Structure and Activity Study of IRW on Improving Insulin Sensitivity

by

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Abstract

Metabolic syndrome, a major public health challenge, is identified as a collection of impaired glucose metabolism, central obesity, elevated blood pressure and dyslipidemia. It is estimated that approximately 25% of the world's population has metabolic syndrome. Insulin resistance, a pathologic state in which target cells fail to respond to normal levels of circulating insulin, is a common feature of most types of metabolic disorders, including type 2 diabetes.

Food protein derived bioactive peptides have attracted extensive attention during the past decades and have great potential for the development of functional foods and/or nutraceuticals for the management and prevention of metabolic syndrome. IRW (Ile-Arg-Trp) was initially identified as an inhibitor of angiotensin converting enzyme (ACE) from egg white protein ovotransferrin. For the past decade, our research group found various activities of IRW. However, the structural requirements of IRW especially which amino acid of IRW is the most important is unknown.

Two dipeptides, IR and RW, three amino acids, I, R and W, as well as eight tripeptides, ARW, IAW, IRA, IWR, RIW, RWI, WIR, and WRI were designed to understand the structure and activity relationship of IRW.

Tumour necrosis factor-α (TNF-α) induced insulin resistant skeletal muscle L6 cell was applied for the study, and IRW was used as the positive control. Among the tested peptides and amino acids, only IRA and IR showed comparable effects as that of IRW: enhanced glucose uptake, improvement in the impaired insulin signaling pathway and increased glucose transporter protein 4 (GLUT4) translocation in TNF-α treated L6 myotubes. This result demonstrated that C-terminal W is not essential to the activity of IRW.

Next, we studied the bioactivity of IR and IRA in a high-fat diet (HFD) induced insulin resistant mouse model, IRW was included as a positive control. Both IRA and IR improved fasting and decreased fasting insulin in HFD induced glucose intolerance mice. While both IRA and IR increased Akt phosphorylation and peroxisome proliferator-activated receptor gamma (PPARγ) abundance in skeletal muscle, only IRA significantly enhanced phosphorylation of 5' AMP-activated protein kinase (AMPK). Our study also showed a reduced local renin angiotensin system (RAS) activity by peptide treatment, but only IR increased mas-receptor (Mas-R) level in skeletal muscle. Our animal study supports the cell experiment's conclusion that C-terminal residue W of IRW is not essential for the activity of IRW, but the mechanisms of action by these two peptides are different.

This thesis supports that the C-terminal residue, W, of IRW is dispensable for regulating the activity of IRW. This result help us to understand the structure and activity relationship of IRW.

PREFACE

This thesis is an original work by Xu Jiang and has been written as per the guidelines given by the Faculty of Graduate Studies and Research, University of Alberta. The concept of the research work in this thesis is originated from my supervisor Dr. Jianping Wu. The figure in this thesis is prepared and drawn by Xu Jiang. The experimental protocol for the animal study was approved by the Animal Care and Use Committee at the University of Alberta (Protocol AUP00001472) in accordance with the guideline issued by the Canada Council on Animal Care.

The thesis consists of five chapters: Chapter 1 provides a general introduction and the objectives of the thesis; Chapter 2 is a literature review on several subjects, including metabolic syndrome, insulin resistance, bioactive peptide, and IRW; Chapter 3 reports the investigation of IRW analog on TNF-α induced insulin resistant skeletal muscle cells; Chapter 4 evaluates effect of tripeptide IRW analogs IRA and IR on improve glucose tolerance in high-fat diet fed mice; Chapter 5 provides overall conclusions and discussion with its significance in food and health management industry for metabolic syndrome treatment.

Dr. Jianping Wu greatly contributed to the experimental design, data interpretation, thesis preparation, and edits. I was responsible for literature search relevant for the above studies, designing and preforming experiments, data collection and analysis, and drafting the thesis.

DEDICATIONS

This thesis is dedicated to my beloved family and friends.

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LIST OF ABBREVIATIONS

AACE- American Association of Clinical Endocrinology

ACE- Angiotensin converting enzyme

ACE2- Angiotensin converting enzyme 2

AHA/NHLBI- American Heart Association/National Heart, Lung, and Blood Institute

Akt/PKB- Protein kinase B

AMPKα- 5' AMP-activated protein kinase-alpha

Ang II- Angiotensin II

AS160- Akt substrate of 160kDa

AT1R- Angiotensin II type 1 receptor

AT2R- Angiotensin II type 2 receptor

AUC- Area under the curve

BMI- Body mass index

BSA-Bovine serum albumin

COX-2- Cyclooxygenase-2

CVD- Cardiovascular disease

DMEM- Dulbecco's modified Eagle medium

EGIR- European group for the study of Insulin Resistance

ERK- Extracellular signal-regulated kinase

FBS- Fetal bovine serum

GLUT4- Glucose transporter 4

Grb2- Growth-factor-receptor-bound- protein-2

HDL- High density lipoprotein

HFD- High fat diet

HOMA-IR- Homeostatic model assessment insulin resistance

HS- Horse serum

iAUC-integrated area under the curve

IDF- International Diabetes Federation

IGT- Impaired glucose tolerance

iNOS- Inducible nitric oxide synthase

IPP- Ile-Pro-Pro

IRS- Insulin receptor substrate

IRK- Insulin receptor kinase

IQW- Ile-Gln-Trp

IRW-Ile-Arg-Trp

ITT- Insulin tolerance test

JNK- C-Jun N-terminal kinases

KHH- Kerbs-Henseleit-Hepes

LFD- Low fat diet

MAPK- Mitogen activated protein kinase

Mas-R- mas-receptor

MetS- Metabolic syndrome

NCEP:ATPIII- National Cholesterol Education Program Adult Treatment Panel III

NF-κB- Nuclear factor kappa B

OGTT- Oral glucose tolerance test

PDK1- Phosphoinositide-dependent kinase 1

PI3K- Phosphatidylinositol-3 kinase

PIP2- Phosphatidylinositol 4,5-bisphosphate

PIP3- Phosphatidylinositol-3,4,5-trisphosphate

PPARγ- Peroxisome proliferator- activated receptor γ

PTEN- Phosphatase and tensin homolog

PTP-1B- Protein-tyrosine phosphatase 1B

RAS- Renin-angiotensin system

RUNX2- Runt-related transcription factor 2

SDS-PAGE- Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM- Standard error of the mean

Shc- Src-homology-2-containing protein

SHIP2- SH2-domain-containing inositol phosphatase 2

SHRs- Spontaneously hypertensive rats

SOS- Son- of -sevenless

TCA- Tricarboxylic acid

TNF-α- Tumor necrosis factor alpha

T2D- Type 2 diabetes

VPP-Val-Pro-Pro

WHO- World Health Organization

CHAPTER 1- GENERAL INTRODUCTION AND THESIS OBJECTIVES

1.1 General Introduction

Metabolic syndrome, a major public health challenge, is identified as a collection of impaired glucose metabolism, central obesity, elevated blood pressure and dyslipidemia. It is estimated that approximately 25% of the world's population has metabolic syndrome [1]. The metabolic syndrome will increase the risk of coronary heart disease, cerebrovascular disease by two-fold [2].

Insulin resistance is a common feature of most types of metabolic disorders, such as obesity, dyslipidemia, hypertension and atherosclerosis, non-alcoholic fatty liver disease, type 2 diabetes and some cases of type 1 diabetes [3]. The beta cells in the pancreas produce insulin to promote glucose absorption. When the muscle cells or adipose cells in the body become less sensitive and finally resistant to insulin, the insulin resistance occur.

Most of the insulin stimulated glucose uptake occurs in skeletal muscle. In normal skeletal muscle cell, insulin binds to insulin receptor that leads to tyrosine phosphorylation of insulin receptor substrate (IRS). IRS protein phosphorylation provides the basis for the subsequent downstream signaling through activation and phosphorylation of phosphatidylinositol 3 kinase (PI3K) and protein kinase B, also known as AKT. AKT plays a central role in connecting insulin signalling with downstream regulators of GLUT4 trafficking [4]. This results in stimulated translocation of glucose transporter protein 4 (GLUT4) from cytosol to the plasma membrane for glucose uptake.

Food protein derived bioactive peptides have attracted extensive attention during the past decades and have great possibility for the development of functional foods and/or nutraceuticals for the management and prevention of metabolic syndrome [5]. IRW (Ile-Arg-Trp) was initially identified as an inhibitor of angiotensin converting enzyme (ACE) from egg white protein ovotransferrin [6]. In Ang II or TNF-α induced insulin resistance L6 cells, IRW could reverse the reduced serine residue phosphorylation but increased the phosphorylation of tyrosine residue in insulin receptor substrate, leading to GLUT4 translocate to the plasma membrane [7, 8]. For the past decade, our research group found various activities of IRW. However, the structural requirements of IRW especially which amino acid of IRW is the most important one is unknown. Structural analog, simply as analog, is a compound having a structure similar to that of another compound. It can differ in one or more atoms, functional groups, or substructures. In peptide/protein analog can differ in one amino acid. Compared with others amino acids, the side chain of alanine only has a methyl. Alanine was chosen to be used as single amino acid scanning substitution [9]. Eight

tripeptides, ARW, IAW, IRA, IWR, RIW, RWI, WIR, and WRI, three amino acids I, R and W, as well as two dipeptides, IR and RW were designed to study the importance of amino acid in IRW.

1.2 Objectives

Since IRW is a tripeptide that only has three amino acids, we hypothesized that IRW analogs may could improve insulin resistance same with the effect as that of IRW.

The overall objectives of the research were to investigate the structure and activity relationship of IRW and understand the importance of different amino acids in IRW. The specific objectives of the research are:

1. To test the effects of IRW analogs on cell viability, glucose uptake assay and insulin signaling pathway using L6 cell line.
2. To test the effects of selected IRW analogs in high fat diet induced insulin resistant animal model.

1.3 Chapter format

There are five chapters in the thesis and a brief description of each chapters given as follows:

Chapter 1 gives a brief introduction to the metabolic syndrome, the growing interest in food-derived bioactive peptide against metabolic syndromes, the thesis hypothesis and objectives.

Chapter 2 provides an insight strategy based on the literature review of the current knowledge on metabolic syndrome, insulin resistance, bioactive peptide against metabolic syndromes and IRW.

Chapter 3 investigates the *in vitro* effect of IRW analogs in TNF-α induced insulin resistance L6 cells. The effects on cell viability were determined by WST-8 kit. The effects on glucose uptake assay were tested by a glucose test kit. The effects on insulin signaling pathways were tested by expression of different biomarkers using the western blot.

Chapter 4 explores the *in vivo* effect of selected IRW analog in a high fat diet induced C57BL/6 mice. OGTT and ITT were used to evaluate the effect of selected IRW analog on improving glucose tolerance. Insulin dependent and independent signaling pathways were tested using western blot. Insulin concentration and plasma components was quantified by commercial ELISA kit.

Chapter 5 provides overall conclusions and discussions of the thesis. The limitations and prospects of this research is also discussed.

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CHAPTER 2-LITERATURE REVIEW

2.1 Metabolic syndrome

Metabolic syndrome was first defined as “syndrome X” in 1988 by Gerald M Reaven, the concept of “syndrome X” is the cluster of insulin resistance, dyslipidaemia, dysglycemia and high blood pressure being the main feature in coronary heart disease and type2 diabetes [1]. After that, many international organizations and groups attempted to define metabolic syndrome. World Health Organization (WHO) defined the metabolic syndrome as presence of insulin resistance or impaired glucose tolerance (IGT) as the essential concept of this disease with at least two of the following parameters: hypertriglyceridemia and/or high density lipoprotein cholesterol, raised blood pressure ($\geqslant$140/90mm Hg), obesity (body mass index (BMI) or waist/hip ratio), and microalbuminuria [2]. In the definition of European Group for the study of Insulin Resistance (EGIR), the hyperinsulinemia was added while the microalbuminuria was deleted from the major component of the disease [3]. Different form both WHO and EGIR, in 2001 the National Cholesterol Education Program Adult Treatment Panel III (NCEP:ATPIII) published a new set of criteria that incuuded waist circumference, blood lipids, blood pressure, and fasting glucose. The insulin resistance was not considered as the essential diagnostic component in NCEP:ATPIII definition [4].

The International Diabetes Federation (IDF) was attempted to define the syndrome more ethnicity specific so that it could be used by different nations and research groups [5]. Same with IDF, the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) also use the waist measurement as a simple evaluation tool to the abdominal obesity, even the waist circumference cut-off points of these two institute was slight different [6]. Different with IDF and AHA/NHLBI, the American Association of Clinical Endocrinology (AACE) considered the IGT was the central feature of metabolic syndrome [7]. The definitions of AACE, WHO and EGIR are largely focused on insulin resistance. In contrast, the NECP:ATPIII and IDP definitions are more focusing on waist circumference (central obesity). The diagnostic components of metabolic syndrome are reduced high density lipoprotein cholesterol, raised triglycerides, blood pressure and fasting plasma glucose, all of this are related to weight gain, specifically intra-abdominal/ectopic fat accumulation and a large waist circumference. Using internationally adopted arbitrary cut-off values for waist circumference offers an effective treatment approach through weight management [8]. According to the new definition of metabolic syndrome in the IDF consensus statements, a

person must have central obesity (defined as waist circumference with ethnicity specific values) and any two of the following four factors: raised triglycerides, reduced high density lipoprotein (HDL), raised blood pressure and raised fasting plasma glucose can be diagnosed have metabolic syndrome.

The average prevalence of metabolic syndrome is 31% and the incidence of metabolic syndrome is expected to increase to approximately 53% at 2035 [9, 10]. However, no matter which criteria are used, the prevalence of metabolic syndrome is increasing worldwide, as the percentage of obesity continue to grow [11]. Using the revised NECP:ATPIII criteria, the estimated prevalence of metabolic syndrome increased up to 5% during the past 15 years. Using the more restrictive WHO criteria, estimated approximately 34% prevalence of metabolic syndrome, while a higher prevalence was estimated using the IDF definition which had a lower cut-off point for waist circumference [12]. The IDF guideline identified a larger prevalence than the NCEP:ATPIII in US adults [13]. While, in Iranian adults the prevalence of metabolic syndrome s higher than western counterparts [14]. The prevalence of metabolic syndrome increases even more obviously along with the BMI increases. In obese females and males compared to normal weight and underweight individuals the prevalence went up to 17 times for females and 32 times for males, respectively [15]. Same trend was found in developing countries, the prevalence of metabolic syndrome is rapidly increasing from 9.8% in male urban North Indians to 42% in female urban Iranians, this may due to different dietary, physical activity and lifestyle [16]. During the last 15 years, the estimated prevalence of metabolic syndrome increased up to 5% using the NCEP:ATPIII criteria [17]. No matter which criteria was used this trend was observed and the transition from the traditional to the Western-like lifestyle contributed to this increase. It should be noted that even the lean individuals can develop metabolic syndrome, further complicating its pathogenesis [18].

According to the IDF statement, people with metabolic syndrome have a fivefold higher risk of developing type 2 diabetes and they are twice as likely to die from and three times as likely to have a heart arrack or stroke compared with people without this syndrome. In addition, the metabolic syndrome is now considered a new driving force for CVD epidemic. Moreover, the metabolic syndrome and its components may increase the possibility to caner development and cancer-related mortality [19]. Therefore, metabolic syndrome is a global challenge in management and prevention.

2.2 Type 2 diabetes

According to Diabetes Canada, "Type 2 diabetes is a disease in which your pancreas does not produce enough insulin, or your body does not properly use the insulin it makes" (https://www.diabetes.ca/about-diabetes/type-2). T2D is the most common type of diabetes, accounting for around 90% of all diabetes cases. T2D affects 422 million adults worldwide and this data will be expected to reach 592 million by 2035 [20]. In countries with a high diabetes incidence, such as those in the Middle East and Pacific, as many as one in four deaths in adults aged between 35 and 64 years is due to this disease. The symptoms of T2D are like those of type 1 diabetes and include: excessive thirst and dry mouth; frequent urination; lack of energy; tiredness; slow healing wounds; recurrent infections in the skin; blurred vision; tingling or numbness in hands and feet (https://www.idf.org/aboutdiabetes/type-2-diabetes.html). These symptoms may be mild or absent and people with T2D diabetes can live several years with the condition before being diagnosed. Except the genetic factors, overnutrition and modern sedentary lifestyle are associated with the onset and development of T2D [21]. A healthy lifestyle including a healthy diet, regular physical activity, no smoking and maintaining a healthy body weight is the cornerstone of managing T2D.

2.3 Insulin and insulin resistance

Insulin, a molecule of only 51 amino acids, was discovered more than 100 years ago [22]. Most of insulin stimulated glucose uptake occurs in skeletal muscle, the adipose tissue only accounts for a small part [23]. Insulin receptor belongs to a subfamily of receptor tyrosine kinases. This receptor consists of two α- and two β-subunits. Insulin binding to the α-subunits leads to derepressing of the activity in the β-subunits, then the β-subunits are phosphorylated and their conformation are changed to increase activity [24]. Once the insulin receptor tyrosine kinase activation recruits and phosphorylates several substrates, including insulin receptor substrate (IRS) [25]. The phosphorylated IRS activates phosphatidylinositol-3 kinase (PI3K) [26]. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) on the 3-position to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3) [27]. PIP3 regulates phosphoinositide-dependent kinase 1(PDK1). PDK1 is one of the serine kinases that phosphorylates and then activates the protein kinase B (Akt/PKB) [28]. Phosphorylated Akt activates Akt substrate of 160kDa (AS160) located in the glucose transporter 4 (GLUT4). GLUT4 is released from the intracellular sites to the plasma

membrane [29]. GLUT4 allows the uptake of extracellular glucose that is stimulated by insulin into adipose tissue and muscle [30]. Independently of PI3K/Akt pathway, another branch of the insulin signaling pathway is mitogen activated protein kinase (MAPK) pathway. This pathway involves the tyrosine phosphorylation of IRS and Src-homology-2-containing protein (Shc). Shc interacts with the adaptor protein growth-factor-receptor-bound- protein-2 (Grb2), recruiting the son- of -sevenless (SOS) exchange protein to the plasma membrane to activate Ras. After activated, Ras acts as a molecular switch, phosphorylating the serine kinase cascade through the activation of Raf, mitogen-activated protein kinase (MAPK/MEK) and extracellular signal-regulated kinase (ERK). Phosphorylated and activated ERK can translocate into nucleus to catalyse the transcription factors phosphorylation [31]. MAPK/ERK plays a crucial role in cell proliferation, differentiation, migration, senescence and apoptosis [32, 33]. While the PI3K/Akt pathway is a major mediator of effects of insulin [34]. The briefly flowchart of PI3K/Akt pathway and MAPK pathway is shown in Figure 2.1 and Figure 2.2.

Insulin resistance is at the core of metabolic syndrome [35]. Insulin resistance is a pathologic state that target cells, like skeletal muscle cell, fail to respond to normal levels of circulating insulin [36]. In this condition, insulin can not to maintain normal glucose and lipid homeostasis. To maintain normal glucose levels a higher concentration of insulin are needed [37]. When cells in the body (liver, skeletal muscle and adipose/fat tissue) become less sensitive and resistant to insulin, insulin resistance occurs [38]. The production of higher amounts of insulin weakens and may eventually wear out the beta cells of pancreas. Once the pancreas is not able to produce enough insulin then a person becomes hyperglycaemic and will be diagnosed with type 2 diabetes [5].

Insulin resistance can be characterized by defects at many different levels, decreasing in receptor concentration and kinase activity, decreasing the concentration and phosphorylation of IRS, PI3K activity, glucose transporter translocation, and the activity of intracellular enzyme [39]. Genetic and many other factors can influence insulin sensitivity. The case of genetic defects in the insulin receptor are rare, but most of the severe forms of insulin resistance are represent by genetic defects, such as leprechaunism, the Rabson Mendenhall Syndrome, and the type A syndrome of insulin resistance [40].

At the molecular level, insulin resistance is the result of impaired insulin signaling that may come from post-translation modification or mutations of the insulin receptor itself or any of its

downstream molecules, including IRS, PI3K, and Akt. Different studies in insulin resistance humans and animal models demonstrated that the impaired insulin signaling is the results of postreceptor perturbations [41]. Multiple degrees of postreceptor defects have been recognized as mechanisms underlying insulin resistance [42]: (a) change ratio of Ser/Thr residue phosphorylation of IRS [43]; (b) increased degradation of IRS [44]; (c) upregulating activity of Tyr-phosphatases in SH2-domain-containing inositol phosphatase 2 (SHIP2), phosphatase and tensin homolog (PTEN), and protein-tyrosine phosphatase 1B (PTP-1B) [45, 46]; (d) downregulating activation of insulin receptor downstream signaling molecules, such as Akt [47]. In animal models and muscle of insulin resistant subjects, impaired insulin receptor autophosphorylation has been demonstrated at the receptor level [48]. Different studies have demonstrated that the major negative regulatory role on insulin resistance is via the increased the ratio of Ser/Thr phosphorylation in IRS [43]. IRS has more than 70 Ser/Thr residues that are potential objectives for phosphorylation. In specific residues Ser/Thr phosphorylation can lead to the dissociation of IRS from the insulin receptor, block Thr phosphorylation sites in IRS, release IRS from intracellular complexes that keep them close to the receptor, induce degradation of IRS, or turn IRS into inhibitors of the insulin receptor kinase (IRK) [49]. Over Ser/Thr phosphorylation of IRS can become adverse for normal insulin signaling pathway, leading to insulin resistance.

2.4 Food proteins derived bioactive peptides

Food protein derived bioactive peptides have attracted extensive attention during the past decades and have great potential for the development of functional food and/or nutraceuticals for the management and prevention of various chronic diseases, such as cardiovascular disease, chronic inflammation disease, cancers, diabetes, osteoporosis [50]. The length of bioactive peptides range from 2 (dipeptides) to about twenty amino acid residues [51]. Much longer peptides with biological activity has also been reported, such as lunasin, a 43 amino acid peptide derived from soy bean has anti-cancer and hypocholesterolemic effects [52, 53]. Numerous bioactive peptides have been identified from various kinds of different food source such as: bovine casein, egg, marine algae, soy, cheese whey, tuna, milk, black soy and so on. Among them, soy, egg, milk, and fish are the most studied protein sources for extraction bioactive peptide. Proteolysis is the most widely used method to prepare bioactive peptides. In addition to proteolysis, bioactive peptides can be formed during food processing, such as fermentation [50, 54]. Diversified structures of bioactive peptides

give them a broad scope of functions ranging from protection against infection, regulating glucose levels and blood pressure, to killing senescent cells for reducing signs of ageing [55-58].

In milk, VPP and IPP are two well-studied casein-derived peptides. *In vivo*, VPP and IPP had shown antihypertensive, vasculo-protective, antioxidant, and anti-inflammatory properties [59-62]. In apolipoprotein E-deficient mice, thirty-one-week intake of VPP and IPP diets attenuated atherosclerosis development. Also, in this animal model, at mRNA level the expression of inflammatory cytokines such as IL-6 and IL-1β and oxidized low-density lipoprotein receptor was reduced [63]. In C57BL/6J mice, 10-week oral administration of 0.3 mg/mL of VPP in high fat diet shown anti-inflammatory effects on adipose tissue[60]. Compared to the control group, the adipose tissue of VPP-administered mice had less pro-inflammatory macrophages and activated monocytes as well as IL-6 and MCP-1gene expression [60]. In 3T3-F442A adipocyte cells, VPP and IPP shown advantageous adipogenic differentiation and insulin mimetic and anti-inflammatory effects [64].

Egg proteins are another important sources to extract bioactive peptides [65, 66]. Egg white protein ovotransferrin derived dipeptide GW had antioxidant and anti-inflammatory effects in human umbilical vein endothelial cells [67]. In literature, Trp have been reported as important amino acid residues in exerting antioxidant and anti-inflammatory properties [68]. Trp was intraperitoneal injected to Wistar rats that significantly reduced TNF-α levels while enhanced IL-10 levels with anti-inflammatory properties [69]. Egg yolk proteins derived pepides: YINQMPQKSRE, YINQMPQKSREA, VTGRFAGHPAAQ, and YIEAVNKVSPRAGQF shown antioxidant, angiotensin converting enzyme (ACE) inhibitory, and antidiabetic activities *in vitro* [70].

Other sources, like marine proteins derived peptides have various biological activities including anticancer, antihypertensive, antimicrobial, antioxidant, antiobesity and immunomodulatory properties [71-73]. Two ACE inhibitory and antioxidant peptides, MVGSAPGVL and LGPLGHQ were identified from skate (*Okamejei Kenojei*) gelation hydrolysate [74]. Sardine muscle hydrolysate dipeptide MY exhibits antioxidant activity by protecting endothelial cells from oxidative stress through induction of heme oxygenase-1 and ferritin [75]. In a zebrafish model, two peptides YSQLENEFDR and YIAEDAER derived from meat and visceral mass effectively protected skin cells against oxidative damage [76]. Tilapia skin gelation hydrolysates identified peptide LSGYGP has been reported to protect endothelial cells against Ang II induced injury. This

peptide inhibited Ang II-stimulated oxidative stress and cytotoxicity, downregulated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) by suppressing the Nuclear factor kappa B (NF-κB) pathway and enhanced the protein levels of antioxidant enzymes in Ang II-treated human umbilical vein endothelial cells [77]. As the oceans and seas cover about 71% of the world's surface area [78]. The marine biosphere including marine derived peptides is a rich source of health-promoting substances [79].

A number of peptides has anti-diabetic effects *via* multiple signaling pathways [80, 81]. Aglycin identified from soybean improved glucose uptake through up-regulated phosphorylation of insulin receptor, IRS, Akt, and translocation of GLUT4 to he plasma membrane [82]. Two *momordia charantia* insulin receptor binding protein (mcIPBP) derived peptides, mcIPBP-19 and mcIPBP-68, enhanced insulin binding to insulin receptor, stimulating phosphorylation of Akt and expression of GLUT4, leading to increased glucose uptake in adipocytes and glucose clearance in diabetic mice [83-85]. AMPK pathway was regarded as another potential therapeutic target for prevention and amelioration of T2D and insulin resistance [86, 87]. Some bioactive peptides have been reported to improve insulin sensitivity through activating AMPK signaling pathways. A casein derived glycomacropeptide, IPPKKNQDKTE, prevented high glucose induced insulin resistance in hepG2 cells by activating AMPK signaling pathway through phosphorylation of AMPK [88]. A dipeptide Trp-His (WH) enhanced glucose uptake in L6 muscle cells *via* activation of AMPK pathway and enhanced GLUT4 translocation to the cell membrane [89]. Soybean peptides improved glucose uptake and insulin sensitivity *via* phosphorylation of AMPK in skeletal muscle L6 cells [90].

2.5 IRW (Ile-Arg-Trp)

Egg is one of the few foods that are used/consumed throughout world due to its protein having a complete amino acid profile, taste good and a affordance source of protein [91, 92]. Eggs consist of 3 main parts: egg white (63%), egg yolk (27.5%) and eggshell (9.5%). Egg white comprises of water (89%), protein (10%), ash (0.5%), carbohydrates (0.4%) and small amount of lipids (0.03%) [91]. The major proteins in egg white are ovalbumin (54%), ovotransferrin (11%), ovomucin (3.5%) and lysozyme (3.4%) [93]. IRW (Ile-Arg-Trp) was predicted as an ACE inhibitory peptide by quantitative structure and activity relationship (QSAR) [94]. After that, IRW was initially

identified as an inhibitor of ACE *in vitro* from egg white protein ovotransferrin in 2011 [95]. Since then, our research group found various activities of IRW.

In spontaneously hypertensive rats (SHRs), oral administration of IRW at a daily does of 15 mg/kg body weight significantly reduced blood pressure [96]. In this animal study, IRW treatment significantly up-regulated the expression of angiotensin converting enzyme-2 (ACE2) [96]. Further, the ACE2 protein levels in both aorta and kidney were significantly enhance in IRW treatment group [97]. These results showed the IRW is a ACE2 activator [97]. Further study in SHRs showed that infusion of MasR antagonist A779 at a dose of 48 μg/kg body weight/h abolished the blood pressure-lowering effect of IRW [98]. This result indicating the antihypertensive effect of IRW was through the ACE2/Ang (1-7)/MasR axis in SHRs. Oral administration of IRW at a 15 mg/kg body weight dose decreased Ang II, increased circulating levels of ACE2 and Ang (1-7) while unaffected ACE [98]. In Ang II stimulated cells, IRW treatment increased the levels of ACE2 and MasR, while both the addition of MasR antagonist and ACE2 knockdown abolished these effects of IRW [99, 100].

In SHRs, IRW treatment activated Akt phosphorylation, indicating the possible role of IRW in improving insulin sensitivity [98]. IRW could significantly reduce serine residue phosphorylation while increase tyrosine residue phosphorylation of IRS, as well as phosphorylation of Akt, leading to enhanced GLUT4 translocation from cytosol to the plasma membrane in Ang II induced insulin resistant L6 skeletal muscle cell model [101]. In TNF-α treated L6 cells, IRW could improve impaired insulin sensitivity by down-regulating the activation of p38 and c-Jun N-terminal kinases (JNK) [102]. In C57BL/6 mice, IRW improves glucose tolerance and body composition in HFD-induced mice and promotes glucose uptake in skeletal muscle through multiple signaling pathways [103].

IRW also play a role in the bone health. IRW could promote osteoblast proliferation, differentiation, and mineralization, via PI3K/Akt pathway and increase expression of Runt-related transcription factor 2 (RUNX2) and its downstream effectors [104]. In mice macrophage RAW 264.7 cells, IRW could inhibit LPS-induced osteoclastogenesis via its anti-inflammatory activity [105]. IRW anti-inflammatory activity was also reported in HUVEC cells and in animals [106, 107].

Taking these into consideration, IRW is tripeptide with various activities [108]. However, the structural requirements of IRW especially which amino acid of IRW is the most important is unknown. Amino acid scanning is widely used to identify the importance of positions and amino acid side chains for protein/peptide function. As the side chain at the β-carbon represents a deletion, alanine was chosen for use as a single amino acid scanning substitution [109]. Structural analog is a compound having a structure similar to that of another compound. In biochemistry analog is differ in one amino acid [110]. IRW is constitute of three amino acids. Two dipeptides, IR and RW, and three amino acids, I, R and W, compound this peptide. The substitution of alanine as well as compound dipeptides and amino acids will be used to study the structure and activity relationship of IRW.

PI3K/Akt pathway

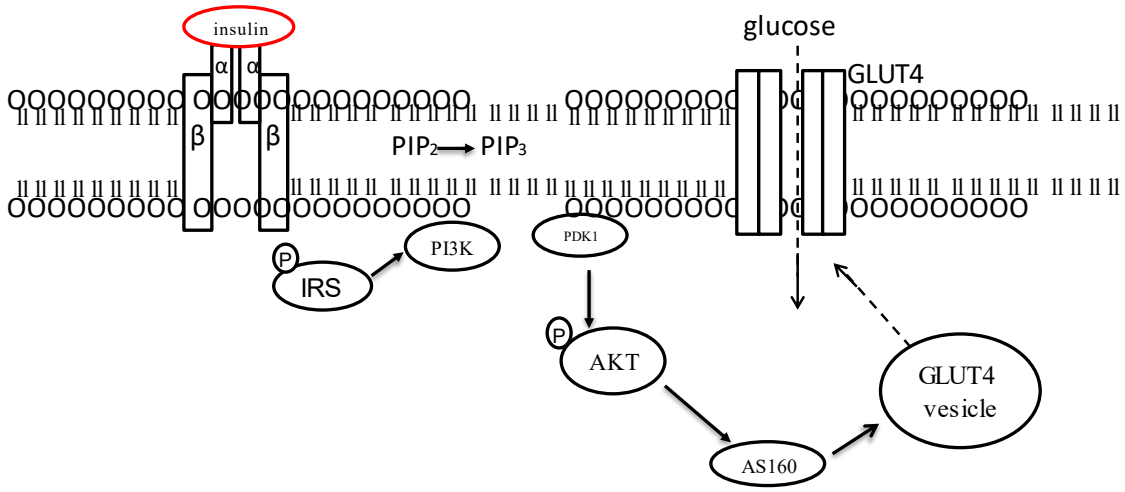

Figure 2.1 PI3K/Akt pathway

When insulin binds to the α subunits of insulin receptor, the β subunits become activated and autophosphorylate themselves on tyrosine residues, once the β subunits become phosphorylated they become activated. The β subunits will actually lead to the phosphorylation and activation of IRS. When IRS is phosphorylated and activated, other proteins including PI3K which will bind to IRS, once PI3K binds to IRS, PI3k will actually phosphorylate PIP2 to PIP3, when PI3K phosphate enough PIP2 to PIP3, PIP3 concentration will increase, this will recruit other proteins toward the plasma membrane including PDK1 and Akt. When PDK1 become activated, then will actually phosphorylate Akt, Akt will phosphate and activate AS160. AS160 will allow the translocation to occur, then the GLUT4 will bebeded into the cellular membrane. The glucose can be transported inside the cell. IRS: Insulin receptor substrate; PI3K: Phosphoinositide 3 kinase; PIP2: Phosphatidylinositol 3,4-bisphosphate; PIP3: Phosphatidylinositol 3,4,5-trisphosphate; PDK1: 3-phosphoinositide-dependent protein kinase; Akt: Protein kinase B; AS160: Akt substrate of 160kDa; GLUT4: glucose transporter 4.

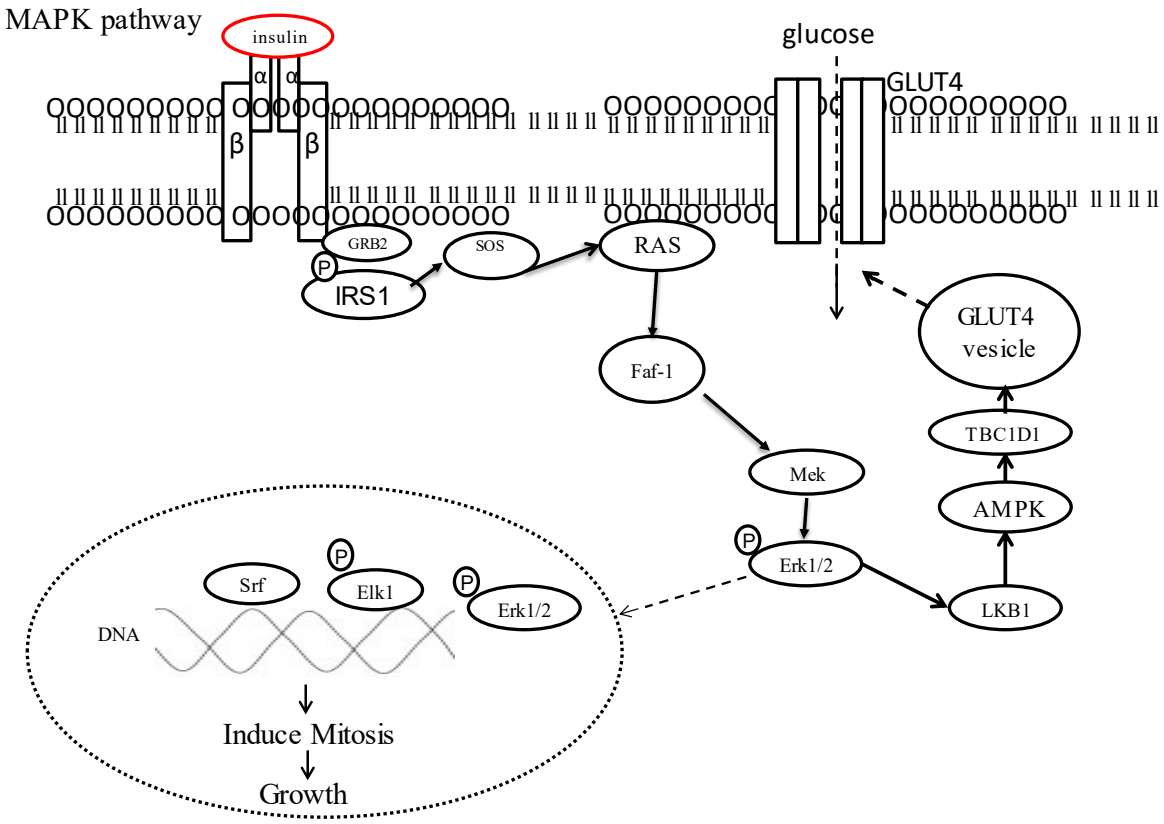

Figure 2.2 MAPK pthway

Binding of insulin to insulin receptor autophosphorylation and activation of the protein kinase that phosphorylates IRS on tyrosine residues. IRS phosphorylation generates binding sites for GRB2. GRB2 recruits the SOS. SOS exchanges protein to the plasma membrane to activate RAS. After activated, Ras acts phosphorylation of the serine kinase cascade through the activation of Raf, MEK and ERK. ERK phosphorylates LKB1. Activated LKB1 will activate TBC1D1, then GLUT4 will translocate to uptake glucose. IRS: Insulin receptor substrate; GRB2: Growth factor receptor-bound protein 2; SOS: Son of sevenless; RAS: rapidly accelerated fibrosarcoma; Mek: mitogen activated protein kinase; Erk: extracellular regulated kinase; LKB1: liver kinase B1; AMPK: 5' adenosine monophosphate-activated protein kinase; TBC1D1: Tre-2/BUB2/cdc1 domain family 1; GLUT4: Glucose transport 4; Elk: ets-like transcription factor; Srf: serum response factor.

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CHAPTER 3- STRUCTURE AND ACTIVITY STUDY OF TRIPEPTIDE IRW IN TNF-α INDUCED INSULIN RESISTANT SKELETAL MUSCLE CELLS

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3.1 Introduction

According to International Diabetes Federation, metabolic syndrome is a cluster of the most dangerous heart attack risk factors: diabetes and prediabetes, abdominal obesity, high cholesterol and high blood pressure [1]. The prevalence of metabolic syndrome is rapidly increasing worldwide and has become a major public and clinical problem [2]. While the pathogenesis of metabolic syndrome and each of its components are complex and are not well understand, insulin resistance is the core underlying mechanism responsible for metabolic syndrome [3]. Insulin resistance is a common feature of most types of metabolic disorders, such as obesity, dyslipidemia, hypertension and atherosclerosis, non- alcoholic fatty liver disease, type 2 diabetes and some cases of type 1 diabetes [4]. Insulin resistance is a pathologic state in which target cells fail to respond to normal levels of circulating insulin. Therefore, insulin is unable to provide normal glucose homeostasis, and higher concentrations of insulin are needed to maintain normal glucose levels [5]. The beta cells in the pancreas produce insulin to promote glucose absorption; insulin resistance occurs when the muscle cells or adipose cells in the body become less sensitive and finally resistant to insulin. As skeletal muscle accounts for up to 75% of insulin-dependent glucose uptake, more attention is being paid to discover bioactive compounds to mitigate insulin resistance in skeletal muscle [6]. Understanding the molecular mechanisms of insulin resistance is important to identify efficient targets, and thus develop new therapeutics to prevent or treat metabolic syndrome [7].

In normal skeletal muscle cells, insulin binds to insulin receptor and leads to tyrosine phosphorylation of insulin receptor substrate (IRS). The IRS protein phosphorylation provides the basis for the subsequent downstream signaling through activation and phosphorylation of phosphatidylinositol 3 kinase (PI3K) and protein kinase B, also known as Akt. This results in stimulated translocation of glucose transporter protein 4 (GLUT4) from cytosol to the plasma membrane for glucose uptake [8]. After uptake, glucose is oxidized through the tricarboxylic acid (TCA) cycle in mitochondria or stored as glycogen in liver or muscle, and plasma glucose remains in a narrow range between 4 and 7 mM in normal individuals [8, 9]. While in state of insulin resistance, the insulin stimulated translocation of GLUT4 is impaired, whereas the expressed of GLUT4 protein is unchanged [10].

Insulin resistance encompasses a wide spectrum of disorders including inflammation [11]. Chronic inflammation is the major contributor to the development of insulin resistance [12, 13]. Tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) and other pro-

inflammatory biomarkers increase in various insulin resistance states. The role of TNF-α in insulin resistance of skeletal muscle has been well documented [14]. Chronic exposure of skeletal muscle to TNF-α impairs insulin stimulated glucose uptake and GLUT4 translocation by increasing the phosphorylation of serine residue of IRS as well as decreasing the phosphorylation of Akt [15-17]. Food protein derived bioactive peptides have attracted extensive attention during the past decades and have great potential for the development of functional foods and/or nutraceuticals for the management and prevention of metabolic syndrome [18, 19]. Soybean peptide aglycin can improve glucose uptake *via* up-regulated phosphorylation of insulin receptor (IR), IRS, and Akt, as well as translocation of GLUT4 to cell membrane in type 2 diabetic mice [20]. A casein glycomacropeptide-derived peptide, IPPKKNQDKTE, prevented high glucose-induced insulin resistance in hepG2 cell by activating both insulin and AMP-activated protein kinase (AMPK) signaling pathways [21]. A dipeptide, WH, activates AMPK pathway and enhances insulin-independent glucose uptake in L6 muscle cell [22]. Soybean identified peptides enhance the glucose uptake by activation of AMPK pathway in L6 myotubes [23]. Egg white protein ovotransferrin derived peptide IRW was initially identified as angiotensin converting enzyme (ACE) inhibitory peptide [24], and later was reported to possess various activities including improves TNF-α or angiotensin II induced insulin resistance [25-29]. IQW, one amino acid residue difference from that of IRW, and the constituent dipeptides or the combination of amino acids of IRW did not exhibit the same antioxidant effects as IRW [28]. Both IRW and IQW had antioxidant activity but the mechanisms are not identical. There are limited structure and activity relationship study of ACE inhibitory peptides [30]. It was suggested that the amphipathicity, molecular wight, net charge and sequence of amino acids affect the activity of a peptide [31]. While the IRW possess promising bioactivities, the structural requirements of IRW especially the significance of each amino acid residue of IRW is unknown. Therefore, the objective of the study was to investigate the structure and activity relationship of IRW in a rat derived skeletal muscle cell line (L6 cells).

3.2 Materials and methods

3.2.1 Materials

Insulin, isoleucine, arginine, tryptophan and Triton X-100 were purchased from Sigma-Aldrich (St Louis, MO, USA). Recombinant rat tumor necrosis factor alpha (TNF-α) was purchased from Biotechne (Minneapolis, MN, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotic–antimycotic solution and horse serum (HS) were purchased from

Gibco/Invitrogen (Carlsbad, CA, USA). The tripeptide IRW, ARW, IAW, IRA, IWR, RIW, RWI, WIR, and WRI, the dipeptide IR and RW, were synthesized by Genscript (Piscataway, NJ, USA). Peptide sequence and purity (99.1%-99.8%) were validated by HPLC-MS/MS.

3.2.2 Peptide design

As the side chain at the β-carbon represents a deletion, alanine was chosen to study the single amino acid substitution [32]. To determine the significance of individual amino acid in IRW, alanine scanning was performed to replace one amino acid at one time, and therefore, three peptides, ARW, IAW, IRA were designed. To study the significance of the order of the peptide sequence, five peptides, IWR, RIW, RWI, WIR, and WRI were designed. Dipeptides IR, RW, and its individual amino acids, I, R, W, were also studied.

3.2.3 Antibodies

Rabbit monoclonal primary antibodies against phospho-insulin receptor substrate (Tyr 895), insulin receptor substrate, phosphor-Akt (Ser 473) and mouse monoclonal primary antibody against Akt ware bought from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit monoclonal primary antibodies against phosphor-insulin receptor substrate (Ser307) was obtained from Santa Cruz Biotechnology Inc. (SantaCruz, CA, USA). Rabbit monoclonal primary antibody against GLUT4 and α-tubulin were purchased from Abcam (Cambridge, MA, USA). Goat anti-rabbit IRDye 680RD secondary antibody and donkey anti-mouse 800CW secondary antibody were bought from Licor Biosciences (Lincoln, NE, USA).

3.2.4 Cell culture

Rat derived L6 muscle cell was bought from American Type Culture Collection (Manassas, VA, USA; ATCC number: CRL-1458). The cells between passage 8 and 20 were used for the experiments. Cells were grown in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution (10,000 units/mL penicillin G, 10 mg/mL streptomycin, and 25 mg/mL amphotericin B) at 37 ℃ and 5% CO_2 until reached 80% confluence. Then, the cells were cultured in DMEM containing 2% HS for 6-7 days to induce differentiation. The medium was changed every 24 h and cells were used at the stage of 60-70% myotubes, which GLUT4 expressed the highest level [33].

3.2.5 Cell Viability

The concentration of IRW was chosen based on our group's previous results. Other peptides and amino acids use the same concentration with IRW. The differentiated L6 myotubes were incubated

with 100 μM of peptides or amino acids in serum free medium for 2 h, followed by treatment with 5 ng/mL TNF-α for 24 h. Next, 10 μL/well of WST-8 solution was added to each well and the plate re-incubated at 37 ℃ and 5% CO_2 protected from the light for another 4 h. The plate was gently mixed and then read on a microplate reader (SpectraMax M3, Molecular devices, CA, USA) at 460 nm and expressed as fold over the control group.

3.2.6 Glucose uptake assay

Glucose uptake assay was performed according to the procedure described previously with small modifications [34, 35]. L6 cell was subcultured into 24-multiwell plates (5×10^4 cells/well) and grown until they formed 60-70% myotubes. The myotubes ware incubated with 100 μM of peptides or amino acid in serum free medium DMEM for 2 h. Then, 5 ng/mL TNF-α was co-incubated for 24 h to induce insulin resistance as described previously [15]. Next, the myotubes were kept for 2 h in Kerbs-Henseleit buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA), 10 mM Hepes, and 2 mM sodium pyruvate (KHH buffer). Then, the myotubes were cultured in KHH buffer containing 11 mM glucose in the absence or the presence of 1 μM of insulin for another 4 h. The concentration of glucose in KHH buffer were measured by Glucose Test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the amounts of cell uptake glucose were calculated according to the differences of glucose concentrations between before and after culture.

3.2.7 Insulin signaling pathway assay

The differentiated L6 myotubes were incubated with 100 μM of peptides or amino acids in serum free medium for 2 h, followed by co-incubated with 5 ng/mL TNF-α for different times. To detect the phosphorylated and total proteins of the interested biomarkers of insulin signaling pathway, the myotubes were kept in KHH buffer (pH 7.4) for 2 h and then incubated in KHH buffer containing 11 mM glucose in the absence or presence of 1 μM insulin for 30 min. At the end of incubation, L6 myotubes were lysed in boiling hot Laemmle's buffer containing 50 mM DTT and 0.2% Triton X-100 to make samples of western blotting. To extract the protein from cell membrane, a Thermo Fisher Scientific Mem -PER™ Plus Membrane Protein Extraction Kit was used. Briefly, cells were washed with cell wash solution and centrifuged at 300 × g for 5 min. After discarding the supernatant, cells were responded and incubated with permeabilization buffer at 4 ℃ for 10 min with constant mixing. Next, permeabilized cells were centrifuged for 15 min at 16,000 × g. The supernatant containing cytosolic proteins was transferred into a new tube and the pellet was incubated with solubilization buffer at 4 ℃ for 30 min with constant mixing. After centrifuging

for 15 min at 16,000 × g, the supernatant containing membrane and membrane-associated proteins was transferred to a new tube for western blotting analysis. The cell lysates were run in 9% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and immunoblotted with primary antibodies. After incubating with secondary antibodies, protein bands were detected by Licor Odyssey BioImager (Licor Biosciences, NE, USA) and quantified by densitometry using Image Studio Lite 5.2.

3.2.8 Statistical analysis

All data are presented as the mean ± SEM (standard error of the mean) of between four and five independent experiments. The data were evaluated by IBM SPSS version 22. Differences between the mean values were assessed using one-way AVOVA, followed by Tukey multiple comparisons test of applicable. Statistical significance was considered for values of $p < 0.05$.

3.3 Results

3.3.1 Effects of IRW Analogs on Cell Viability and Glucose Uptake on L6 Myotubes

Cell viability was performed by cell counting kit 8. At the concentrations of 100 μM, none of the tripeptides, dipeptides, or amino acids affected the cell viability (Fig. 3.1). To test the effect of IRW analogs on insulin independent glucose uptake in L6 myotubes, IRW analogs was co-incubated with KHH buffer containing glucose for 4 h in the absence of insulin. IRW analogs did not affect basal level of glucose uptake (Fig. 3.2). IRW analogs were incubated with insulin for 4 h to investigate whether these peptides or amino acids have sensitizing effect with insulin. Compared with insulin alone, adding peptides or amino acids did not affect glucose uptake in the presence of insulin (Fig. 3.3). TNF-α was reported to decrease insulin stimulated glucose uptake in skeletal muscle [15, 16]. IRW can reverse TNF-α induced insulin resistance [25]. Therefore, we examined whether IRW analogs have same effect as that of IRW. As shown in Figure 3.4, TNF-α significantly decreased glucose uptake in comparison with insulin only. However, pre-treatment with IRA or IR for 2 h significantly prevented the decrease of glucose uptake in TNF-α treated L6 myotubes. Thus, in addition to IRW, IRA and IR can also protect the action of insulin from TNF-α treatment.

3.3.2 Effects of IRW Analogs on Insulin Signaling Pathway in TNF-α Treated L6 Myotubes

Previous studies showed that TNF-α acts on the insulin signaling pathway by inhibiting tyrosine residue phosphorylation of IRS [15, 36, 37]. To test whether the increased glucose uptake by IRW, IR and IRA are due to activating the insulin signaling pathway, we investigated the effect of

peptides and amino acid on Akt and IRS phosphorylation on TNF-α treated L6 myotubes. IRW analogs treatment did not influence Akt phosphorylation in the absence or the presence of insulin (Fig. 3.5, 3.6, 3.7, 3.8). This is consistent with the results of glucose uptake. Phosphorylation of Akt was significantly decreased by TNF-α compared with the insulin treated myotubes, whereas it was improved by IRW, IR and IRA (Fig. 3.9, 3.10). TNF-α treatment induced serine residue phosphorylation of IRS (Fig. 3.11) while reduced tyrosine phosphorylation of IRS (Fig. 3.12) when comparison with insulin treated cells. This is consistent with the state of insulin resistance [38]. Adding IRW, IR and IRA significantly decreased the phosphorylation of serine residue of IRS, while the phosphorylation of tyrosine residue was increased when comparison with TNF-α group (Fig. 3.11, 3.12). Based on the effects on insulin signaling pathway and glucose uptake, IRW, IR and IRA were further studied on GLUT4 translocation.

3.3.3 Effect of IRW, IR and IRA on GLUT4 Translocation in TNF-α Treated L6 myotubes

TNF-α treatment significantly decreased GLUT4 level in cell membrane while compared with insulin treatment cells. IRW, IR and IRA treatment significantly increased the level of membrane GLUT4 (Fig. 3.13). Pre-treatment IRW, IR and IRA reverse the TNF-α decreased GLUT4 translocation.

3.4 Discussion

Insulin resistance precedes the onset of metabolic syndrome. As the skeletal muscle is the major tissue for insulin stimulated glucose absorption, rat derived L6 skeletal muscle cell is widely used to study insulin resistance [39, 40]. In our previous study, IRW can reverse TNF-α induced insulin resistance in L6 skeletal muscle cells [25]. In this study, effects of IRW analogs on improving TNF-α induced insulin resistance were studied to understand its structure and activity relationship. TNF-α treatment significantly decreased insulin stimulated glucose uptake, impaired insulin signaling pathway and GLUT4 translocation. However, adding IR, IRA and IRW 2 h prior to TNF-α treatment significantly reversed these outcomes. Pre-treatment with IR, IRA and IRW prevented the decrease of glucose uptake in TNF-α treated L6 myotubes. IRW, IR and IRA improved the decrease of phosphorylation of Akt induced by TNF-α. The phosphorylation of serine residue of IRS was decreased while that of tyrosine residue was increased significantly in the presence of IRW, IR and IRA, compared with TNF-α group. The GLUT4 level in cell membrane was decreased by TNF-α. IRW, IR and IRA treatment significantly increased the level of membrane

GLUT4. Our study reveals IRW, IR and IRA improved the GLUT4 translocation from cytosol to cell membrane.

IRW was initially identified as ACE inhibitory peptide [41], after which it showed anti-hypertensive effect in spontaneously hypertensive rats (SHR) as well as decreased oxidative/pro-inflammatory stress markers [42]. IRW was recently shown to improve TNF-α or Ang II induced insulin resistance [24, 25]. In comparison, IQW did not enhance insulin stimulated glucose uptake and did not show any improvement on impaired insulin signaling induced by Ang II [24], similar to our previous studies on vascular function *in vivo* and *in vitro* [27, 41, 42]. Because both IRW and IQW have the same N-terminal Ile(I) and C-terminal Trp (W), the difference was possible due to the presence of a positively charged amino acid of Arg(R) in IRW but not in IQW. Our previous study suggested that the integrity of IRW and IQW is essential for inhibiting the TNF-α induced increased expression of ICAM-1 and VCAM-1 [27]. Their constituting dipeptides and amino acids with the exception of R and amino acid combination (I+R+W) did not affect TNF-α induced levels of ICAM-1 and VCAM-1. However, the significance of each amino acid remains elusive, and the positional effect has not been studied. Glucose uptake was inhibited in the presence of TNF-α, however IRA, but not ARW and IAW, could reverse TNF-α induced decrease of glucose uptake to a level comparable to that of IRW (Fig 3.4), indicating W is not essential to the activity of IRW. Peptides with altered order of IRW did not reverse TNF-α induced decrease of glucose uptake, suggesting as critical role of the peptide sequence. The constituting dipeptide IR, but not RW and its constituting amino acids, showed a similar biological activity as that of IRW, further supporting the C-terminal W is not an essential contributor to the activity of IRW. IR was also identified as ACE inhibitory peptide from pea protein hydrolysate [43]. It should be noted that single amino acid I along with L are well-known for their ability of improving glucose uptake via upregulation of translocation of glucose transporters [44]. Our insulin signalling pathway study further confirmed the structure and activity relationship of IRW.

Bioactive peptides can be released from their parent protein by fermentation, enzymatic treatments or other processing under controlled conditions of pH and temperature. Increasing number of food-derived bioactive components such as bioactive peptides have been tested for their potential use in the prevention or treatment of type 2 diabetes [45]. These bioactive peptides can improve glucose homeostasis and insulin resistance via various mechanisms of action, such as through inhibition of α-glucosidase and/or α-amylase to inhibit carbohydrate digestion [46], affecting gut

hormone release [47], improving insulin secretion [48] and function [49], promoting glucose uptake [20], activating AMPK pathway [22], inhibitors of DPP-IV [50], and adipose differentiation [51]. The complex mechanisms of action imply the challenges of structure and activity relationship study of peptides towards type 2 diabetes as the structural requirements for different mechanisms differ greatly. Further, the peptides identified in literature are limited, in comparison to massive database of ACE inhibitory peptides [52], whose structure and activity relationship are better understood [30, 31]. IPP and VPP are two well-known ACE inhibitory peptides [53], and we reported their insulin-mimetic adipogenic activities in adipocytes [51]. In our recent structure and activity study of IPP and VPP, we found that the presence of a negatively charged N-terminal amino acid residue such as E is beneficial, while the presence of amino acid residue P either in the middle or C-terminal positions is detrimental to their glucose uptake activities [54]. Nevertheless, our understanding of the structure and activity relationship of bioactive peptides for improving insulin sensitivity is limited. This study is the first to report the structure and activity relationship of IRW analogs for improving insulin sensitivity. While IRA and IR showed similar effect and underlying mechanism as that of IRW in L6 cells, whether IRA and IR have same *in vivo* activity as IRW or not needs to be tested.

In conclusion, the present study shows the significance of peptide IRW sequence, especially the constituent dipeptide IR. The C-terminal W is not essential to the activity of IRW. Both IRA and IR could improve glucose uptake and impaired insulin signaling pathway in TNF-α treated rat derived skeletal muscle cells to the same extent as that of IRW.

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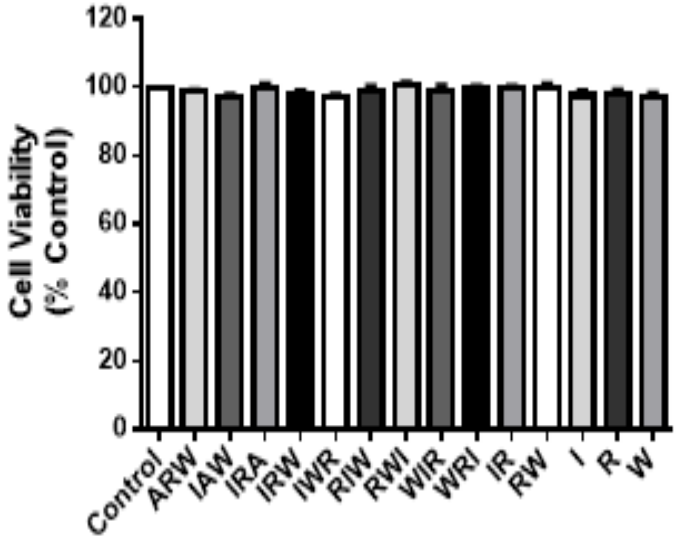

Figure 3.1 Effects of IRW analogs on cell viability

The differentiated myotubes were incubated with 100 μM of IRW analogs or analogs 2 h prior to the treatment of 5ng/mL TNF-α for 24 h. Cell viability was performed by cell counting kit 8.

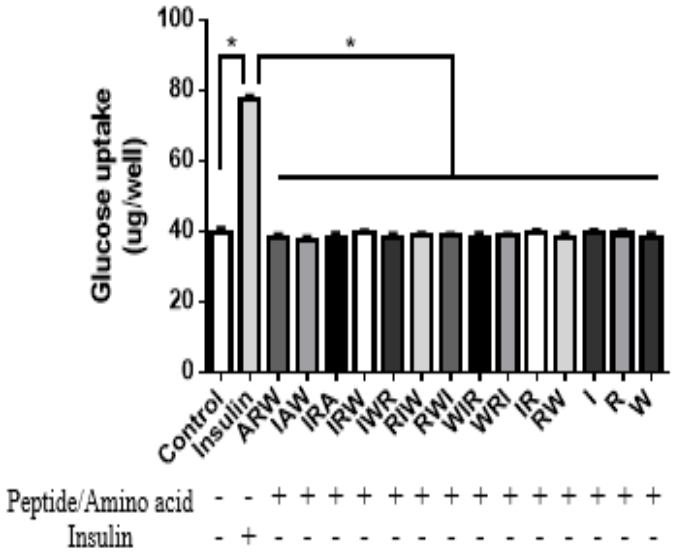

Figure 3.2 Effects of IRW analogs on glucose uptake in the absence of insulin.

The differentiated myotubes was incubated in Krebs-Henseleit-Hepes buffer (KHH buffer) without glucose for 2 h. Then, they were incubated in KHH buffer containing 11 mm glucose without insulin and 100 μM of IRW analogs or analogs for 4 h, and then the glucose uptake was measured using a Glucose Test Kit. Each value represents the mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group.

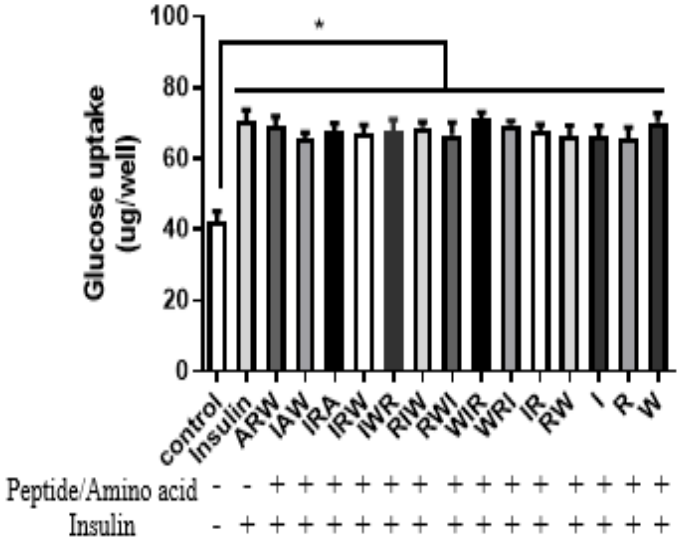

Figure 3.3 Effects of IRW analogs on glucose uptake in the presence of insulin.

The differentiated myotubes was incubated in Krebs-Henseleit-Hepes buffer (KHH buffer) without glucose for 2 h. Then, they were incubated in KHH buffer containing 11 mm glucose with 1 μM of insulin and 100 μM of IRW analogs or analogs for 4 h, and then the glucose uptake was measured using a Glucose Test Kit. Each value represents the mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group.

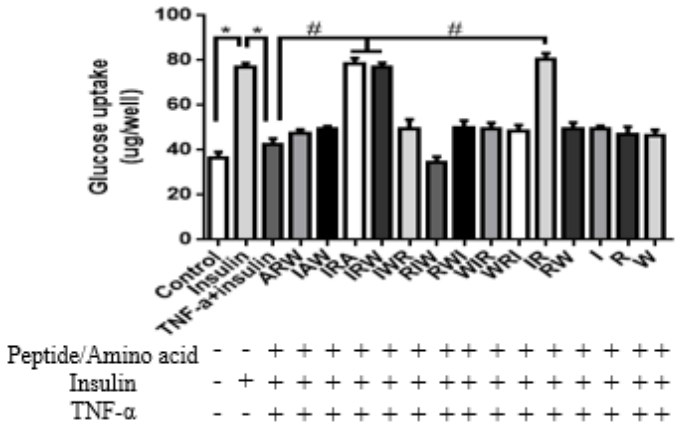

Figure 3.4 Effect of IRW analogs and on insulin stimulated glucose uptake in TNF-α treated L6 myotubes.

The differentiated myotubes were incubated with 100 μM of IRW analogs or analogs 2 h prior to the treatment of 5 ng/mL TNF-α for 24 h. Next, the myotubes were kept for 2 h in KHH buffer. The myotubes were then cultured in KHH buffer containing 11 mM glucose in the presence of 1 μM of insulin for another 4 h, and then the glucose uptake was measured using a Glucose Test Kit. Each value represents the mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group. #Indicates $p < 0.05$ as compared to the TNF-α treated group.

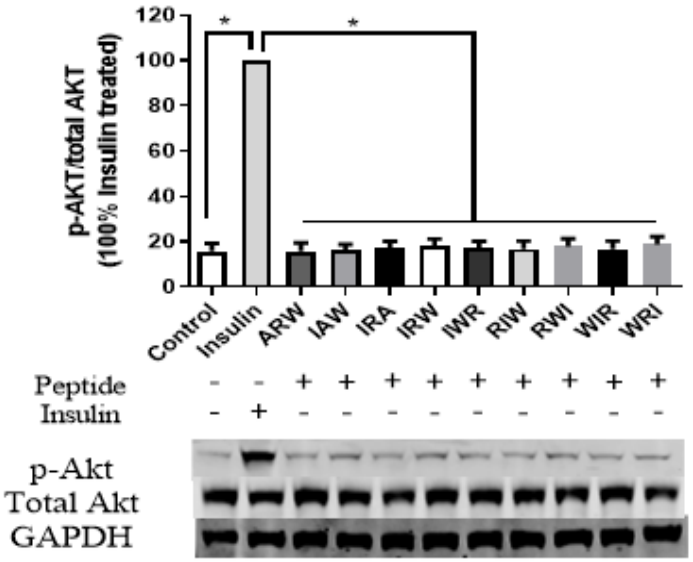

Figure 3.5 Effect of IRW analogs (tripeptides) on insulin signaling in the absence of insulin The myotubes were treated with 100 μM of IRW analogs without insulin for 30 min. The cells were lysed, and western blotting of the lysates was performed with antibodies against p-Akt, total Akt, and GAPDH (loading control). A set of representative images is shown. Data are presented as mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group.

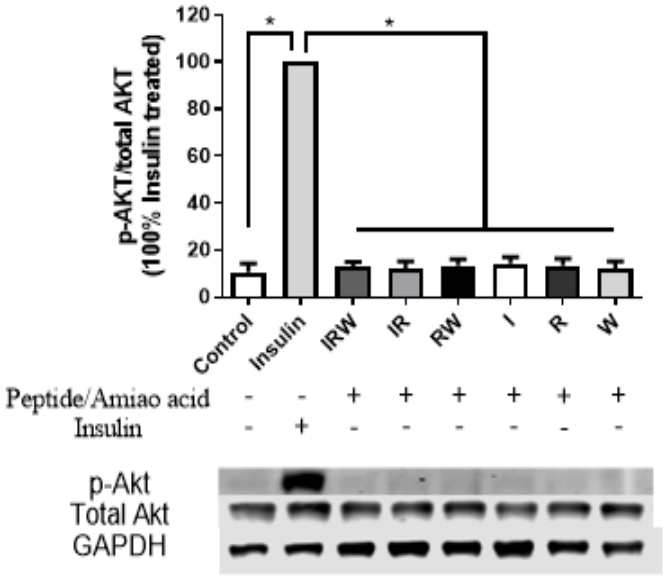

Figure 3.6 Effect of IRW analogs (dipeptides and amino acids) on insulin signaling in the absence of insulin

The myotubes were treated with 100 μM of IRW analogs without insulin for 30 min. The cells were lysed, and western blotting of the lysates was performed with antibodies against p-Akt, total Akt, and GAPDH (loading control). A set of representative images is shown. Data are presented as mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group.

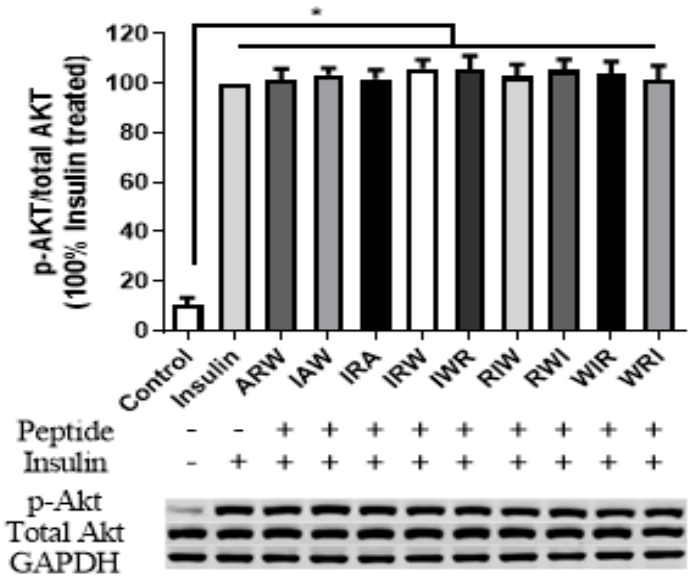

Figure 3.7 Effects of IRW analogs (tripeptides) on insulin signaling in the presence of insulin The myotubes were treated with 100 μM of IRW analogs with 1 μM of insulin for 30 min. The cells were lysed, and western blotting of the lysates was performed with antibodies against p-Akt, total Akt, and GAPDH (loading control). A set of representative images is shown. Data are presented as mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group.

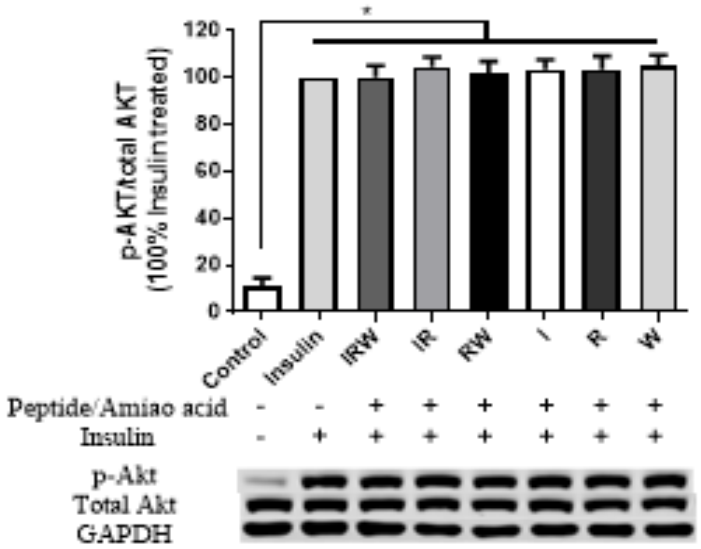

Figure 3.8 Effects of IRW analogs (dipeptides and amino acids) on insulin signaling in the presence of insulin

The myotubes were treated with 100 μM of IRW analogs with 1 μM of insulin for 30 min. The cells were lysed, and western blotting of the lysates was performed with antibodies against p-Akt, total Akt, and GAPDH (loading control). A set of representative images is shown. Data are presented as mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group.

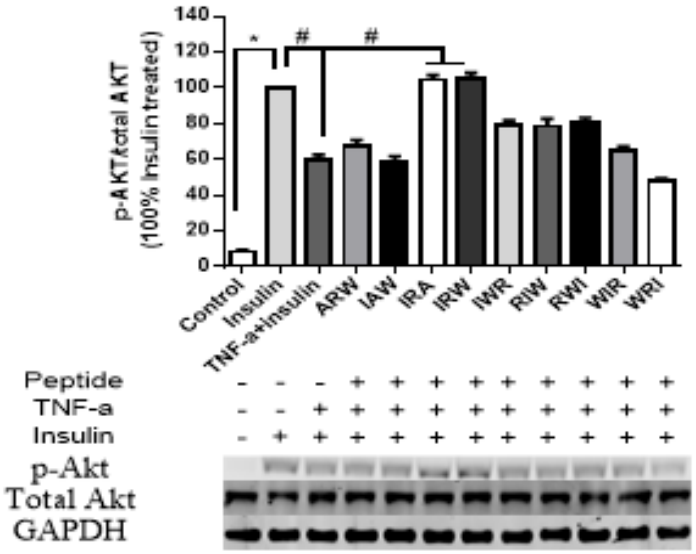

Figure 3.9 Effects of IRW analogs (tripeptides) on insulin signaling in TNF-α treated L6 myotubes.

The myotubes were treated with 100 μM of IRW analogs for 2 h followed by treatment with 5 ng/ml of TNF-α for 24 h. L6 myotubes were preincubated in KHH buffer for 2 h. They were then incubated in KHH buffer containing 11 mM glucose without or with 1 μM of insulin for 30 min. The cells were lysed, and western blotting of the lysates was performed with antibodies against p-Akt, total Akt and GAPDH (loading control). A set of representative images is shown. Data are presented as mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group. #Indicates $p < 0.05$ as compared to the TNF-α treated group.

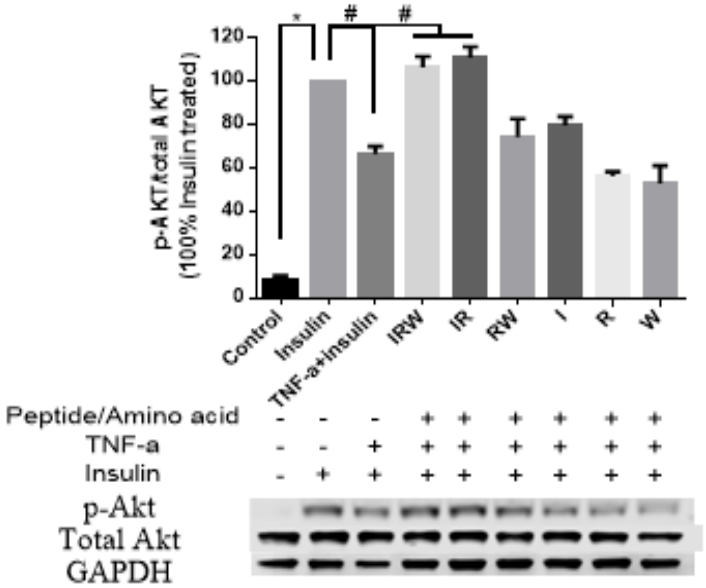

Figure 3.10 Effects of IRW analogs (dipeptides and amino acids) on insulin signaling in TNF-α treated L6 myotubes.

The myotubes were treated with 100 μM of IRW analogs for 2 h followed by treatment with 5 ng/ml of TNF-α for 24 h. L6 myotubes were preincubated in KHH buffer for 2 h. They were then incubated in KHH buffer containing 11 mM glucose without or with 1 μM of insulin for 30 min. The cells were lysed, and western blotting of the lysates was performed with antibodies against p-Akt, total Akt and GAPDH (loading control). A set of representative images is shown. Data are presented as mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group. #Indicates $p < 0.05$ as compared to the TNF-α treated group.

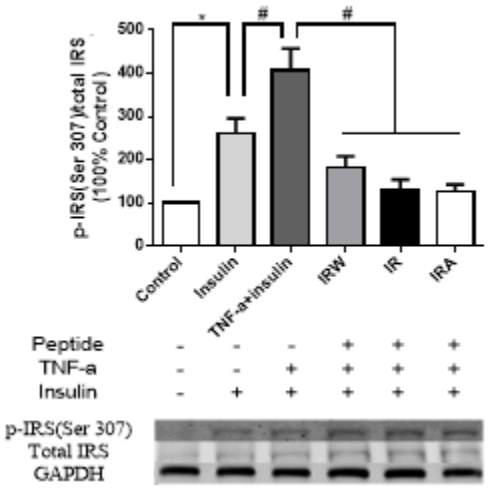

Figure 3.11 Effects of IRW/IR/IRA analogs on insulin signaling (p-IRS Ser 307) in TNF-α treated L6 myotubes.

The myotubes were treated with 100 μM of IRW/IR/IRA for 2 h followed by treatment with 5 ng/ml of TNF-α for 24 h. L6 myotubes were preincubated in KHH buffer for 2 h. They were then incubated in KHH buffer containing 11 mM glucose without or with 1 μM of insulin for 30 min. The cells were lysed, and western blotting of the lysates was performed with antibodies against p-IRS (Ser 307), total IRS, and GAPDH (loading control). A set of representative images is shown. Data are presented as mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group. #Indicates $p < 0.05$ as compared to the TNF-α treated group.

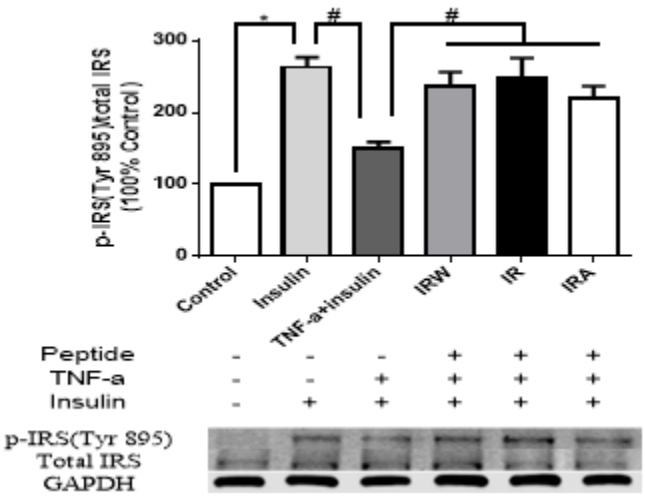

Figure 3.12 Effects of IRW/IR/IRA analogs on insulin signaling (p-IRS Tyr 895) in TNF-α treated L6 myotubes.

The myotubes were treated with 100 μM of IRW/IR/IRA for 2 h followed by treatment with 5 ng/ml of TNF-α for 24 h. L6 myotubes were preincubated in KHH buffer for 2 h. They were then incubated in KHH buffer containing 11 mM glucose without or with 1 μM of insulin for 30 min. The cells were lysed, and western blotting of the lysates was performed with antibodies against p-IRS (Tyr 895), total IRS, and GAPDH (loading control). A set of representative images is shown. Data are presented as mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group. #Indicates $p < 0.05$ as compared to the TNF-α treated group.

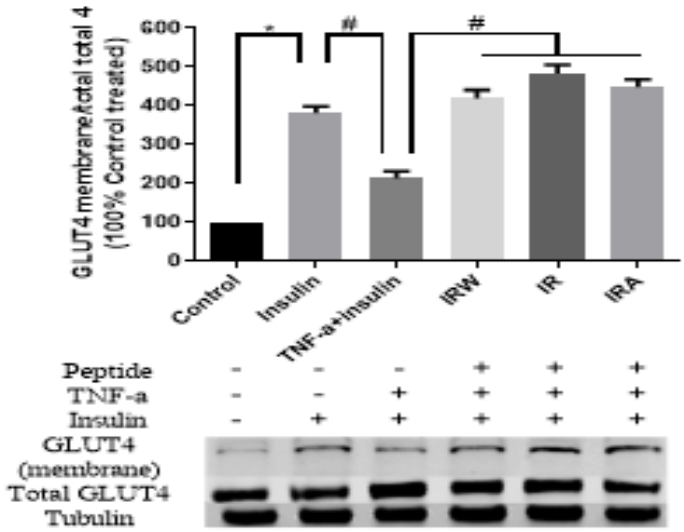

Figure 3.13 Effects of IRW, IR or IRA on GLUT4 translocation in TNF-α treated L6 myotubes. Membrane protein were extraction from the cells. The expression of GLUT4 was tested by western blot. Each value represents the mean ± SEM. *Indicates $p < 0.05$ as compared to the insulin group. #Indicates $p < 0.05$ as compared to the TNF-α treated group.

CHAPTER 4- TRIPEPTIDE IRW ANALOGS IRA AND IR IMPROVE GLUCOSE TOLERANCE IN HIGH-FAT DIET FED C57BL/6 MICE VIA ACTIVATION OF INSULIN DEPENDENT AND INDEPENDENT PATHWAYS IN SKELETAL MUSCLE

4.1 Introduction

Metabolic syndrome (MetS), a major public health challenge, is a cluster of impaired glucose metabolism, central obesity, elevated blood pressure and dyslipidemia. It is estimated that about 25% of the world's population has metabolic syndrome [1]. Metabolic syndrome was estimated to increases the risk of coronary heart disease, cerebrovascular disease by two-fold [2]. MetS involves the renin- angiotensin system (RAS), where angiotensin converting enzyme (ACE) plays a core role in the formation of angiotensin II (Ang II) from Ang I, while the rise of Ang II level leads to vasoconstriction through angiotensin II type 1 receptor (AT1R) [3]. Animal models with targeted inactivation of RAS genes exhibit improved insulin sensitivity and are protected from high fat diet induced obesity and insulin resistance. Also, there is evidence for RAS overactivation in obesity [4]. RAS overactivation is linked to obesity and insulin resistance [5]. Besides the systemic RAS, there is an independent local RAS in various tissues including skeletal muscle, influencing the tissue's response to insulin [6]. ACE inhibitors and AT1R blockers reduce IR in animal models and improve insulin sensitivity in humans [7-9]. In addition, RAS blockade reverses the detrimental effect of Ang II on skeletal muscle mitochondria and improves glycemic control in mice [10].

High-fat diet (HFD) is commonly used to induce insulin resistance [11]. Briefly, HFD feeding will lead to weight gain, hyperglycemia, and then hyperinsulinemia finally inducing insulin resistance [12]. When start the HFD feeding, the murine adipose tissue expands and stores the excess calories. During the chronic HFD feeding the adipose can not store the excess calories and the excess lipids are deposited into other organs [12]. After the HFD feeding, lipotoxicity occurs in multiple tissues: heart, skeletal muscle, liver pancreas, and kidneys. Then, after chronic HFD mice and individuals become metabolically inflexible, which is characterized by the decreased fat oxidation during fasting and a reduced ability to upregulate carbohydrate oxidation during feeding [13] [14]. Meanwhile, inflammation is involved in the HFD induced pathologies including the cardiovascular system [15, 16]. HFD is widely used to mimic the western diet, which is considered a major contributing factor to obesity and type 2 diabetes (T2D) in North America [17]. In addition to the HFD induced insulin resistance animal model, there are many animal models including high carbohydrate diet induced model, thermal/physical injury induced model as well as genetic modified models [18]. Although the HFD requires a long period of dietary treatment, it is cheaper

than genetic modified models and our research group have used HFD induced insulin resistance models successfully [19]. In this study, HFD induced insulin resistance model was used.

Food protein derived bioactive peptides have attracted extensive attention for their great potential in the management and prevention of metabolic syndrome [20, 21]. Aglycin, a 37-amino-acid polypeptide isolated from soybean, could enhance oral glucose tolerance and control hyperglycemia in streptozotocin (STZ)/high-fat diet induced type 2 diabetic mice at a dose of 50 mg/kg/day. Aglycin improved glucose uptake via upregulating phosphorylation of protein kinase B (Akt) and insulin receptor substrate 1(IRS-1), as well as translocation of glucose transporter protein 4 (GLUT4) to the cell membrane in type 2 diabetic mice [22]. In the tumor necrosis factor induced insulin resistance 3T3-F442A adipocytes cells, casein derived tripeptides VPP and IPP enhanced insulin signaling [23]. Treatment of L6 myotubes with dipeptide WH significantly increased glucose uptake via phosphorylation of 5' AMP-activated protein kinase-alpha (AMPK α), and translocation of GLUT4 to the plasma membrane [24]. Peptides derived from soy glycinin , IAVPGEVA, IAVPTGVA, and LPYP, have been reported to increase glucose uptake via activation of Akt and AMPK pathways in hepatic cells [25]. In a double-blind randomized placebo-controlled trial in Korean adults with prediabetes (fasting glucose ≥110 mg/dL), black soy peptide treatment improved glucose tolerance and reduced fasting glucose [26].

IRW (isoleucine-arginine-tryptophan) was initially identified as an inhibitor of ACE from egg white protein ovotransferrin [27]. In spontaneously hypertensive rats (SHRs) the mechanism of blood pressure reduction by IRW is via up-regulation of ACE2 [28]. In skeletal muscle L6 cells, Ang II or TNF-α treatment could increase the phosphorylation of insulin receptor substrate serine residue and decrease the phosphorylation of insulin receptor substrate tyrosine residue [29, 30]. While IRW could reverse the effect of Ang II or TNF-α and lead to GLUT4 translocate to the plasma membrane [29, 30]. IRW supplementation prevented body weight and fat mass gain, protected lean body mass loss, improved glucose tolerance, decreased fasting blood glucose and insulin levels, and enhanced insulin-dependent and -independent signaling governing glucose uptake in skeletal muscle of HFD fed mice [19]. In order to understand the structure and activity relationship of IRW, we investigated the effect of IRW analogs on TNF-α induced insulin resistant skeletal muscle L6 cell. IRA and IR showed similar effects to that of IRW: enhanced glucose

uptake, improved the impaired insulin signaling pathway and increased the GLUT4 translocation [31].

In this study, we aimed to compare the effects of IRA and IR with IRW on improving glucose tolerance and enhancing insulin-dependent and -independent signaling pathways using a HFD-induced insulin resistant C57BL/6 mouse model. Due to the crosstalk between obesity, hypertension and IR, we hypothesized that IRW analogs can improve glucose intolerance by inhibiting local RAS in skeletal muscle, increasing insulin signaling and enhancing GLUT4 translocation to the plasma membrane.

4.2 Materials and methods

4.2.1 Animals, diet, and body weight measurements

The animal experimental protocol was approved by the Animal Care and Use Committee of the University of Alberta (Protocol # 1472) in accordance with guidelines issued by the Canadian Council on Animal Care. Forty 4-week-old male C57BL/6 mice were purchased from Charles River Canada (St. Constant, QC, Canada) and housed 4 per cage with ad libitum access to standard chow and water for one week as acclimatization. The mice were in 12:12-hour cycle of light: dark with controlled humidity and temperature. Eight mice were fed with low fat diet (LFD, 10% kcal from fat, Envigo, Indianapolis, IN, USA, TD06415) and the remaining rest 32 mice with high fat diet (HFD, 45% kcal from fat, Envigo, Indianapolis, IN, USA, TD110675) for 6 weeks to induce obesity and glucose intolerance [32]. After this period, HFD fed mice were randomly assigned to 4 groups (n=8 per group): HFD control, IRW (45 mg/kg BW + HFD), IRA (34 mg/kg BW +HFD), IR (27.3 mg/kg BW + HFD). The peptides were mixed with small amount of powdered HFD first and then mixed with larger amount of HFD to make the diet homogeneous. To make sure the peptide is stable, the diet was prepared weekly. The dose of IRW was selected based on our previous study [19]. The dose of IRA and IR was calculated based on their different molecular weight compared to IRW. These diets continued for another 8 weeks with ad libitum access to food and water. IRW, IRA and IR were synthesized by Genscript (Piscataway, NJ, USA). Food consumption was measured three times and BW twice per week. Body composition was evaluated before and after peptides treatment using Echo MRI^{TM} (Echo Medical Systems LLC, Houston, TX, USA).

4.2.2 Oral glucose tolerance and insulin tolerance tests

After 6 weeks of LFD and HFD feeding, oral glucose tolerance test (OGTT) was performedto assess glucose tolerance. After 7 and 8 weeks of peptides treatment, insulin tolerance test (ITT) and OGTT were performed respectively as previously described [33] with the following modifications: for ITT, 1.5 IU/kg BW insulin was injected intraperitoneally; for OGTT, 40% glucose solution at a dose of 1 g glucose/kg BW was given via oral gavage. In both tests, blood glucose was measured before and after 15, 30, 60, 90, and 120 min of injection or gavage. Blood glucose was measured from the tail vein using a Contour® Next glucometer (Mississauga, ON, Canada). Fasting glucose and fasting insulin measurements in the tissue collection day were used to calculate homeostatic model assessment insulin resistance (HOMA-IR) using the formula [34]: [fasting glucose (mmol/L)] *[fasting insulin (μU/mL)]/22.5].

4.2.3 Tissue collection

At the end of the study, all mice were fasted for 12 h and half of them was injected with insulin (2 IU/kg BW) intraperitoneally to stimulate insulin signaling 10 min before euthanasia. Animals were euthanized using CO_2 and blood was collected via cardiac puncture. Blood was centrifuged at 3000 g for 15 min to obtain plasma, which was stored at -80 ^oC for further analysis. The soleus muscle was collected, snap frozen and stored at -80 ^oC.

4.2.4 Protein extraction and western blotting

Skeletal muscle total protein was extracted using a lysis buffer containing phosphatase and protease inhibitor cocktail (Cell Signaling Technology Inc, Danvers, MA, USA). Membrane protein was extracted as previously described using a commercial kit (Thermo Fisher Scientific, Waltham, MA, USA)[29]. Total protein content was measured using the bicinchoninic acid assay. Western blotting was performed as previously described [31] with the following modifications: protein was separated on a 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to a nitrocellulose membrane, which was incubated overnight with antibodies against AKT (Cell Signaling Technology (CST) Inc, Danvers, MA, USA), p-AKT (CST), p-IRS(Y895) (CST), PPARγ (CST), p-AMPKα (CST), AMPKα (CST), GAPDH (CST) IRS (Santa Cruz Biotechnology Inc, Dallas, TX, USA), p-IRS(S307) (Santa Cruz Biotechnology Inc, Dallas, TX, USA) GLUT4 (Abcam, Cambridge, MA, USA), ACE (Abcam), AT2R (Abcam), ACE2 (Proteintech Group Inc, Rosement, IL, USA), AT1R (Invitrogen, Ottawa, ON, Canada) and Mas receptor (Norus Biologicals, Toronto, ON, Canada). After incubating with appropriate fluorescent-conjugated secondary antibodies (Li-cor Biosciences, Lincoln, NE, United Stated) for

1 hour at room temperature, protein bands were quantified by densitometry using Image Studio Lite 5.2 (Li-Cor Biosciences).

4.2.5 Plasma RAS components and insulin

Plasma concentration of RAS components was quantified by mouse-specific commercial ELISA kits according to the manufacturers' instructions as follows: ACE (Aviva System, San Diego, CA, USA), ACE2 (Abcam), Angiotensin II (Enzo Life Science, Burlington, ON, Canada), Angiotensin (1-7) (Aviva System). Insulin was measured using an ELISA kit from Abcam (Cambridge, MA, USA).

4.2.6 Statistics

All data presented were expressed as mean ± SEM of 'n' mice from each treatment group as indicated in the figure and table legends. Data was checked for normal distribution by the Shapiro-Wilk test. Statistical analysis was performed using GraphPad Prism 7.0. Outliers indicated by the statistical software were removed before the data evaluation. Data were evaluated by one-way ANOVA or two-way ANOVA when appropriate as indicated in the figure legends. Post hoc analysis was done using Bonferroni's test. A value of $p < 0.05$ or 0.01 was considered as statistically significant, and $p < 0.001$ was considered as highly significant.

4.3 Results

4.3.1 Body composition and OGTT before peptides treatment

After 6 weeks of feeding, the absolute and relative fat mass (vs body weight) of HFD group were significantly higher than LFD group (Figure 4.1, 4.2). While the absolute lean mass of LFD group was higher (p=0.0567) than HFD but it's not significant (Figure 4.3), the relative lean mass was higher than HFD group (Figure 4.4). During the OGTT, the blood glucose of HFD group was higher than LFD, and significant difference was observed at times of 15 min and 30 min (Figure 4.5). The results indicated the model was induced successfully.

4.3.2 Body weight, food intake and water intake

At the beginning of the experiment, the body weight of all the groups was not different. At the end of experiment, the body weight of the HFD group was higher than all peptide groups (Figure 4.6). Body weight gain showed a similar trend as the body weight. The weight gain in the HFD group (23.5±0.8g) was greater than peptides group (IRW: 20.8±0.8g, IRA: 21.9±0.7g, IR: 19.7±1.2g).

The two-way ANOVA showed that the peptide treatment had a significant effect. IR, IRW and IRA were similar to LFD whereas HFD was consistently higher from 10-13 weeks (Figure 4.7). When weight gain was expressed as % of the first week of treatment, IRW and IRA groups had consistently lower weight gain from weeks 10-13 than the other groups (Figure 4.8); food intake (Kcal) and water intake in all the groups were not different (Figure 4.10, 4.11). LFD group had significant lower absolute fat mass and relative fat mass than all the other groups (Figure 4.12, 4.13); compared to the HFD, only IR group reduced the absolute fat mass (Figure 4.12). The absolute lean mass of all the groups were almost same (Figure 4.14). For relative lean mass, LFD and IR group were significantly high than HFD group (Figure 4.15).

4.3.3 ITT and OGTT after peptides treatment

For ITT, IRA and IR group had significantly lower 4 hours fasting blood glucose compared with HFD. After insulin injection, blood glucose of LFD group started getting lower, which is consistent with what we expected. However, after injecting insulin, the blood glucose of HFD went higher in 15 and 30 min. Maybe the HFD group was so resistant to insulin that delayed the effect of insulin. All peptide groups had a significantly lower blood glucose than HFD group (Figure 4.16). After 12 hours fasting, all peptide groups and LFD group had a significantly lower fasting blood glucose than HFD group. During OGTT, HFD group had a higher blood glucose than other groups. Peptide groups had a significantly lower circulating glucose at 15 min compared with HFD group (Figure 4.17). The area under the curve (AUC) analysis showed significant differences between all the peptide groups compared to the HFD group (Figure 4.18), while the significant difference disappeared in integrated AUC (Figure 4.19).

4.3.4 Plasma insulin and insulin signaling

After 8 weeks of peptide supplementation, all peptide groups and LFD had lower fasting plasma insulin than HFD while only LFD and IRW groups had significant difference when compared to HFD group (Figure 4.20). HOMA-IR was significantly lower in all peptide groups compared to HFD group (Figure 4.21). Insulin stimulated phosphorylation of AKT (Ser 473) in skeletal muscle was significant higher in peptides groups compared to the HFD group (Figure 4.22). For non-injected insulin group, phosphorylation of AKT was not detected (data was not shown). Peptides IRW and IRA significantly reversed the HFD-induced decrease of IRS phosphorylation (Y895) (Figure 4.23). LFD as well as peptides IRW and IR significantly decreased the phosphorylation of

IRS(S307) when compared with HFD group (Figure 4.24). Consistent with the result of phosphorylation of AKT, more GLUT4 translocated from cytosol to the plasma membrane in skeletal muscle, except for the IR group (Figure 4.25).

4.3.5 PPARγ and AMPKα abundance in soleus muscle from fasted mice

About ~ 4-fold increase in peroxisome proliferator- activated receptor γ (PPARγ) abundance was found in all peptide groups when compared to the HFD group (Figure 4.26). Except IR, peptides IRW and IRA increased phosphorylation of AMPKα, by ~7- and ~5-fold respectively, compared with the HFD group (Figure 4.27). For GLUT4 translocation from non-injected insulin mouse, more GLUT4 translocated to the plasma membrane to uptake glucose (Figure 4.28).

4.3.6 RAS components

In skeletal muscle, there were no significant difference in ACE2, AT1R among all the groups (Figure 4.29, 4.30). However, a significant lower ACE abundance was found in all peptide groups when compared with the HFD control (Figure 4.31). Also, angiotensin II type 2 receptor (AT2R) abundance was increased more than 2-fold in IRW group and IRA group compared to the HFD control ($P<0.01$) (Figure 4.32). IRW and IR increased the Mas receptor abundance while only the IR group showed a significant effect ($P<0.01$) (Figure 4.33).

The plasma ACE activity and Ang II level were not different among all the groups (Figure 4.34, 4.35). The peptide groups showed a trend of increased ACE2 activity, but only the IRW group was significantly higher than the HFD group (Figure 4.36). For plasma Ang (1-7), IRA group increased the concentration significantly compared to the HFD group (Figure 4.37).

4.4 Discussion

MetS including obesity, and type 2 diabetes (T2D) are a global health challenge. Diabetes prevalence is expected to rise up to 10.2% globally by 2030, affecting about 578.4 million adults [35]. Food- derived bioactive peptides exhibit beneficial effects against metabolic disease and its complications [20, 36]. In this study, we compared the effects of two peptide IRW analogs, IRA and IR using an obese, insulin resistant rodent model. Our results showed that IRW, and its analogs IRA and IR, improved fasting and decreased fasting insulin in HFD induced glucose intolerance mice. Consistent with our previously study [19], IRW reduced fasting blood glucose, fasting insulin, HOMA-IR and had a lower blood glucose during OGTT. IRA and IR group had similar

results with IRW group. Peptides treatment improved OGTT in this study. This appears to be mainly because of the effect on fasting blood glucose. However, this study can not rule out that the improved response was due to glucose stimulation. Future experiment using a hyperglycemic clamp would help us to answer this question. Our results suggest that peptides IRA and IR had comparable beneficial effect as that of IRW in managing glucose homeostasis and improving fasting insulin sensitivity, supporting the C-terminal residue W is not critical for the activity of IRW [31].

Same with our group previously result, IRW supplementation had a lower body weight and body weight gain than HFD group [19]. The possible reason is that peptide treatment increase energy expenditure, which we did not measure in this study. Interestingly, consistent with previously study [19], the body weight of peptide groups before the peptide treatment were lower than HFD group. For future animal experiment, it would be better to avoid confounder (body weight) in analyzing weight change and insulin sensitivity.

Skeletal muscle accounts for up to 75% of insulin-dependent glucose uptake [37], meanwhile insulin-independent signaling pathway also occurs in skeletal muscle. Insulin stimulates PI3K-AKT pathway leading to the translocation of GLUT4 from cytosol to the plasma membrane to uptake glucose. IRW, and its analog IRA, but not IR, increased the phosphorylation of AKT and IRS (Y895) compared to the HFD control. While only IRW and IR decreased the phosphorylation of IRS (S307). Furthermore, both IRW and IRA also significantly increased the AMPKα phosphorylation in skeletal muscle, suggesting the involvement of insulin-independent signaling pathway. However, as we do not have enough sample to extract the membrane protein, we only did a pilot study using one sample from injected and non-injected insulin group respectively to test the GLUT4 translocation. Even the data of GLUT4 translocation in injected insulin group is consistent with phosphorylation of AKT, more experiments are needed to draw a conclusion . These results suggested that IRA, but not IR, shared a similar mechanism of improved glucose metabolism via insulin-dependent and -independent glucose uptake. AMPK is one of the insulin-independent pathways involved in glucose uptake and glucose homeostasis [38]. Activation of AMPK via insulin-independent mechanism can lead to increases in GLUT4 translocate from intracellular pool to the plasma membrane [39, 40]. IRW and IRA may be a candidate in managing type 2 diabetes as AMPK has been identified as an important target in the prevention and treatment

of this disease. However, as the body weight of peptide groups were lower than HFD group, the improvements in insulin signaling cannot be attributed to a direct effect of IRW-related peptides on skeletal muscle because any intervention that results in weight loss can improve insulin signaling. This is a limitation of this study.

Insulin sensitivity has also been linked to RAS modulation. Peptides did not significantly lower systematic ACE activity in plasma. Plasma ACE2 activity was increased in all peptides group, consistent with our group previous study administered IRW to SHRs by oral enhanced circulating ACE2 activity and abundance [41]. Interestingly, in this study only IRA treated group increased the Ang (1-7) level. Even IRW was identified as an ACE inhibitory peptide *in vitro* [27], we found no effect of IRW on systemic ACE activity in SHR rats [41] and in insulin resistant model [19]. As IRA and IR has similar structural with IRW, the same trend was found in this study (Figure 4.34). Consistent with previous results that oral supplementation of IRW enhanced ACE2 circulating level, plasma ACE2 activity was significantly increased in IRW treated group. The ACE2/Ang-(1-7)/Max axis can enhance glucose tolerance and improve insulin sensitivity by protecting pancreatic β cells to increase insulin secretion, improving glucose metabolism in adipose tissue, and enhancing glucose uptake in skeletal muscle [42]. While all the peptides reduced ACE protein abundance in skeletal muscle, ACE2 level was not affected locally (Figure 4.29, 4.31).

Previous studies suggested that the improvement in insulin signaling is associated with reduced local RAS activity [27, 41, 43, 44]. In skeletal muscle, AT1R and AT2R play a role in regulate insulin action locally, systemic AT2R blockade impaired insulin-stimulated Akt phosphorylation, whole body glucose uptake, and muscular microvascular function, while systemic AT1R blockade restored muscle insulin signaling [6]. In skeletal muscle, AT2R is believed to cause the opposing effects to AT1R activation in blood vessels with interplay regulating blood flow and glucose utilization [45]. The improved glucose metabolism and insulin sensitivity in IRA and IRW treated mice was association with increased AT2R abundance in skeletal muscle (Figure 4.32). Increased AT2R abundance in skeletal muscle allows increased binding of Ang II to AT2R in the capillary endothelium, which improve blood flow to the tissue and facilitate the access of insulin to the muscle cells to uptake glucose [46].

PPARγ plays a central role in regular insulin-mediated signaling in muscle, maintaining in normal glucose disposal [47-49]. For mice with a PPARγ knock-out in the skeletal muscle, there was presence of insulin resistance in the whole body [50]. All peptide groups significantly increased PPARγ protein abundance (Figure 4.26), suggesting these three peptides treatment improved the HFD induced insulin resistance via PPARγ. PPARγ activation has insulin-sensitizing effect. PPARγ agonists potentiate phosphorylation of AKT in skeletal muscle [51].

In conclusion, our study showed both IRW analogs, IRA and IR, could improve glucose intolerance in HDF fed mice, supporting that the C-terminal of IRW is not essential for the activity of IRW. While both analogs could increase the phosphorylation of Akt, only IRA significantly increased the phosphorylation of AMPKα, suggesting a difference in the mechanisms of action by these two peptides. Our study also showed a reduced local RAS activity in insulin stimulated tissue, but only IR increased Mas level in skeletal muscle. In addition to the difference in the mechanisms of action, further study is needed to answer the question if any tripeptide IRX (X means any amino acids) has comparable activity as that of IRW.

4.5 References

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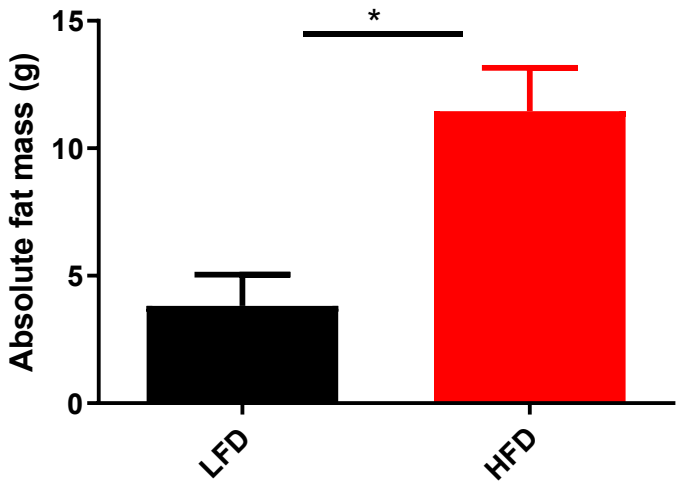

Figure 4.1 Absolute fat mass of LFD and HFD group before peptides treatment

Data is shown as the Mean±SEM for n=6 mice. The normally distributed data was analyzed by two-tailed t-test. * $P < 0.05$ between LFD and HFD. HFD: high fat diet; LFD: low fat diet.

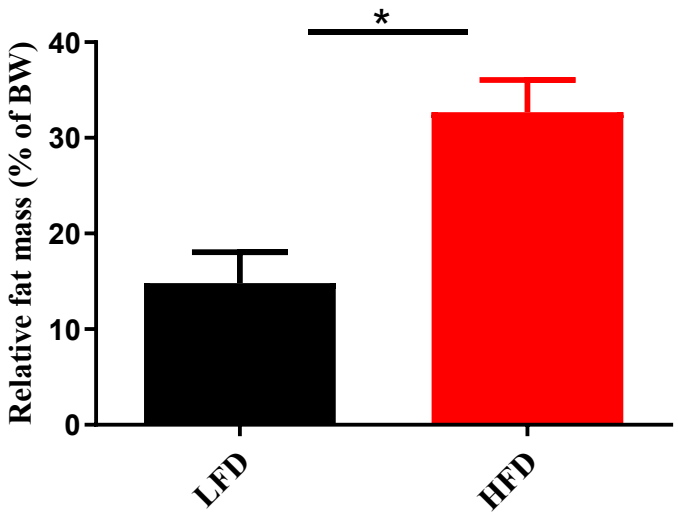

Figure 4.2 Relative fat mass of LFD and HFD group before peptides treatment

Data is shown as the Mean±SEM for n=6 mice. The normally distributed data was analyzed by two-tailed t-test. * $P < 0.05$ between LFD and HFD. HFD: high fat diet; LFD: low fat diet.

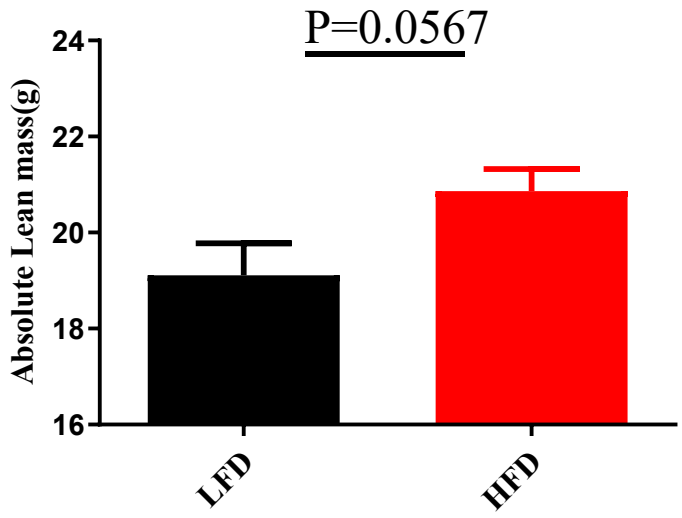

Figure 4.3 Absolute lean mass of LFD and HFD group before peptides treatment

Data is shown as the Mean±SEM for n=6 mice. The normally distributed data was analyzed by two-tailed t-test. HFD: high fat diet; LFD: low fat diet.

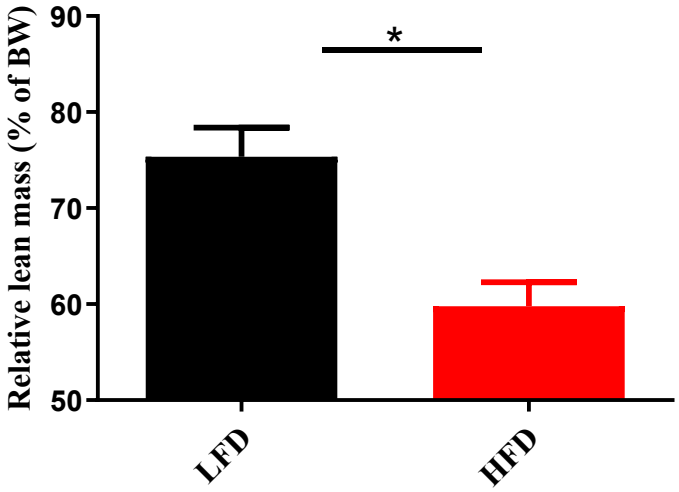

Figure 4.4 Relative lean mass of LFD and HFD group before peptides treatment

Data is shown as the Mean±SEM for n=6 mice. The normally distributed data was analyzed by two-tailed t-test. * $P < 0.05$ between LFD and HFD. HFD: high fat diet; LFD: low fat diet.

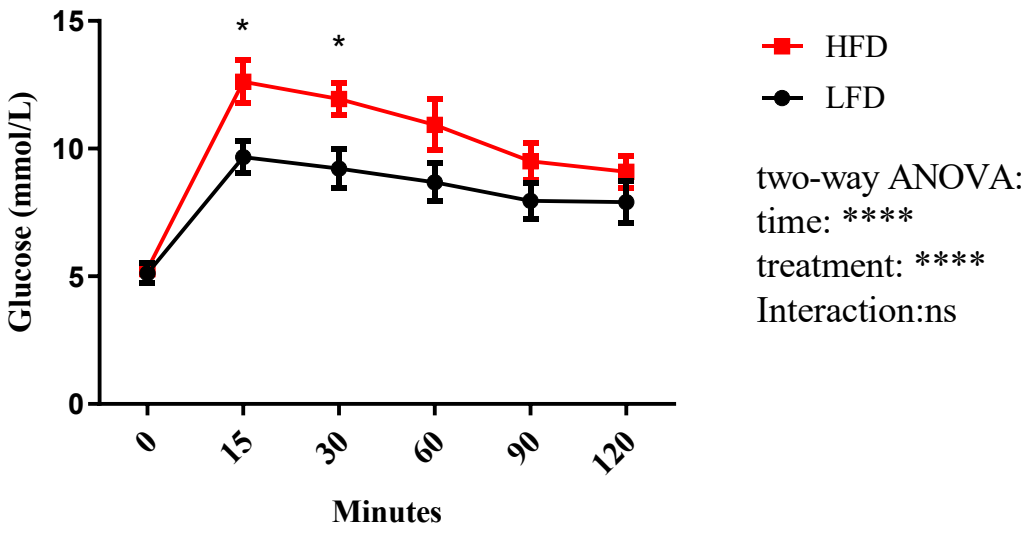

Figure 4.5 OGTT before peptides treatment

Oral glucose tolerance test was performed on mice after a 12 hour fast. Data is shown as the Mean±SEM for n=6 mice. The normally distributed data was analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$ between LFD and HFD. HFD: high fat diet; LFD: low fat diet.

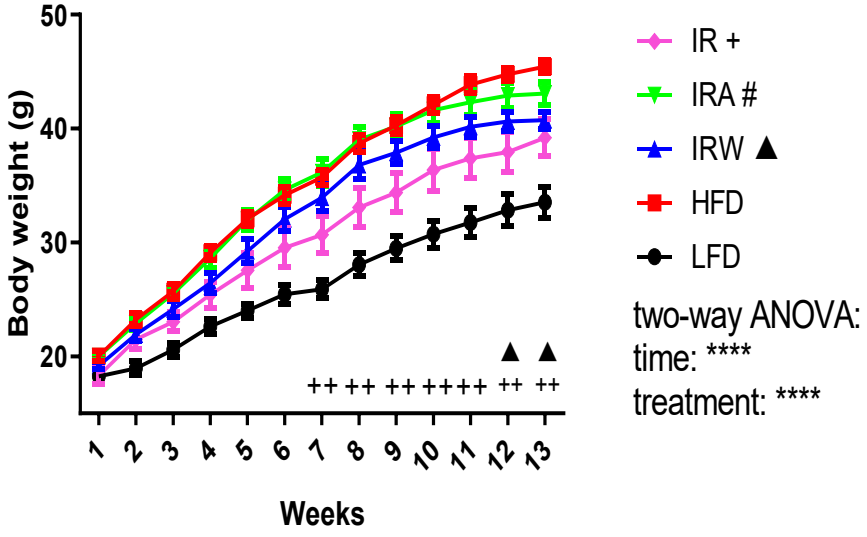

Figure 4.6 Effect of peptides feeding on body weight in mice

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. ▲$P < 0.05$ between IRW and HFD. + $P < 0.05$, ++ $P < 0.01$ between IR and HFD. HFD: high fat diet; LFD: low fat diet.

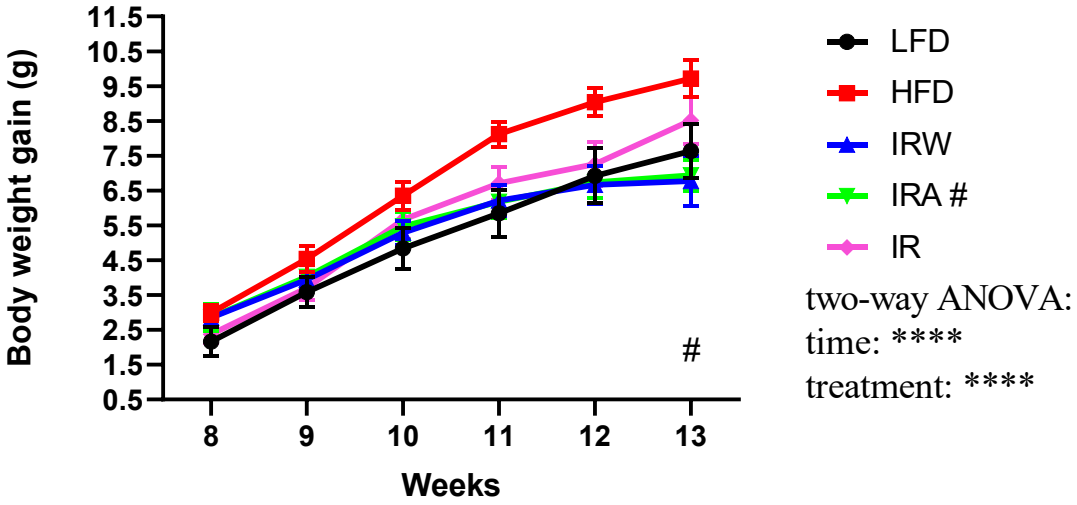

Figure 4.7 Effect of peptides feeding on body weight change (g) in mice since the peptide treatment started

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. #$P < 0.05$ between IRA and HFD. HFD: high fat diet; LFD: low fat diet.

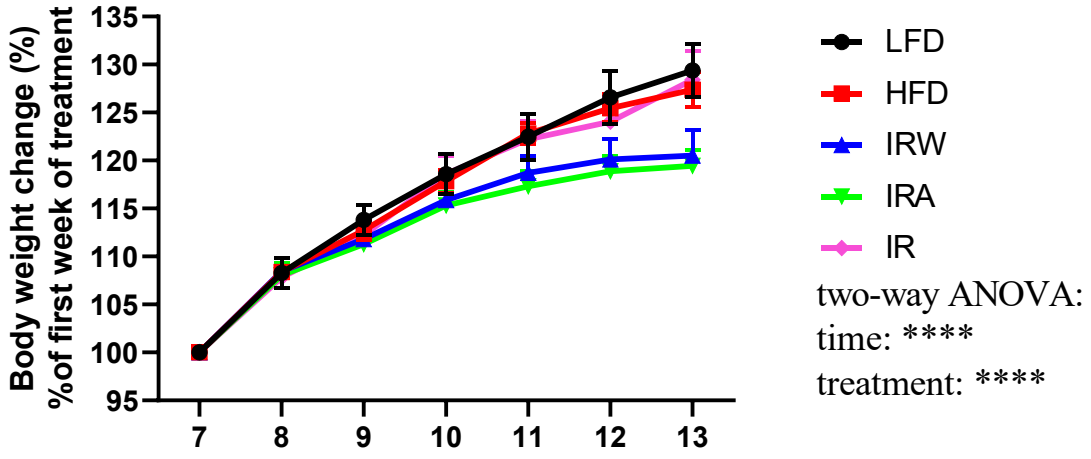

Figure 4.8 Effect of peptides feeding on body weight change (%) in mice since the peptide treatment started

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. HFD: high fat diet; LFD: low fat diet.

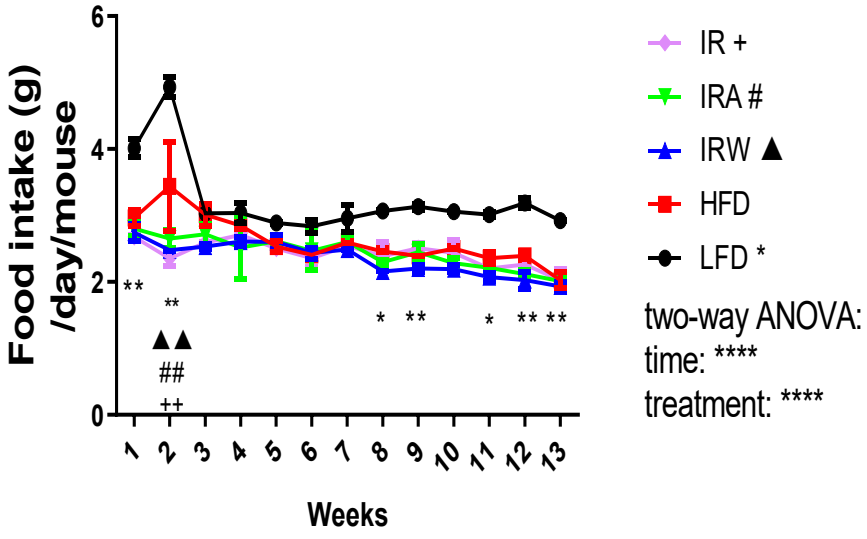

Figure 4.9 Effect of peptides feeding on food intake(g) in mice

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$, ** $P < 0.01$bewteen LFD and HFD. ▲▲$P < 0.01$ between IRW and HFD. ## $P < 0.01$ between IRA and HFD. ++ $P < 0.01$ between IR and HFD. HFD: high fat diet; LFD: low fat diet.

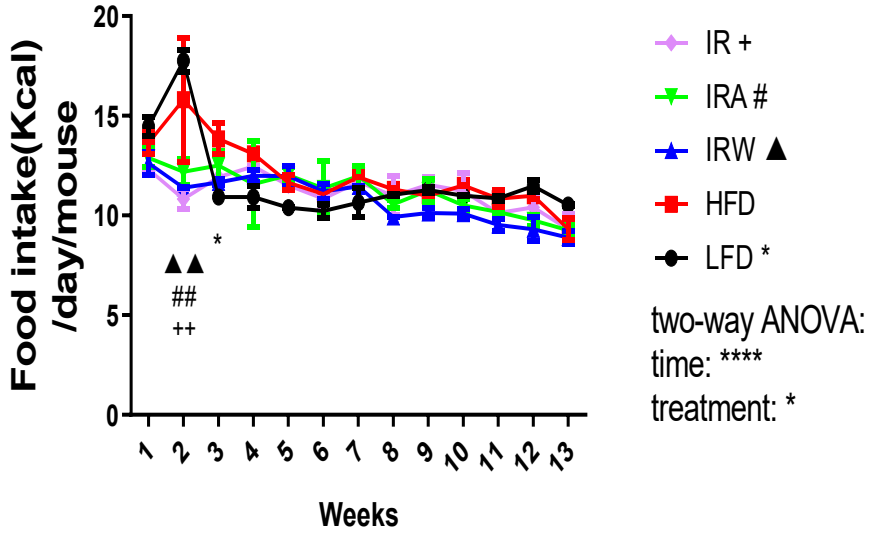

Figure 4.10 Effect of peptides feeding on food intake (Kcal) in mice

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$ between LFD and HFD. ▲▲$P < 0.01$ between IRW and HFD. ## $P < 0.01$ between IRA and HFD. ++ $P < 0.01$ between IR and HFD. HFD: high fat diet; LFD: low fat diet.

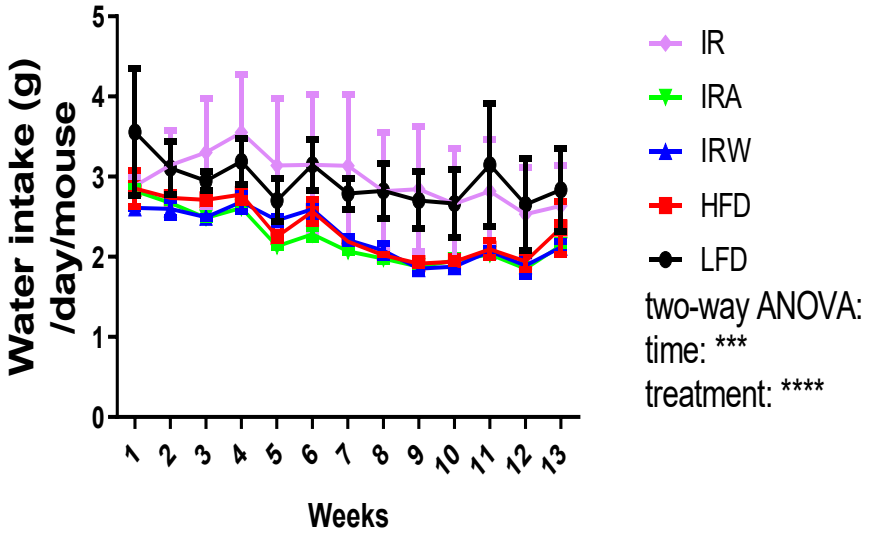

Figure 4.11 Effect of peptides feeding on water intake (g) in mice

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. HFD: high fat diet; LFD: low fat diet.

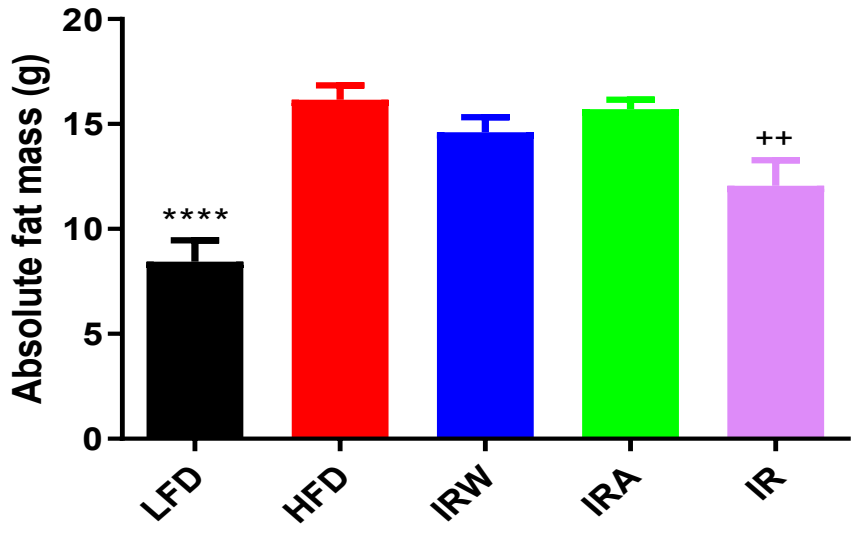

Figure 4.12 Effect of peptides feeding on absolute fat mass

Data is shown as the Mean±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA. **** $P < 0.0001$ between LFD and HFD. ++ $P < 0.01$ between IR and HFD. HFD: high fat diet; LFD: low fat diet.

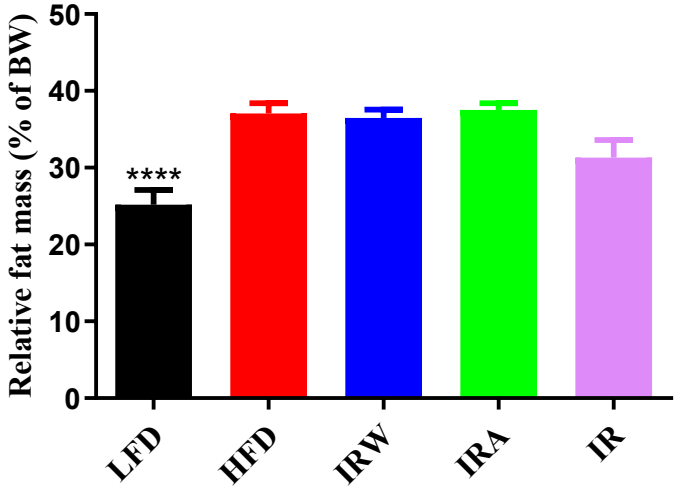

Figure 4.13 Effect of peptides feeding on relative fat mass

Data is shown as the Mean±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA. **** $P < 0.0001$ between LFD and HFD. HFD: high fat diet; LFD: low fat diet.

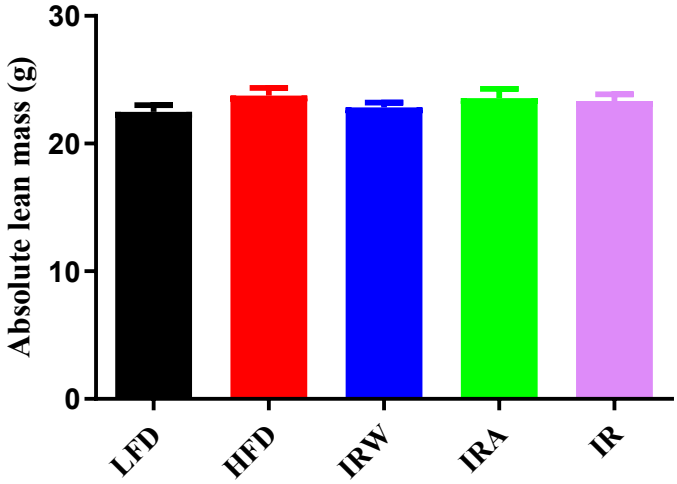

Figure 4.14 Effect of peptides feeding on absolute lean mass

Data is shown as the Mean±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA. HFD: high fat diet; LFD: low fat diet.

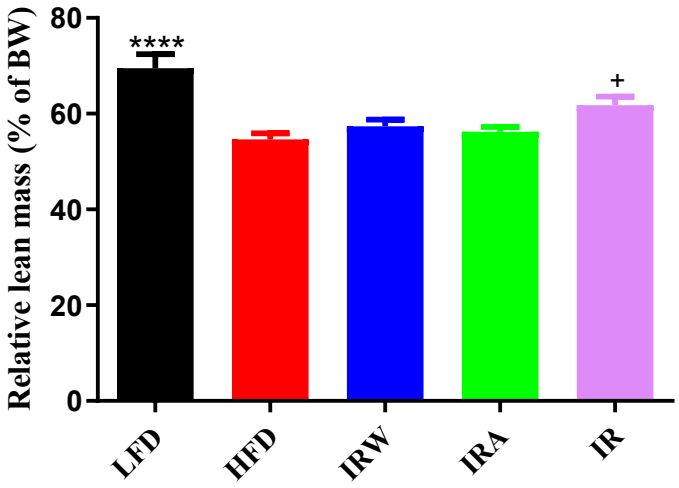

Figure 4.15 Effect of peptides feeding on relative lean mass

Data is shown as the Mean±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA. **** $P < 0.0001$ between LFD and HFD. HFD: high fat diet; LFD: low fat diet.

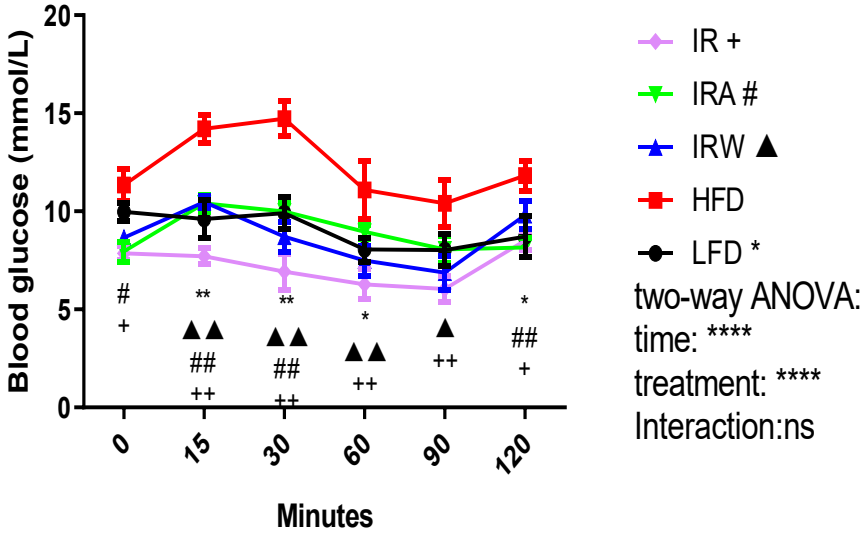

Figure 4.16 Effect of peptides treatment on ITT

Insulin tolerance test was performed on mice after a 4h fast. Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$, ** $P < 0.01$ between LFD and HFD. ▲$P < 0.05$, ▲▲$P < 0.01$ between IRW and HFD. ## $P < 0.01$ between IRA and HFD. + $P < 0.05$, ++ $P < 0.01$ between IR and HFD. HFD: high fat diet; LFD: low fat diet.

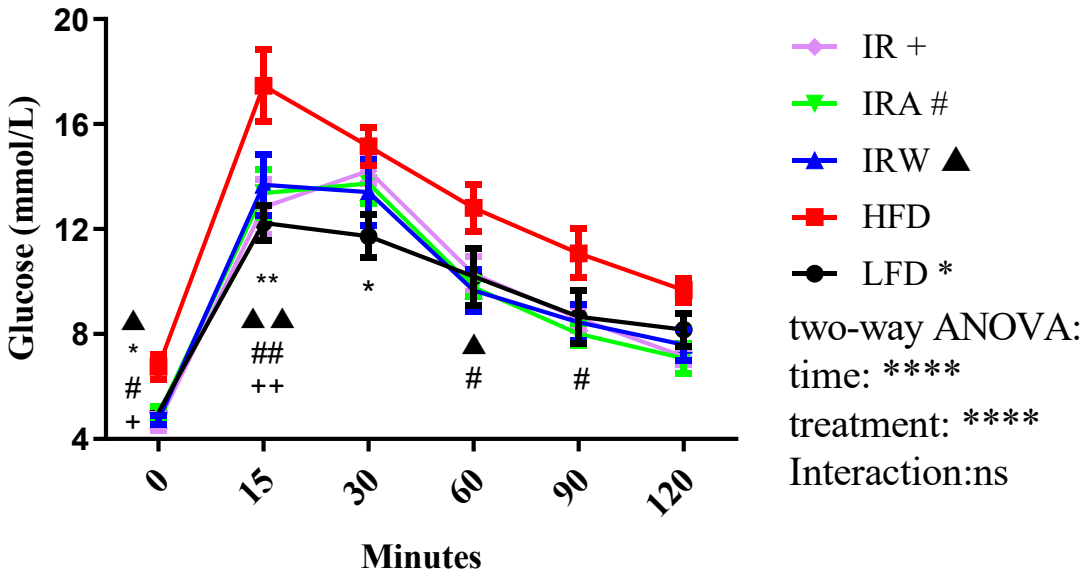

Figure 4.17 Effect of peptides treatment on OGTT

Oral glucose Insulin tolerance test was performed on mice after a 12h fast. Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by two-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$, ** $P < 0.01$ between LFD and HFD. ▲$P < 0.05$, ▲▲$P < 0.01$ between IRW and HFD. # $P < 0.05$, ## $P < 0.01$ between IRA and HFD. ++ $P < 0.01$ between IR and HFD. HFD: high fat diet; LFD: low fat diet.

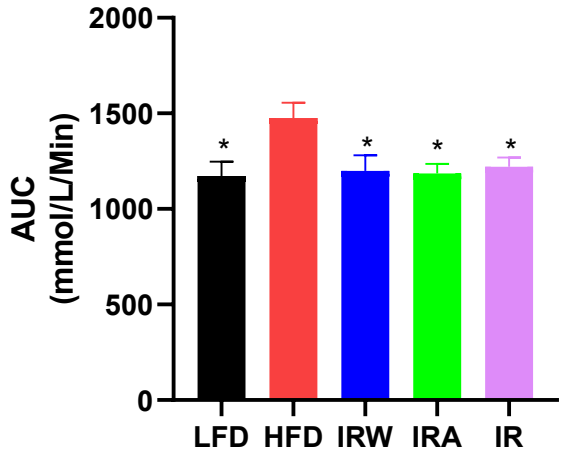

Figure 4.18 Effect of peptides treatment on AUC

Area under the curve for OGTT. Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

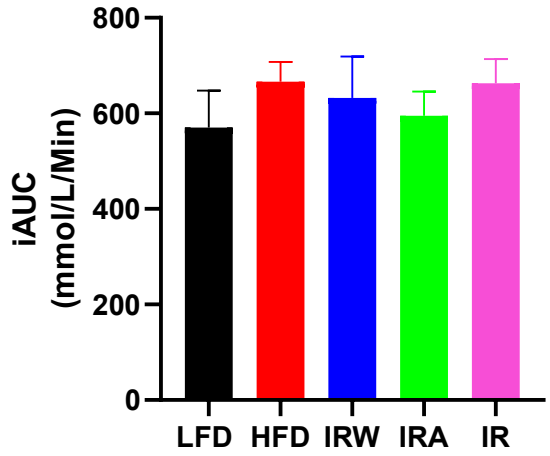

Figure 4.19 Effect of peptides treatment on iAUC

Integrated area under the curve (iAUC). Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. HFD: high fat diet; LFD: low fat diet.

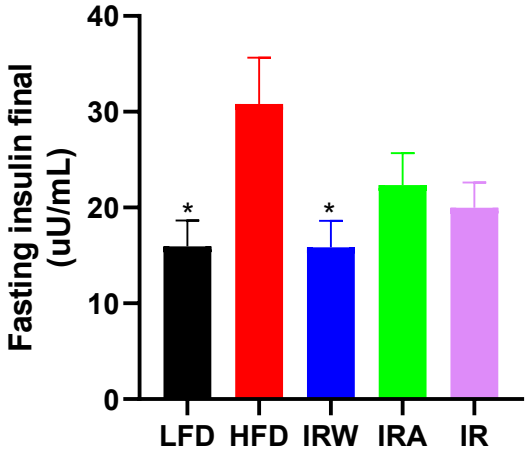

Figure 4.20 Effect of peptides treatment on plasma insulin levels

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

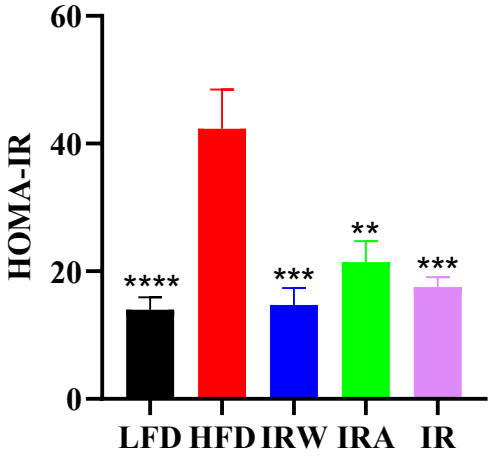

Figure 4.21 Effect of peptides treatment on HOMA-IR

HOMA-IR, homeostatic model assessment insulin resistance (fasting glucose(mmol/L) X fasting insulin (μU/L)/22.5. The fasting glucose from OGTT and fasting insulin from euthanasia (blood collected via cardiac puncture) was used to calculate the HOMA-IR. Data is expressed as mean ± SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

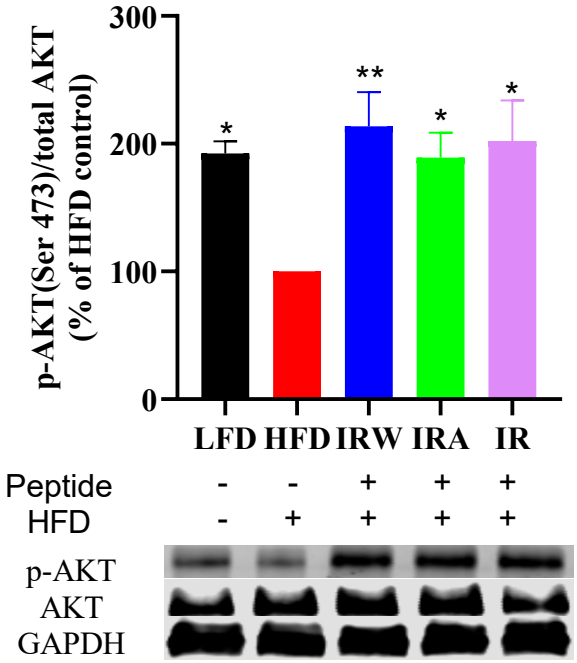

Figure 4.22 Effect of peptides treatment on skeletal muscle insulin signaling (p-Akt/Akt)

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$, ** $P < 0.01$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

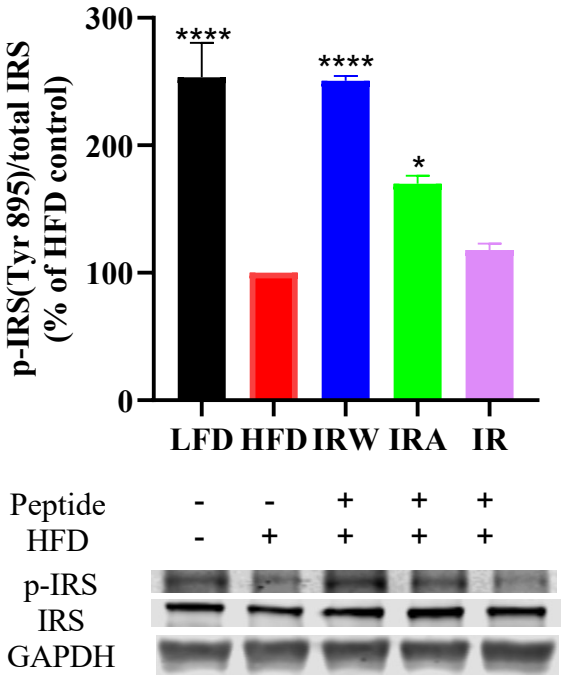

Figure 4.23 Effect of peptides treatment on skeletal muscle insulin signaling (p-IRS(Y895)/IRS)

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$, **** $P < 0.0001$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

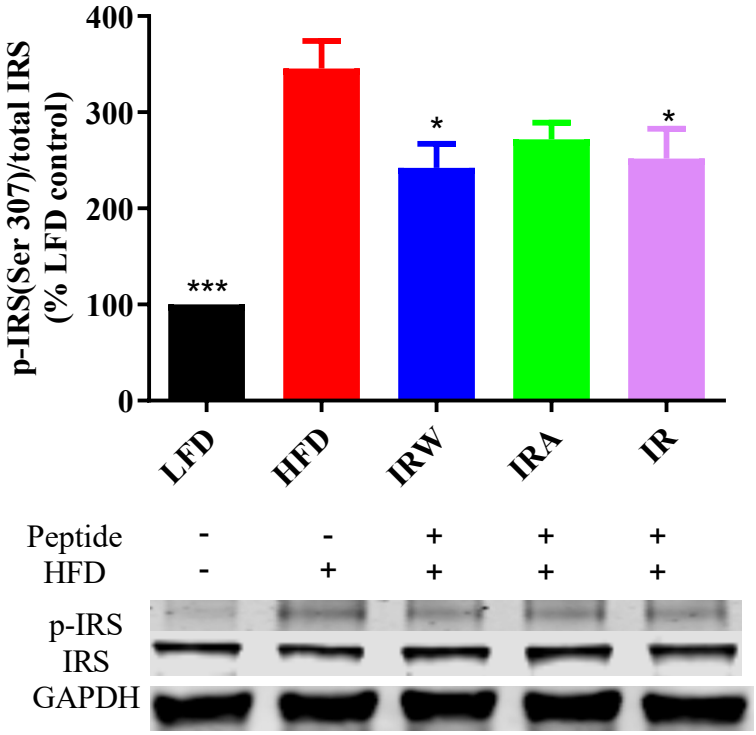

Figure 4.24 Effect of peptides treatment on skeletal muscle insulin signaling (p-IRS(S307)/IRS)

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.05$, *** $P < 0.001$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

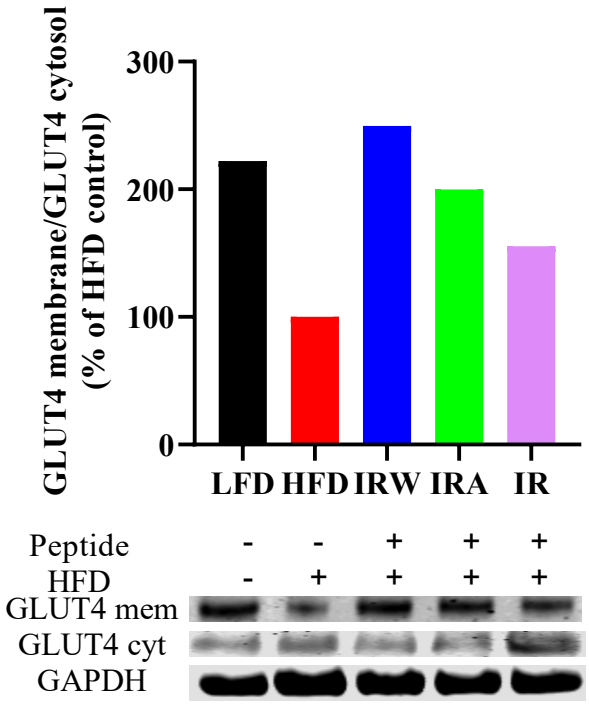

Figure 4.25 Effect of peptides treatment in injected insulin skeletal muscle insulin signaling (GLUT4)

Data is expressed as mean for one mouse. HFD: high fat diet; LFD: low fat diet.

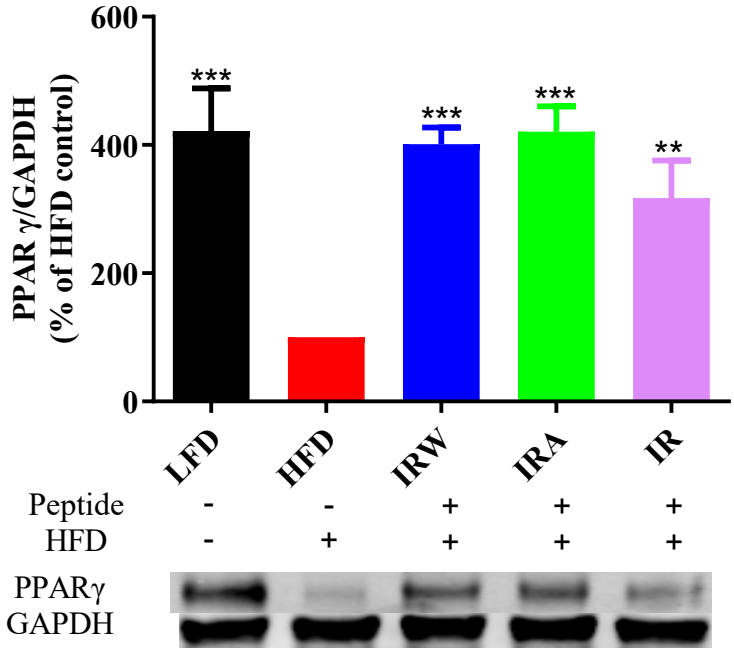

Figure 4.26 Effect of peptides treatment on skeletal muscle PPARγ abundance

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. ** $P < 0.01$, *** $P < 0.001$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

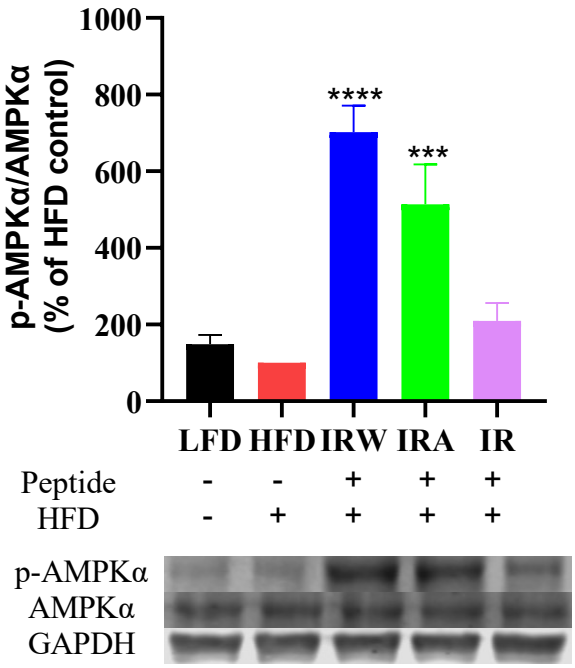

Figure 4.27 Effect of peptides treatment on skeletal muscle AMPKα abundance

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. *** $P < 0.001$, **** $P < 0.0001$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

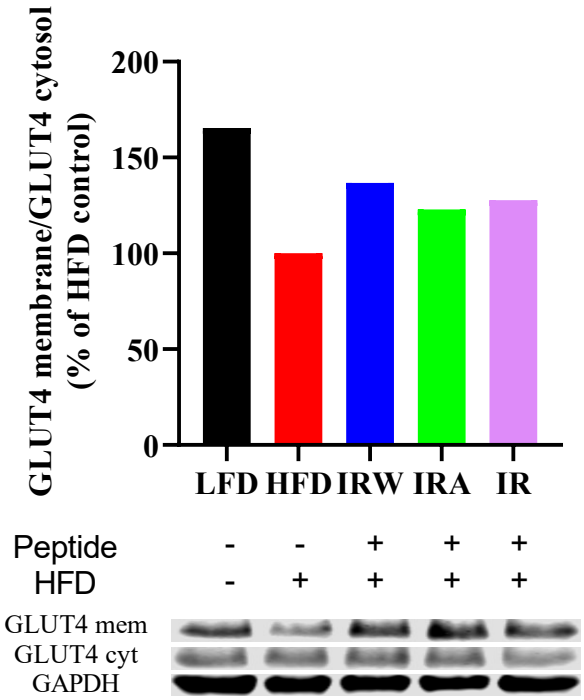

Figure 4.28 Effect of peptides treatment in non-injected insulin skeletal muscle insulin independent signaling (GLUT4)

Data is expressed as mean for one mouse. HFD: high fat diet; LFD: low fat diet.

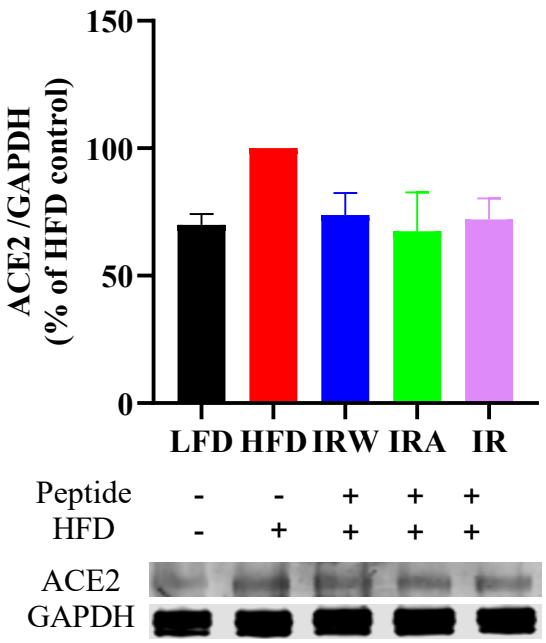

Figure 4.29 Effect of peptides treatment on skeletal muscle ACE2 abundance

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. HFD: high fat diet; LFD: low fat diet.

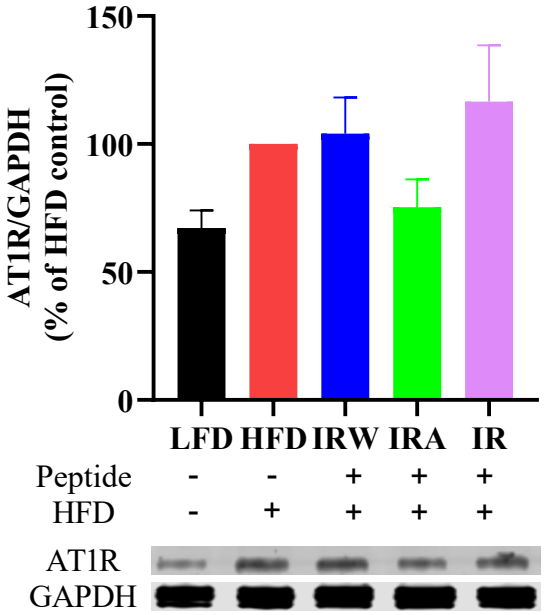

Figure 4.30 Effect of peptides treatment on skeletal muscle AT1R abundance

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. HFD: high fat diet; LFD: low fat diet.

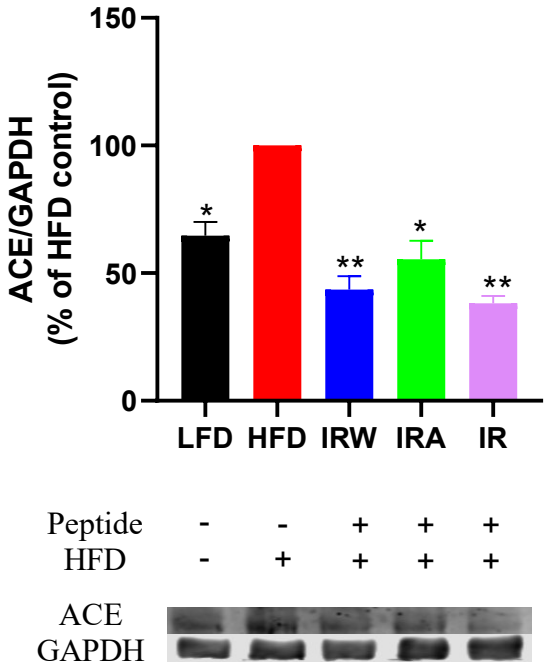

Figure 4.31 Effect of peptides treatment on skeletal muscle ACE abundance

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.5$, ** $P < 0.01$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

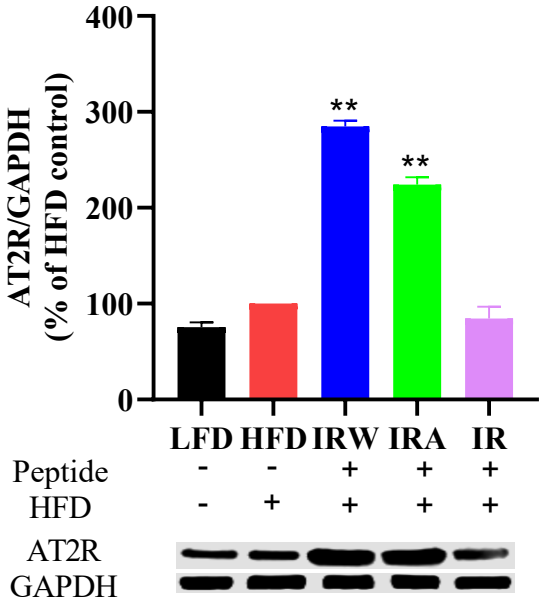

Figure 4.32 Effect of peptides treatment on skeletal muscle AT2R abundance

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. ** $P < 0.01$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

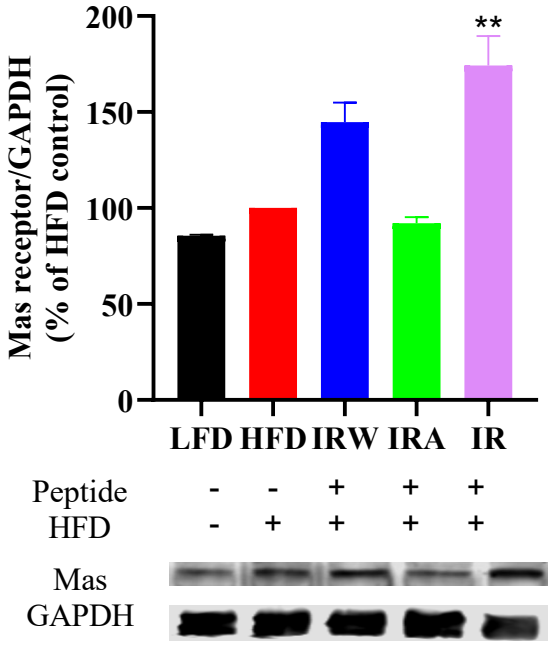

Figure 4.33 Effect of peptides treatment on skeletal muscle MasR abundance

Data is expressed as mean ±SEM for n=3 mice. Data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. ** $P < 0.01$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

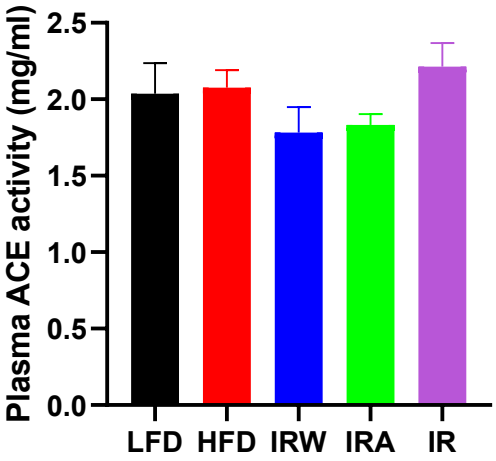

Figure 4.34 Effect of peptides treatment on plasma ACE activity

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. HFD: high fat diet; LFD: low fat diet.

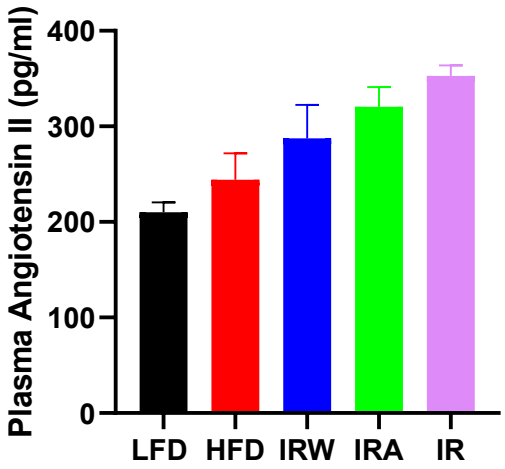

Figure 4.35 Effect of peptides treatment on plasma Angiotensin II activity

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. HFD: high fat diet; LFD: low fat diet.

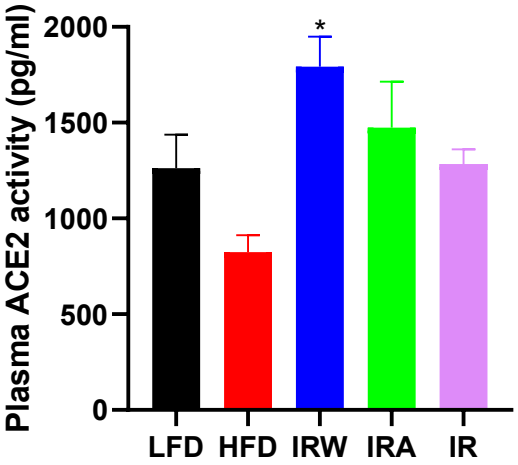

Figure 4.36 Effect of peptides treatment on plasma ACE2 activity

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.5$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

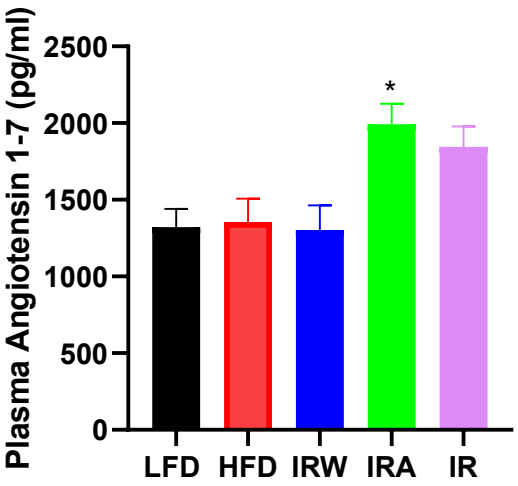

Figure 4.37 Effect of peptides treatment on plasma Angiotensin1-7 activity

Data is expressed as mean ±SEM for n=8 mice. The normally distributed data was analyzed by one-way ANOVA followed by Bonferroni's post-hoc comparison test. * $P < 0.5$ when compare between groups and HFD. HFD: high fat diet; LFD: low fat diet.

CHAPTER 5- THESIS SUMMARY AND DISCUSSION

5.1 Key findings of the present research

The overall objectives of this study were to investigate the activity of IRW analogs *in vivo* and *in vitro* to understand the importance of different amino acids in IRW. The key findings of each study are listed below:

5.1.1 IRA and IR showed the same effects as that of IRW in TNF-α treated L6 myotubes

Insulin resistance precedes the onset of metabolic syndrome. As the skeletal muscle is the major tissue for insulin stimulated glucose absorption, rat derived L6 skeletal muscle cell is widely used to study insulin resistance [1, 2]. In this study, effects of IRW analogs on improving TNF-α induced insulin resistance were studied to understand its structure and activity relationship. TNF-α treatment significantly decreased insulin stimulated glucose uptake, impaired insulin signaling pathway and GLUT4 translocation. However, adding IR, IRA and IRW 2 h prior to TNF-α treatment significantly reversed these outcomes. Pre-treatment with IR, IRA and IRW prevented the decrease of glucose uptake in TNF-α treated L6 myotubes. IRW, IR and IRA improved the decrease of phosphorylation of Akt caused by TNF-α. The phosphorylation of serine residue of IRS was decreased significantly when adding the IRW, IR and IRA. IRW, IR and IRA increased the phosphorylation of tyrosine residue of IRS when comparison with TNF-α group. The GLUT4 level in cell membrane was decreased by TNF-α. IRW, IR and IRA treatment significantly increased the level of membrane GLUT4. This means IRW, IR and IRA improved the GLUT4 translocation from cytosol to cell membrane. This study shows the significance of peptide IRW sequence, especially the constituent dipeptide IR. The C-terminal W is not essential to the activity of IRW. Both IRA and IR could improve glucose uptake and impaired insulin signaling pathway in TNF-α treated L6 cells to the same extent as that of IRW.

5.1.2 IRA and IR improve glucose tolerance in high fat diet fed C57BL/6 mice

Metabolic syndrome including obesity, and type 2 diabetes (T2D) are a global health challenge. Diabetes prevalence is expected to rise up to 10.2% globally by 2030, affecting about 578.4 million adults [3]. Skeletal muscle accounts for up to 75% of insulin-dependent glucose uptake [4], meanwhile insulin-independent signaling pathway also occurs in skeletal muscle. In this study, we compared the effects of two peptide IRW analogs, IRA and IR using an obese, insulin resistant rodent model. In skeletal muscle, IRA and IR increased the Akt phosphorylation, while only IR significantly decreased the phosphorylation of IRS(Ser307), IRA significantly increased the phosphorylation of IRS(Tyr895). Both peptides enhanced peroxisome proliferator-activated

receptor gamma (PPARγ) abundance and only IRA significantly increased the phosphorylation of 5' AMP-activated protein kinase (AMPK). IRW, and its analogs IRA and IR, improved fasting glucose decreased fasting insulin in HFD induced glucose intolerance mice. Our results suggest that peptides IRA and IR had comparable beneficial effect as that of IRW in managing glucose homeostasis and improved fasting insulin sensitivity, supporting the C-terminal residue W is not essential for the activity of IRW.

IRW and IRA are prone to trypsin degradation as the presence of Arg in the middle of them. IRW and IRA should be degraded into the dipeptide IR after gastrointestinal digestion [5]. There is high possibly that IRW and IRA retained biological activity *in vivo* because they were mixed into the HFD, which can protect them from digestive enzymes' degradation [6]. Absorption, distribution, metabolism and excretion properties of peptides can lead to low peptide bioavailability [7], which can cause different effect *in vitro* and *in vivo*. The different result of IRA and IR *in vitro* and *in vivo* may be due to their different bioavailability. This question should be answer in our group future experiment.

In conclusion, this thesis studied the structure and activity relationship of IRW to understand the importance of difference amino acid of IRW *in vitro* and *in vivo*.

5.2 Significance of this research

For the past decade, our research group found various activities of IRW. However, the structural requirements of IRW especially which amino acid of IRW is the most important is unknown. To our best knowledge, this study is the first to report the structure and activity relationship of IRW analogs for improved fasting insulin sensitivity. While IRA and IR showed a similar effect and the underlying mechanism as that of IRW in L6 cells. *In vivo*, the effect of IRA and IR were slight difference: IRA significantly increased the phosphorylation of IRS(Tyr895), while only IR increased Mas level in skeletal muscle. Both in vitro and in vivo results showed the C-terminal residue W is not essential for the activity of IRW. This thesis helps us to understand the structure and activity relationship of IRW.

5.3 Future research prospect

It should be noted that the thesis also has some limitations in its research work. The reason why we chose alanine as the single amino acid scanning substitution, because it represents a deletion of the side chain at the β-carbon [8]. However, there are at least 20 amino acids presenting naturally in proteins. According to the property of side chains in the α-position of amino acids, they are

classified into five groups: (1) basic amino acids; (2) acidic amino acids; (3) aromatic amino acids; (4) polar, uncharged amino acids; (5) nonpolar, aliphatic amino acids. It's better to choose one amino acid from each group to test the conclusion that C-terminal residue is not important for the activity of IRW. Interestingly, in this thesis and our group previously result [9], before the peptide treatment the body weight of IRW/peptide groups were lower than HFD group. As the body weight is a confounder of reduced body weight and insulin sensitivity, this limitation should be avoided when designed the experiment.

Based on the key findings and the limitations of this research work, the prospective of future studies are outlined below:

1. Both IRA and IR showed the same effect with IRW in cell model and similar effect with IRW in animal model. Whether IRA and IR can be identified from egg white hydrolysate or other food protein hydrolysate is a question that needs to be answer. Also, the targets of IRA and IR either as a receptor on the cell membrane or in the nucleus are not clear.
2. As the presence of Arg in the middle of IRW. IRW is prone to trypsin degradation. Our ongoing study is to look the ability of IRW to be absorbed and used in animal model. The ability of IRA and IR to be absorbed and used in animal model are also needed to be tested as the dipeptide and tripeptide may have different half-life and cell permeability [10].
3. Other IRW analogs that the W is replaced by one amino acid from each group are needed to test the importance of C-terminal of IRW.
4. In the future animal experiment, before peptide treatment make sure the peptide group and HFD control have the same body weight to avoid different body weight as a confounder.

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