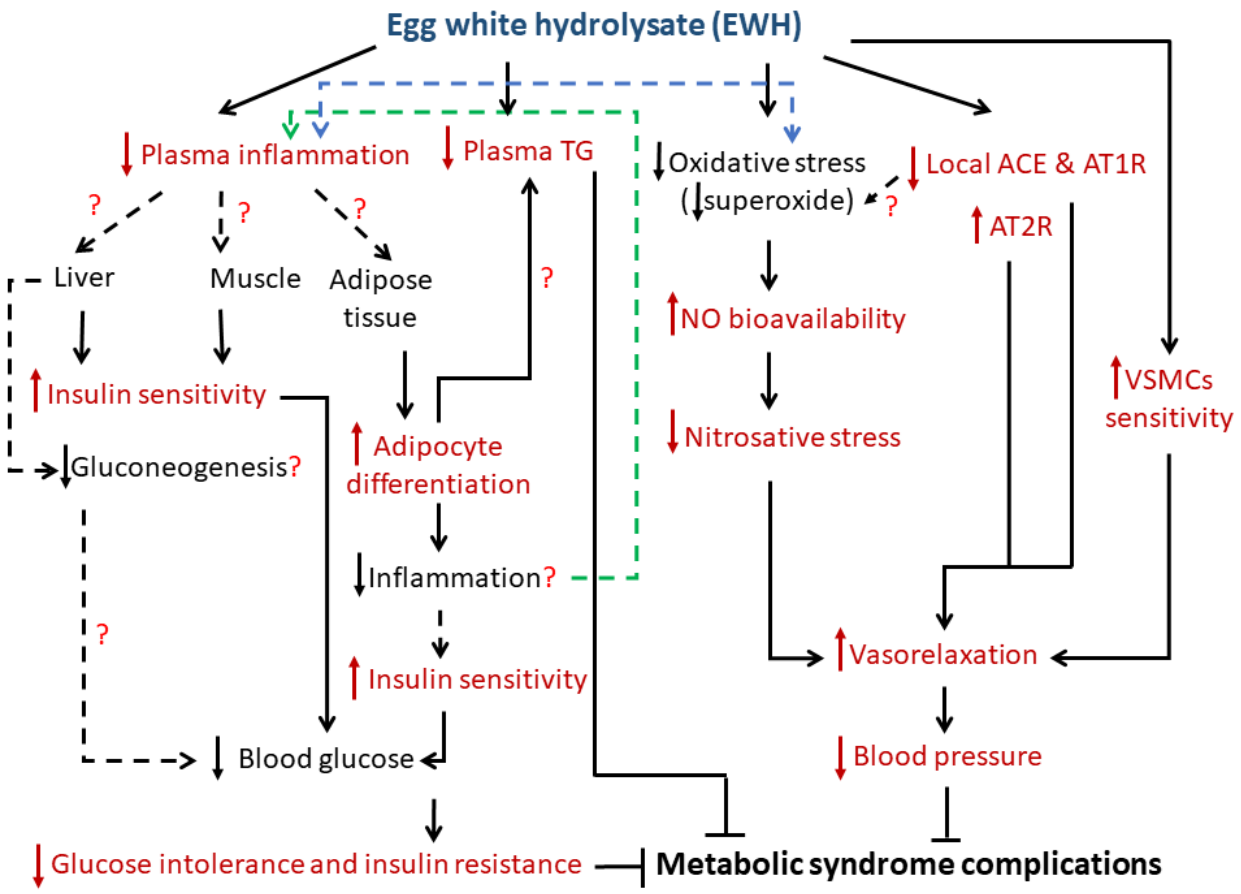

Figure 8- 1. A schematic representation of the proposed mechanism of action of EWH on MetS complications. EWH positively affects several components of the MetS namely hypertension,

dyslipidemia, inflammation, and insulin resistance. EWH exerts anti-hypertensive effects by reducing vascular ACE and AT1R expression while enhancing AT2R expression. It further reduces oxidative stress, increases NO bioavailability and increases endothelium-dependent vascular relaxation in mesenteric arteries of rats. Moreover, EWH enhances VSMC sensitivity contributing to the endothelium-independent vascular relaxation as another mechanism for reducing blood pressure. Additionally, EWH reduces plasma triglyceride level exerting lipid lowering effects and reduces systemic inflammation. This may affect insulin signaling in major insulin sensitive tissues namely liver, muscle and adipose tissue. The enhanced insulin sensitivity in these tissues results in the improved glucose tolerance and insulin tolerance. Glucose production in liver and adipocyte differentiation are likely affected by the EWH as other contributors to the improved glucose tolerance. In summary, EWH reduces blood pressure, plasma triglycerides, inflammation, and enhances insulin sensitivity in adipose tissue, liver, and muscle collectively contributing to the improved glucose tolerance and insulin sensitivity in animal models of hypertension and MetS.

The key findings of the present research are outlined in the following sections.

8.1. Egg white ovotransferrin-derived antioxidant peptides did not exert antioxidant and anti-inflammatory effects in endothelial cells.

Experiments in Chapter 3 (published in *J. Agric. Food Chem*., 2016, 64 (1), 113–119) were essential to evaluate the 1) stability of the previously identified antioxidant peptides from ovotransferrin hydrolysate (with *in vitro* ORAC activity) against GID and 2) anti-inflammatory and antioxidant effects of these peptides and their generated digests in TNF-α induced HUVEC monolayers.

Oxidative stress, defined as an imbalance between the formation of reactive oxygen species (ROS) and the body's antioxidant system, linked to inflammation is a major contributor to various disease pathologies including hypertension, insulin resistance, diabetes, etc. (Park, Gross et al. 2009, Rains and Jain 2011, Gutowski and Kowalczyk 2013, Ramalingam, Menikdiwela et al. 2017). Oxidative stress has been shown to impair insulin signaling and glucose uptake in

skeletal muscle and adipose tissue (Rudich, Kozlovsky et al. 1997, Rudich, Tirosh et al. 1998, Kim, Saengsirisuwan et al. 2006, Dokken, Saengsirisuwan et al. 2008, Yuzefovych, Wilson et al. 2010) contributing to the etiology of MetS. Increased oxidative stress also underlies the pathophysiology of hypertension and atherosclerosis through enhancing vascular dysfunction (Schulz, Gori et al. 2011, Gonzalez, Valls et al. 2014). Regulation of ROS levels therefore, is essential for enhancing or maintaining normal metabolic functions in the body. Therapies targeted against free radicals have been shown to be useful on hypertension (Virdis, Neves et al. 2004, Rodrigo, Prat et al. 2008) and insulin resistance (Teachey, Taylor et al. 2003).

Dietary intake of antioxidant compounds may also improve the body's oxidant status (Ardalan and Rafieian-Kopaei 2014). Therefore, there is increasing interest in food-derived compounds including bioactive peptides as potential candidates for use as antioxidants. Indeed, there is a large body of literature on identification and characterization of antioxidant peptides from various food sources based on *in vitro* chemical-based assays including ORAC assay (Shen, Chahal et al. 2010, Zhang, Duan et al. 2012, Ji, Sun et al. 2014, Chi, Wang et al. 2015, Nimalaratne, Bandara et al. 2015, Wattanasiritham, Theerakulkait et al. 2016). These methods however, lack biological relevance by not considering the complexity of *in vivo* systems and as a result, antioxidant indices determined through *in vitro* methods may not reflect corresponding *in vivo* efficacy in more biological-relevant systems. Therefore, it is imperative to assess the bioactivity of such peptides in cells or *in vivo*.

The outcome of our study failed to correlate the ORAC-based antioxidant peptides with their anticipated effects in endothelial cells; none of the peptides showed any anti-inflammatory and antioxidant activities in endothelial cells. Furthermore, all 16 studied peptides degraded to shorter fragments after the simulated GID, where several peptide digests significantly reduced

the expression of TNF-α-induced pro-inflammatory ICAM-1 and VCAM-1 to different extents. It was only GWNI digest, composed of GWN and GW, exerting both anti-inflammatory and antioxidant effects in HUVECs. Between the two fragments, only GW reduced TNF-α-induced VCAM-1 expression (64.3 ± 20.6%) significantly compared to the TNF-α treated cells, whereas, both GW and GWN showed antioxidant effects in vascular smooth muscle cells. We concluded that ORAC lacked biological relevance in assessing bioactive peptides.

Although we did not observe any significant effects of *in vitro* antioxidant peptides on oxidative stress and inflammation in endothelial cells, egg contains several proteins as the potential sources for the production of bioactive peptides with diverse physiological effects as reported by our group (Huang, Chakrabarti et al. 2010, Jahandideh, Majumder et al. 2014, Majumder, Chakrabarti et al. 2015), and others (Miguel, Alonso et al. 2007, Garces-Rimon, Gonzalez et al. 2016, Garces-Rimon, Lopez-Exposito et al. 2016). Therefore, we considered selecting an alternative egg derived compound for our studies on bioactive peptides based on the literature and our knowledge on egg peptides.

Individual egg white proteins contain bioactive peptides with antioxidant, anti-inflammatory, and anti-hypertensive effects (Matoba, Usui et al. 1999, Miguel and Aleixandre 2006, Shen, Chahal et al. 2010, Memarpoor-Yazdi, Asoodeh et al. 2012, Majumder, Chakrabarti et al. 2013, Abeyrathne, Lee et al. 2014, Majumder, Liang et al. 2015). The link between hypertension (RAS impairment) and insulin resistance as key features of MetS has also been published in literature (Yusuf, Gerstein et al. 2001, Braga and Leiter 2009), therefore, we decided to study the potential physiological benefits of whole egg white hydrolysate composed of a complex array of bioactive peptides on hypertension and insulin resistance for the following chapters. Using whole EWH rather than individual proteins or bioactive peptides has economic advantages. Moreover, the

emergence of peptides with diverse physiological effects with potential synergistic effects among them may add to the physiological benefits of EWH (Liu, Oey et al. 2017).

EWH was previously shown to have *in vitro* ACE-inhibitory properties in our lab (unpublished data). However, the physiological effect of this treatment on blood pressure is not known *in vivo*. Therefore, in Chapter 4, we investigated the potential effects of EWH in SHRs, as the animal model of essential hypertension.

8.2. Egg white hydrolysate reduces blood pressure through enhancing vascular relaxation and renin angiotensin system (RAS) modulation in spontaneously hypertensive rats (SHRs).

Male SHRs develop persistent hypertension at about 12-14 weeks of age and remain hypertensive throughout their lives (Trippodo & Frohlich, 1981; Zicha & Kunes, 1999). Increased RAS activity, oxidative stress and pro-inflammatory phenotype, and impaired vasodilation are the characteristics of adult SHRs (16-17 weeks old) (Liu, 2009; Reaves, Beck, Wang, Raizada & Katovich, 2003; Sriramula, Cardinale, Lazartigues & Francis, 2011; Wu & Juurlink, 2002).

Findings from Chapter 4 (published in *J Funct Foods*, 2016, 27, 667-673) revealed the anti-hypertensive effects of EWH at 1000 mg/kg BW (equal to approximately 3% of daily food intake) in adult male SHRs; both systolic and diastolic blood pressures were reduced at different time points following oral administration of the treatment to SHRs. The significant difference in mean arterial blood pressure compared to control rats was observed after 9 days of EWH treatment.

Hyperactivity of the RAS, inflammation, oxidative stress, and impaired vascular relaxation are some of the pathologies underlie the onset and long-term persistence of hypertension (Giachini, Callera et al. 2008, Schulz, Gori et al. 2011). To elucidate the mechanisms by which EWH reduces blood pressure in SHRs, we performed vascular function studies on the mesenteric arteries of SHRs, as the main vascular bed affecting systemic vascular resistance (Pannirselvam, Wiehler, Anderson, & Triggle, 2005), through wire-myograph studies *ex vivo*. We did not observe any changes in the vasoconstriction response to phenylephrine between the EWH treated and untreated control SHRs, while, vasodilation responses to MCh and SNP were significantly enhanced in EWH treated group compared to the untreated rats. MCh mediated vasodilation is multifactorial; nitric oxide (NO), prostaglandins and endothelial derived hyperpolarizing factor (EDHF) are the potent mediators in this process (Morton, Rueda-Clausen et al. 2010). We showed the role of NO in MCh-dependent vasodilation in EWH treated SHRs compared to the untreated group in the presence/absence of the NOS inhibitor L-NAME. The increase in NO-dependent vasodilation may be due to the enhanced NO bioavailability (through scavenging of free radicals) or increased NO production in the vasculature.

One of the novel findings of this study was the potential involvement of endothelium-independent mechanisms (in addition to endothelium-dependent one) to the observed vasodilatory effects of EWH in SHRs compared to untreated group. Indeed, the vascular relaxation to SNP, an exogenous NO donor, was also significantly enhanced in EWH treated compared to the untreated group. Our results on the effect of EWH treatment on nitrosative stress in aortic sections of SHRs revealed the potential enhanced vascular NO bioavailability in EWH-treated SHRs compared to untreated group. Further exploration of endothelium-independent

mechanisms for the observed vasodilation effects of EWH in SHRs would help to gain a more comprehensive picture on the benefits of EWH on vasorelaxation responses in SHRs.

Although EWH initially showed ACE-inhibitory properties *in vitro*, we did not observe any changes in Ang II levels in circulation after oral administration of EWH to SHRs, which can be an indication of lack of ACE-inhibitory activity *in vivo*. Although our lab previously reported the *in vivo* ACE-inhibitory effect of IRW (identified initially as an *in vitro* ACE-inhibitory peptide) by reducing plasma Ang II and inhibiting bradykinin degradation, different mechanism of action could be involved in the observed anti-hypertensive effect of EWH compared to the tri-peptide IRW. SHRs functionally express ACE on vascular endothelial and smooth muscle cells (SMCs) (Arnal, Battle et al. 1994, Fukuda, Hu et al. 1999). ACE expression on SMCs increases local Ang II production which impairs vascular function leading to increased blood pressure (Fukuhara, Geary et al. 2000). EWH reduced the expression level of aortic ACE in EWH treated SHRs compared to untreated group. Moreover, EWH enhanced vasorelaxation response in SHRS, therefore, it is plausible to suggest that reduced vascular Ang II level also contributed to the observed effects of EWH on vascular dysfunction and hypertension in SHRs. Indeed, further *ex vivo* experiments on Ang II production in aortic rings in the presence/absence of EWH can specifically address this question; whether or not, the EWH enhanced vasorelaxation through reduced Ang II production in vasculature. Therefore, we believe that modulation of local RAS as opposed to the systemic RAS (lack of change in plasma Ang II levels) is one of the mechanisms by which EWH reduces blood pressure in SHRs. Indeed, similar evidence on the contribution of local RAS rather than the circulating RAS has been reported by other researches in a variety of animal models of hypertension and cardiovascular disease (Shiota, Miyazaki et al. 1992, Zhang, Li et al. 2011, Koyama, Hattori et al. 2014). Another novel finding of this study was the fact that

EWH found to modulate the Ang II receptors expression differentially in vasculature; a reduced AT1R expression and an enhanced AT2R expression in aortic sections was evident in EWH treated group compared to the untreated SHRs. This means that, although rats in the EWH and untreated groups had almost the same levels of circulating Ang II, the antihypertensive effects observed upon EWH feeding were due mainly to different expression levels of Ang II receptors. Interaction of Ang II with AT1R induces oxidative stress by upregulation of reactive oxygen species which can contribute to the impaired vascular functions in hypertension. It is plausible to suggest that EWH-mediated reduced oxidative stress in vasculature was also effective on the observed enhanced vasorelaxation (as evident by the enhanced NO bioavailability in mesenteric arteries and the reduced nitrosative stress in aorta) and blood pressure lowering effects of EWH in SHRs.

In addition to regulating blood pressure, the RAS also contributes to the pathophysiology of insulin resistance (Olivares-Reyes, Arellano-Plancarte et al. 2009, de Kloet, Krause et al. 2010, Underwood and Adler 2013). Adipose tissue with a central role in lipid and glucose metabolism is a major insulin sensitive organ (Ruan and Lodish 2003). Under normal metabolic conditions insulin promotes differentiation of pre-adipocytes into mature adipocytes through upregulation of peroxisome proliferator associated receptor gamma (PPARγ) leading to generation of small insulin sensitive adipocytes with capacity to store lipids (Lu, Wang et al. 2006, Harwood 2012). RAS blockade has also shown favorable effects on adipose tissue and insulin sensitivity (Iwai and Horiuchi 2009, Jing, Mogi et al. 2013). Valsartan and olmesartan, the ARBs, have been reported to enhance adipocyte differentiation with enhanced expression of adiponectin and PPARγ, reduction of inflammatory markers, increased adipose tissue insulin stimulated glucose uptake, and improved insulin sensitivity in both diabetic rodents and human subjects (Furuhashi,

Ura et al. 2004, Tomono, Iwai et al. 2008, Pscherer, Heemann et al. 2010, Goossens, Moors et al. 2012).

Since we observed RAS modulating properties of EWH in SHRs, and due to the potential link between RAS and adipocyte differentiation, in Chapter 5, we further studied the effects of EWH on adipocyte differentiation and insulin signaling *in vitro* in 3T3-F442A pre-adipocytes.

8.3. Egg white hydrolysate enhances adipocyte differentiation, and exerts insulin mimetic and sensitizing effects in 3T3-F442A cells

The experiments in Chapter 5 (published in PLoS One. 2017 Oct 3;12(10): e0185653) elucidated the potential benefits of EWH on pre-adipocytes differentiation, insulin signaling, and inflammation in adipocytes.

Effect of EWH on pre-adipocyte differentiation was evident by increased lipid accumulation, and the concomitant increased adiponectin level. Both EWH and insulin, the physiological adipogenic hormone in the body, enhanced pre-adipocyte differentiation in 3T3-F442A pre-adipocytes. Furthermore, the enhanced adipocyte differentiation in EWH treated 3T3-F442A cells also accompanied by increased expression of transcriptional regulators, PPARγ and C/EBP-α, demonstrating the successful induction of adipogenic differentiation event at the molecular level.

We further demonstrated the concentration-dependent effects of EWH on enhanced PPARγ expression; EWH at concentrations of 2.5, 5 and 10 mg/mL enhanced PPARγ expression significantly as compared to untreated cells. Since the observed effects of EWH on adipocyte differentiation were similar to insulin, we further investigated the effects of EWH on key insulin signaling pathways in these cells. Surprisingly, we observed the differential effects of EWH on

ERK1/2 and Akt phosphorylation (and activation) in 3T3-F442A cells in the presence/absence of insulin. Treatment with EWH alone increased ERK1/2 phosphorylation in pre-adipocytes, an indication of insulin mimetic effects of the treatment, whereas, insulin-induced ERK1/2 phosphorylation in EWH-treated cells was comparable to that observed in EWH-free cells. In contrast to the observed insulin-mimetic on ERK1/2, EWH alone had no effects on Akt phosphorylation, while insulin actions on Akt phosphorylation were enhanced in EWH-treated cells over and above the response seen in control cells. Interestingly, a significant interaction between EWH and insulin effects on Akt phosphorylation was observed which interpreted as a novel insulin sensitizing action for EWH in these cells.

Our data also demonstrated the partial involvement of insulin receptor in the observed effects of EWH on ERK1/2 (but not the Akt) phosphorylation in 3T3-F442A cells. Indeed, blocking of insulin receptor by using an insulin receptor antagonist (S961) abolished the effects of both insulin and EWH on ERK1/2 phosphorylation comparable to the basal levels. While our data supports the insulin receptor-mediated effects of EWH on ERK1/2 phosphorylation in adipocytes, targets downstream to IRS-1 may potentially be involved for the observed insulin sensitizing effects of EWH on Akt phosphorylation which needs further exploration in future studies.

Adipose tissue inflammation plays a critical role in the development of a variety of cardiometabolic disorders including MetS and type 2 diabetes (Coppack, Mohamed-Ali et al. 2005). EWH upregulated the expression of anti-inflammatory molecules such as PPARγ and adiponectin as discussed earlier, and also prevented the TNF-α-mediated induction of the pro-inflammatory enzyme COX-2, a molecule that contributes to the pathologic complications of

MetS (Renna, Diez et al. 2013). Our data with EWH further supports its role as a regulator of adipose functions with additional anti-inflammatory benefits.

Collectively, findings presented in Chapter 5 demonstrated for the first time that EWH with RAS modulating properties effectively promoted adipocyte differentiation through a combination of insulin mimetic and insulin sensitizing actions on 3T3-F442A cells, increased expression of the anti-inflammatory hormone adiponectin, and suppressed cytokine mediated inflammatory response in these cells. Considering the fundamental role of adipose tissue in the pathogenesis of hypertension, inflammation, insulin resistance, and MetS, EWH may have potential benefits in the prevention and management of MetS complications. Therefore, in Chapter 6, we aimed to evaluate the effects of EWH on glucose and insulin tolerance as the final readouts for the potential benefits of EWH on MetS in an animal model of insulin resistance.

8.4. Egg white hydrolysate enhances glucose tolerance and insulin sensitivity in diet induced insulin resistant rats

The experiments in Chapter 6 illustrated the efficacy of EWH on glucose tolerance and insulin sensitivity in high fat diet (HFD) induced insulin resistant Sprague Dawley rats. Supplementing a HFD with 4% EWH (equivalent to 1.2 g/Kg BW) improved glucose tolerance as determined by oral glucose tolerance test. Although we did not observe any changes in the postprandial insulin between the treatment and control groups, overall insulin sensitivity as determined by insulin tolerance test (ITT) was enhanced in EWH-treated rats. Interestingly, our data showed an enhanced Akt phosphorylation in liver, muscle and adipose tissue in the fasting state. Furthermore, we showed a significant increase in insulin sensitivity through enhanced Akt phosphorylation in EWH treated adipocytes after stimulating cells with insulin previously. Therefore, we designed an experiment to specifically study the potential effects of EWH on

muscle and fat insulin sensitivity *in vivo* by insulin intraperitoneal injection 10 min before euthanizing rats. Consistent with our *in vitro* data in 3T3-F442A adipocytes, EWH enhanced insulin sensitivity in muscle and adipose tissue (retroperitoneal fat). Therefore, we concluded that enhanced insulin sensitivity was one of the mechanisms by which EWH improved glucose tolerance in HFD induced insulin resistant rats. Our ITT data also indicated the potential effects of EWH on reduced hepatic glucose production which needs further experiments.

As mentioned earlier, pharmacological RAS blockade has been shown to enhance insulin sensitivity (Lee, Song et al. 2008, Weisinger, Stanley et al. 2009) and prevent adipose tissue dysfunction by enhancing adipocyte differentiation and reducing adipose tissue inflammation. Despite the enhanced muscle insulin sensitivity, we did not observe any changes in muscle RAS component expression between the treatment and control groups indicating that the observed muscle enhanced insulin sensitivity may be independent to muscle RAS expression.

Our results also showed some preliminary indications for the enhanced adipocyte differentiation in adipose tissue as EWH was shown to enhance PPARγ2 expression in epididymal fat pad consistent with our previous data in adipocytes *in vitro*. Whole body inflammation was also reduced in EWH-treated rats compared to control group as shown by reduced inflammatory markers including MCP-1, IL-1α, and IL-β. Considering the role of MCP-1 in development of inflammation through macrophage accumulation and activation, the potential role of EWH on macrophage infiltration worth further investigation in future studies. Overall, these data points to the potential effects of EWH on adipose tissue functions. Further confirmatory studies on adipose tissue will shed more light to the potential of EWH on modulating adipose tissue in HFD induced insulin resistant rats.

The potential effect of EWH on modulating RAS components in adipose tissue was not studied in this research. Based on the literature and our data on the enhanced differentiation and insulin sensitivity in adipose tissue both *in vivo* and *in vitro* especially when we observed a reduced AT1R expression in 3T3-F442A adipocytes in EWH-treated cells, EWH may also modulate RAS components in adipose tissue *in vivo* which needs further experiments.

In summary, this study evaluating the effects of EWH in HFD-induced insulin resistant rats showed the positive effects of EWH on inflammatory profile, glucose tolerance, and insulin sensitivity in HFD-induced insulin resistance rats. The enhanced tissue insulin sensitivity is one of the mechanisms by which EWH exerted its novel effects on glucose tolerance. We also observed some evidence for the enhanced adipocyte differentiation or reduced hepatic glucose production in EWH-treated rats which needs further experiments. Since EWH is a mixture of peptides, it is essential to identify potent bioactive peptide(s) responsible for the observed advantageous effects on adipocyte differentiation. Therefore, in the last chapter of this thesis, we identified the most effective peptides on adipocyte differentiation through *in vitro* experiments on 3T3-F442A cells as reported in Chapter 5.

8.5. Purification and Identification of Adipogenic Differentiating Peptides from Egg White Hydrolysate

The experiments done in Chapter 7 were critical in identifying the bioactive peptides in EWH with beneficial effects on adipocyte differentiation. This data is pivotal for future structure-function studies of bioactive peptides. Our data on the purification and characterization of peptides from EWH with PPARγ stimulatory activity in 3T3-F442A cells identified 42 potent peptides from 2 major fractions of this hydrolysate. We successfully validated the stimulatory

effects of several novel peptide sequences including WEKAFKDED, QAMPFRVTEQE, and VFKGL from the major egg white proteins ovalbumin and ovotransferrin. ERYPIL identified from ovalbumin was another potent peptide with stimulatory effects on PPARγ expression. For the first time, adipogenic differentiating peptides have been characterized from the hydrolysate of a food-derived protein. Our findings suggested that Asp, Leu, and Lys residues in peptide sequences may contribute to the PPARγ stimulatory activity in 3T3-F442A pre-adipocytes. Although reports on the identification of adipogenic differentiating bioactive peptides from food proteins are scarce, a growing body of reports on novel peptide sequences similar to our reported data, and structure-function relationship studies would contribute to building a database of peptides exhibiting adipogenic differentiating properties. Constructing such data base is essential for the establishment of targeted strategy (in oppose to the conventional technology) which involves the use of bioinformatics technology, such as the quantitative structure–activity relationship modelling (QSAR) and in silico digestion (Wu, Aluko et al. 2006) for the prediction of bioactive peptides with a specific activity.

In conclusion, findings from this thesis demonstrated the potential of egg white hydrolysate in ameliorating several complications of the MetS; a cluster of abnormalities including hypertension, insulin resistance, dyslipidemia, glucose intolerance, and abdominal obesity, a major public health challenge worldwide (Wong 2007). Through different animal and cell models we demonstrated the beneficial effect of EWH on hypertension, inflammation, adipose tissue, glucose intolerance and insulin resistance. Due to the high and growing prevalence of MetS and its association with cardiovascular disease and type 2 diabetes (Grundy 2008, Kaur 2014), EWH and its bioactive peptides as novel ingredients for the development of functional foods and/or nutraceuticals may be potentially effective in the prevention and management of the

complications of the MetS. Indeed, EWH with potential benefits on the multi-faceted nature of MetS, may serve as an alternative option to the several synthetic drugs targeting individual component of MetS with associated side effects. Further studies are required to establish the clinical efficacy, mode of action, and safety of EWH for long-term use in humans.

8.6. The Implications of Research Findings

Egg is a nutrient-dense whole food and a common source of high quality dietary proteins, essential fatty acids, antioxidants, choline, vitamins, and minerals. Egg also contains cholesterol; a single large egg contains approximately 275 mg of cholesterol. The potential relationship between dietary cholesterol and CVD risk has been the subject of debate for decades. While early studies reported strong correlations between cholesterol intake and heart disease, recent studies showed no or little association between these factors (Kanter, Kris-Etherton et al. 2012). There are inconsistencies in the reported effects of egg consumption on CVD and mortality, and incidence of diabetes. Although most studies show no association between egg consumption (up to one egg per day) and CVD risk in healthy population (Nakamura, Iso et al. 2006, Iqbal, Anand et al. 2007, Qureshi, Suri et al. 2007, Scrafford, Tran et al. 2011, Rong 2013, Alexander, Miller et al. 2016), others suggest an increased risk of CVD and heart failure with higher egg consumption (≥7 eggs per week) (Nakamura, Okamura et al. 2004, Djousse and Gaziano 2008, Nettleton, Steffen et al. 2008, Khawaja, Singh et al. 2017). However, even in studies where no association between egg consumption (up to one egg per day) and CVD in healthy population has been observed, the CVD risk was increased in participants who became diabetic during the course of the study (Hu, Stampfer et al. 1999, Qureshi, Suri et al. 2007). With respect to the incidence of diabetes, most studies (Djousse, Gaziano et al. 2009, Shi, Yuan et al. 2011, Radzeviciene and Ostrauskas 2012, Li, Zhou et al. 2013, Shin, Xun et al. 2013), but not all

(Djousse, Kamineni et al. 2010, Zazpe, Beunza et al. 2013) suggest an increased association between egg consumption and diabetes incidence.

There is also controversy in the current guidelines for recommendations on dietary cholesterol and/or egg intake for both the general population and those at higher risk of CVD. These guidelines differ between countries and even within a country between different specialist societies (Fuller, Sainsbury et al. 2015).

There are fewer published reports in literature regarding the association between MetS and egg consumption. Interestingly, the current reports indicate a positive effect of egg consumption on MetS complications; consumption of more than 3 eggs per week by Korean adults was associated with less abdominal obesity in both men and women compared to non-egg users (Woo, Choi et al. 2016). Furthermore, consumption of more than 7 eggs/week by Korean adults was associated with lower odds of MetS risk compared to those who consumed less than one egg/week in women (Shin, Lee et al. 2017). An inverse association between higher egg consumption and elevated waist circumference, triglyceride, blood pressure, fasting glucose, and reduced HDL-C in women as well as reduced HDL-C in men was observed (Shin, Lee et al. 2017). More studies in other ethnic groups on the relationship between egg consumption and MetS risk is required. Indeed, animal studies have shown that consumption of whey protein at 15% w/w diet but not 5% w/w (Tong, Li et al. 2014) and fish protein at 23% w/w diet (Lavigne, Tremblay et al. 2001) in the original form (non-hydrolyzed form) has the potential to reduce insulin resistance and increase muscle glucose uptake. Since very high dosage of protein is required to observe the positive effect on insulin resistance in animal studies, it may not be feasible to translate these effects in human studies. We tested the potential effects of 4% egg white protein (non-hydrolyzed) on glucose tolerance in the HFD-induced insulin resistant rats in

a preliminary experiment but we did not observe any effects of this treatment while the same dose of egg white hydrolysate (4% EWH) increased glucose tolerance and improved insulin sensitivity in these rats. We concluded that the generated bioactive peptides play the major role for the positive effects of EWH on glucose uptake and insulin sensitivity in this animal model of MetS.

It has also been recently shown that daily intake of whole egg rather than the yolk-free egg substitute in a moderately carbohydrate-restricted diet provides further improvements in the atherogenic lipoprotein profile in individuals with MetS (Blesso, Andersen et al. 2013). Egg consumption reduced LDL-C/HDL-C ratio from baseline, primarily by increasing plasma HDL-C. Authors have attributed the observed improvements in HDL-C to the additional phospholipids consumed by the whole egg group compared to the yolk-free egg substitute group (Blesso, Andersen et al. 2013). While the yolk-free egg substitute mainly containing egg white proteins lacked any effects on dyslipidemia, feeding insulin resistant rats with 4% EWH reduced plasma TG levels significantly compared to the control group. This shows the potential effects of bioactive peptides on lipid metabolism and/or tissue partitioning. Moreover, EWH also reduced blood pressure in SHRs which further highlights the effectiveness of this treatment in complicated metabolic diseases.

EWH exerting positive effects on glucose tolerance, insulin sensitivity, and lipidemia made about 16% of the total protein of the diet fed to insulin resistant rats. Adults should get about 20 percent of their total calories from protein. Considering that an adult needs 2000 Kcal/day energy and the fact that 1 g protein equals to 4 Kcal energy, an adult would need about 100 g protein daily. From the practical point of view, incorporating 16% of the total daily protein intake with

EWH which equals to 16 g, although high, seems feasible. Moreover, cutting proteins from other sources would help not exceeding the maximum am ount of daily protein intake.

Although incorporating 16 g of EWH into a product in the diet seems doable, sensory characteristics of the final product may be altered. Therefore, finding a good medium for incorporating EWH without compromising sensory and textural properties of the product is of immense importance.

Recommendations for Future Research

Based on the results of present research, the recommended future experiments and studies from different perspectives to address the limitations of this research are as follow:

1. Recommendations regarding the effects of EWH on hypertension
    a) Although the poteintail benefits of EWH in a male hypertensive rat model have been reported in this thesis (Chapter 4), further research is essential to target the limitations of this study. For example, it is not known if this treatment is effective on female rats or would show preventative effects on hypertension; incorporating a longer-term treatment period with younger hypertensive as well as normotensive rats with animals of both sexes would better address these questions.

2. Recommendations for future studies regarding the effects of EWH on insulin resistance
    a) While our data in HFD-induced insulin resistance rats suggested the beneficial effects of EWH in these rats, one of the pitfalls of this study was the fact that in the first animal trial, only half of the rats were euthanized in the fasted state which lead to n=4 for some of the experiments including plasma inflammatory markers and lipid profile. Despite a low n number (n=4), however, some of the

inflammatory markers in EWH treated rats were significantly reduced compared to the HFD-treated rats indicative of the anti-inflammatory effects of EWH *in vivo*.

b) The potential effects of GID on the peptide profile of EWH and its effectiveness can be determined through the *in vitro* simulated GID. Both the gastro-intestinal digest of EWH and unprocessed EWH can be used in cell experiments i.e 3T3-F442A pre-adipocytes to verify the effects of GID on the bioactivity of EWH *in vitro* and potentially in vivo.

c) It is not known whether EWH could normalize the impaired postprandial glucose and insulin levels observed in HFD treated rats to the normal level; using normal chow fed rats along with the HFD-fed rats would better show the effects of EWH on insulin resistance state in rats.

d) EWH appears to positively affect adipose tissue dysfunction as shown by the enhanced insulin sensitivity and upregulated PPARγ2 expression, however, additional experiments specifically in adipose tissues derived from HFD and EWH+HFD treated groups would better show the potential benefits of EWH in this organ. Assessing adipsoe tissue inflammatory profile, gene expression, adipose tissue macrophage infiltration, and plasma adiponectin content is essential in exploring the potential effects of EWH on adipose tissue dysfunction (if any) in this isulin resistance rat model.

e) We observed that plasma triglyceride (TG) levels were diminished significantly in HFD+4% EWH tretaed rats compared to the control group (HFD). It is critical to measure lipid content in muscle and liver to see if the reduced TG level in plasma

is not due to accumulation of fats in isulin sensitive tissues. Exploration of mechanisms involved in lipid metabolism may also further contribute to understanding of how EWH works in insulin resistance state.

f) Our data suggested the potential benefits of EWH on hepatic glucose production. This mechanism of action needs further exploration by measuring the activity and expression of responsible proteins in this process such as glucagon, and phosphoenolpyruvate carboxykinase (PEPCK).

g) Based on the current data on the effects of EWH on adipose tissue insulin sensitivity and PPARγ2 expression, it is interesting to measure local RAS components in adipose tissue of HFD and HFD+4% EWH treated rats in order to see if adipose tissue RAS components have been affected by the treatment.

h) Further studies on tissue insulin signaling especially in muscle and adipose tissue would help undermine the mechanism by which EWH enhances insulin sensitivity in these organs. Furthermore, measurement of tissue GLUT4 is essential to determine if this process contributes to the reduced postprandial glucose in insulin resistant rats.

i) Major EWH peptides on adipocyte differentiation have been identified in this study; evaluating their effectiveness on insulin signaling and glucose uptake in muscle and adipose tissue *in vitro* would help identify the most effective peptide(s) for future animal experiments.

j) Stability of the identified peptides can be measured through the *in vitro* simulted GID in order to determine if these peptides are stable through the gastro-intestinal digestion.

k) The absorption of peptides can also be further studied by the intravenous injection of peptides in rats *in vivo*. This may help understand better how EWH works *in vivo*.

3. Recommendations regarding EWH effectiveness *in vivo*
    a) Since we showed the effectiveness of EWH on hypertension and insulin resistance in 2 animal models, it will be interesting to study the potential effects of this treatment in an animal model exerting both complications together.
    b) Long-term studies are essential to evaluate the safety profile of EWH by chronic administration.
    c) Human clinical trials are essential to determine the efficacy and dose-response effects of EWH on hypertension and insulin resistance.

Results from these recommended studies will help better justify the use of EWH as a naturally-derived multi-functional agent in commercialized functional foods or nutraceuticals products for the prevention and management of complications of MetS.

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