Food Protein-Derived Peptides Targeting Angiotensin Converting Enzyme 2

By

Wang Liao

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ABSTRACT

Hypertension is a worldwide health challenge, afflicting about 25% of adults. Antihypertensive drugs are available, but prolonged use is associated with side-effects. Food protein-derived antihypertensive peptides have gained substantial interests during the past decades as a natural substitute to antihypertensive drugs. Given the central role of angiotensin converting enzyme (ACE) in generating angiotensin II (Ang II), a potent vasoconstrictor, most antihypertensive peptides have been characterized as ACE-inhibitory peptides by *in vitro* chemical assay. However, results from a mechanistic study indicated ACE-inhibitory peptides might have other targets in addition to ACE *in vivo*.

Notably, our previous study found that egg white ovotransferrin-derived ACE-inhibitory peptide IRW (Ile-Arg-Trp) could up-regulate mRNA level of angiotensin converting enzyme 2 (ACE2) in mesenteric artery of spontaneously hypertensive rats (SHRs). ACE2 is a homolog of ACE, but functions in an opposite way. Ang II could be cleaved by ACE2 into angiotensin (1-7) (Ang (1-7)) and protective effects could be further exerted via mas receptor (MasR). ACE2 has been considered as a novel target for cardiovascular therapy.

With the discovery of the potential of IRW in targeting ACE2, the specific objectives of this thesis were to 1) evaluate the ACE2-activating potential of IRW in different systems. 2) to test the beneficial effects of IRW-mediated ACE2-activation and up-regulation in VSMCs and SHRs. 3) to identify peptides which can up-regulate ACE2 from food protein sources.

IRW could increase activity of human recombinant ACE2 in an *in vitro* chemical assay. ACE2 activity and expressions in A7r5 cells, a VSMC cell-line, were increased by IRW treatment. Protein expressions of ACE2 in aorta and kidney of SHRs were also enhanced by IRW.

Beneficial effects of IRW on intracellular events of VSMCs and the relationship with ACE2 up-regulation were studied using Ang II-stimulated A7r5 cells. Results suggested that IRW treatment could inhibit aberrant proliferation and abrogate oxidative stress in Ang II-stimulated A7r5 cells. Expressions of cyclooxygenase 2 (COX2), inducible nitric oxide synthase (iNOS) and metalloproteinase 9 (MMP9) were down-regulated by IRW treatment in Ang II-stimulated A7r5 cells, indicating anti-inflammatory and anti-migrant activities of IRW in VSMCs. Signaling pathways including nuclear factor κB (NF- κB) and p38 mitogen-activated protein kinases (P38 MAPK) were involved in the modulatory effects of IRW in Ang II-stimulated A7r5 cells. Further study found that IRW treatment increased the levels of ACE2 and MasR in Ang II-stimulated cells. While, ACE2 knockdown and the addition of MasR antagonist abolished the antioxidant and anti-inflammatory activities of IRW, demonstrating beneficial effects from IRW-mediated ACE2 up-regulation at least in cellular level.

The contribution of ACE2-activation by IRW treatment to blood pressure reduction was evaluated in SHRs. Infusion of MasR antagonist A779 (48 μg/kg body weight/h) in SHRs abolished blood pressure-reducing effect of IRW, indicating the hypotensive effect of IRW in SHRs was via the ACE2/Ang (1-7)/MasR axis. Further analysis found that IRW oral administration (15 mg/kg body weight) increased circulating levels of ACE2 and Ang (1-7) as well as decreased circulating Ang II level. However, ACE level was unaffected. Infusion of MasR antagonist also affected the protein kinase B (Akt)/endothelial nitric oxide synthase (eNOS) signaling and expressions of inhibitory κB α (IκBα) and COX2 in aorta of SHRs, suggesting that enhanced endothelium-dependent vasorelaxation and reduced vascular inflammation were underlying the mechanisms of IRW as an ACE2 activator in SHRs.

Pea protein was used for identification of ACE2 up-regulating peptide after thermoase and pepsin digestion. The peptide was characterized based on activity-guided fractionation. A peptide with the sequence of AKSLSDRFSY was characterized by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Activity of AKSLSDRFSY in up-regulating ACE2 expression was validated in A7r5 cells. It was found that AKSLSDRFSY up-regulated ACE2 and MasR expressions, but down-regulated COX2 expression in Ang II-stimulated A7r5 cells. While, the addition of MasR antagonist could restore the expression of COX2, suggesting the AKSLSDRFSY-mediated ACE2/MasR up-regulations in A7r5 cells could further contribute to anti-inflammatory potential of this peptide.

Taken together, the present study demonstrated the ACE2-activating effect of egg white-derived tripeptide IRW. Besides, a peptide with the sequence of AKSLSDRFSY was identified from pea protein, which could increase the level of ACE2. Findings from this study advance the knowledge on molecular targets of food protein-derived antihypertensive peptides.

PREFACE

This thesis is an original work done by Wang Liao and has been written according to the guidelines provided by the Faculty of Graduate Studies and Research at the University of Alberta. The concept of the thesis originated from my supervisor Dr. Jianping Wu. Research grants of this thesis were funded by Alberta Livestock Meat Agency (ALMA) and Natural Sciences and Engineering Research Council (NSERC) of Canada to Dr. Wu. The experimental protocols for animal studies in Chapters 3 and 5 of this thesis were approved by the Animal Care and Use Committee at the University of Alberta Protocol # 611/09/10D and Protocol # AUP00001571, respectively, in accordance with the guidelines issued by the Canada Council on Animal Care and also adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

This thesis is consisted of seven chapters. Chapter 1 provided a general introduction on the research backgrounds, hypotheses and objectives of the thesis. Chapter 2 is the literature review. A revision of Chapter 2 will be submitted to *Critical Reviews in Food Science and Nutrition* as a review article. Chapter 3 has been published as "Egg white-derived tripeptide IRW (Ile-Arg-Trp) is an activator of angiotensin converting enzyme 2" in *Journal of Agricultural and Food Chemistry*. Chapter 4 has been published as "Modulatory effects of egg white ovotransferrin-derived tripeptide IRW (Ile-Arg-Trp) on vascular smooth muscle cells against angiotensin II stimulation" and "Egg white-derived antihypertensive peptide IRW (Ile-Arg-Trp) inhibits angiotensin II-stimulated migration of vascular smooth Muscle cells via angiotensin type I receptor" in *Journal of Agricultural and Food Chemistry*. Chapter 5 entitled as "Egg white-derived antihypertensive peptide IRW (Ile-Arg-Trp) reduces blood pressure in spontaneously hypertensive rats via the ACE2/Ang (1-7)/Mas receptor axis" will be submitted to *Molecular*

Nutrition and Food Research. Chapter 6 entitled as "Identification of angiotensin converting enzyme 2 up-regulating peptide from pea protein hydrolysate" will be submitted to *Food Chemistry* for consideration. The last Chapter 7 provides discussions and conclusion remarks on this thesis, as well as recommendations for future research.

Drs. Jianping Wu and Sandra T. Davidge contributed to experimental design, data interpretation and manuscript edits. Dr. Subhadeep Chakrabarti has assisted me in experimental design and provided technical support in cell culture experiments (Chapters 3 and 4). Mr. Khushwant S. Bhullar has assisted me in the molecular docking study (Chapter 3). Mrs. Sareh Panahi provided technical support in animal studies including animal surgery, animal care and isolation of mesenteric arteries. Mr. Hongbing Fan has assisted me in animal husbandry (Chapter 5) and liquid chromatography (Chapter 6). I was responsible for literature search required for the study, experimental designs, performing experiments, data collection and analysis, and drafting the manuscripts.

DEDICATION

This thesis is dedicated to my beloved family members.

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Abbreviation used:

ACE: Angiotensin converting enzyme

ACE2: Angiotensin converting enzyme 2

Angiotensin (1-7): Ang (1-7)

Angiotensin (1-9): Ang (1-9)

Ang I: Angiotensin I

Ang II: Angiotensin II

Akt: Protein kinase B

ApoE: Apolipoprotein E

AT1R: Angiotensin type I receptor

AT2R: Angiotensin type II receptor

COX2: Cyclooxygenase 2

DHE: Dihydroethidium

eNOS: endothelial nitric oxide synthase

ECE: Endothelin-converting enzyme

ECM: Extracellular matrix

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

ETs: Endothelins

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

HPLC: High-performance liquid chromatography

hrACE2: Human recombinant ACE2

IκBα: Inhibitory κBα

iNOS: Inducible nitric oxide synthase

ICAM-1: Intercellular adhesion molecule 1

IL-6: Interleukin 6

JNK: c-Jun N-terminal kinases

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

MasR: Mas receptor

MCh: Methacholine

mRNA: Messenger RNA

MMP9: Matrix metallopeptidase 9

MCP1: Monocyte chemoattractant protein 1

MAPK: Mitogen-activated protein kinases

NADPH: Nicotinamide adenine dinucleotide phosphate hydrogen

NF-κB: Nuclear factor κB

NO: Nitric oxide

PE: Phenylephrine

PI3K: Phophatidylinositol-3-kinase

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

RAS: Renin angiotensin system

ROS: Reactive oxygen species

SHR: Spontaneously hypertensive rat

TNF- α: Tumor Necrosis Factor-α

VCAM-1: Vascular cell adhesion molecule 1

VSMC: Vascular smooth muscle cell

XNT: Xanthenone

List of amino acids

A (Ala): Alanine

C (Cys): Cysteine

D (Asp): Aspartic acid

E (Glu): Glutamic acid

F (Phe): Phenylalanine

G (Gly): Glycine

H (His): Histidine

I (Ile): Isoleucine

K (Lys): Lysine

L (Leu): Leucine

M (Met): Methionine

N (Asn): Asparagine

P (Pro): Proline

Q (Glu): Glutamine

R (Arg): Arginine

S (Ser): Serine

T (Thr): Threonine

V (Val): Valine

W (Trp): Tryptophan

Y (Tyr): Tyrosine

CHAPTER 1 - General Introduction and Thesis Objective

1.1 Hypertension and the renin angiotensin system (RAS)

Hypertension is a global health challenge, affecting ~ 25% of adults worldwide (Perkovic, Huxley, Wu, Prabhakaran, & MacMahon, 2007) . The lowering cut-off, from 140/90 to 130/80 mmHg for the diagnosis of hypertension by the American College of Cardiology/American Heart Association on the prevention, detection, evaluation, and management of high blood pressure (BP) in adults, implies a significant higher prevalence than previously reported (Whelton et al., 2017). For example, this represents an additional 31 million individuals considered as hypertensive. Besides, ~ 53% of the current medicated hypertensive subjects are in need of improved treatment to meet the newly defined targets (Hoare, Kingwell, & Jennings, 2018). Hypertension is also known as a risk factor of cardiovascular diseases, which are the leading cause of mortality worldwide (Dahlöf, 2010).

There are two types of hypertension including essential hypertension and secondary hypertension. While, essential hypertension is the major type as it accounts for 95% of all hypertension cases, which is developed from an interaction of genetic and environmental factors (Carretero & Oparil, 2000). Despite the complex pathophysiology of essential hypertension, an altered RAS is considered as the major player (Beevers, Lip, & O'Brien, 2001). As a vasoconstrictor in the RAS, angiotensin II (Ang II) is mainly formed with the action of angiotensin converting enzyme (ACE) and functions via angiotensin type I receptor (AT1R); thus, ACE inhibitor and AT1R antagonist are the most common targets for treating essential hypertension. However, Ang II can be degraded by angiotensin converting enzyme 2 (ACE2), an analogous of ACE, into Ang (1-7), which was identified in 2000 (Donoghue et al., 2000). Ang (1-7) functions via mas receptor

(MasR), therefore counterbalancing effects against the ACE/Ang II/AT1R axis could be exerted (Santos et al., 2003). Various study have demonstrated beneficial roles of the ACE2/Ang(1-7)/MasR axis in cardiovascular functions and this axis has been considered as a novel target for cardiovascular therapy (Der Sarkissian, Huentelman, Stewart, Katovich, & Raizada, 2006).

1.2 Overview of antihypertensive peptides

Bioactive peptides are oligopeptides liberated from food proteins, which can exert physiological functions. Bioactive peptides have been implicated with potentials to prevent or treat chronic diseases, including hypertension (Li-Chan, 2015). Given the central role of ACE in producing Ang II, ACE is a main target to identify antihypertensive peptides. The first ACE-inhibitory peptide was identified from snake venom more than four decades ago (Ondetti et al., 1971), triggering extensive research on antihypertensive peptides. Various antihypertensive peptides have been identified, followed by demonstrations of their *in vivo* blood pressure-lowering effect (Udenigwe & Aluko, 2011). Mechanisms of antihypertensive peptides have also been explored, including modulations of expressions of the ACE/Ang II/AT1R axis, improvement of endothelial dysfunction and modulation of vascular remodelling (Majumder & Wu, 2015; Wu, Liao, & Udenigwe, 2017). However, interactions between antihypertensive peptides and the ACE2/Ang (1-7)/MasR axis is rarely reported.

It is noteworthy that our group previously found egg white ovotransferrin-derived antihypertensive peptide IRW (Ile-Arg-Trp) could increase mRNA level of ACE2 in mesenteric artery of spontaneously hypertensive rats (SHRs) upon 18-day oral administration (Majumder et al., 2015). This discovery raised the feasibility that targeting ACE2 might be a novel mechanism of antihypertensive peptides.

1.3 Hypotheses and objectives

IRW was found to increase the expression of ACE2 mRNA in SHR (Majumder et al., 2015), however, effects of IRW on ACE2 activity and protein level have not been studied. The contribution of ACE2 activation by IRW to reducing blood pressure in SHRs is unknown. In addition, it is unclear whether IRW is the only bioactive peptide which possesses the potential to target ACE2.

It was hypothesized that IRW can activate ACE2, which significantly contributed to its blood pressure-lowering effect in SHRs. Besides, peptides which can target ACE2 can be identified from food protein sources. The specific objectives of this study are given below:

1. Evaluate the effects of IRW on ACE2 activity and protein level in suitable cell-free system, cell culture model as well as animal model.
2. Investigate the role of IRW in modulating intracellular events in vascular smooth muscle cells (VSMCs) against Ang II stimulation and to understand the relationship between these modulatory effects and ACE2-activation by IRW.
3. Test the contribution of ACE2-activation by IRW to the blood pressure-lowering effect of IRW in suitable animal model and explore the possible molecular mechanisms involved in.
4. Identify ACE2 up-regulating peptides from food protein sources.

To achieve these specific objectives, a commercially available rat aortic VSMC cell line A7r5 was used for cell culture studies. SHR, a hypertensive rat model mimetic to human essential

hypertension (Trippodo & Frohlich, 1981), was used for *in vivo* studies. Brief descriptions of each chapter are as follows:

Chapter 2 provides a literature review covering topics relevant to this thesis including an introduction on the RAS, roles of the ACE2/Ang (1-7)/MasR axis in regulating cardiovascular functions. Molecular mechanisms of food protein-derived antihypertensive peptides were also discussed.

Chapter 3 investigated effects of IRW on the activity of human recombinant ACE2 (hrACE2), expressions and activity of ACE2 in A7r5 cells, as well as protein level of ACE2 in tissues of SHRs. In an *in vitro* assay using hrACE2, IRW directly activated ACE2 in a dose-dependent manner ranging from 10^{-7} M to 10^{-3} M; the EC_{50} value (the concentration to elicit 50% of the maximum response) of IRW activation on hrACE2 is 7.24×10^{-5} M. In A7r5 cells, IRW treatment (50 μM) significantly increased the mRNA level, protein expression and activity of ACE2. Following oral administration of IRW to SHRs, the ACE2 protein levels were significantly enhanced in both kidney and aorta. Thus, ACE2-enhancing property of IRW was demonstrated in different systems ranging from *in vitro* ACE2 to cellular ACE2 and *in vivo* ACE2.

Chapter 4 studied the modulatory effects of IRW in Ang II-stimulated intracellular events of VSMCs. Results indicated that treatment of IRW could mitigate aberrant proliferation, abolish superoxide generation, reduce inflammation and inhibit migration of Ang II-stimulated A7r5 cells. Nuclear factor κB and p38 mitogen-activated protein kinases (MAPK) signalling appeared to be involved in these actions of IRW. More interestingly, the antioxidant and anti-inflammatory effects of IRW in Ang II-stimulated A7r5 cells were exerted via the ACE2/Ang (1-7)/MasR axis. Hence, beneficial effects from IRW-mediated ACE2 up-regulation, including antioxidant and anti-inflammatory effects, were demonstrated at least at the cellular level.

Chapter 5 evaluated the contribution of ACE2-activation by IRW to reducing blood pressure in SHRs. MasR antagonist A779 (48 μg/kg body weight/h) was infused for 7 days prior to starting IRW oral administration (15 mg/kg body weight) to block the ACE2/Ang (1-7)/MasR axis. The co-treatment period of A779 and IRW was 7 days. Results indicated that blocking of MasR abolished blood pressure-reducing effect of IRW. Circulating ACE2 and Ang (1-7) were increased but Ang II was decreased by IRW treatment. However, circulating ACE level was unaffected by the peptide treatment. IRW-mediated up-regulation of protein kinase B (Akt)/endothelial nitric synthase signaling in aorta was deactivated by A779 infusion. IRW-reduced circulating interleukin 6 and monocyte chemoattractant protein 1, along with the down-regulation of cyclooxygenase 2 in aorta were resorted by A779 infusion. In conclusion, IRW reduced blood pressure in SHRs via the ACE2/Ang (1-7)/MasR axis. Mechanisms pertaining to IRW as an ACE2 activator *in vivo* included enhanced endothelium-dependent vasorelaxation and reduced vascular inflammation.

Chapter 6 tried to identify ACE2 up-regulating peptides from pea protein. Thermoase and pepsin were used to digest pea protein, the protein hydrolysate was purified based on activity-guided fractionation. A peptide with the sequence of AKSLSDRFSY was identified. ACE2 protein expression in A7r5 cells could be up-regulated by AKSLSDRFSY treatment. AKSLSDRFSY also up-regulated ACE2 and MasR expressions in Ang II-stimulated A7r5 cells, which could further contribute to the anti-inflammatory activity of this peptide. Results from this study showed pea protein was a promising commodity to identify ACE2 up-regulating peptides.

Findings from this thesis suggest the potential of food protein-derived antihypertensive peptides in targeting ACE2, which advances the knowledge on mechanisms of antihypertensive peptides and provides new target for identification of antihypertensive peptides from food proteins.

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CHAPTER 2 - Literature Review[1]

Hypertension is a global health pandemic, afflicting ~1/4 adults worldwide and costing billions of dollars in healthcare service (Perkovic, Huxley, Wu, Prabhakaran, & MacMahon, 2007). Antihypertensive drugs are available, but are associated with side-effects, in particular during prolonged use (Bardage, 2000; Messerli, 2002). Thus, there is a growing interest in developing food-derived, cost-effective alternative therapies for hypertension.

Food bioactives, either present naturally in the food or released during food processing, can beneficially affect human health beyond their nutritional value. Food protein-derived bioactive peptides are one of the representatives, with vast potential in treatment or prevention of various chronic diseases including hypertension (Cicero, Fogacci, & Colletti, 2016).

2.1 The renin angiotensin system (RAS)

2.1.1 Classical RAS and updated RAS

Hypertension is a risk factor for cardiovascular diseases (Chockalingam, Campbell, & George Fodor, 2006). In 2017, hypertension was redefined to be above 130/80 mmHg rather than 140/90 mmHg for systolic/diastolic blood pressure by the American Heart Association and the American College of Cardiology (Whelton et al., 2018), resulting in a higher prevalence. Essential hypertension, also known as primary hypertension, accounts for 95% of all hypertension cases around the world (Carretero & Oparil, 2000).

Although etiology of essential hypertension is not clearly understood as it develops from a complex interaction of genetic and environmental factors, an altered RAS is considered to play

[1] A revision of this chapter will be submitted to *Critical reviews in Food Science and Nutrition* as a review article.

the central role (Beevers, Lip, & O'Brien, 2001). In 1898, a heat-reliable substance in crude extracts of rabbit renal cortex was found to cause a sustainably increase in arterial pressure. The substance was then proposed as "renin" (Piepho & Beal, 2013). While, it was later found that the renin's pressor activity was not exerted directly in blood vessels, instead, it was through a proteolytic action on a substrate termed as angiotensinogen (Atlas, 2007). Afterwards, in 1954, two peptides were identified as angiotensin I (Ang I) and angiotensin II (Ang II) (Ferrario, 2006). It was further demonstrated that Ang I was cleaved by angiotensin converting enzyme (ACE) to form Ang II (Skeggs, Kahn, Lentz, & Shumway, 1957). Potent vasoconstricting effect of Ang II is mediated via angiotensin type I receptor (AT1R) (Hunyady, Balla, & Catt, 1996; Ito et al., 1995). On the contrary, another G protein-coupled receptor, namely angiotensin type II receptor (AT2R), is responsible for the counterbalancing effects against Ang II/AT1R axis (AbdAlla, Lother, Abdel-tawab, & Quitterer, 2001). Ang II plays an important role in various biological functions, particularly in blood pressure regulation. Increased level of circulating Ang II can cause vasoconstriction in blood vessels directly (Ferrario, 2006).

While, the RAS is also found in tissues, known as local RAS, mainly in kidney, heart, brain and adrenal gland (Bader & Ganten, 2008). Local RAS also plays a role in increasing blood pressure through regulations of physiological functions in the specific organs. In kidney, Ang II has an impact on sodium reabsorption which is through synergistic effect at renal vascular and tubular sites. Additionally, Ang II has a direct effect on tubular sodium transport by enhancing proximal tubule Na^+/H^+ exchanger. The net effect of these renal vascular and tubular actions of Ang II is to increase sodium retention, which then increases cardiac output and blood pressure (Carey & Siragy, 2003). The RAS in the sympathetic nervous system participates in the development and maintenance of hypertension as well. Activated sympathetic nervous system stimulates the

release of catecholamines (Tsuru, Tanimitsu, & Hirai, 2002), which further increases the activity of β-adrenoceptors, resulting in the conversion of pro-renin to renin and increased Ang II (Currie, Freel, Perry, & Dominiczak, 2012). In heart, Ang II stimulates the growth of fibroblasts and myocytes, which is thought to induce hypertrophy and fibrosis (Bader & Ganten, 2008) and eventually to regulate blood pressure by altering cardiac functions. Taken together, Ang II plays a central role in the RAS. Hence, ACE inhibitors and AT1R blockers are conventional therapies for hypertension based on the central principle of mitigating the effect of Ang II.

In 2000, our view on the RAS has been evolved with the identification of angiotensin converting enzyme 2 (ACE2), a homologue of ACE. ACE2 was initially identified from a human heart failure ventricle cDNA library (Donoghue et al., 2000), followed by study reporting its expressions in blood vessels (Sluimer et al., 2008; Zulli et al., 2006), kidney (Tikellis et al., 2003), liver (Paizis et al., 2005) and brain (Doobay et al., 2007). The ACE2 protein is encoded by the ACE2 gene located on chromosome Xp22. Like ACE, ACE2 is a type I membrane protein with the catalytic domain on the extracellular surface (Donoghue et al., 2000; Tipnis et al., 2000). ACE2 shares approximately 40% of identical sequence with ACE in the N-terminal catalytic domain but functions in an opposite way (Donoghue et al., 2000; Tipnis et al., 2000). In contrast to ACE, which is responsible for Ang II generation, ACE2 cleaves the carboxyl-terminal phenylalanine of Ang II to form angiotensin (1-7) (Ang (1-7)) (Vickers et al., 2002). Alternatively, ACE2 hydrolyzes Ang I to form angiotensin (1-9) which can be further converted by ACE to Ang (1-7) (Donoghue et al., 2000; Vickers et al., 2002). Nevertheless, the ACE2/Ang (1-7) pathway is 400-fold higher than the latter one (Vickers et al., 2002). Thus, cleaving Ang II into Ang (1-7) is postulated as the main action of ACE2 (Wysocki et al., 2010). The receptor mediating the effect of Ang (1-7) has also been characterized, which is known as mas receptor

(MasR) (Santos et al., 2003). ACE2/Ang (1-7)/MasR axis is considered as a counter-regulatory arm of the RAS, which can exert beneficial effects against ACE/Ang II/AT1R axis (Ferrario, Trask, & Jessup, 2005) (Figure 2.1). ACE2/Ang (1-7)/MasR axis has gained substantial attentions since the identification of ACE2, numerous studies reported the potential of this axis to modulate functions within or even beyond cardiovascular system, which will be introduced in detail in the next part.

2.1.2 Functions of the ACE2/Ang (1-7)/MasR axis

2.1.2.1 Decrease of blood pressure

The relationship between ACE2 and blood pressure was realized since the observation that mRNA and protein of ACE2 were remarkably reduced in spontaneously hypertensive rats (SHRs), as compared with the normative rats (Crackower et al., 2002). It was later reported that basal blood pressure in ACE2 knockout (ACE2 KO) mice was 10 mm Hg higher than in wild type-mice. Additionally, ACE2 KO mice was found to be more sensitive during Ang II infusion, as the pressor response was significantly enhanced in ACE2 KO mice compared with the wild-type (Allred, Donoghue, Acton, & Coffman, 2002). Numerous further studies were performed using various models (summarized in Table 2.1) to test the hypothesis that blood pressure could be modulated by ACE2 (Yagil & Yagil, 2003).

Efforts have also been made to identify the contribution of Ang (1-7) to reducing blood pressure. Documentation of the antihypertensive effect of Ang (1-7) could be traced back to 1995 (Benter, Ferrario, Morris, & Diz, 1995), even prior to the identification of ACE2. The antihypertensive effect of Ang (1-7) is mainly through enhancing the release of nitric oxide (NO) from endothelium (Ferrario, Chappell, Tallant, Brosnihan, & Diz, 1997; Li, Chappell, Ferrario, &

Brosnihan, 1997; Pörsti, Bara, Busse, & Hecker, 1994; Sampaio et al., 2007). It is also noteworthy that increase in Ang (1-7) was involved in the antihypertensive mechanisms of some ACE inhibitor and AT1R blocker (Iyer, Ferrario, & Chappell, 1998), further confirming the essential role of Ang (1-7) in regulating blood pressure.

Given the increasing evidence showing the relationship between the ACE2/Ang (1-7)/MasR axis and blood pressure. ACE2 activation has been considered as a new strategy for hypertension therapy (Der Sarkissian, Huentelman, Stewart, Katovich, & Raizada, 2006; Jiang et al., 2014). Indeed, xanthenone (XNT), a drug identified from structure-based screening, was claimed as an ACE2 activator, showing blood pressure-reducing effect in SHRs (Hernández Prada et al., 2008). However, blood pressure of Ang II-induced hypertensive mice could not be decreased in an ACE2-dependent manner (Haber et al., 2014), indicating the variability of the effect of XNT among different animal models. As biological function of Ang (1-7) is mediated via MasR, development of MasR agonist appears to be another way to activate the ACE2/Ang (1-7)/MasR axis and contributes to reducing blood pressure. CGEN-856, a peptide composed of 24 amino acids, displays high specificity for the MasR. Its derivative, CGEN-856S, was reported with MasR activating effect, which contributed to its vasodilating effect (Lautner et al., 2010). However, identification of ACE2-activating agent or MasR agonist is still at infancy, more efforts are warranted to increase their availabilities.

2.1.2.2 Mitigation of vascular inflammation

Vascular inflammation contributes to the initiation and progression of atherosclerosis as well as hypertension-induced vascular complications. The inflammatory response in blood vessels can be mainly divided into 3 steps: (1) increase in vascular permeability; (2) infiltration of leukocytes; and (3) tissue remodelling (Cheng, Vapaatalo, & Mervaala, 2005). Indeed, Ang II is more than a

potent vasoconstrictor, it is also a pro-inflammatory factor, which is able to induce vascular injury independent of its haemodynamic effect (Schiffrin, 2002). Ang II is involved in all the 3 steps of vascular inflammation. Ang II could regulate vascular permeability via pressure-mediated mechanical injury to the endothelium. Meanwhile, Ang II could also locally influence the permeability by increasing prostaglandins and endothelial cell growth factor (Suzuki et al., 2003). Migration of leukocytes from the bloodstream through the vascular wall is the next step in vascular inflammation. In endothelial cells, Ang II up-regulates cell adhesion molecules and induces leukocyte-endothelium interactions to allow activated leukocytes bind firmly to adhesion molecules (Marchesi, Paradis, & Schiffrin, 2008). As part of the inflammatory response, injured blood vessels undergo recovery through vascular remodelling. The growth-promoting effects of Ang II to induce cell proliferation and hypertrophy are mainly mediated by autocrine and paracrine growth factors (Cheng et al., 2005). Despite various signalling involved in, AT1R is considered as the major effector mediating Ang II-induced pro-inflammatory effects in all the 3 stages (Cheng et al., 2005; Marchesi et al., 2008).

As a counter-regulatory arm in the RAS, increasing evidence showed the anti-inflammatory effect of the ACE2/Ang (1-7)/MasR axis to counterbalance the effect of the Ang II/AT1R axis (Simões e Silva, Silveira, Ferreira, & Teixeira, 2013; Zhang, Chen, Zhong, Gao, & Oudit, 2014). As documented, administration of Ang (1-7) or AVE 0991 (MasR agonist) contributed to reduced rolling and adhesion of leukocytes to microvascular endothelium in an arthritis model, indicating the ACE2/Ang (1-7)/MasR axis could counter-regulate the actions of the Ang II/AT1R axis in the context of leukocyte recruitment (Silveira et al., 2010; Simões e Silva et al., 2013). Ang (1-7) was also shown to reduce the inflammatory response in macrophages by downregulating the expression of pro-inflammatory cytokines, which suggested the potential of

Ang (1-7) to retard the progression of atherosclerosis (Libby, 2009; Souza & Costa-Neto, 2011). Overexpression of ACE2 could inhibit expression of monocyte chemoattractant protein 1 (MCP1) in Ang II-stimulated macrophages, which appeared to be mediated by increased level of Ang (1-7) (Guo, Li, Wu, Xie, & Cui, 2008). On the contrary, loss of ACE2 would lead to a remarkable increase in Ang II-stimulated aortic expressions of pro-inflammatory cytokines and chemokines including MCP1, interleukin-1β and interleukin-6 (Jin et al., 2012). In ApoE KO mice, genetic deficiency of ACE2 led to increased expressions of adhesion molecules and pro-inflammatory cytokines, which would further accentuate atherosclerosis (Thomas et al., 2010). Hence, the ACE2/Ang (1-7)/MasR axis is considered to play a significant role in mitigating vascular inflammation.

Moreover, activated ACE2/Ang (1-7)/MasR axis could present anti-inflammatory effect beyond vasculature. It was reported that brain infusion of Ang (1-7) could suppress inflammation by inhibiting nuclear factor κB (NF-κB) pathway in a rat model with permanent cerebral ischaemia (Jiang et al., 2012). Additionally, treatment of bioencapsulated ACE2/Ang (1-7) decreased retina inflammation in experimental autoimmune uveoretinitis (Shil et al., 2014).

2.1.2.3 Amelioration of vascular oxidative stress

Free radicals are atoms, molecules or ions with unpaired electrons, which can be generated through reactions during respiration in physiological condition (Evans, Goldfine, Maddux, & Grodsky, 2003). Reactive oxygen species (ROS), reactive nitrogen species and reactive sulphur species are considered as major types of free radicals (Lu, Lin, Yao, & Chen, 2009). Under normal conditions, free radicals generated in the body can be removed by antioxidant defense system in enzymatic and non-enzymatic-mediated patterns (Johansen, Harris, Rychly, & Ergul, 2005). While, oxidative stress is resulted from the failure of the endogenous defense system to

protect the body against reactive radicals (Sarmadi & Ismail, 2010). There is accumulating evidence suggesting that nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase-mediated excessive production of ROS in endothelial cells and vascular smooth muscle cells (VSMCs) is associated with aberrant vascular function (Cai & Harrison, 2000; Frey, Ushio-Fukai, & Malik, 2008; Irani, 2000; Lassègue & Griendling, 2010; Taniyama & Griendling, 2003).

It is noteworthy that there is a potential relationship between the ACE2/Ang (1-7)/MasR axis and ROS-mediated oxidative stress. It was reported that the ROS level was increased in the aorta of MasR-deficient mice. Further study found that the increased oxidative stress in MasR-deficient mice was associated with up-regulation of gp91phox, which is a main catalytic subunit of NADPH oxidase (Xu et al., 2008). Similar finding was observed in ACE2-null mice. As compared with the wild-type, lipid peroxidation was significantly increased but the activity of superoxide dismutase was remarkably decreased in the aorta of ACE2-null mice (Rabelo et al., 2016). In addition, in a high-salt-fed rat model, acute and chronic treatments of Ang (1-7) could reduce oxidative stress in mesenteric arteries (Raffai, Durand, & Lombard, 2011). In human brain microvascular endothelial cells, Ang (1-7) treatment suppressed Ang II-induced pro-apoptotic activity and over-production but restored NO production. However, the addition of MasR antagonist abolished the modulatory effects of Ang (1-7) in endothelial cells against Ang II (Xiao et al., 2015), which elucidated the specific relationship between the ACE2/Ang (1-7)/MasR axis and ROS at least in cellular level. Indeed, attenuation of oxidative stress by Ang (1-7) could further contribute to abrogating Ang II-induced intracellular events. More recently, Zhang and co-workers reported that Ang (1-7) abrogated Ang II-stimulated proliferation, migration and inflammation in VSMCs through ROS-mediated signaling pathways (Zhang, Ren, Zhao, Zhou, & Han, 2016). Nevertheless, the contribution of reduced oxidative stress by

activated the ACE2/Ang (1-7)/MasR axis to blood pressure reduction is unclear, which is warrant for future study.

2.1.2.4 Modulation of cardiac remodelling

Cardiac remodelling, which is characterized by cardiac hypertrophy and dilatation as well as conformational changes in the shape of heart, is a response of heart to an injury or increase in wall stress (Iwata, Cowling, Yeo, & Greenberg, 2011). Cardiac remodelling can progress the deterioration of cardiac functions and eventually leads to heart failure. Despite efforts and advances in the treatment, heart failure remains the most common cause of death and disability (Iwata et al., 2011; Patel, Zhong, Grant, & Oudit, 2016). Hence, novel strategies which can inhibit cardiac remodelling may be helpful to improve the therapeutic outcomes of heart failure (Iwata et al., 2011).

ACE2 was initially identified in the heart, extensive research has been performed to explore the role of the ACE2/Ang (1-7)/MasR axis in cardiac functions since this identification. Evidence from "gain" and "loss" of ACE2 function (summarized in Table 2.2) suggested a critical counter-regulatory role of ACE2 in cardiac remodelling. Indeed, signaling pathways involving in regulatory effects of the ACE2/Ang (1-7)/MasR axis in cardiac remodelling have also been discussed, which mainly include three aspects: 1) activation of phophatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)/endothelial nitric oxide synthase (eNOS) to increase the release of NO from cardiomyocytes (Dias-Peixoto et al., 2008); 2) inhibition of mitogen-activated protein kinases (MAPK) phosphorylation to ameliorate heart hypertrophy (Ferreira et al., 2011; Giani et al., 2010); 3) downregulation of collagen expression to limit cardiac fibrosis (Giani et al., 2010; Huentelman et al., 2005).

The role of the RAS in developing cardiac remodelling, which is mediated by the Ang II/AT1R axis has been universally accepted (Billet, Aguilar, Baudry, & Clauser, 2008). Pharmacological antagonism of the RAS using ACE inhibitor or AT1R blocker is a conventional therapy for heart failure (Patel et al., 2016). Interestingly, it was found that increase in ACE2 was a mechanism of some ACE inhibitors or AT1R blockers in treating heart failure (Ferrario, Jessup, et al., 2005; Ocaranza et al., 2006), indicating the potential contribution of the activated ACE2/Ang (1-7)/MasR axis in the therapeutic process. Further study found that Ang (1-7) showed an equivalent cardioprotective effects as AT1R blocker in pressure-overload-induced heart failure in ACE2 KO mice (Patel et al., 2012), which shed light on the potential of ACE2/Ang (1-7)/MasR axis as an alternative target in treating heart failure.

It is important to note that clinical data are available to support that the ACE2/Ang (1-7)/MasR axis is involved in human heart failure as increased cardiac or circulating ACE2 activity has been reported in patients with heart failure (Burrell et al., 2005; Epelman et al., 2008; Goulter, Goddard, Allen, & Clark, 2004; Patel et al., 2014; Zisman et al., 2003), which is considered as a compensatory response. However, more translational studies are required to evaluate the cardioprotective effects of the activated ACE2/Ang (1-7)/MasR axis by therapeutic agents.

2.1.2.5 Modulations of cardiovascular functions through central regulator

The existence of brain RAS was first postulated with the identification of renin activity in the central nervous system (Ganten et al., 1971). Brain RAS contains the same elements as the other tissue RAS, but it has a main feature of distinction is brain RAS physically separated from the circulating RAS due to the presence of blood-brain barrier which prevents the diffusion of circulating RAS components into the brain (Schelling, Hutchinson, Ganten, Sponer, & Ganten, 1976). Nevertheless, brain RAS also participates in regulating cardiovascular functions, which is

acting as a central regulator. Upregulations of angiotensinogen, ACE, Ang II and AT1R in the brain are correlated with the development and maintenance of hypertension and heart failure (Xu, Sriramula, & Lazartigues, 2010). Specifically, the Ang II/AT1R axis in the brain mediates pathological changes of cardiovascular functions, which is through promoting salt appetite (Weisinger, Blair-West, Denton, & Tarjan, 1997), vasopressin secretion (Matsukawa, Keil, & Reid, 1991) and sympathetic outflow (Reid, 1992).

As a counter-regulatory arm in the RAS, roles of the activated brain ACE2/Ang (1-7)/MasR axis in modulating pathological changes of cardiovascular functions have been documented. Brain-targeted ACE2 overexpression could attenuate deoxycorticosterone acetate-salt-induced hypertension and cardiac hypertrophy via reducing brain oxidative stress and inflammation (Sriramula, Xia, Xu, & Lazartigues, 2015). Brain-selective overexpression could also exert protective effects in cardiac functions through attenuating sympathetic outflow to retard chronic heart failure (Xiao, Gao, Lazartigues, & Zucker, 2011) as well as reduce Ang II-induced cardiac hypertrophy (Feng, Hans, McIlwain, Varner, & Lazartigues, 2012). Indeed, beneficial effects of elevated brain Ang (1-7) in cardiovascular functions have been investigated as well. Infusion of Ang (1-7) into the brain could reduce blood pressure in different hypertensive rodent models (Chaves, Caligiorne, Santos, Khosla, & Campagnole-Santos, 2000; Guimaraes et al., 2012). While, treatment of MasR antagonist A779 abrogated the Ang (1-7) effect (Chaves et al., 2000), which further confirmed the distinguished role of the brain ACE2/Ang (1-7)/MasR axis in regulating blood pressure as a central regulator.

In addition to cardiovascular diseases, the ACE2/Ang (1-7)/MasR axis also exerts protective effects in cerebrovascular disease. During the past years, various studies demonstrated the potential for prevention and treatment of ischemic stroke by targeting the ACE2/Ang (1-7)/MasR

axis (Jiang et al., 2012; Lu, Zhang, & Shi, 2008; Mecca et al., 2011; Regenhardt et al., 2013). Increased formation of bradykinin (Lu et al., 2008) and decreased inflammation and oxidative stress were implicated as the mechanisms (Jiang et al., 2012).

2.1.2.6 Regulation of blood pressure through nephron functions

kidney participates in regulation of blood pressure via controlling body fluid volumes (Ferrario & Varagic, 2010). Elevated intrarenal formation of Ang II modulates solute and water transport across the renal tubules and the filtration of proteins through the glomerular barrier, which causes reduction in sodium excretion and leads to progressive hypertension (Ferrario & Varagic, 2010; Navar, Kobori, & Prieto-Carrasquero, 2003).

Indeed, the activated ACE2/Ang (1-7)/MasR axis was shown to modulate water and electrolyte transports in the nephron, which is, at least partially, associated with its blood pressure-reducing effect. Intrarenal infusion of Ang (1-7) increased urinary sodium excretion (Bürgelová et al., 2002; Heller, Kramer, Malý, Červenka, & Horáček, 2000), but the effect was blocked by administration of MasR antagonist A779 (Baracho, Simões-e-Silva, Khosla, & Santos, 1998; Bürgelová et al., 2002). On the other hand, Ang (1-7) could enhance water transport in rat inner medullary collecting duct and the effect was also abolished by A779 (Magaldi, Cesar, de Araújo, Simões e Silva, & Santos, 2003). Mechanisms of modulatory roles of the ACE2/Ang (1-7)/MasR axis in renal electrolyte transport have been studied as well. Ang (1-7) could stimulate the Na^+-ATPase activity via MasR mediated by G_q protein-phosphatidylinositol phospholipase C beta-protein kinase C pathway (Caruso-Neves, Lara, Rangel, Grossi, & Lopes, 2000; Lara et al., 2002; Lara, Correa, Lavelle, Lopes, & Caruso-Neves, 2008).

In addition, Ang (1-7) was suggested to regulate intrarenal cell growth pathways (Dilauro & Burns, 2009). Ang II-stimulated phosphorylation of MAPK and production of profibrotic cytokine transforming growth factor-β1 in rat proximal tubular cells could be inhibited by Ang (1-7) (Gava et al., 2009; Su, Zimpelmann, & Burns, 2006), indicating the protective potential of Ang (1-7) against pathologic changes in the kidney.

2.1.2.7 Modulations of functions beyond cardiovascular system

As the RAS is a main regulator of blood pressure, extensive research has been performed to investigate the protective roles of the ACE2/Ang (1-7)/MasR axis in regulating cardiovascular functions. While, accumulative evidence in recent years indicate that the ACE2/Ang (1-7)/MasR axis may have beneficial effects even beyond cardiovascular system.

Protective roles of the ACE2/Ang (1-7)/MasR axis in lung functions have been reported. Overexpression of Ang (1-7) or ACE2 conferred cardiopulmonary protection against lung fibrosis (Shenoy et al., 2010). Besides, ACE2 has been identified as a functional receptor of severe acute respiratory syndrome coronavirus (Hamming et al., 2004; Li et al., 2003). Additionally, treatment of recombinant ACE2 could protect mice from acute respiratory distress syndrome, the most severe form of acute lung injury (Imai et al., 2005).

The RAS components including ACE2 are also expressed in retina (Sjølie & Chaturvedi, 2002; Tikellis et al., 2004). More interestingly, it was documented that overexpression of ACE2 or Ang (1-7) exerted protective effects against development of diabetic retinopathy (Dominguez et al., 2016; Verma et al., 2012). Overexpression or activation of ACE2 was also associated with ameliorating inflammation in retinal pigment epithelium (Fu, Lin, Qiu, Yu, & Lei, 2017; Tao et al., 2016). On the contrary, loss of ACE2 worsened diabetic retinopathy (Duan et al., 2018).

Together, these findings indicated the potential in targeting the ACE2/Ang (1-7)/MasR axis to serve as a novel therapeutic strategy for diabetic retinopathy, which is the most common diabetic vascular complication (Sjølie & Chaturvedi, 2002).

It is noteworthy that ACE2 has also been associated with gut health.. Susceptibility to epithelial damage-induced intestinal inflammation in ACE2 KO mice was significantly increased as compared with the wild-type mice. Transplantation of the altered microbiota from ACE2 KO mice to the wild-type hosts was able to transmit the increased propensity to develop severe colitis (Hashimoto et al., 2012). On the other hand, ACE2 is involved in regulating amino acid uptake (Kleta et al., 2004), as expression of B^0AT1, a main epithelial neutral amino acid transporter in small intestine (Perlot & Penninger, 2013), is dependent on ACE2 (Camargo et al., 2009).

In conclusion, the ACE2/Ang (1-7)/MasR axis has been implicated in diverse experimental models within or beyond cardiovascular system (Figure 2.2), enabling this axis as a new therapeutic target of various human diseases (Ingelfinger, 2006; Raizada & Ferreira, 2007; Shenoy, Qi, Katovich, & Raizada, 2011; Teng, Li, Jie, & Zhang, 2013).

2.2 Mechanisms of food protein-derived antihypertensive peptides

Food protein-derived bioactive peptides can exert physiological functions beyond their nutritional value. Bioactive peptides are inactive until they are released from their parent proteins by enzymatic digestion or food processing (Vermeirssen, Camp, & Verstraete, 2004). Peptides identified from various food proteins have been shown diverse activities, including antihypertensive, anti-inflammatory, antioxidant, anti-cancer, and opiate-like actions. These peptides have been implicated in preventing or treating different chronic diseases (De Mejia & De Lumen, 2006; Erdmann, Cheung, & Schröder, 2008; Korhonen, 2009; Udenigwe & Aluko,

2011). As a major category of bioactive peptide, antihypertensive peptides have gained substantial interests during the past decades as food-derived alternative to antihypertensive drugs (Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). Abundant work has been concentrated on identification of antihypertensive peptides from food proteins (Martínez-Maqueda et al., 2012). While, mechanistic studies are emerging in recent years to elucidate the molecular mechanisms of antihypertensive peptides (Majumder & Wu, 2015; Wu, Liao, & Udenigwe, 2017). However, as regulation of blood pressure in the body is a complex process involving various molecules and signalling pathways, to fully understand the mechanisms of food protein-derived antihypertensive peptides requires more extensive work in this area. Our evolving understanding of the RAS over the years, in particular the regulatory role of the ACE2/Ang (1-7)/MasR axis (introduced in 2.2), provides new perspectives in exploring the mechanisms of antihypertensive peptides. In the next section, mechanisms of food protein-derived antihypertensive peptides based on the current knowledge, if any, in relationship with the ACE2/Ang (1-7)/MasR axis, will be introduced.

2.2.1 Animal models used for studies on antihypertensive peptides

To understand the molecular mechanisms of food protein-derived antihypertensive peptides, it is crucial to select an appropriate animal model. A number of genetic or non-genetic hypertensive rodent models have been developed to study hypertension from different perspectives (Table 2.3). As essential hypertension accounts for ~ 95% of human hypertensive cases (Carretero & Oparil, 2000), SHR, which is mimetic to human essential hypertension, is the most popular animal model used for mechanistic studies of antihypertensive peptides. SHR is an experimental hypertensive model which was firstly bred by mating a male Wistar Kyoto rat with spontaneous hypertension with a female rat of the same strain with a slightly high blood pressure. Blood

pressure of SHR starts to rise at around 6-week of age until reaching ~ 180 mmHg of systolic pressure (Okamato & Aoki, 1963). As generally there is a higher prevalence of essential hypertension among males than age-matched females before menopause (Cheng, Xanthakis, Sullivan, & Vasan, 2012), young male SHR is the most universal animal model used for studies on antihyertensive peptides.

However, studies based on male SHR model can only offer a general view on the mechanisms of antihypertensive peptides. In the future research, female SHR is also suggested to be included to evaluate the differential responses between genders. In addition, various research was conducted on ACE2 KO mice model to investigate the functions of ACE2 as discussed in 2.2. Therefore, to apply the food protein-derived antihypertensive peptides on ACE2 KO mice model is recommended to characterize the specific roles of antihypertensive peptides-mediated ACE2 up-regulation /activation in lowering blood pressure.

2.2.2 Peptides modulating the RAS components

As a key enzyme in generating Ang II, ACE is a common target for identification of antihypertensive peptides from food proteins, which is similar as development of antihypertensive drug. *In vitro* ACE-inhibition assay is the most common assay for characterization of ACE-inhibitory peptides, in which, hippuryl-histidyl-leucine is hydrolyzed by ACE to produce hippuric acid and histidyl-leucine. The mixture of the reaction is then separated by liquid chromatography, activity of ACE is evaluated based on the amount of hippuric acid generated (Wu, Aluko, & Muir, 2002). The activity of the peptide is always expressed as IC_{50} value (the concentration of the peptide to inhibit 50 % of ACE activity).

Followed by the dentification, blood pressure-reducing effect of many ACE-inhibitory peptides have been demonstrated in SHRs. However, the potency of blood pressure-reducing effect sometime is incomparable with the IC_{50} value (Fujita, Yokoyama, Yoshikawa, & Angiotensin, 2000; Liao, Jahandideh, Fan, Son, & Wu, 2018; Miguel, Manso, et al., 2007). It is also very surprising that many ACE-inhibitory peptides did not show ACE inhibition effect *in vivo*, despite their ability to reduce blood pressure in SHRs. The inconsistence between results from *in vitro* ACE-inhibitory assay and mechanistic study suggested *in vivo* antihypertensive mechanisms of ACE-inhibitory peptides might be beyond ACE inhibition (Wu et al., 2017). Indeed, this notion has been demonstrated by some study reporting expressions of the RAS components could be modulated by ACE inhibition. Egg white protein hydrolysate was shown to down-regulate protein expression of AT1R but upregulate AT2R in aorta of SHRs (Jahandideh et al., 2016). Egg white -derived RVPSL was reported to modulate mRNA levels of renin, AT1R and AT2R in SHRs to exert its blood pressure-reducing effect (Yu, Yin, Zhao, Chen, & Liu, 2014). Results from "omics" study showed the potential of ACE-inhibitory peptides in altering expressions of molecules beyond the RAS to exert their antihypertensive activity. Microarray data showed that milk-derived ACE-inhibitory peptides IPP and VPP modified the expressions of genes in the RAS, vascular function, arachidonic acid, blood coagulation system and cytokines in aorta of SHR to exert their blood pressure-reducing effects (Yamaguchi, Kawaguchi, & Yamamoto, 2009). Another study from our group using RNA-Seq found that egg white-derived IRW could reduce blood pressure in SHRs via regulating expressions of genes related the RAS, leukocyte recruitment and vascular inflammation (Kaustav Majumder, Liang, et al., 2015).

Interestingly, the potential impact of ACE-inhibitory peptides on the ACE2/Ang (1-7)/MasR axis has been implicated. It was reported that Ang (1-7) production could be augmented by IPP

treatment in an *ex vivo* study, mediated the vasorelaxation effect of the peptide (Ehlers, Nurmi, Turpeinen, Korpela, & Vapaatalo, 2011). However, effect of IPP on ACE2 is ambiguous. More recently, a study from our group found that mRNA level of ACE2 in mesenteric artery of SHRs could be significantly up-regulated by 18-day oral administration of IRW, suggesting the potential of antihypertensive peptides in targeting ACE2 (Majumder, Liang, et al., 2015). While, the contribution of ACE2 increase to reducing blood pressure by IRW treatment was not shown in this study.

Taken together, the *in vitro* ACE-inhibitory assay used for identifying antihypertensive peptides needs to be modified into a more biologically relevant assay to improve the confidence of *in vivo* efficacy of the identified peptides. On the other hand, targeting ACE2 may be a new opportunity for identification of food protein-derived antihypertensive peptides.

2.2.3 Peptides improving endothelial dysfunction

In vascular endothelium, endothelins (ETs) are a family of vasoconstricting peptides synthesized by endothelial cells. The family of ETs consists of 3 peptides including ET-1, ET-2 and ET-3 (Sudano et al., 2006). Endothelial dysfunction is commonly referred to an impairment of endothelium-dependent vasorelaxation due to an interrupted balance between vasodilator (NO) and vasoconstrictor (ET1). Endothelial dysfunction manifests adverse cardiovascular events (Cai & Harrison, 2000). Food protein-derived antihypertensive peptides have been shown to improve endothelial dysfunction either by stimulating production of NO or reducing generation of ET1.

It was reported that egg white-derived antihypertensive peptide RADHP showed a dose-dependent relaxing effect in the isolated rat aortic ring. While, removal of the endothelium abolished the vasorelaxation effect of the peptide, preincubation of the aortic ring with L-N^G-

Nitroarginine methyl ester, an inhibitor of nitric oxide synthase, blocked the peptide activity as well. These results indicated that RADHP could increase endothelial-dependent vasodilation and increase in the bioavailability of NO appeared to be involved in the mechanism (Miguel, Alvarez, López-Fandiño, Alonso, & Salaices, 2007). However, molecular mechanisms mediating the improved bioavailability of NO was unknown.

eNOS is expressed by the endothelium, which converts L-arginine to L-citrulline to produce NO (Palmer, Ashton, & Moncada, 1988). Reduced eNOS expression is associated with diminished level of bioavailable NO (Napoli & Ignarro, 2009). Indeed, up-regulation of eNOS expression is indicated in the mechanism underlying the endothelial-dependent vasorelaxation activity of some food protein-derived antihypertensive peptides. Long-term (6 weeks) treatment of casein hydrolysate containing peptides RYLGY and AYFYPEL significantly up-regulated protein expression of eNOS in aorta of SHRs and retarded consistent blood pressure progressing as compared with the untreated control (Sánchez et al., 2011). Treatment of casein-derived VPP and IPP could significantly increase NO production in cultured endothelial cells (Hirota et al., 2011). Oral administration of VPP and IPP for 5 days could increase mRNA level of eNOS in aorta of SHR, coupled with a 30 mmHg reduction of systolic pressure (Yamaguchi et al., 2009). Another example is that 18-day oral administration of egg white-derived IRW could upregulate protein expressions of eNOS in mesenteric artery and aorta of SHRs to exert its blood pressure-reducing effect (Majumder, Chakrabarti, Morton, et al., 2013). However, egg white-derived antihypertensive peptides IQW and LKP also showed effects in enhancing endothelium-dependent vasorelaxation in SHRs, but expression of eNOS was not altered by either of the treatment, suggesting that possible action might be through endothelin pathways (Majumder, Chakrabarti, et al., 2015).

ET1 is a powerful vasoconstrictor synthesized by endothelin-converting enzyme (ECE). An increased production and biological activity of ET1 is an important alteration in endothelial dysfunction (Böhm & Pernow, 2007). ECE inhibition appears to be involved in the antihypertensive mechanism of some food protein-derived peptides. Traced back to 1995, Okitsu and co-workers reported that pepsin digests of bonito protein exhibited ECE-inhibitory activity in an *in vitro* assay (Okitsu, Morita, Kakitani, Okada, & Yokogoshi, 1995). Milk-derived peptides have also been reported with ECE-inhibitory activity. ALPMHIR derived from β-lactoglobulin suppressed ET1 release in cultured endothelial cells (Maes et al., 2004). A set of eight peptides identified from lactoferricin B showed ECE-inhibitory effects in an *ex vivo* study using isolated rabbit carotid artery segments (Fernández-Musoles et al., 2010). In addition, GILRPY and REPYFGY identified from lactoferrin hydrolysate were also reported with ECE-inhibitory activity (Fernández-Musoles et al., 2013). However, *in vivo* evidence is lacking, which needs to be further investigated. Meanwhile, ET1 acts through the activation of G_i-protein-coupled ET_A and ET_B receptors and development of endothelin receptor antagonist is another strategy for cardiovascular therapy (Lüscher & Barton, 2000). However, food protein-derived ET1 receptor blocker has not been reported yet, which needs future exploration.

2.2.4 Peptides ameliorating vascular inflammation

As introduced previously, vascular inflammation is a milestone in progressing vascular remodelling, which consequently leads to increasing peripheral resistance (Renna, de las Heras, & Miatello, 2013). Anti-inflammatory effects of food protein-derived antihypertensive peptides in vascular cells and tissues have been reported as a mechanism of these peptides. Milk-derived VPP was shown to exhibit activity in modulating adhesion of monocyte to vascular endothelium via attenuation the c-Jun N-terminal kinases (JNK) pathway, indicating the feasibility of VPP in

prevention of atherosclerosis (Aihara, Osaka, & Yoshida, 2014). In a later study, it was further demonstrated that treatment of VPP in ApoE KO mice down-regulated mRNA levels of proinflammatory cytokines (Nakamura et al., 2013). IRW derived from egg white was reported with the anti-inflammatory activity in vascular endothelial cells via down-regulating TNF-α-induced expressions of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Majumder, Chakrabarti, Davidge, & Wu, 2013). Down-regulations f ICAM-1 and VCAM-1 were also observed in mesenteric artery of SHR upon 18-day oral administration of IRW (Majumder, Chakrabarti, Morton, et al., 2013). These results suggested that amelioration of vascular endothelial inflammation was involved in the antihypertensive mechanisms of IRW.

VSMC is another essential regulator present in the vascular wall in addition to vascular endothelial cells in regulating blood vessel diameter and blood flow (Rudijanto, 2007). Inflammation in VSMCs resulted from over stimulation of Ang II in VSMCs is an event in development of vascular remodelling (Touyz & Schiffrin, 2000). Indeed, it was found that IRW also showed anti-inflammatory activity in VSMCs against Ang II stimulation via down-regulations of cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) expressions; NF-κB pathway was responsible to mediate the anti-inflammatory effect of IRW in Ang II-stimulated VSMCs (Liao, Chakrabarti, Davidge, & Wu, 2016).

2.2.5 Peptides reducing oxidative stress in vasculature

Vasculature is a rich source of ROS, increased oxidative stress is a mediator of cardiovascular pathologies including hypertension (Touyz, 2004). Although abundant antioxidant peptides have been characterized from various food proteins, peptides which can target vascular tissues to

reduce vascular oxidative stress and further contribute to lowering blood pressure are rarely reported except for some egg-derived peptides.

Egg white hydrolysate prepared by pepsin digestion decreased blood pressure in SHRs upon 5-week treatment. The radical-scavenging capacity of plasma from the treated rats was increased. The level of malondialdehyde, a marker of oxidative stress, in aorta was decreased by the hydrolysate treatment (Manso, Miguel, Even, Aleixandre, & Lopez-Fandino, 2008). Thermoase and pepsin-digested egg white hydrolysate could also reduce blood pressure in SHRs via attenuating oxidative stress in aorta (Jahandideh et al., 2016). Egg white-derived antihypertensive peptides IRW and IQW could reduce TNFα-induced oxidative stress in vascular endothelial cells, (Majumder, Chakrabarti, Davidge, et al., 2013). In addition, IRW also showed antioxidant activity in Ang II-stimulated VSMCs (Liao et al., 2016), suggesting the potential of this peptides in reducing oxidative stress in different vascular components. Antioxidant activity of the above-mentioned antihypertensive peptides has been demonstrated in vascular cells or tissues, which was implicated as an antihypertensive mechanism of these peptides. However, if these peptides exert their antioxidant activity via suppressing ROS production or accelerating ROS scavenging is unknown.

2.3 General conclusion

Multiple regulatory roles of the ACE2/Ang (1-7)/MasR axis have been recognized, which makes this axis as an emerging target for development of new cardiovascular therapy. Food protein-derived bioactive peptides have shown the potential to be incorporated into functional foods or nutraceuticals to mitigate certain type of chronic disease. ACE inhibitory peptides have been extensively studied over the past three decades. The recognition of the presence of peptides target ACE2 may not only help address the inconsistency between ACE inhibition and blood

pressure lowering activity, but also offers new ways to further explore antihypertensive peptides from food proteins. Future research is suggested to focus on the following aspects:

- Mechanisms of some food protein-derived antihypertensive peptides have been elucidated (Figure 2.3). While, knowledge on the involvement of the ACE2/Ang (1-7)/MasR axis is scarce. Only IRW was reported with the potential to target ACE2 (Majumder, Liang, et al., 2015). As IRW was initially characterized as an ACE inhibitor (Majumder & Wu, 2011), it is recommended to investigate if ACE2 activation is a common feature of ACE-inhibitory peptides, which can help to advance our knowledge on mechanisms of antihypertensive peptides. If any, a structure and activity study is also recommended.

- Although IRW was reported to increase mRNA expression of ACE2 in vasculature of SHRs (Majumder, Liang, et al., 2015), effects of IRW on protein expression of ACE2 as well as circulating ACE2 have not been shown yet. In addition, the contributions of ACE2 increase to mitigating pathological alterations in vasculature and reducing blood pressure is warranted to be studied as well, which can unlock the significance of the activation of ACE2/Ang (1-7)/MasR axis by food protein-derived peptides in controlling blood pressure.

- It is also worthwhile to identify novel peptides targeting ACE2 from other food protein sources, which can contribute to our understanding of the structure and activity relationships of these peptides. These peptides will also provide new opportunities for future development of hypotensive functional foods or nutraceuticals.

- As the activated ACE2/Ang (1-7)/MasR axis has beneficial effects within or beyond cardiovascular functions, peptides targeting ACE2 may also possess other activities in preventing or treating various chronic diseases in addition to hypertension. Thus, it is suggested to expand the activity study of these peptides, which may broaden the application potentials of these peptides.

The present study tries to address some of these issues to fill the knowledge gap between the ACE2/Ang (1-7)/MasR axis and antihypertensive peptides. Results from this study may deepen our view on mechanisms of antihypertensive peptides as well as provide new opportunity for identification of antihypertensive peptides from various food proteins.

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Table 2.1: Functions of ACE2 in regulating blood pressure

Animal model	Intervention	Major findings	Reference
Ang II-induced hypertensive mice	ACE2 knockout (KO)	ACE2 KO enhanced susceptibility to Ang II-induced hypertension	(Gurley et al., 2006)
Ang II-induced hypertensive mice	ACE2 overexpression	ACE2 overexpression reduced the pressor response to Ang II infusion	(Feng et al., 2008)
Ang II-induced hypertensive mice	ACE2 overexpression	Prevention of hypertension by ACE2 overexpression was reversed by blockade of the Ang (1-7) receptor	(Wysocki et al., 2010)
SHR	ACE2 overexpression	ACE2 overexpression decreased blood pressure	(Yamazato, Yamazato, Sun, C., Diez-Freire, & Raizada, 2007)
Ang II-induced hypertensive rat	ACE2 overexpression	ACE2 overexpression decreased blood pressure	(Sriramula, Cardinale, Lazartigues, & Francis, 2011)
Stroke-prone SHR	Vascular smooth muscle-specific ACE2 gene transfer	ACE2 gene transfer improved endothelial function and decreased blood pressure	(Rentzsch et al., 2008.)
Ang II-induced hypertensive mice	Infusion of recombinant ACE2	ACE2 infusion reduced the pressor response to Ang II infusion	(Ye et al., 2012)

Table 2.2: Functions of ACE2 in cardiac remodelling

Animal model	Intervention	Major findings	Reference
Rat with permanent coronary artery ligation	ACE2 overexpression	Complete rescue of cardiac output in 6 weeks after surgery	(Der Sarkissian et al., 2008)
SHR	ACE2 overexpression	Reduction in left ventricular wall thickness; Attenuation of periventricular fibrosis	(Díez-Freire et al., 2006)
Rat with Ang II-induced cardiac hypertrophy and fibrosis	ACE2 overexpression	Attenuation of cardiac hypertrophy and myocardial fibrosis	(Huentelman et al., 2005)
Rat with acute hypoxic exposure	ACE2 overexpression	Reduced hypoxia-induced production of collagen	(Grobe et al., 2007)
Mice with Ang II infusion	Infusion of recombinant ACE2	Amelioration of hypertrophic response	(Zhong et al., 2010)
Rat with Ang II infusion	Infusion of recombinant ACE2	Reduced myocardial hypertrophy	(Lo et al., 2012)
SHR	Infusion of recombinant ACE2	Reduced production of reactive oxygen species and corrected pathological signaling in the heart	(Lo et al., 2012)
Mice with Ang II infusion	ACE2 KO	Worsened cardiac fibrosis and hypertrophy	(Zhong et al., 2010)
Mice with coronary artery ligation	ACE2 KO	Adverse ventricular remodelling	(Kassiri et al., 2009)
High-fat diet-induced obesity mice	ACE2 KO	Abnormal myocardial metabolism	(Patel et al., 2015)
Ren-2 hypertensive rat	Infusion of ACE2 inhibitor	Increased cardiac Ang II level, left ventricular wall thickness and cardiomyocyte hypertrophy	(Trask et al., 2010)

Table information gathered from (Patel, Zhong, Grant, & Oudit, 2016)

Table 2.3 Rodent models widely used for hypertension study

Name	Features	Reference
SHR	A model which simulates essential hypertension with impaired endothelium-dependent vasorelaxation, cardiac hypertrophy and renal dysfunction.	(Lin, Lee, Chan, & Tse, 2016)
TGR(mREN2)27 rat	The murine Ren-2 gene is incorporated into the rat genome to overexpress the gene of renin in kidney, which is used for studying the role of local RAS in hypertension.	(Langheinrich et al., 1996)
Deoxycorticosterone acetate (DOCA) salt hypertensive rat	DOCA, which is a mineralocorticoid, is used with salt to increase fluid reabsorption and induce hypertension. This model can be used for identifications of sodium-dependent mechanisms in mediating hypertension.	(Lin et al., 2016)
Ang II-infusion model	Chronic systematic infusion of Ang II can result in increase in arterial pressure in both f normotensive mice and rat.	(Wang et al., 2001; Reckelhoff et al., 2000)
N-nitro-L-arginine methyl ester (L-NAME)-infusion model	Chronic infusion of L-NAME which is a nitric oxide synthase inhibitor leads to decreased glomerular filtration and increased arterial pressure.	(Ribeiro, Antunes, de Nucci, Lovisolo, & Zatz, 1992)
Two kidney one clip model	One of the two renal arteries is constricted by a clip, which eventually leads to chronically elevated plasma renin activity. This model can be used for study on renovascular hypertension.	(Lin et al., 2016)

Figure 2.1: Classical RAS and updated RAS. In the classical RAS, ACE is the key enzyme as it functions to generate vasoconstrictor Ang II. Pathological effects of Ang II are mainly mediated by AT1R. In the updated RAS, ACE2 is in the counter-regulatory arm of the RAS to counterbalance the harmful effects of Ang II by generating Ang (1-7) coupled with MasR, which is the main pathway of ACE2.

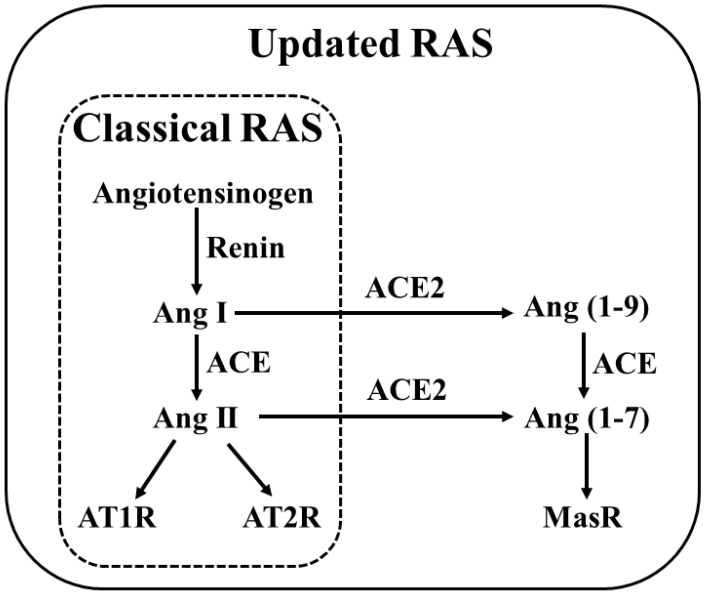


Figure 2.2: Functions of the activated ACE2/Ang (1-7)/MasR axis. The activated ACE2/Ang (1-7)/MasR axis could directly decrease blood pressure by increasing NO bioavailability. But also, activation of this axis could attenuate vascular inflammation and oxidative stress, modulate the brain RAS and modulate water and electrolyte transport in kidney to lower blood pressure. The activated ACE2/Ang (1-7)/MasR axis could also ameliorate cardiac remodelling and retard progression of heart failure. In addition, the activated ACE2/Ang (1-7)/MasR axis could modulate functions beyond cardiovascular system mainly including to repair lung injury, mitigate diabetic retinopathy and modify gut microbiota.

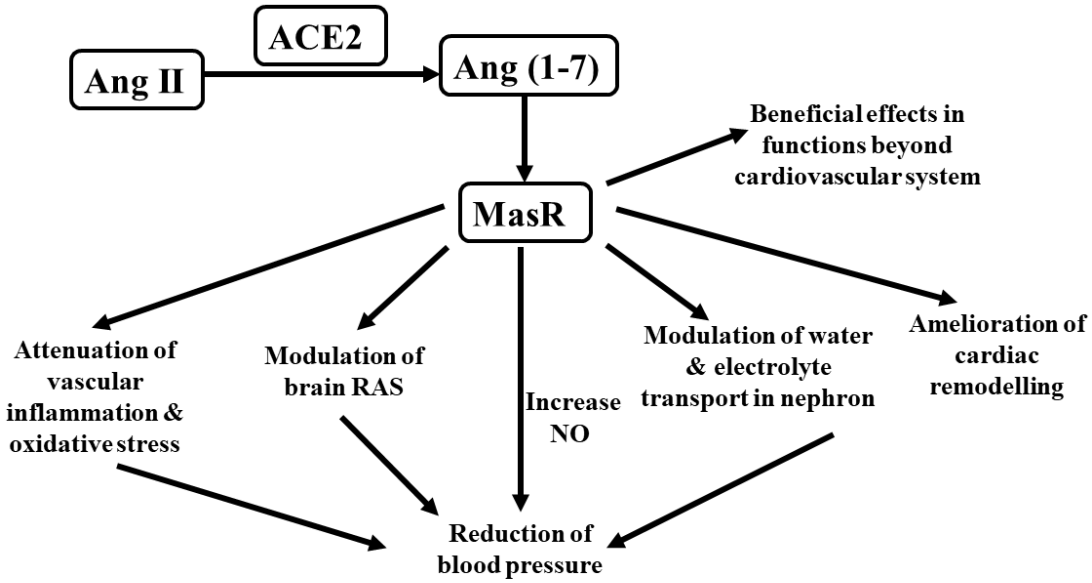
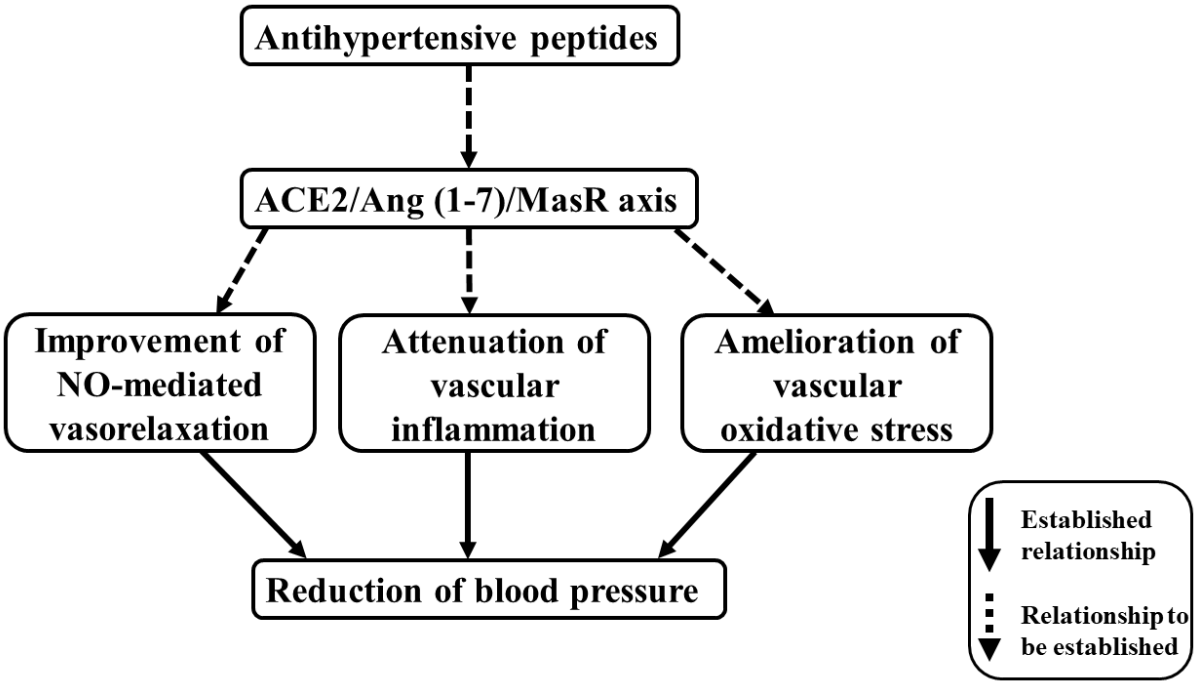


Figure 2.3: Mechanisms of antihypertensive peptides. Improvement of NO-mediated vasorelaxation, attenuation of vascular inflammation and oxidative stress are involved in the mechanisms of antihypertensive peptides. As these effects could be mediated by the ACE2/Ang (1-7)/MasR axis, it is warranted to investigate the relationship between the activation of ACE2/Ang (1-7)/MasR axis and reduction of blood pressure by antihypertensive peptides.



CHAPTER 3[2] - Egg White-Derived Tripeptide IRW (Ile-Arg-Trp) Is an Activator of Angiotensin Converting Enzyme 2

[2] A revision of this chapter has been accepted: Liao, W., Bhullar, K. S., Chakrabarti, S., Davidge, S. T, & Wu, J. (2018). Egg white-derived tripeptide IRW (Ile-Arg-Trp) is an activator of angiotensin converting enzyme 2. *Journal of Agricultural and Food Chemistry*, *66*(43), 11330-11336.

3.1 Introduction

Hypertension is a major risk factor for cardiovascular, neural and renal diseases, and is present in approximately one-quarter of the adult population (Chockalingam, 2008). Hyperactive renin angiotensin system (RAS), a classic endocrine system, is a common feature of hypertension and related pathologies (Sarzani, Salvi, Dessì-Fulgheri, & Rappelli, 2008). While angiotensin converting enzyme (ACE) is responsible for the generation of angiotensin II (Ang II), one of the most potent vasoconstrictors in the body, another RAS enzyme, angiotensin converting enzyme 2 (ACE2) is involved in its conversion into angiotensin (1-7) (Ang (1-7)). Ang (1-7) is a smaller peptide with pronounced anti-inflammatory and antihypertensive properties (Rice, Thomas, Grant, Tuener, & Hooper, 2004). As such, ACE2 is considered as a negative regulator of RAS pathology in cardiovascular diseases (Tikellis & Thomas, 2012). Therefore, increased expression and activity of ACE2 has been correlated with improvements in cardiovascular health while down-regulation of ACE2 is associated with adverse outcomes in multiple experimental models (Díez-Freire et al., 2006; Patel et al., 2014; Soler et al., 2007; Sriramula, Cardinale, Lazartigues, & Francis, 2011; Tikellis et al., 2008).

Reducing Ang II formation through ACE inhibition or blockage of angiotensin type I receptor (AT1R), has been the major therapeutic approaches to control RAS overactivity and consequently hypertension; indeed, a range of ACE inhibitors and AT1R antagonists have been developed and widely prescribed in clinical settings over the past two decades (Contreras et al., 2003; Donnelly, 1992). However, these antihypertensive agents are generally associated with significant side-effects over prolonged use, while many cases of essential hypertension only show modest benefits upon their use (Khanna, Lefkowitz, & White, 2008; Moser & Franklin, 2007). Therapeutic options, particularly using food-derived products or nutraceuticals, targeting

ACE2 are rare. Moreover, rationally designed ACE2 activators are still emerging on a languid pace. Some established anti-hypertensive drugs such as losartan and telmisartan have shown concomitant up-regulation of ACE2 expression (Klimas et al., 2015; Zhang et al., 2014; Zhong et al., 2011), while synthetic compounds like diminazine (4-[2-(4-carbamimidoylphenyl) iminohydrazinyl]benzenecarboximidamide dihydrochloride) and XNT (1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyloxy]-9H-xanthene-9-one) have been claimed as specific ACE2 activators (Hernández Prada et al., 2008; Ibrahim, Froemming, Omar, & Singh, 2014). Indeed, diminazine administration appears to reduce the severity of cardiovascular diseases in animal models suggesting the therapeutic potential of ACE2 activator (Fraga-Silva et al., 2015; Qi et al., 2013). However, these promising approaches have been insufficiently investigated for their safe therapeutic applications.

Food proteins are a rich source of bioactive peptides which may beneficially affect our health through modulation of various physiological systems including the RAS pathway (Wu, Liao, & Udenigwe, 2017). Indeed, previous work from our lab has identified several egg-derived ACE inhibitory peptides (Majumder & Wu, 2010). One of these peptides, IRW (Ile-Arg-Trp), exhibits additional anti-inflammatory and antioxidant effects in both cells and SHRs (Majumder, Chakrabarti, Morton, et al., 2013; Majumder, Chakrabarti, Davidge, & Wu, 2013). Its antihypertensive activity is associated with reduced Ang II and inflammation, as well as enhanced nitric oxide-mediated vasorelaxation (Majumder, Chakrabarti, Morton, et al., 2013). It was found by a transcriptomics study that IRW could significantly up-regulate the mRNA level of ACE2 in mesenteric artery, thus supporting its candidacy as an ACE 2 activator (Majumder et al., 2015). However, effect(s) of IRW on the ACE2 protein expression and enzyme activity remain elusive. Therefore, to bridge this research gap, this study was conducted to understand if

IRW could affect the protein expression and activity of ACE2. Experimentally, the potential of IRW as an ACE2 activator was first studied using purified human recombinant ACE2 (hrACE2, *in situ* cell free system), followed by rat aortic vascular smooth muscle cells (VSMCs), and in tissues of SHRs. A molecular docking study was also performed to investigate the interaction between IRW and ACE2.

3.2 Material and Methods

3.2.1 Reagents

Dulbecco's phosphate buffered saline (PBS) and dithiothreitol (DTT) were purchased from Sigma Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Penicillin-Streptomycin as well as Gentamicin was from Life Technologies (Carlsbad, CA, USA). Triton-X-100 was from VWR International (West Chester, PA, USA). The tripeptide IRW was synthesized by Genscript (Piscataway, NJ, USA). Peptide sequence and purity were validated by HPLC-MS/MS at the minimum requirements of 99% purity. The peptide was dissolved in PBS at a stock concentration of 10 mM, aliquoted and stored at -20 ℃ until further use.

3.2.2 *In vitro* (cell-free) ACE2 activity assay

In vitro ACE2 activity under cell-free system was measured as described by Hernández Prada *et al* (Hernández Prada et al., 2008). hrACE2 (R&D systems, Minneapolis, MN, USA) was incubated with different concentrations of IRW for 5 min before 50 µM of the ACE2 fluorogenic substrate Mca-Dnp-OH (R&D systems) was added. The reaction was performed in a total volume of 100 µL containing 10 nM hrACE2, 1 M NaCl, 75 mM Tris-HCl and 0.5 µM $ZnCl_2$, at the physiological pH (7.4). All assays were performed in triplicates and read at the interval of 30

sec for 30 min at 37 ℃ using SpectraMax M3 spectrophotometer (Molecular devices, Sunnyvale, CA, USA). Background of each measurement was subtracted from the observed readings. Relative fluorescence unit (RFU) of each time point was plotted to generate the kinetic curves. Maximum response under each IRW concentration was expressed as maximum RFU in percentage, with respect to the corresponding control and plotted to fit into sigmoidal concentration response curve, followed by calculation of EC_{50} value (the concentration eliciting 50% of maximal response) using PRISM 5 statistical software (Graph Pad Software, La Jolla, CA, USA).

3.2.3 Cell culture

Cell culture was performed according to our previous studies (Chakrabarti, Liao, Davidge, & Wu, 2017; Lin, Liao, Bai, Wu, & Wu, 2017; Wang, Liao, Nimalaratne, Chakrabarti, & Wu, 2018). A commercially available rat aortic VSMC cell-line, A7r5, was purchased from ATCC (cat# CRL-1444, Manassas, VA, USA). The cells between passage 3 and 10 were grown in DMEM supplemented with 10% FBS and 1% antibiotics (Penicillin-Streptomycin and Gentamicin) until they reached ~70% confluence. The confluent cells were then placed in a quiescing medium (DMEM + 1% FBS + 1% antibiotics) and treated with different concentrations of IRW (10 μM or 50 μM) for 24 hours.

3.2.4 Cellular ACE2 activity assay

ACE2 activity of A7r5 cells was measured by SensoLyte®390 ACE2 activity assay kit (Anaspec, Fremont, CA, USA) according to the manufacturer's instructions with slight modifications. Briefly, the cultured cells were detached and lysed using assay buffer containing 0.1% Triton-X100. Supernatant from the cell lysates was collected for analysis. ACE2 activity was measured

in a reaction system with 50 µL of sample and 50 µL of ACE2 substrate solution. All assays were read every 5 min for 30 min at 37 °C using SpectraMax M3 spectrophotometer (Molecular devices). Auto-fluorescence of each assay was subtracted from the measured values to generate final results. RFU of each sample was normalized to the corresponding total protein concentration, which was measured by Pierce™ BCA protein assay kit (Thermo Scientifc, Carlsbad, CA, USA).

3.2.5 Quantitative reverse transcription PCR (qRT-PCR)

TRIzol Reagent (Ambion, Carlsbad, CA, USA) was used for total RNA extraction from A7r5 cells *in vitro*. RNA from cell lysates was separated and precipitated by chloroform and isopropanol, respectively. Finally, RNA pellets were washed with 80% ethanol and solubilized in 50 µL of nuclease-free water. Quantity and quality of RNA were evaluated by Qubit® 3.0 Fluorometer (Invitrogen) and Agilent 2200 TapeStation (Agilent technologies, Santa Clara, CA), respectively. Only RNA samples with integrity number higher than 7.0 were eligible for the subsequent applications. SuperScript® II Reverse transcriptase (Invitrogen) was used for reverse transcription to synthesize complementary DNA. qPCR was performed by StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using TaqMan® gene expression assay (Applied Biosystems). Primers and probes (Table 3.1) were designed by Primer Express 3.0 (Applied Biosystems). Thermal conditions were as follow: 95 °C for 20 sec; 40 cycles at 95 °C for 1 sec, followed by annealing/extension for 20 sec at 60 °C. Fold change of ACE2 expression was calculated by using Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene based on cycle of threshold (CT) as follows: ΔCT= [CT (ACE2) - CT (GAPDH)], $\Delta\Delta CT$= [Δ CT (IRW) - Δ CT (Untreated)], fold change= $2^{-\Delta\Delta CT}$.

3.2.6 Western blotting

At the end of the experimental intervention(s), cell culture medium was removed, and the cells were lysed in boiling hot Laemmle's buffer containing 50 μM DTT (a reducing agent) and 0.2% Triton-X-100 to prepare samples for western blotting. These cell lysates were then run using 9% SDS-PAGE gel electrophoresis, transferred to nitrocellulose membranes and immunoblotted with antibodies against ACE2 (rabbit polyclonal antibody from Abcam, Cambridge, MA, USA) and α-tubulin (rabbit polyclonal antibody from Abcam). Anti-α-tubulin was used at 0.4 μg/mL, while anti-ACE2 was incubated at concentration of 1.5 μg/mL. Goat anti-rabbit IRDye 680RD or Donkey anti-rabbit IRDye 800CW (Licor Biosciences, Lincoln, NE, USA) were used as the secondary antibodies. The protein bands were detected by a Licor Odyssey BioImager (Licor Biosciences) and quantified through densitometry using the corresponding Odyssey v3.0 software (Licor Biosciences). Each ACE2 band was normalized to its corresponding band of the loading control (α-tubulin). Lysates from untreated cells were loaded onto every gel for comparative analysis. Data were expressed as the percentage change with respect to untreated control.

3.2.7 Immunostaining

Animal experiment was performed in SHR as described previously (Majumder, Chakrabarti, Morton, et al., 2013). In brief, male SHRs (16-17 weeks old) obtained from Charles River (Senneville, QC, Canada) were randomly assigned into two groups, untreated (vehicle, n=5) and IRW (15 mg/kg body weight, n=5); after 18 days of treatment, the rats were sacrificed, and the tissue/organs were collected for further analysis. The experimental procedures were approved by the University of Alberta Animal Welfare Committee (Protocol # 611/09/10/D) in accordance with the guidelines issued by the Canada Council on Animal Care and also adhered to the Guide

for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Kidney and aorta sections were prepared under -20 °C by Leica CM1900 Cryostat (Leica Biosystems, Heidelberger, Nussloch, Germany) and mounted on glass slides for analysis. During the experiment, tissue sections were fixed by cold acetone and blocked by 1% bovine serum albumin (BSA) in PBS for 1 hour. The sections were immunostained with anti-ACE2 antibody (rabbit polyclonal antibody from Abcam) at a final concentration of 3 µg/mL in PBS overnight at 4 °C. Upon washing, the sections were incubated with goat anti-rabbit IRDye 680RD (Licor Biosciences) secondary antibody at room temperature for 30 min. Later, the sections were washed and mounted on a coverslip with PBS. The slides were then scanned by Odyssey Sa Imaging System (Licor Biosciences) at a resolution of 20 µm. The intensity (maximum and minimum) of all the images were adjusted to a similar level by Image Studio lite Ver 5.0 software (Licor Biosciences). The data obtained were expressed as mean fluorescence intensity (MFI) as measured by Image J software (http://imagej.net/Welcome). For each image, MFI of the corresponding secondary antibody control was subtracted to acquire normalized results.

3.2.8 Molecular docking

The structure of the human ACE2 (PDB: Q9BYF1) was retrieved from the PDB (Towler et al., 2004). The protein was loaded and analyzed using University of California, San Francisco Chimera version 1.9 software (University of California, San Francisco, CA) (Pettersen et al., 2004). The torsional root and branches of the ligands were chosen with the same program, allowing flexibility for all rotational bonds (Pettersen et al., 2004). Docking calculations with respect to the active zinc site were performed using the AutoDock Vina software (Molecular Graphics Lab at The Scripps Research Institute) (Trott & Olson, 2009). A grid box of 12Å^3 and

centered at the binding site was used to calculate the atom types needed for the calculation. A total of five runs were performed with exhaustiveness of 50. The best binding mode of each molecule was selected based on the lowest binding free energy and best peptide binding score. The 3D figures were generated using PyMOL Molecular Graphics System (DeLano Scientific LLC, Palo Alto, CA, USA).

3.2.9 Statistical analysis

Data were presented as mean ± SEM (standard error of mean) of 3 to 5 independent experiments. For 2 groups, data were analyzed by an unpaired *t*-test, while for 3 or more groups, a one way analysis of variance (ANOVA) with the Turkey's post-hoc test was used for determination of statistical significance. For the cell-free and cellular ACE2 kinetics studies, two way ANOVA was used with Turkey's post-hoc test. The PRISM 5 statistical software (GraphPad Software) was used for all analyses. The $P<0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 IRW activates ACE2 under cell-free conditions *in vitro*

Different concentrations of IRW (10^{-7} M to 10^{-3} M) were used to evaluate their effect on hrACE2 activity under cell-free conditions. ACE2 activity was enhanced *in vitro* by IRW in a dose-dependent manner ranging from 10^{-7} M to 10^{-3} M (Figure 3.1A). The maximum response of hrACE2 was not affected by IRW additions at concentrations ranging from 10^{-10} M to 10^{-7} M, but increased linearly from 10^{-4} M to 10^{-13} M, and then started to saturate at the higher IRW concentrations (Figure 3.1B). The EC_{50} value of IRW for hrACE2 activation was 7.24×10^{-5} M.

3.3.2 IRW enhances ACE2 activity in cultured VSMCs

A7r5 cells, a rat aortic VSMC cell line, was used to investigate the effects of IRW treatment on cellular ACE2 activity. IRW dosages of 10 and 50 μM were selected based on our previous study (Majumder, Chakrabarti, Davidge, et al., 2013). Fluorescence formation from the ACE2-mediated hydrolysis of fluorogenic substrate was increased within 30 min and the accumulative fluorescence levels were significantly higher in both 10 μM and 50 μM IRW treated groups, compared with the untreated control group. However, there was no difference noted between 10 μM and 50 μM treated groups (Figure 3.2).

3.3.3 IRW up-regulates ACE2 mRNA and protein expressions in VSMCs

Since increased cellular ACE2 activity could be due to up-regulation of ACE2 expression as well as direct activation of pre-existing ACE2, we then examined the effects of IRW treatment on ACE2 expression. It was found that both the mRNA and protein levels of ACE2 in A7r5 cells could be significantly up-regulated by 50 μM of IRW treatment; while, lower concentration of IRW (10 μM) did not have effect (Figures 3.3A and 3.3B). Our results suggested that up-regulation of ACE2 protein expression in A7r5 cells was likely mediated by an increased mRNA expression.

3.3.4 IRW-treated rats show increased tissue ACE2 expression *in vivo*

Finally, we examined the effects of 18-day oral administration of IRW on ACE2 expression in SHRs. Tissues collected from these animals (kidney and aorta) showed a significant up-regulation of ACE2 expression upon IRW treatment (Figures 3.4A and 3.4B). These results demonstrated that the oral administration of ACE could enhance the tissue specific expression of ACE2.

3.3.5 Interaction of IRW with ACE2 is mediated by hydrogen bond of ACE2 subdomain

As documented, a potent ACE2 inhibitor, MLN-4760 binds with the active Zn site, which offers us an insight of ACE2 structure and function (Joshi, Balasubramanian, Vasam, & Jarajapu, 2016; Towler et al., 2004). While, our results showed that IRW, unlike MLN-4760, could interact via hydrogen bonding with subdomains of ACE2 instead of the active metal site (Figures 5A and 5B), which might explain the observed *in vitro* and *in vivo* ACE2 activating potential. Specifically, IRW might interact with subdomain I near the active site, composed of key residues 19-102, 290-397, and 417-430, which modulate its activity (Joshi, Balasubramanian, Vasam, & Jarajapu, 2016). Such interaction might be mediated by the hydrogen bonds as shown in Figure 5C (i: 2H-bonds, ii: 3H-bonds and iii: 6H-bonds).

3.4 Discussion

The RAS plays an essential role in the pathogenesis of hypertension. ACE is the key enzyme for the generation of Ang II, a well-known vasoconstrictor contributing to increased vascular tone and blood pressure. In the year of 2000, the classical view on RAS was evolved with the identification of ACE2 (Donoghue et al., 2000). Structurally, ACE2 contains a single HEXXH zinc-binding domain which is 42% identical to the corresponding domain of ACE (Towler et al., 2004). However, ACE2 functions in contrast to ACE as it metabolizes Ang II into smaller non-hypertensive metabolites such as Ang (1-7) and Ang (1-9) (Donoghue et al., 2000). As indicated in multiple studies, ACE2 is involved in counterbalancing the detrimental effects of Ang II and exerting protective effects such as antihypertensive, antioxidant and anti-inflammatory functions within and beyond the cardiovascular system (Imai et al., 2005; Silveira et al., 2010; Zhang, Chen, Zhong, Gao, & Oudit, 2014). Taken together, ACE2 is an important negative regulator of RAS pathology which could be a novel therapeutic target for hypertension and related diseases.

IRW, a bioactive tripeptide derived from the egg white protein ovotransferrin, was initially characterized as an ACE inhibitor through *in vitro* assay (Majumder & Wu, 2011). Subsequent studies further established its antioxidant, anti-inflammatory and antihypertensive potential (Huang et al., 2010; Majumder, Chakrabarti, Morton, et al., 2013). Findings from the current study demonstrate additional novel effects of IRW as a direct regulator of ACE2. IRW could activate purified ACE2 with an EC_{50} value of 7.24×10^{-5}M (Figure 3.1), which is within the same magnitude as the synthetic ACE2 activator XNT with an EC_{50} value of 2.01×10^{-5}M (Hernández Prada et al., 2008). As ACE2-activating effect of IRW was demonstrated by hrACE2, molecular docking was then conducted to understand its interaction with ACE2. At the molecular level, there are two subdomains, I and II, in the active site domain (residues 19–611) of ACE2 (Towler et al., 2004). These subdomains form a deep cleft that is proposed to be the active site for substrate binding and catalysis. Unlike MLN-4760, an ACE2 inhibitor, which binds to both subdomains, causes the hinge to flex and shrinks the cleft, thus preventing substrate binding (Towler et al., 2004), IRW only binds to subdomain I (Figure 3.5). The lack of shrinking of cleft due to single domain binding may be the mechanism of IRW-mediated ACE2 activation. The difference in the molecular interaction of IRW and MLN-4760, an established ACE2 antagonist, suggests the use of IRW as a lead molecule towards the development of other ACE2 protagonists. Indeed, a few ACE2-activating agents have been reported to date. Although diminazene and XNT, the two drugs claimed as ACE2 activators were identified by structure-based compound screening, however, the effects of these pharmaceutical agents on ACE2 activation remain controversial (Haber et al., 2014). Among food-derived peptides, Ile-Pro-Pro (IPP), a casein-derived tripeptide with anti-hypertensive and anti-diabetic potential, was shown to improve Ang (1-7) levels in mesenteric artery of SHR, but a potential inclusive role of ACE2

was not examined (Ehlers, Nurmi, Turpeinen, Korpela, & Vapaatalo, 2011). The ACE2-activating effect of IRW was further demonstrated on A7r5 cells, an aortic VSMC cell line. Enzymatic activity, mRNA and protein levels of ACE2 were enhanced in A7r5 cells following IRW treatment (Figures 3.2 and 3.3). As VSMCs are vital participants in blood pressure regulation (Touyz & Schiffrin, 2000), increased ACE2 activity, and expression in these cells may have a direct beneficial effect on vascular tone and consequently on blood pressure. In our previous study, IRW was shown to exert anti-inflammatory and antioxidant effects on vascular endothelial cells, suggesting its role in modulating inflammation related endothelial dysfunction, a vital contributor to both hypertension and vascular damage (Majumder, Chakrabarti, Davidge, et al., 2013). Enhancement of ACE2 levels in A7r5 cells by IRW indicates the positive effect of this peptide on VSMCs, which warrants a greater range of its beneficial effects in different components of the vascular system. According to the *in silico* study, IRW could activate ACE2 via the interaction with the subdomain of ACE2 through hydrogen bond. The increase in ACE2 activity could accelerate the degradation of Ang II, thus mitigating the detrimental effect of Ang II. On the other hand, IRW could up-regulate ACE2 expression in A7r5 cells. Indeed, up-regulation of ACE2 is associated with attenuation of oxidative stress and inflammation (Rodrigues Prestes, Rocha, Miranda, Teixeira, & Simoes e Silva, 2017). Hence, further studies aim to explore the contribution of ACE2 up-regulation towards anti-oxidant and anti-inflammatory activities of IRW in both VSMCs and SHRs.

Further, it is essential to note that a significant increase in ACE2 protein expression was also observed in both kidneys and aorta of SHRs following oral administration of IRW (Figure 3.4). The importance of kidneys in maintaining normal blood pressure has been well recognized (Dilauro & Burns, 2009), as they are important sites for the action of ACE2 on Ang II

metabolism and Ang (1-7) generation. Increased ACE2 expression in the kidneys of SHRs suggested the possible role of IRW in shifting the balance from vasoconstrictor/pro-inflammatory actions of Ang II towards cytoprotective Ang (1-7). IRW was also shown to increase ACE2 protein expression in the aorta of SHRs, which further confirmed its effects on vascular cells and *in vivo*. In addition to increased expression of ACE2 mRNA level by IRW treatment (Majumder et al., 2015), this study further confirmed ACE2 protein was also significantly increased upon IRW treatment. These results show that the enhancement of ACE2 expression in vascular tissues of SHRs may directly contribute to the reduction in blood pressure.

In conclusion, findings from this study demonstrate the ability of IRW to activate ACE2 as well as directly up-regulate ACE2 expression *in vivo*. This study presents IRW as the first food-derived bioactive peptide identified till date as a direct ACE2 activator. ACE2 activation appears to be a novel mechanism underlying antihypertensive effect of tripeptide IRW, indicating the potential to develop new opportunities for management of hypertension. However, the direct contribution of increased ACE2 activity/expressions in SHRs towards the reduction of blood pressure remains unclear. Thus, the understanding of the underlying molecular mechanisms related to the IRW-ACE2 mediated reduction of blood pressure in SHRs requires further investigation.

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Table 3.1: Primers and probes sequences for qRT-PCR

Genes	Primers/Probes	Sequences
ACE2	Forward primer	5'-TTGAACCAGGATTGGACGAAA-3'
	Reverse primer	5'-GCCCAGAGCCTACGATTGTAGT-3'
	Probe	5'-FAM-AATGGCAACAAGCACA-MGB-NFQ-3'
GAPDH	Forward primer	5' -CCTGGAGAAACCTGCCAAGTAT-3'
	Reverse primer	5'-CTCGGCCGCCTGCTT-3'
	Probe	5'-FAM-ATGACATCAAGAAGGTGG-MGB-NFQ-3'

Figure 3.1: IRW activates ACE2 under cell-free *in vitro* conditions. (A) Dose-dependent hrACE2 activity in the presence of IRW. Data represented as mean±SEM values of 4 independent experiments. (B) Maximal response of hrACE2 with different concentrations of IRW. Results were expressed as mean±SEM of 4 independent experiments; maximal response was calculated as percentage ratio in comparison to the control (in the absence of IRW). * and **** indicate P<0.05 and P<0.0001, respectively, as compared to the control.

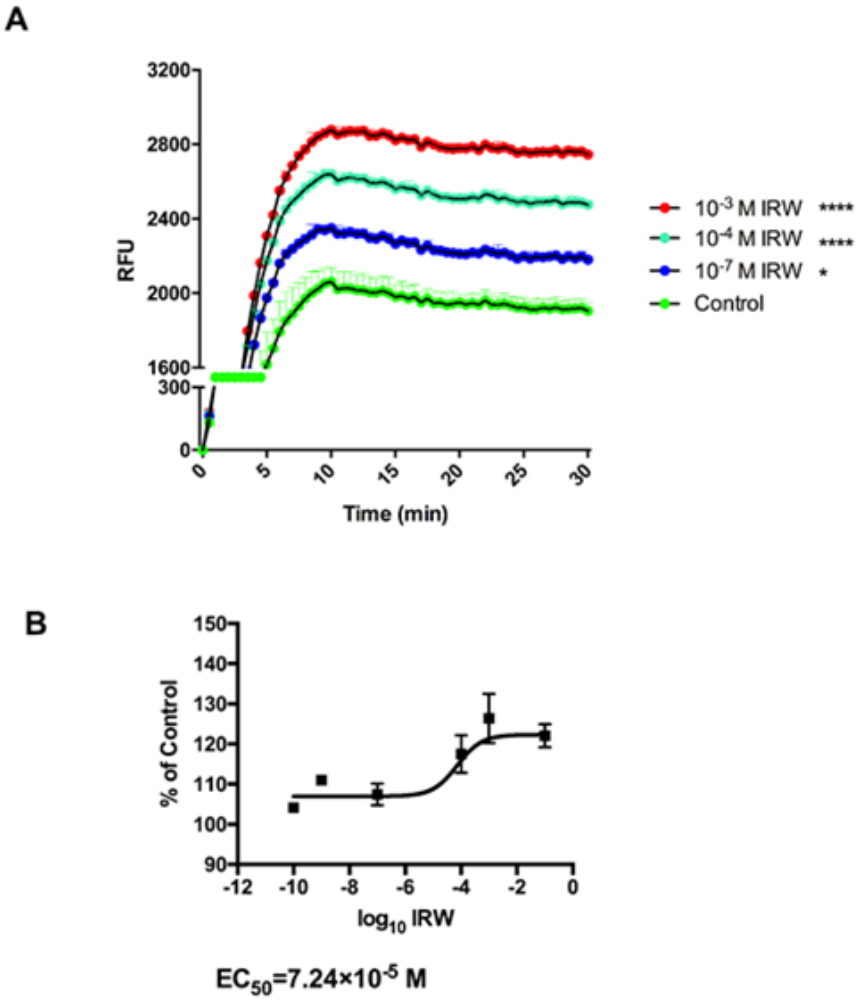


Figure 3.2: IRW increases ACE2 activity in A7r5 cells. Confluent monolayers of A7r5 cells were treated with different doses of IRW for 24 hrs prior to cell lysis for assessing ACE2 activity. RFU normalized by total protein concentration at each time point was plotted to represent ACE2 activity of A7r5 cells. Data represented as mean±SEM of 4 independent experiments. * and **indicate P<0.05 and P<0.01, respectively, as compared to the untreated control.

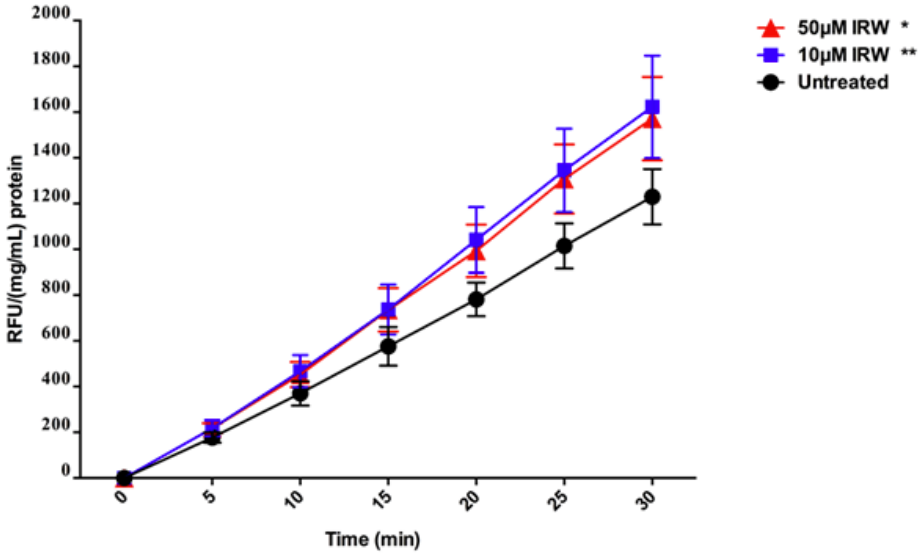


Figure 3.3: ACE2 expression of A7r5 cells is enhanced by IRW treatment. Confluent monolayers of A7r5 cells were treated with different doses of IRW for 24 hrs. (A) RNA was extracted from these cells and used in qRT-PCR for estimation of ACE2 gene upregulation. Results were normalized to corresponding values of the housekeeping gene and expressed as fold change over the untreated control. (B) Cells were lysed at the end of the experimental period and lysates were used in western blotting for ACE2 and α-tubulin (loading control). Bands were quantified by densitometry, normalized to the loading control and results were expressed as % of the untreated group value. Data represented as mean±SEM from 4 independent experiments. * and ** indicate P<0.05 and P<0.01, respectively, as compared to the untreated control.

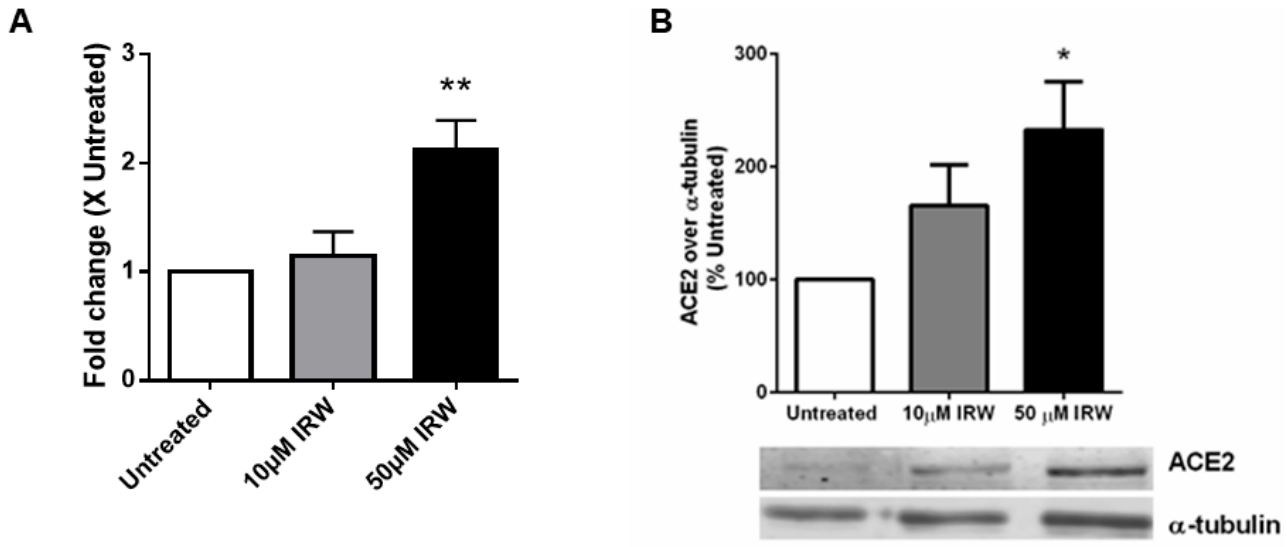


Figure 3.4: Oral IRW administration increases ACE2 expression in kidney and aorta of SHRs. SHRs were orally fed with IRW (15 mg/kg BW) for 18 consecutive days. Following sacrifice, kidney and aorta were collected, frozen, sectioned and immunostained for ACE2. A set of representative images from kidney (A) and aortic (B) sections are shown. Data represented as mean±SEM of fluorescence intensity from sections of 3-5 animals per treatment group. * and *** indicate P<0.05 and P<0.001 respectively, as compared to the untreated group.

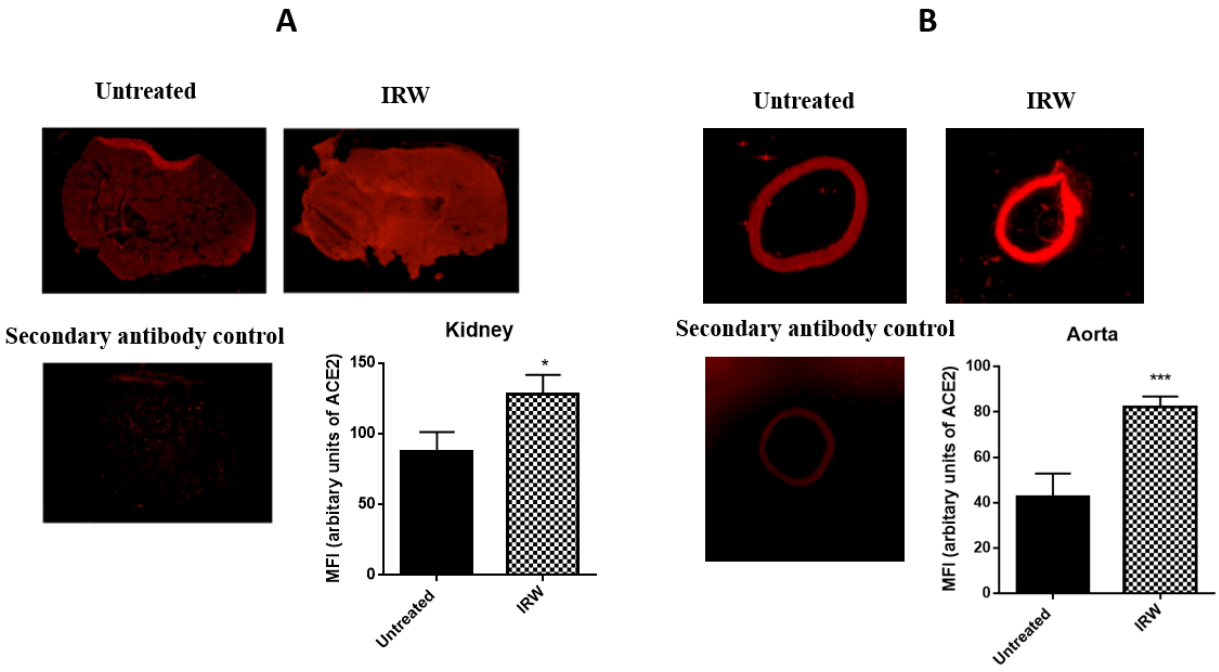
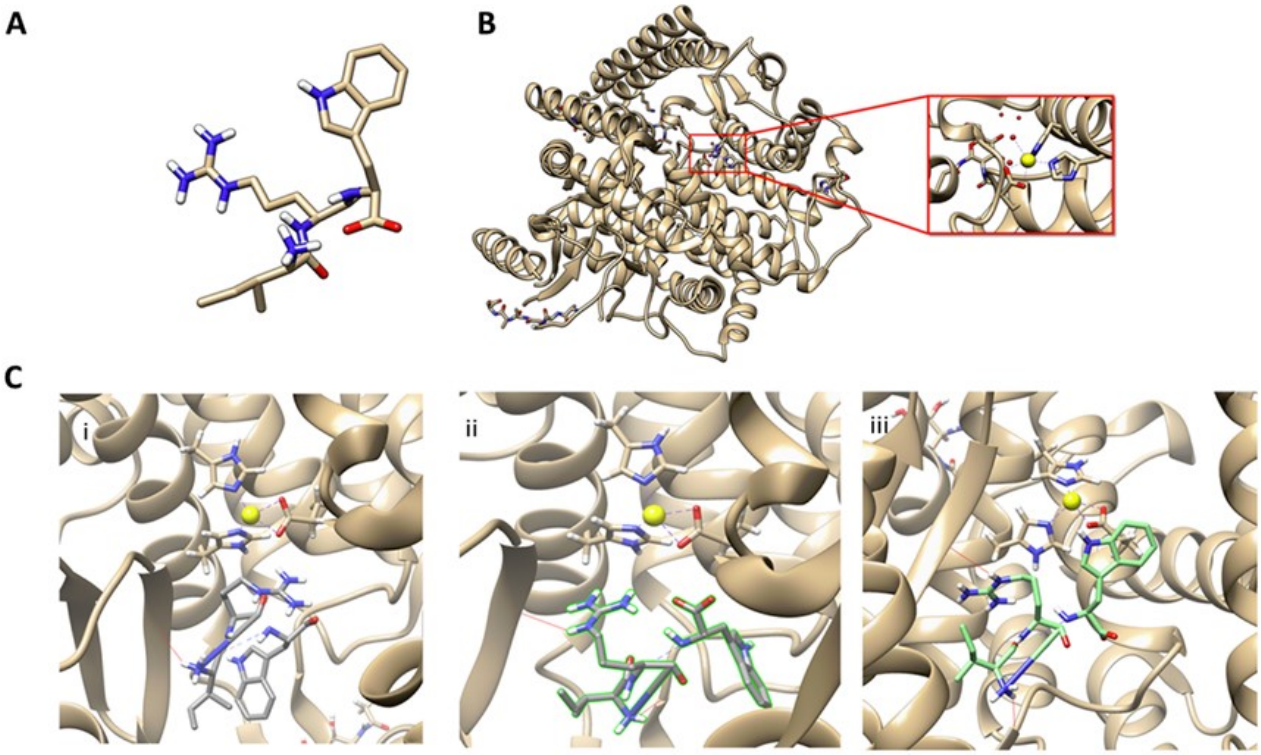


Figure 3.5: Interaction of IRW with ACE2 is mediated by hydrogen bond of ACE2 subdomain. (A) Structure of IRW (B) Schematic of the ACE2 active site (C) Representation of the active site zinc ion is shown as a yellow sphere. Three predicted binding modes of IRW (elemental colors) based on hydrogen bonding with hinge region near active site (i), IRW interacts near the active site of ACE2 with 2 hydrogen bonds (red), RMSDu.b_5.258 (ii), three hydrogen bonds (red), RMSDu.b_2.36 (iii) and six hydrogen bonds, RMSDu.b_5.073. All hydrogens were omitted for clarity. The dotted lines represent distances for the formation of hydrogen bonds.



CHAPTER 4[3] - Modulatory Effects of IRW on Intracellular Events in Vascular Smooth Muscle Cells against Angiotensin II Stimulation

[3] This chapter has been published: Liao, W., Chakrabarti, S., Davidge, S. T., & Wu, J. (2016). Modulatory effects of egg white ovotransferrin-derived tripeptide IRW (Ile-Arg-Trp) on vascular smooth muscle cells against angiotensin II stimulation. *Journal of Agricultural and Food Chemistry*, *64*(39), 7342-7347.

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4.1 Introduction

Vascular smooth muscle cell (VSMC) is a highly specialized cell in vasculature. By contraction and relaxation, VSMC can alter the luminal diameter, enabling blood vessels to maintain an appropriate blood pressure (Douglas & Channon, 2018). Physiologically, synthetic VSMC and quiescent VSMC represent the two ends of a spectrum of VSMCs with intermediate phenotypes. In normal conditions, quiescent VSMC is the predominant phenotype. While, in response to stimulus, VSMC could transform from quiescent phenotype to synthetic phenotype, leading to vascular remodelling (Rudijanto, 2007), which is a common feature among major cases of hypertension (Owens, Kumar, & Wamhoff, 2004). Excessive proliferation, inflammation, oxidative stress and migration are typical intracellular events in VSMCs during vascular remodelling (Touyz & Schiffrin, 2000).

The renin angiotensin system (RAS) plays an essential role in blood pressure regulation, in which, angiotensin II (Ang II) is a potent vasoconstrictor in the RAS. Indeed, Ang II plays multiple roles in controlling the functional and structural integrity of the vascular wall (Higuchi et al., 2007). In VSMCs, over stimulation of Ang II could lead to aberrant proliferation (Petrovic et al., 2005), excessive superoxide production (Jinno et al., 2004), inflammation (Higuchi et al., 2007), and migration which would further promote vascular remodelling, playing pivotal roles in atherosclerotic diseases (Touyz & Schiffrin, 2000). Effects of some angiotensin type I receptor (AT1R) antagonists in mitigating vascular remodelling have been reported (Jinno et al., 2004; Petrovic et al., 2005).

Functional food and nutraceuticals developed from food protein-derived bioactive peptides may provide new opportunities for treatment and prevention of cardiovascular diseases (Udenigwe &

Aluko, 2011; Cicero, Fogacci, & Colletti, 2016). IRW (Ile-Arg-Trp), characterized from egg white ovotransferrin by our lab is one of the most potent antihypertensive peptides (Majumder & Wu, 2011). Followed by the validation of its blood pressure-lowering effect in spontaneously hypertensive rats (SHRs), mechanisms underlying its antihypertensive activity have been addressed mainly including ameliorations of vascular oxidative stress and inflammation, as well as improvement of endothelial dysfunction (Majumder, Chakrabarti, Morton, et al., 2013). Specific roles of IRW in regulating functions of vascular endothelial cells have been elucidated as well including reducing oxidative stress and attenuating inflammation through nuclear factor κB (NF- κB) pathway (Majumder, Chakrabarti, Davidge, & Wu, 2013). However, effects of IRW on VSMCs have not been investigated.

As discussed in Chapter 3, IRW could activate and up-regulate ACE2 in VSMCs, which appears to be a novel antihypertensive mechanism of IRW. As ACE 2 up-regulation is associated with antioxidant and anti-inflammatory effects in vasculature (Zhang, Chen, Zhong, Gao, & Oudit, 2014), it is unknown if the IRW-mediated ACE2 up-regulation in VSMCs could contribute to down-stream beneficial effects against Ang II stimulation.

Given this background, it was aimed to investigate effects of IRW on proliferation, oxidative stress inflammation and migration in Ang II-stimulated VSMCs in this study. The involvement of ACE2 up-regulation in the modulatory effects of IRW were studied as well.

4.2 Materials and Methods

4.2.1 Reagents

Dulbecco's phosphate buffered saline (PBS), Ang II and dithiothreitol (DTT) were from Sigma Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), Opti-MEM and

fetal bovine serum (FBS) were purchased from Gibco/ Invitrogen (Carlsbad, CA, USA). Penicillin-Streptomycin as well as Gentamicin was from Life Technologies (Carlsbad, CA, USA). Triton-X-100 was from VWR International (West Chester, PA, USA). The tripeptide IRW was synthesized by Genscript (Piscataway, NJ, USA). Peptide sequence and purity (99.8 %) were validated by HPLC-MS/MS. The peptide was dissolved in PBS at a stock concentration of 10 mM, aliquoted and stored at -20^{o}C until use.

4.2.2 Cell culture

Rat aortic VSMC cell line A7r5 was purchased from ATCC (cat# ATCC CRL-1444, Manassas, VA, USA). The cells were grown in DMEM supplemented with 10% FBS and antibiotics (Penicillin-Streptomycin and Gentamicin) until they reached 80% confluence. For experiments, the confluent cells were placed in a quiescing medium (DMEM + 1% FBS + antibiotics) and then treated with 50 µM of IRW with or without AT1R antagonist losartan potassium (Tocris, Oakville, ON, Canada) or mas receptor (MasR) antagonist A779 (Cambridge, MA, USA) 1 h prior to the addition of 1 µM of Ang II for different time periods. Cells between passages 4 and 11 (since receiving) were used for all experiments.

4.2.3 ACE2 knockdown

Lipofectamine 2000 transfection reagent (Invitrogen) and small interference RNA (siRNA) (Invitrogen) were used for silencing ACE2 in A7r5 cells. Cells with 50% of confluence were placed in non-antibiotic DMEM with 10% FBS for overnight. Then, the cell culture media was replaced with serum-reduced Opti-MEM media. ACE2 siRNA (80 pmol) containing 0.8 µL of transfection reagent was treated to each targeting well for ACE2 knockdown. For the other wells, same amount of a random siRNA (Invitrogen) was treated. After incubation for 6 hours, the

serum-reduced Opti-MEM media was replaced by non-antibiotic DMEM with 10% FBS for 24 hours. The knockdown efficiency was at least 70%. After the ACE2 knockdown assay, cells were placed in the non-antibiotic quiescing medium (DMEM + 1% FBS) and then treated with 50 μM of IRW 1 h prior to 1 μM of Ang II for different time periods.

4.2.4 Cell proliferation assay

Cell proliferation was evaluated by bromodeoxyuridine (BrDU) incorporation assay. A7r5 cells were seeded into a 48 well plate and grown in DMEM with 10% of FBS for overnight. After the medium was replaced by quiescing medium, 50 μM of IRW was added 1 h prior to stimulation of Ang II (1 μM). The co-treatment period with IRW and Ang II was 23 h. Afterwards, BrDU reagent (Invitrogen) diluted in quiescing medium at a final concentration of 1% was added for 1 h incubation at 37 ℃. Then the cells were fixed by 70% ethanol (20 min, room temperature). The antigen was exposed by incubation with 1M hydrochloric acid (20 min, room temperature) followed by permeabilization by 0.1% Triton-X- 100 in PBS (5 min, room temperature) and finally, blocking was done in 1% bovine serum albumin (BSA) in PBS (60 min, room temperature). Mouse monoclonal antibody against BrDU (1:1000; Cell Signaling, Beverly, MA, USA) was diluted in PBS with 0.1% BSA and incubated at 4 °C overnight. Upon washing, cell was treated with anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA) for 30 minutes in the dark. Nuclei were stained with the Hoechst33342 nuclear dye (Molecular Probes). Visualization was performed by Olympus IX81 fluorescent microscope (Carson Scientific Imaging Group; Markham, ON, Canada). For each data point, 3 fields were randomly selected, and the number of nuclei counted. The percentage of nuclei positive for BrDU stain was noted in each field and the mean value was calculated.

4.2.5 Superoxide detection

Cellular superoxide generation was detected by dihydroethidium (DHE) staining similar to our previous work (Huang et al., 2010). Reactive oxygen species (ROS) could react with DHE to form ethidium, which then binds to nuclear DNA and release nuclear fluorescence (Peshavariya, Dusting, & Selemidis, 2007). A7r5 cells were seeded into 48 well plate and grown in DMEM with 10% of FBS until confluent. After the medium was replaced by quiescing medium, 50 μM of IRW was added 1 h prior to stimulation of Ang II (1 μM). The co-treatment period of IRW and Ang II was 30 min. Then cells were treated with 20 μM of DHE and incubated in dark for 30 min. Afterwards, the cells were washed 3 times. Fluorescence signal was detected by Olympus IX81 fluorescent microscope (Carson Scientific Imaging Group). For each data point, images from 3 random fields were taken. Mean fluorescence intensity (MFI) of each image was quantified by ImageJ software (http://imagej.net/Welcome). MFI/cell was calculated based on the cell number in each field. Results were presented as % of the untreated group.

4.2.6 Western blotting

At the end of experiment, the A7r5 cells were lysed in boiling hot Laemmle's buffer containing 50 mM DTT (a reducing agent) and 0.2% Triton-X-100 to prepare samples for western blotting. These cell lysates were then run in 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and immunoblotted with antibodies. Protein bands for cyclooxygenase 2 (COX2, Abcam), inducible nitric oxide synthase (iNOS, BD Biosciences, San Jose, CA, USA), matrix metallopeptidase 9 (MMP9, NOVUS Biologicals, Littleton, CO, USA), inhibitory κBα (IκBα, Cell Signaling, Danvers, MA, USA), ACE2 (Abcam), and MasR (NOVUS Biologicals) were normalized to the loading control α-tubulin (Abcam).

For intracellular signalling study, the co-treatment time of IRW and Ang II was 15 min. Protein samples were harvested and run in SDS-PAGE, followed by membrane transfer as indicated above. Phospho-p65 (p-p65, Cell Signaling), phospho-extracellular signal-regulated kinases 1/2 (p-ERK1/2, Cell Signaling), phspho-p38 (p-p38, NOVUS Biologicals) and phospho-c-Jun N-terminal Kinase (p-JNK, NOVUS Biologicals) were normalized to the corresponding total form "total" (Total p65, Santa Cruz Biotechnology, Dallas, TX, USA; Total ERK1/2, Cell Signaling; p38, Santa Cruz Biotechnology; JNK, NOVUS Biologicals). Goat anti-rabbit IRDye 680RD or Donkey anti-mouse 800CW from Licor Biosciences (Lincoln, NE, USA) was used as the secondary antibody. Protein bands were detected by Licor Odyssey BioImager (Licor Biosciences) and quantified by densitometry using Image Studio Lite 5.2 (Licor Biosciences). Cell lysates from untreated cells were loaded on every gel and all data were expressed as % of the corresponding untreated.

4.2.7 Cell migration assay

Migration of A7r5 cells was evaluated by CytoSelect^{TM} wound healing assay kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manual. Briefly, cells were seeded into a 24-well plate with a wound healing insert in the middle of each well. Cells were cultured for overnight until the monolayer forms. The wound healing insert was removed to create a 0.9 mm-wound healing area. Cells then were treated with 50 μM of IRW combined with 100 μM of AT1R antagonist losartan potassium (Tocris) 1 h before the addition of 1 μM Ang II. The co-treatment period of IRW, AT1R antagonist and Ang II was 4 h (Greene, Lu, Zhang, & Egan, 2001). Afterwards, cells were fixed by fixation solution and cell nucleus were stained by DAPI fluorescence stain. Cells were visualized by Olympus IX83 fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan). Distance of the wound area was measured by ImgeJ software

(https://imagej.net/Welcome). Less distance indicated a higher migration ability of the cells. Results of each treatment group were normalized to the corresponding untreated.

4.2.8 Statistics

All data were presented as mean ± SEM (standard error of mean) of 4 to 6 independent experiments. Data were analyzed by one way analysis of variance (ANOVA) coupled with Tukey post-hoc test. The PRISM 5 statistical software (GraphPad Software, Inc., La Jolla, CA, USA) was used for all analyses. $P<0.05$ was considered to be significant.

4.3 Results

4.3.1 Modulatory effects of IRW on intracellular events in Ang II-stimulated A7r5 cells

As expected, stimulation with 1 μM of Ang II for 23 h significantly increased the proliferation of A7r5 cells (Figure 4.1A). Pre-treatment with IRW significantly decreased the percentage of BrDU positive cells (Figure 4.1A), indicating the capability of this peptide in inhibiting the accelerated proliferation of VSMCs after Ang II stimulation.

Next, effects of IRW on the intracellular superoxide generation with Ang II stimulation was examined. The ROS mediated fluorescence was significantly enhanced by Ang II stimulation, which could be abolished by IRW pre-treatment (Figure 4.1B). Interestingly, IRW alone did not show a significant effect on the superoxide level in A7r5 cells (Figure 4.1B). These results suggested IRW could exert antioxidant effect in VSMCs against Ang II stimulation without affecting the basal ROS level.

Then, anti-inflammatory effect of IRW in Ang II-stimulated A7r5 cells was tested, using COX2 and iNOS as pro-inflammatory biomarkers (Hirafuji et al., 2002). Expressions of both COX2

and iNOS were up-regulated by Ang II stimulation (Figure 4.1C). Treatment of IRW could inhibit Ang II-stimulated up-regulations of both pro-inflammatory molecules (Figure 4.1C), indicating the anti-inflammatory effect of IRW in Ang II-stimulated VSMCs.

MMP9, a key member in MMP family, mediates the degradation of extracellular matrix (ECM) surrounding VSMCs and facilitates migration of VSMCs (Jin et al., 2008). Therefore, MMP9 was used as the biomarker to evaluate the anti-migrant potential of IRW in Ang II-stimulated A7r5 cells. As expected, Ang II could significantly increase the expression of MMP9 in A7r5 cells (Figure 4.1D). While, treatment of IRW could abolish the up-regulation of MMP9 by Ang II (Figure 4.1D). The down-regulation of MMP9 by IRW in Ang II-stimulated A7r5 cells indicated the potential of IRW in inhibiting migration of VSMCs against Ang II stimulation.

Taken together, IRW showed anti-proliferative, antioxidant, anti-inflammatory and anti-migrant activities in Ang II-stimulated A7r5 cells.

4.3.2 Nuclear factor κB (NF-κB) and p38 mitogen-activated protein kinases (p38 MAPK) signaling were involved in the modulatory effects of IRW in Ang II-stimulated A7r5 cells

MAPKs are involved in the Ang II-mediated signalling in VSMCs (Touyz & Schiffrin, 2000). In the next, signal transduction of MAPK in A7r5 cells was investigated. Stimulation of Ang II could increase phosphorylation ratios of ERK1/2, p38 and JNK significantly (Figure 4.2A), which are key members of MAPKs. However, treatment of IRW could only reduce the phosphorylation of p38 (Figure 4.2A), which suggested that p38 MAPK was, at least partially, involved in the modulatory effects of IRW in Ang II-stimulated A7r5 cells.

COX2 and iNOS are pro-inflammatory molecules associated with NF-κB signaling (Tak & Firestein, 2001). It was examined IRW's effect on the modulation of the key components of NF-

κB signaling. IRW could restore the Ang II-stimulated degradation of IκBα (Figure 4.2B) as well as decrease phosphorylation of p65 (Figure 4.2B). Since IκBα degradation and p65 phosphorylation are both critical steps in NF-κB signaling (Viatour, Merville, Bours, & Chariot, 2005), these results indicated NF-κB signaling was attenuated by IRW in Ang II-stimulated VSMCs, which was analogous to its anti-inflammatory effect on vascular endothelial cells as we found in our previous study (Majumder, Chakrabarti, Davidge, et al., 2013).

4.3.3 IRW up-regulated ACE2 and MasR expressions in Ang II-stimulated A7r5 cells

As discussed in Chapter 3, IRW could upregulate mRNA and protein levels of ACE2 in non-stressed A7r5 cells. While, it is not known if IRW could show comparable effects in Ang II-stimulated A7r5 cells. It was found in this chapter that Ang II could slightly increase the protein expression of ACE2 in A7r5 cells, which might be a compensatory response of the cells towards Ang II stimulation (Figure 4.3A). While, treatment of IRW could further boost the expression of ACE2, contributing to a significant difference (Figure 4.3). More importantly, consistent trends were observed on the expression of MasR (Figure 4.3), which indicated the up-regulation of MasR by IRW treatment might be mediated by the increased expression of ACE2. The above results suggested that both of ACE2 and MasR expressions in Ang II-stimulated A7r5 cells could be up-regulated by IRW treatment, which might contribute to downstream beneficial effects against Ang II stimulation found in this chapter.

4.3.4 IRW up-regulated ACE2 contributed to antioxidant and anti-inflammatory activities of IRW in Ang II-stimulated A7r5 cells

As up-regulation of ACE2 is associated with antioxidant, anti-inflammatory and anti-migrant effects in vasculature (Zhang et al., 2014). It was then examined the contribution of ACE2-up-

regulation to antioxidant, anti-inflammatory and anti-migrant activities of IRW in Ang II-stimulated A7r5 cells. It was found that the addition of MasR antagonist A779 abolished the anti-oxidant activity of IRW (Figure 4.4A). Besides, expression of COX2, but not iNOS, was restored by the addition of A779 (Figure 4.4B), indicating up-regulation of ACE2 partially contributed to the anti-inflammatory activity of IRW in Ang II-stimulated A7r5 cells. However, treatment of A779 did not affect MMP9 expression (Figure 4.4C), suggesting the down-regulation of MMP9 by IRW was independent from the up-regulation of ACE2.

4.3.5 ACE2 knockdown abolished antioxidant and anti-inflammatory activities of IRW in Ang II-stimulated A7r5 cells

The correlations of IRW-upregulated ACE2 with antioxidant and anti-inflammatory activities were further evaluated by ACE2 knockdown. It was found that ACE2 knockdown totally abolished the antioxidant activity of IRW. Meanwhile, ACE2 knockdown could only restore the expression of COX2, but not iNOS (Figure 4.5A), in Ang II-stimulated A7r5 cells upon IRW treatment. These results were consistent with findings from the experiments using MasR antagonist A779 treatment (Figures 4.4 A&B), further confirming that up-regulation of ACE2 by IRW contributed to the antioxidant and anti-inflammatory activities of the peptide in Ang II-stimulated A7r5 cells.

4.3.6 IRW inhibited migration of Ang II-stimulated A7r5 cells in AT1R-dependent manner

Detrimental effects of Ang II are mainly exerted through AT1R (Higuchi et al., 2007). It was then examined if the anti-migrant activity of IRW was via AT1R. It was found that the addition of AT1R antagonist (losartan potassium) could restore the MMP9 expression in Ang II-stimulated A7r5 cells (Figure 4.6A). Results from migration assay suggested that treatment of

IRW could restore the distance of the wound healing area in Ang II-stimulated A7r5 cells (Figure 4.6B), but such an effect could be abolished by the addition of AT1R antagonist (Figure 4.6B). Results from migration assay further demonstrated that IRW exhibited anti-migrant activity in Ang II-stimulated A7r5 cells in AT1R-dependent manner.

4.4 Discussion

VSMC is an essential cellular component in vascular wall, which functions as a regulator of blood vessel diameter and blood flow (Owens et al., 2004). Upon Ang II stimulation, aberrant proliferation, oxidative stress, inflammation and migration would occur in VSMCs, which are considered as milestones in development of vascular remodelling and ultimately lead to increasing peripheral resistance (Fortuño, José, Moreno, Díez, & Zalba, 2005; Rudijanto, 2007). Modulation of intracellular events in VSMCs to retard vascular remodelling is included as a mechanism of some antihypertensive drugs (Jinno et al., 2004; Kawasaki et al., 2000; Petrovic et al., 2005). As a promising alternative to antihypertensive drug, food protein-derived antihypertensive peptides were also shown the ability to modulate intracellular events in vascular cells. However, previous study focused on effects of antihypertensive peptides in vascular endothelial cells (Gu & Wu, 2016; Huang et al., 2010; Maes et al., 2004; Majumder, Chakrabarti, Davidge, et al., 2013). Knowledge on regulatory roles of these peptides in VSMCs is lacking.

In the present study, IRW, a potent antihypertensive peptide, was shown to possess the activity to modulate intracellular events in Ang II-stimulated VSMCs. Upon Ang II stimulation, excessive proliferation would occur in VSMCs, which is a pathophysiological contributor to atherosclerosis (Rudijanto, 2007). Results from this study showed that IRW could inhibit such abnormal proliferation, indicating the potential of this peptide in retarding vascular lesion during development of hypertension. It was reported that stimulus like Ang II on VSMCs could induce a

rapid increase in intracellular H_2O_2 that is involved in accelerated cell proliferation and hypertrophic response (Sorescu, Szöcs, & Griendling, 2001). Similar to what was found in vascular endothelial cells (Huang et al., 2010; Majumder, Chakrabarti, Davidge, et al., 2013), IRW also showed anti-oxidant activity in VSMCs upon Ang II stimulation.

COX2 is an enzyme catalyzing the production of prostanoids from arachidonic acid. COX2-mediated prostanoids could induce the release of inflammatory cytokines and lead to inflammatory response (Ricciotti & FitzGerald, 2011). iNOS catalyzes the oxidative deamination of L-arginine to produce NO, a pro-inflammatory mediator (Aktan, 2004). Down-regulation of Ang II-stimulated COX2 and iNOS expressions by IRW treatment in VSMCs was observed in this study, which indicated the anti-inflammatory actions of IRW in vascular tissues might not only via modulating inflammation in endothelial cells (Majumder, Chakrabarti, Davidge, et al., 2013), but also in VSMCs.

MMP9 is a type of gelatinase that degrades the intimal ECM, mainly targeting on fibronectin, laminin, nidogen, proteoglycan link protein and versican (Newby, 2006). VSMCs are a major source of MMP9. Upon Ang II stimulation, MMP9 could be up-regulated which would further facilitate migration of VSMCs. Thus, inhibition of MMP9 secreted by VSMCs is considered as a strategy to retard vascular remodelling (Guo, Yang, Wang, Liu, & Wang, 2008). In the present study, IRW was found to down-regulate MMP9 expression in Ang II-stimulated A7r5 cells, which indicated the potential of IRW in inhibiting VSMC migration.

MAPKs play the central role in mediating Ang II-induced signaling in VSMCs (Touyz & Schiffrin, 2000). Involvements of ERK1/2, p38 and JNK activations in Ang II-induced intracellular events in VSMCs have all been documented (Irani, 2000; Kyaw et al., 2004; Mugabe et al., 2010; Ushio-Fukai, Alexander, Akers, & Griendling, 1998). Surprisingly, current

study found that IRW could only inhibit phosphorylation of p38 in Ang II-stimulated A7r5 cells, suggesting a selective role of IRW in regulating MAPKs. Our previous study indicated that soy protein-derived ACE-inhibitory peptide LSW (Leu-Ser-Trp) could inhibit phosphorylation of tyrosine kinase and ERK1/2 in Ang II-stimulated A7r5 cells (Lin, Liao, Bai, Wu, & Wu, 2017). Along with results from the current study, it further enhances the notion that ACE inhibition is not the only mechanism of ACE-inhibitory peptides; intracellular signaling pathways are involved in the mechanism of action of these peptides. In addition, peptides with different structures could differentially regulate the intracellular signalling pathways (Wu, Liao, & Udenigwe, 2017).

NF-κB signaling plays a vital role in inflammation through its ability to induce transcription of pro-inflammatory genes including COX2 and iNOS (Dinarello, 2000). Given the contributions of IRW in decreasing expressions of COX2 and iNOS, it was next examined effects of IRW on degradation of IκBα and phosphorylation of p65, two pivotal steps during NF-κB activation (Viatour et al., 2005). Comparable to what was found in endothelial cells (Majumder, Chakrabarti, Davidge, et al., 2013), IRW could regulate the action of p65 by inhibiting its phosphorylation in Ang II-stimulated VSMCs. More interestingly, Ang II-mediated loss of IκBα could be restored by IRW in VSMCs, suggesting a novel regulatory role of IRW in NF-κB signaling.

In addition to antiproliferative, antioxidant, anti-inflammatory and anti-migrant activities, IRW also showed ACE2-enhancing property in Ang II-stimulated VSMCs. The expression of MasR could also be up-regulated. As documented, up-regulation of the ACE2/Ang (1-7)/MasR axis is associated with antioxidant, anti-inflammatory and anti-migrant effects (Zhang et al., 2014). It was then to explore the relationship between IRW-mediated ACE2 up-regulation and modulatory

effects in Ang II-stimulated VSMCs. It was found that antioxidant activity of IRW was dependent on the up-regulation of the ACE2/Ang (1-7)/MasR axis, demonstrating the IRW-mediated ACE2 up-regulation could further contribute to beneficial effect at least in cellular level. The upregulation of the ACE2/Ang (1-7)/MasR axis by IRW also partially contributed to the anti-inflammatory activity of the peptide as only COX2 expression but not iNOS could be altered by MasR antagonist treatment or ACE2 knockdown. Although both of COX2 and iNOS are in the downstream of Ang II-mediated signalling in VSMCs (Hirafuji et al., 2002), differential regulations of these two pro-inflammatory biomarkers by IRW-upregulated ACE2 expression indicated IRW might play multiple roles in exerting anti-inflammatory effect in VSMCs against Ang II stimulation, which is warranted for further study.

Surprisingly, down-regulation of MMP9 was independent from the up-regulation of the ACE2/Ang (1-7)/MasR aixs by IRW treatment. Further study indicated that the anti-migrant activity of IRW was mediated by AT1R. Although several food protein-derived peptides were shown to down-regulate AT1R expression (Jahandideh et al., 2016; Son, Chan, & Wu, 2017.; Yu, Yin, Zhao, Chen, & Liu, 2014), this is the first report characterizing distinguished contribution of the down-regulated AT1R by food protein-derived antihypertensive peptide to modulating vascular abnormality.

In conclusion, this study demonstrated the potential of IRW in modulating Ang II-stimulated intracellular events in VSMCs. More importantly, antioxidant and anti-inflammatory activities of IRW in VSMCs were dependent on the up-regulation of the ACE2/Ang (1-7)/MasR axis, indicating up-regulation of ACE2 by IRW could further contribute to beneficial effects in VSMCs.

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Zhang, Z., Chen, L., Zhong, J., Gao, P., & Oudit, G. Y. (2014). ACE2/Ang-(1-7) signaling and vascular remodeling. *Science China Life Sciences*, *57*(8), 802-808.

Figure 4.1A: IRW inhibited Ang II-stimulated proliferation of A7r5 cells. Cells were pre-treated with 50 µM of IRW for 1 h prior to 23 h stimulation with 1 µM of Ang II. Cells were treated with 1% BrDU for 1 hour, then fixed and immunostained for BrDU. Data are presented as % of BrDU positive cells. Mean ± SEM of 4 independent experiments are shown.

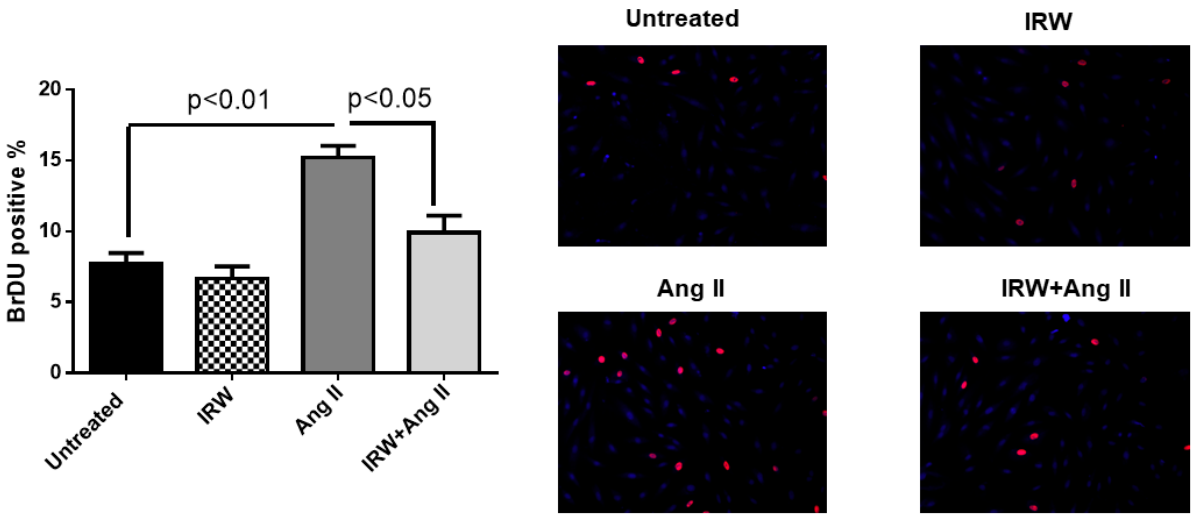


Figure 4.1B: IRW abolished Ang II-stimulated oxidative stress in A7r5 cells. Cells were pre-treated with 50 µM of IRW for 1 h prior to 30 min stimulation with 1 µM of Ang II. Cells were treated with 10 µM of DHE for 30 min, then visualized by fluorescent microscopy. MFI per cell was then calculated and the data were expressed as % of the untreated from 4 independent experiments. Mean ± SEM are shown.

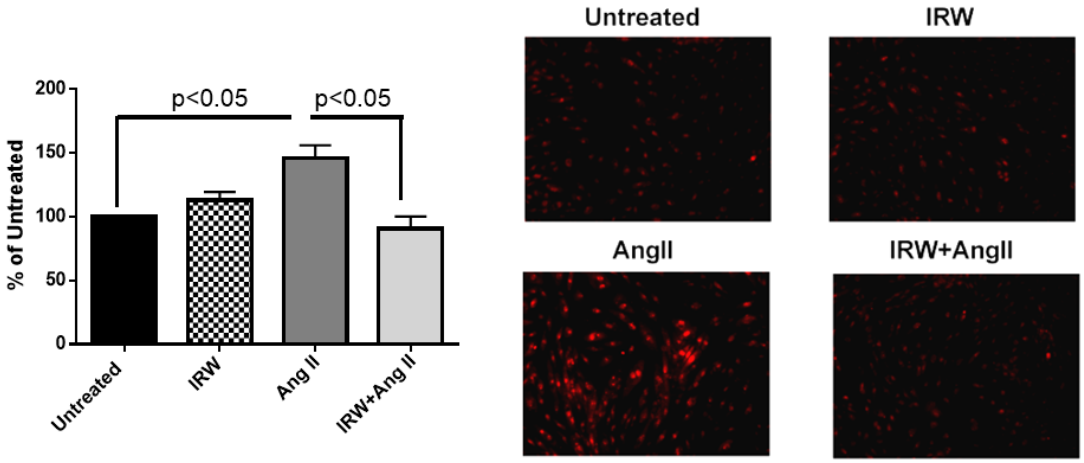


Figure 4.1C: IRW decreased Ang II-stimulated inflammation in A7r5 cells. Cells were pre-treated with 50 μM of IRW for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for COX2 or iNOS and α-tubulin (loading control). Protein bands were quantified by densitometry and normalized to their respective loading controls. Data are expressed as mean ± SEM of 5 independent experiments.

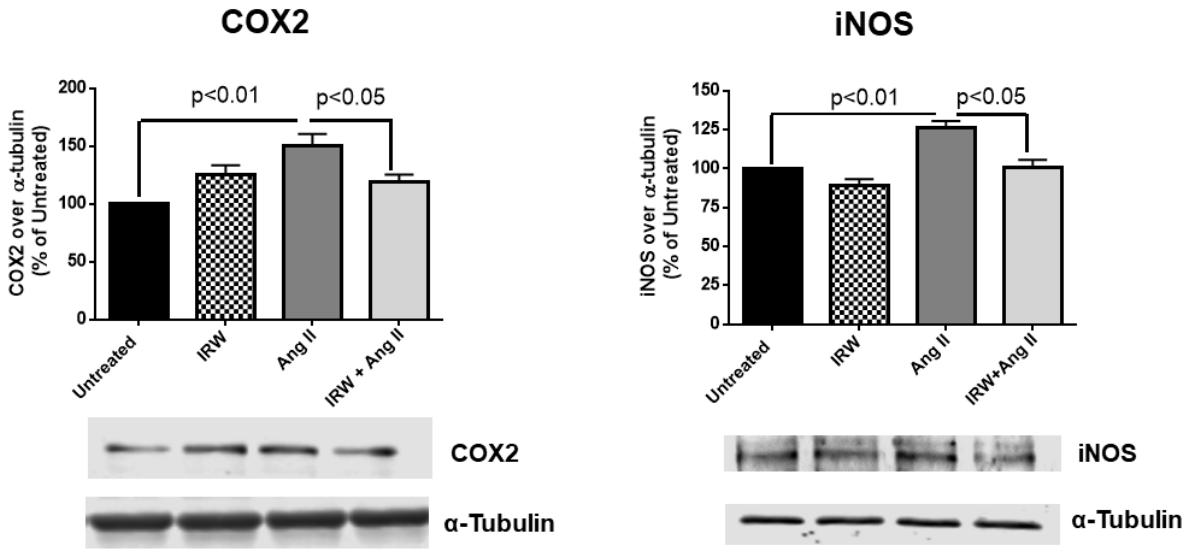


Figure 4.1D: IRW down-regulated MMP9 expression in Ang II-stimulated A7r5 cells. Cells were pre-treated with 50 μM of IRW for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for MMP9 and the loading control α-tubulin. Protein bands were quantified by densitometry and normalized to their respective loading controls. Data are expressed as mean ± SEM of 4 independent experiments.

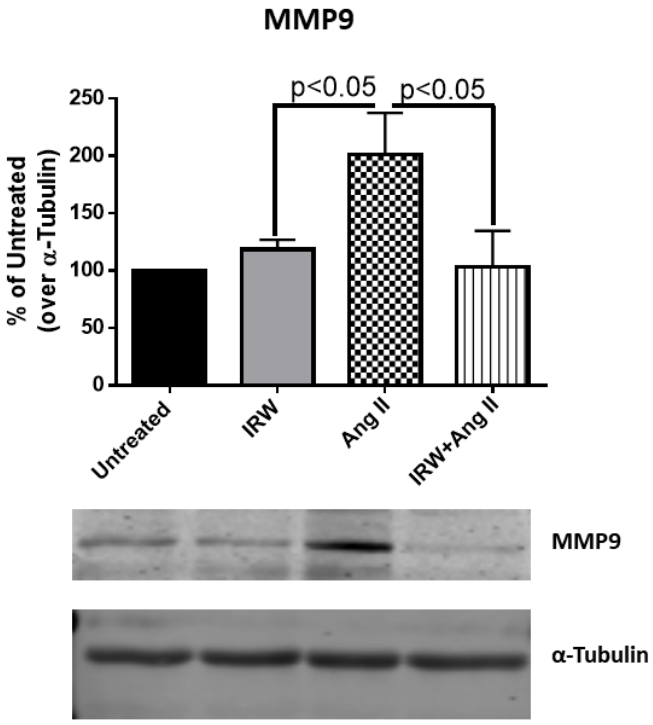


Figure 4.2A: P38 MAPK was involved in the modulatory actions of IRW in Ang II-stimulated A7r5 cells. Cells were pre-treated with 50 μM of IRW for 1 h prior to 15 min stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for ERK1/2 and total ERK1/2; p38 and total p38; JNK and total JNK. Protein bands were quantified by densitometry and normalized to the corresponding total form. Data are expressed as mean ± SEM of 4-5 independent experiments.

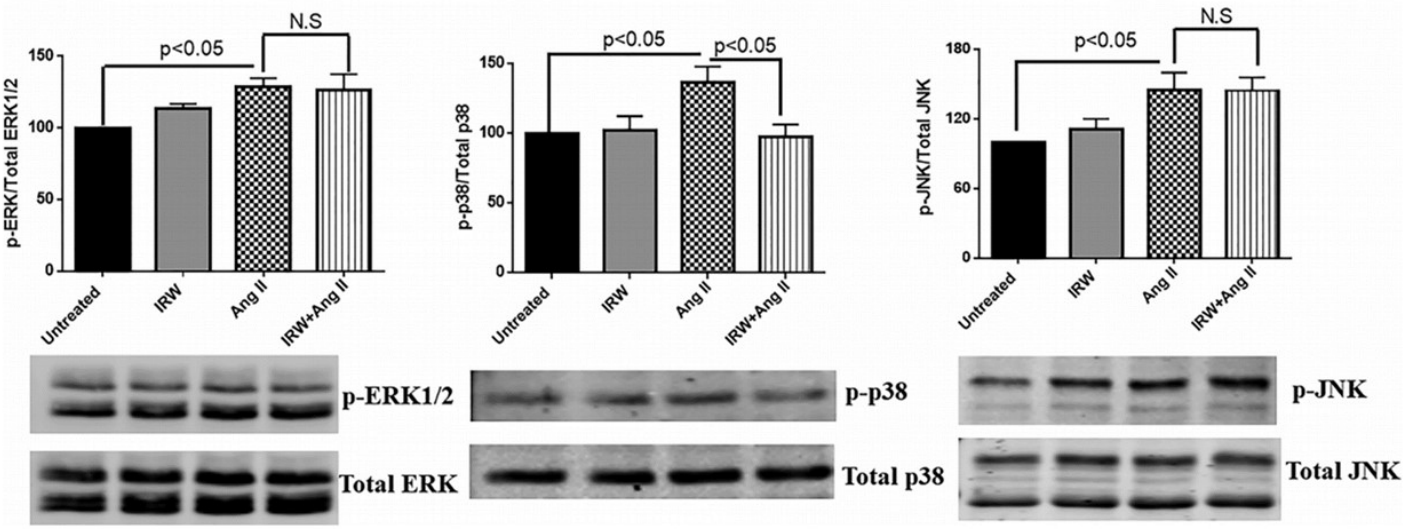


Figure 4.2B: NF-κB signaling was involved in the anti-inflammatory action of IRW against Ang II stimulation in A7r5 cells. Cells were pre-treated with 50 μM of IRW for 1 h prior to 10 min stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for IκBα and α-tubulin or phosphorylated and total forms of p65. Protein bands of IκBα were quantified by densitometry and normalized to their respective loading controls. Phosphorylated protein bands of p65 were quantified by densitometry and normalized to their respective 'total' forms. Data are expressed as Mean ± SEM of 4-6 independent experiments, normalized to the untreated control.

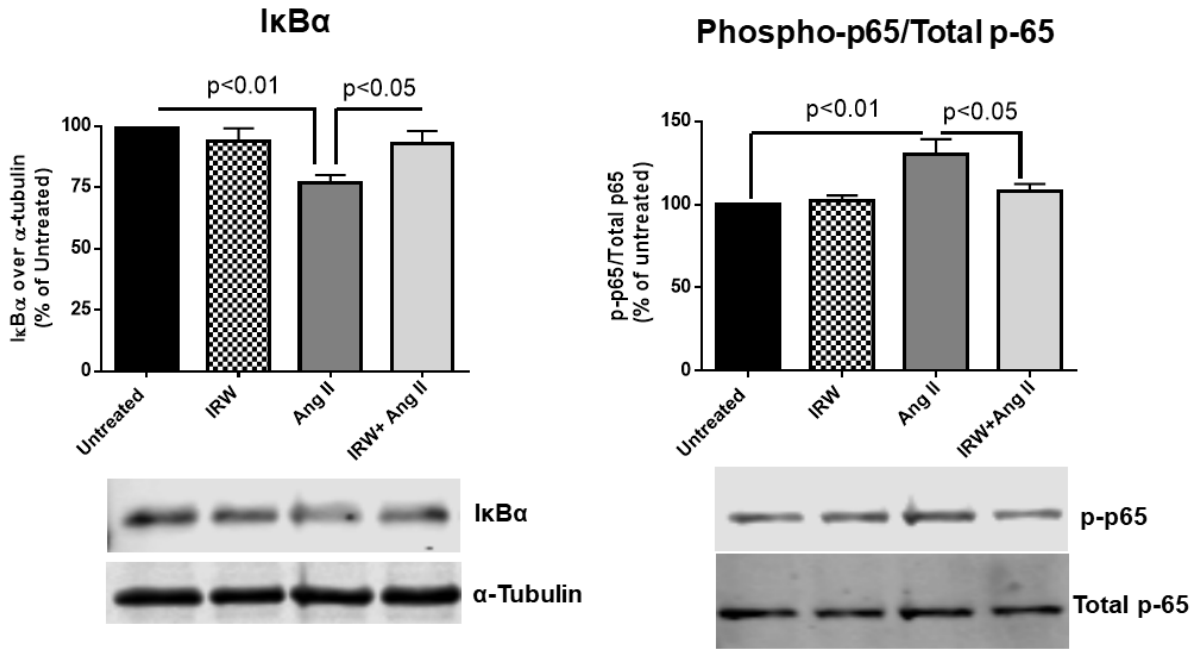


Figure 4.3: IRW up-regulated ACE2 and MasR expressions in Ang II-stimulated A7r5 cells. Cells were pre-treated with 50 μM of IRW for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for ACE2 or MasR and α-tubulin (loading control). Protein bands were quantified by densitometry and normalized to their respective loading controls. Data are expressed as mean ± SEM of 5 independent experiments.

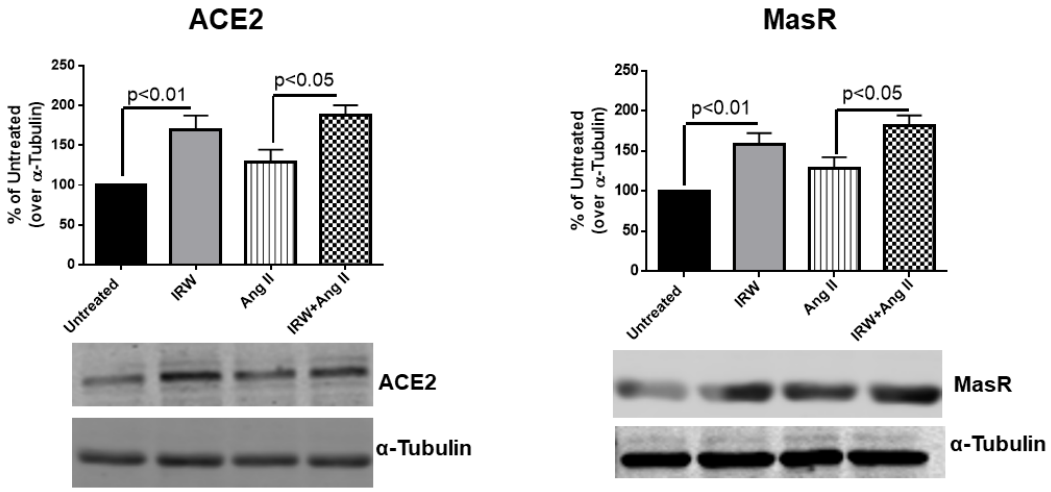


Figure 4.4A: The addition of MasR antagonist A779 abolished antioxidant activity of IRW in Ang II-stimulated A7r5 cells. Cells were pre-treated with 50 µM of IRW combined with or without 1 µM of MasR antagonist for 1 h prior to 30 min stimulation with 1 µM of Ang II. Cells were treated with 10 µM of DHE for 30 min, then visualized by fluorescent microscopy. MFI per cell was then calculated and the data were expressed as % of the untreated from 4 independent experiments. Mean ± SEM are shown.

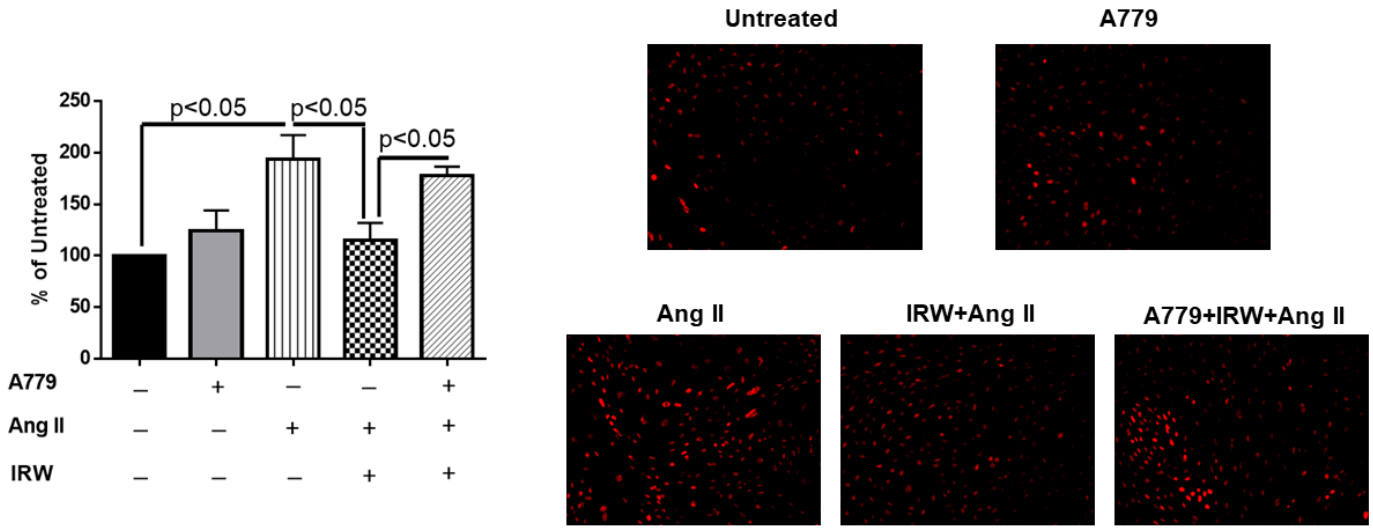


Figure 4.4B: The addition of MasR antagonist A779 partially abolished anti-inflammatory activity of IRW in Ang II-stimulated A7r5 cells. Cells were pre-treated with 50 μM of IRW combined with or without 1 μM of MasR antagonist for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for COX2 or iNOS and α-tubulin (loading control). Protein bands were quantified by densitometry and normalized to their respective loading controls. Data are expressed as mean ± SEM of 4 independent experiments.

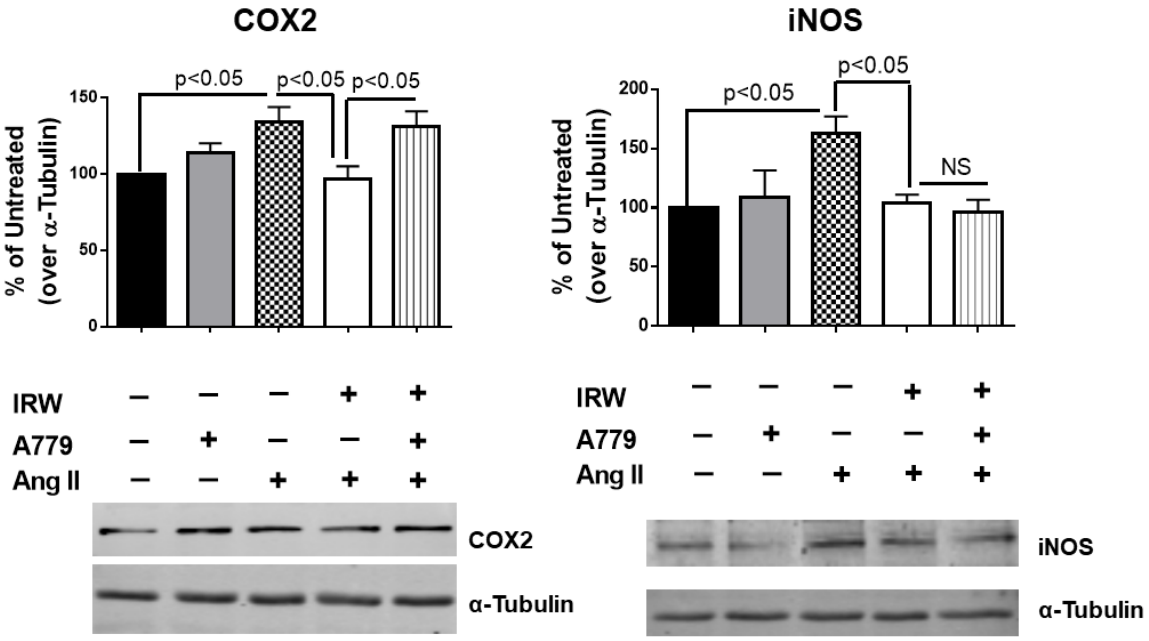


Figure 4.4C: The addition of MasR antagonist A779 did not affect MMP9 expression in Ang II-stimulated A7r5 cells upon IRW treatment. A7r5 cells were pre-treated with 50 μM of IRW combined with or without 1 μM of MasR antagonist for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for MMP9 and the loading control α-tubulin. Protein bands were quantified by densitometry and normalized to their respective loading controls. Data are expressed as mean ± SEM of 4 independent experiments.

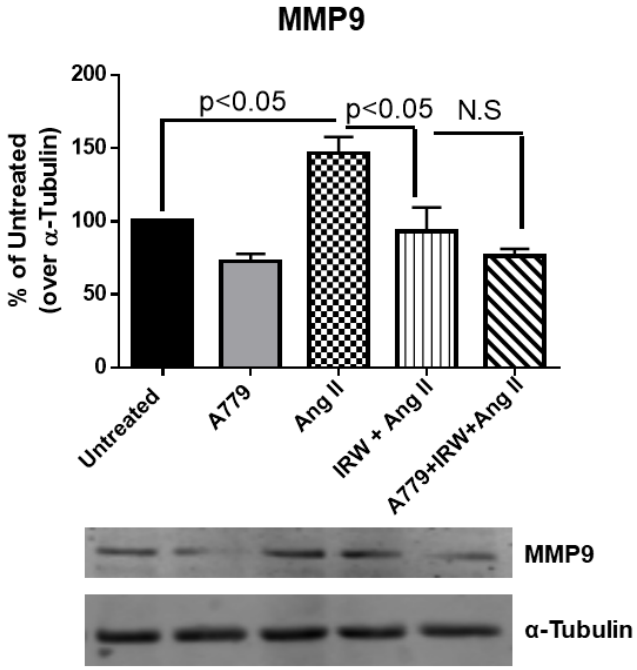


Figure 4.5A: ACE2 knockdown abolished antioxidant activity of IRW in Ang II-stimulated A7r5 cells. ACE2 knockdown in A7r5 cells were conducted using ACE2 siRNA. Afterwards, cells were pre-treated with 50 µM of IRW for 1 h prior to 30 min stimulation with 1 µM of Ang II. Cells were treated with 10 µM of DHE for 30 min, then visualized by fluorescent microscopy. MFI per cell was then calculated and the data were expressed as % of the untreated from 4 independent experiments. Mean ± SEM are shown.

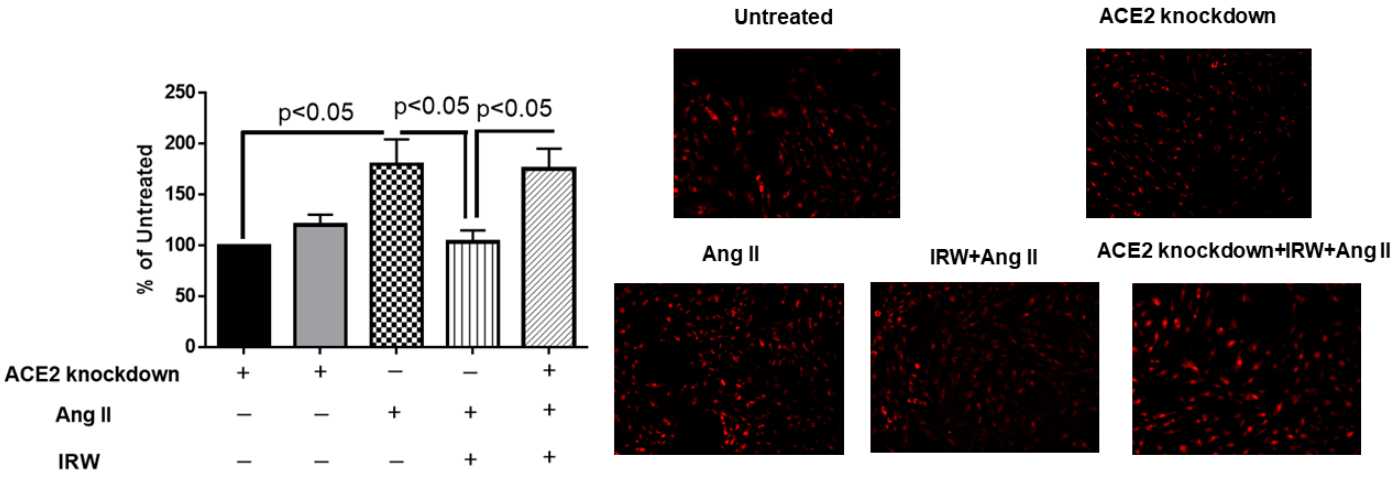


Figure 4.5B: ACE2 knockdown partially attenuated anti-inflammatory activity of IRW in Ang II-stimulated A7r5 cells. ACE2 knockdown in A7r5 cells were conducted using ACE2 siRNA. Afterwards, cells were pre-treated with 50 μM of IRW for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for COX2 or iNOS and α-tubulin (loading control). Protein bands were quantified by densitometry and normalized to their respective loading controls. Data are expressed as mean ± SEM of 4 independent experiments.

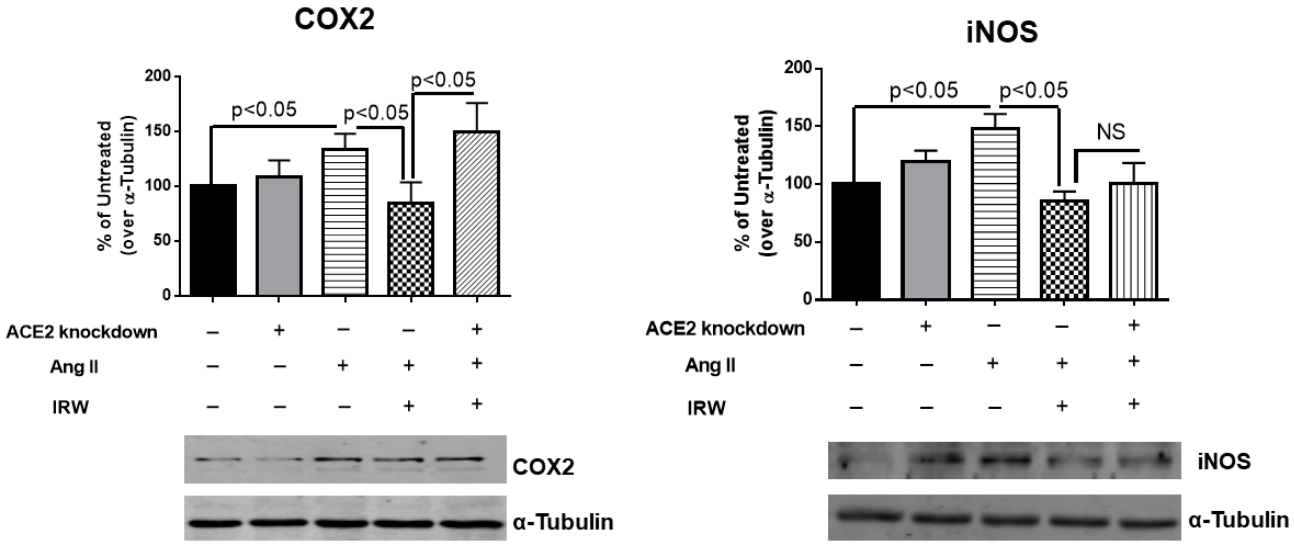


Figure 4.6A: Down-regulation of MMP9 by IRW in Ang II-stimulated A7r5 cells was via AT1R. A7r5 cells were pre-treated with 50 μM of IRW combined with 100 μM of AT1R antagonist for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for MMP9 and the loading control α-tubulin. Protein bands were quantified by densitometry and normalized to their respective loading controls. Data are expressed as mean ± SEM of 4 independent experiments.

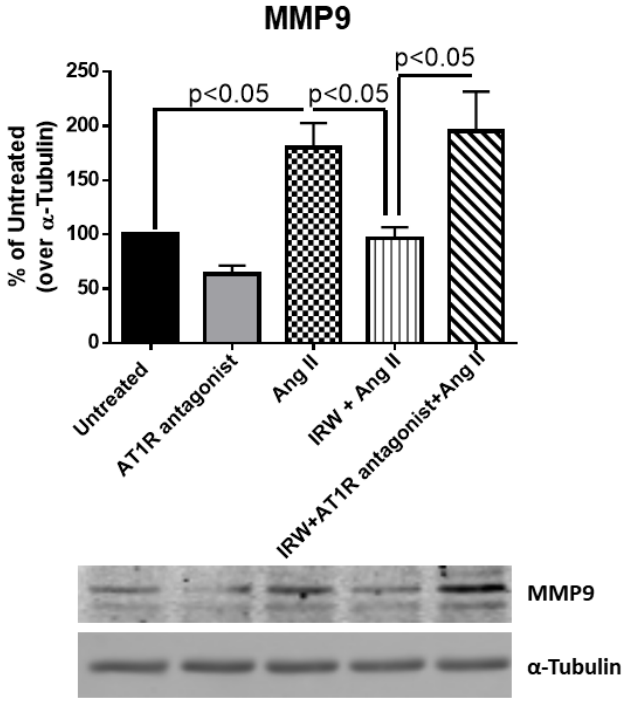
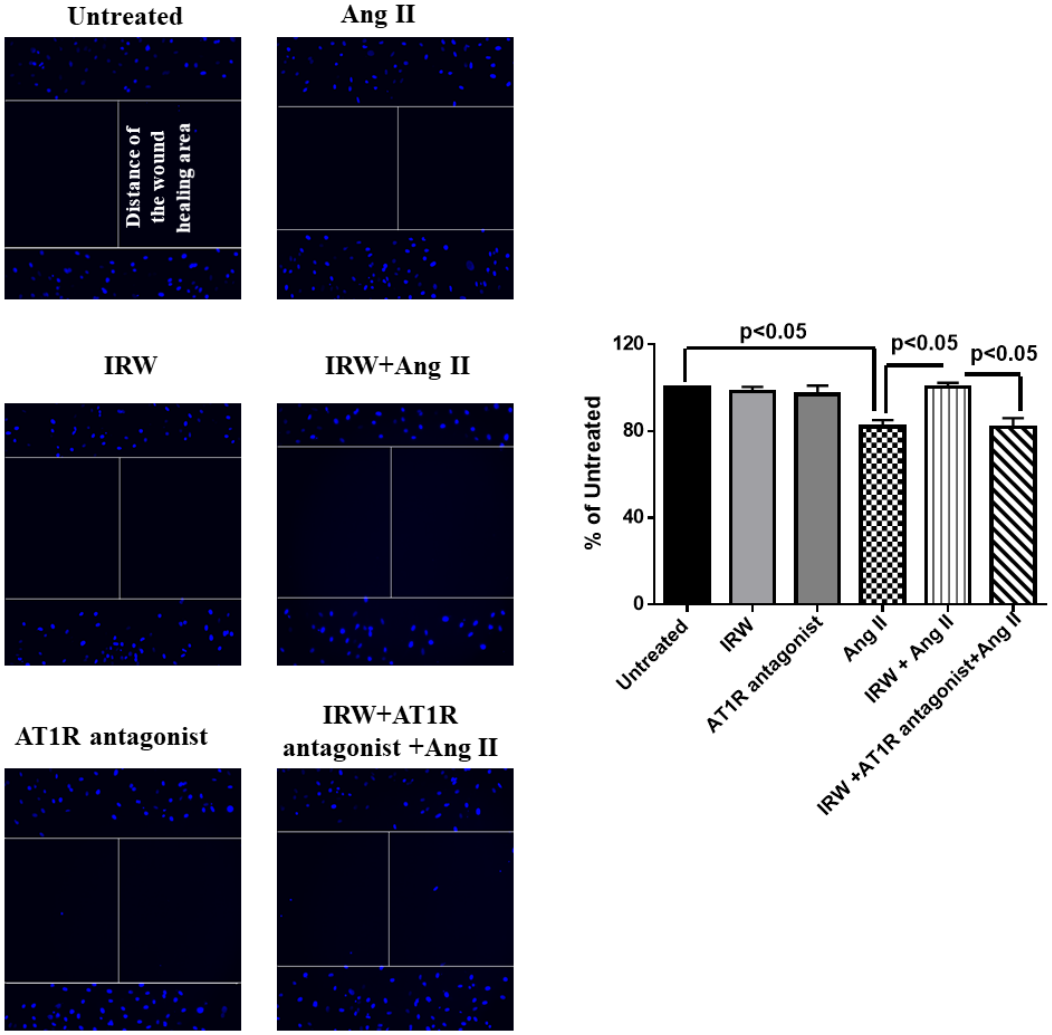


Figure 4.6B: IRW inhibited migration of Ang II-stimulated A7r5 cells in AT1R-dependent manner. A7r5 cells were pre-treated with 50 μM of IRW and/or 100 μM of AT1R antagonist for 1 h prior to 4 h stimulation with 1 μM of Ang II. Cell migration was evaluated by wound healing assay. Distance of the wound healing area was measured by ImageJ software. Result of each treatment group was normalized to the corresponding control. Data are expressed as mean ± SEM of 5 independent experiments.



CHAPTER 5[4] - Egg White-Derived Antihypertensive Peptide IRW Reduces Blood Pressure in Spontaneously Hypertensive Rats via the ACE2/Ang (1-7)/Mas Receptor Axis

[4] A version of this chapter will be submitted to "*Molecular Nutrition and Food Research*" for publication.

5.1 Introduction

Hypertension is a risk factor for cardiovascular diseases, affecting about 25% of adults worldwide (Chockalingam, Campbell, & George Fodor, 2006). In 2017, hypertension was redefined to be above 130/80 mmHg rather than 140/90 mmHg for systolic/diastolic blood pressure by the American Heart Association and the American College of Cardiology (Whelton et al., 2017), resulting in a higher prevalence. Essential hypertension, also known as primary hypertension, accounts for 95% of all hypertension cases around the world (Carretero & Oparil, 2000).

Although the pathophysiology of essential hypertension is multifactorial and complicated, an over activated renin angiotensin system (RAS) is considered as a major factor, given the essential role of the RAS in regulating blood pressure (Esteban & Ruiz-Ortega, 2001). Angiotensin II (Ang II) is a vasoconstricting component in the RAS, which is converted from angiotensin I (Ang I) with the action of angiotensin converting enzyme (ACE) (Esteban & Ruiz-Ortega, 2001). Indeed, Ang II is more than a vasoconstrictor, it also controls the structure and function of vascular wall. An excessive level of Ang II is associated with aberrant proliferation and migration of vascular cells, oxidative stress and inflammation of vascular wall, which further promotes vascular remodelling and increases peripheral resistance (Touyz & Schiffrin, 2000). Hence, to reduce the generation of Ang II by inhibiting ACE activity is a common strategy for hypertension therapy (Te Riet, van Esch, Roks, van den Meiracker, & Danser, 2015).

The RAS got evolved in 2000 with the identification of angiotensin converting enzyme 2 (ACE2). As a homolog of ACE, ACE2 shares 42% of identical sequence with ACE but functions in an opposite way (Donoghue et al., 2000; Tipnis et al., 2000). ACE2 cleaves the carboxyl-terminal phenylalanine of Ang II to form angiotensin (1-7) (Ang (1-7)), which further

counterbalances the harmful effects of Ang II via mas receptor (MasR) (Re, 2004; Santos et al., 2003). Contributions of increased ACE2 to modulating various cardiovascular functions have been recognized (Patel, Zhong, Grant, & Oudit, 2016). In spontaneously hypertensive rats (SHRs), an animal model mimetic to human essential hypertension, up-regulation of ACE2 expression is associated with decreased blood pressure, attenuated oxidative stress and cardiac remodelling (Díez-Freire et al.,. 2006) While, supplementation of ACE2 could abolish Ang II-induced hypertrophic response, confirming the beneficial roles of ACE2 in cardiovascular functions (Alghamri et al., 2012). Thus, targeting ACE2 is considered as a novel therapeutic strategy for hypertension (Ferreira et al., 2010).

Given the side effects generated by prolonged use of antihypertensive drugs, food protein-derived antihypertensive peptides have attracted substantial interests as a safer alternative (Torruco-Uco et al., 2008). Similar to antihypertensive pharmaceuticals, most of antihypertensive peptides were characterized as ACE-inhibitory peptides (Wu, Liao, & Udenigwe, 2017). While, peptide with the potential to target ACE2 has been rarely reported. Our lab previously identified an antihypertensive peptide with the sequence of Ile-Arg-Trp (IRW) from egg white ovotransferrin (Majumder & Wu, 2011). Blood pressure-reducing effect of IRW was demonstrated in SHRs (Majumder, Chakrabarti, Morton, et al., 2013). Molecular mechanisms of IRW in modulating intracellular events of vascular cells as well as in reducing blood pressure of SHRs have also been explored (Liao, Chakrabarti, Davidge, & Wu, 2016; Liao, Fan, & Wu, 2018; Majumder, Chakrabarti, Davidge, & Wu, 2013). Interestingly, a transcriptomic study found that oral administration of IRW could up-regulate mRNA of ACE2 in mesenteric artery of SHRs (Majumder et al., 2015). Further study found IRW could activate *in vitro* ACE2 as well as cellular ACE2 activities (Chapter 3). In addition, oral administration of

ACE2 could up-regulate protein expressions of ACE2 in aorta and kidney of SHRs (Chapter 3). Taken together, IRW was shown the potential of an ACE2 activator. However, the contribution of ACE2-activation by IRW to reducing blood pressure in SHRs is ambiguous.

In the present study, relationship between the activated ACE2/Ang (1-7)/MasR axis by IRW and the reduced blood pressure in SHRs was investigated. Mechanisms pertaining to IRW as an ACE2 activator were studied as well.

5.2 Materials and Methods

5.2.1 Animal model and ethics statement

Twelve to fourteen-week old male SHRs (290.0±10 g) were obtained from Charles River (Senneville, QC, Canada). Animals were kept in the University of Alberta animal core facility for a week of acclimatization, where they were exposed to 12:12 hour of light: dark cycle with controlled humidity and temperature. All SHRs were given standard rat chow and water ad libitum. This protocol was approved by the University of Alberta Animal Welfare Committee (Protocol # AUP 00001571) in accordance with the guidelines issued by the Canada Council on Animal Care and also adhered to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

5.2.2 Experimental protocol

Thirteen to fifteen-week old male SHRs were surgically implanted with telemetry transmitters (HD-S10, DSI, St. Paul, MN, USA) for chronic blood pressure monitoring. The catheter of the telemetry probe was inserted into the left femoral artery of the rat and advanced up to the aorta. The transmitter was placed in a subcutaneous pocket in the left leg. After the surgery, the rats were allowed for a week of post-surgery recovery. The animals were then randomly assigned

into four groups: Untreated (n=6), IRW (15 mg/kg BW, n=6), MasR antagonist A779 (48 µg/kg BW/h, n=6)(Sullivan, Bhatia, Yamamoto, & Elmarakby, 2010) and IRW+A779 (n=6). Basal blood pressure (Day -7) was recorded, and then an osmotic pump (ALZET, Cupertino, USA) loaded with A779 was implanted subcutaneously into the animal. An osmotic pump (ALZET) filled with saline was implanted into animals without A779 treatment. A779 was infused for 7 days prior to the start of IRW treatment. IRW was administered orally the same as we described previously (Majumder, Chakrabarti, Morton, et al., 2013). IRW was dissolved in 20 mL of Ensure (Abbott Nutrition, QC, Canada) and administered once per day for 7 days.

5.2.3 Telemetry recording

Real-time blood pressure was monitored during the IRW treatment period. Each rat was caged individually and placed on the top of a RPC-1 receiver (ADI instruments, CO, USA). An atmospheric-pressure monitor (Model APR-1, ADI instruments) was also installed to normalize the pressure values received from the transmitters, which provided the actual values irrespective of changes in atmospheric pressure. Signals were recorded and output by Dataquest 4.3.6 (DSI). The average value of blood pressure/heart rate was calculated based on 24 h-recording (10 seconds of every 30 seconds).

5.2.4 Vascular function

Endothelium-dependent vasorelaxation using mesenteric artery was evaluated as we described previously (Majumder, Chakrabarti, Morton, et al., 2013). Mesenteric arteries (diameter: 150-250 µm, length: ~ 2mm) were mounted on two tungsten wires (Fine Wire Company, California, USA) and attached to a wire-myograph (DMT, Ann Arbor, MI, USA). After normalization and equilibration, arteries were exposed to 10 µM of phenylephrine (PE, Sigma-Aldrich, St. Louis,

MO, USA) twice, followed by a single dose of methacholine (MCh, 3 μM, Sigma-Aldrich) to evaluate the integrity of the vessel. A cumulative concentration response curve to PE ranging from 10^{-8} M to 10^{-4} M was performed. Afterwards, the vessel was pre-constricted to 80% of maximum using PE. A cumulative concentration response curve to MCh (10^{-10} to 10^{-6} M) was performed to assess the endothelium-dependent vasorelaxation.

5.2.5 Plasma biomarker analysis

Blood samples were collected at the end point, followed by centrifugation under 1,000 Xg for 15 min at 4 ℃. Plasma was stored at -80 ℃ until analysis. Levels of Ang II, ACE2, Ang (1-7) and ACE were quantified by respective ELISA kit (CUSABIO TECHNOLOGY, TX, USA) according to the manual. Estimations of cytokine samples were performed using a commercially available rat cytokine strips (Signosis, Santa Clara, CA, USA) (Majumder, Chakrabarti, Morton, et al., 2013).

5.2.6 Western blotting

Total proteins were extracted from aortas by a protein extraction buffer (20 mM Tris, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM sodium fluoride and 1% NP-40) containing 1% (v/v) protease inhibitor cocktail (Sigma, St. Louise, MO). The homogenate was centrifuged at 10, 000 Xg for 10 min at 4 ℃. Protein concentration was quantified by BCA assay (ThermoFisher, Waltham, MA, USA).

Total protein was loaded on a 9% SDS-PAGE and then transferred to a nitrocellulose membrane. Bands of phospho-protein kinase B (p-Akt, Cell Signalling, Danvers, MA, USA), phospho-endothelial nitric oxide synthase (p-eNOS, Cell Signalling) and phospho-extracellular signal-regulated kinases1/2 (p-ERK1/2, Cell Signalling) were normalized to the corresponding “total”

form (Akt, Cell Signalling; eNOS, BD Biosciences, Franklin Lakes, NJ, USA; ERK1/2, Cell Signalling). Bands of inhibitory κBα (IκBα, Cell Signalling), cyclooxygenase 2 (COX2, NOVUS Biologicals, Littleton, CO, USA) and matrix metallopeptidase 9 (MMP9, NOVUS Biologicals) were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Abcam, Cambridge, UK). Bands were developed by Goat anti-rabbit IRDye 680RD or Donkey anti-mouse 800CW from Licor Biosciences (Lincoln, NE, USA) as the secondary antibody, and detected by Licor Odyssey BioImager (Licor Biosciences). Quantification of protein bands were performed using Image Studio Lite 5.2 (Licor Biosciences).

5.2.7 Statistics

Statistical analysis was performed using GraphPad Prism (La Jolla, CA, USA). All data were presented as mean ± SEM (standard error of mean) from 6 animals. Blood pressure and heart rate data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test. MCh curves were fitted by nonlinear regression and analyzed by two-way ANOVA coupled with Tukey's multiple comparisons test. Results of western blotting and plasma biomarker analysis were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. A p value < 0.05 was considered as statistically significant.

5.3 Results

5.3.1 IRW decreases blood pressure in SHRs via the ACE2/Ang (1-7)/MasR axis

IRW oral administration reduced blood pressure in SHRs significantly within 7 days of treatment (Figures 5.1A-C), which was consistent as we observed in previous study (Majumder, Chakrabarti, Morton, et al., 2013). However, the blood pressure-reducing effect of IRW was blocked by A779 infusion, indicating the effect was via ACE2/Ang (1-7)/MasR-dependent

manner. Regardless the decrease of blood pressure, none of the treatment affected heart rate of SHRs (Figure 5.1D).

5.3.2 IRW modulates levels of circulating RAS components

IRW treatment increased circulating ACE2 and Ang (1-7) levels but decreased the concentration of Ang II significantly (Figures 5.2A-C), which implicated the potential of IRW in increasing circulating ACE2 to decrease blood pressure in SHRs. On the contrary, IRW treatment did not show a significant effect on ACE level (Figure 5.2D), which further demonstrated ACE2, instead of ACE, was the target of IRW *in vivo*.

5.3.3 Activation of the ACE2/Ang (1-7)/MasR axis by IRW treatment contributes to improving endothelium-dependent vasorelaxation

MCh-mediated vasodilation is associated with endothelium-derived hyperpolarizing factor, which hence was used for assessing endothelium-dependent vasorelaxation (Pretorius, Rosenbaum, Vaughan, & Brown, 2003). As illustrated by Figure 3A, MCh-mediated vasodilation was enhanced significantly by IRW treatment. However, the effect was abolished by A779 infusion. Akt/eNOS signaling plays a central role in regulating endothelium-dependent vasodilation (Shiojima & Walsh, 2002). It was found that the enhanced phosphorylation of Akt and eNOS in aorta by IRW treatment were mitigated by A779 infusion (Figures 5.3B&C). The above results indicated activation of the ACE2/Ang (1-7)/MasR axis by IRW in SHRs could enhance Akt/eNOS-mediated endothelium-dependent vasorelaxation, which, at least partially, contributed to reducing blood pressure in SHRs.

5.3.4 Activation of the ACE2/Ang (1-7)/MasR axis by IRW treatment contributes to attenuating vascular inflammation

As we found in our previous study, amelioration of vascular inflammation was underlying antihypertensive mechanism of IRW in SHRs (Majumder, Chakrabarti, Morton, et al., 2013). Whereas, results from the present study indicated levels of pro-inflammatory cytokines interleukin 6 (IL-6) and monocyte chemoattractant protein 1 (MCP1) in circulation were restored with A779 infusion (Figures 5.4A&B). In addition, up-regulation of IκBα by IRW was abrogated and down-regulation of COX2 was restored in aorta of SHRs with A779 infusion (Figures 5.4C&D). Taken together, activation of the ACE2/Ang (1-7)/MasR axis by IRW treatment was associated with mitigating vascular inflammation in SHRs. Nuclear factor κB (NF-κB) signaling was involved in the anti-inflammatory effect of IRW as an ACE2 activator in SHRs.

5.3.5 IRW treatment did not affect ERK1/2 or MMP9 expression in aorta of SHRs

Down-regulation of MMP9 via deactivating ERK1/2 mitogen-activated protein kinases (MAPK) signalling is another beneficial effect of increased Ang (1-7) level, which is further implicated in retarding atherosclerotic plaque formation.(Yang, et al., 2013; Rodrigues Prestes, Rocha, Miranda, Teixeira, & Simoes e Silva, 2017) However, IRW treatment showed no significant effect in regulating phosphorylation of ERK1/2 or expression of MMP9 in aorta of SHRs (Figures 5.5A&B).

5.4 Discussion

In this study, it was found that blood pressure-reducing effect of IRW could be blunt by infusion of MasR antagonist A779 in SHRs, indicating the mechanism of blood pressure lowering activity of IRW is via the ACE2/Ang (1-7)/MasR axis. A direct activation of ACE2 by IRW treatment

was evident as levels of circulating ACE2 and Ang (1-7) were increased, but Ang II level was reduced. The infusion of A779 also abolished the IRW-enhanced endothelium-dependent vasorelaxation as well as the IRW-mediated attenuation of vascular inflammation, suggesting the actions of IRW *in vivo* as an ACE2 activator were via modulations of endothelial dysfunction and vascular inflammation.

Blood pressure recording data of this study demonstrated a predominant role of the ACE2/Ang (1-7)/MasR axis in mediating the antihypertensive effect of IRW in SHRs. Followed by the previous studies, which showed the potential of IRW in activating ACE2 *in vitro* and in vascular smooth muscle cells (Chapter 3), results from this study further indicated the *in vivo* efficacy of IRW as an ACE2 activator. Indeed, xanthenone (XNT), a drug identified from structure-based screening, was claimed as an ACE2 activator, blood pressure-reducing effect of XNT was validated in SHRs (Hernández Prada et al., 2008). However, antihypertensive effect of XNT could not be achieved in ACE2 knockout mice (Haber et al., 2014), indicating the variability of the effect of ACE2 activator among different animal models. Thus, to investigate the ACE2-activating effect of IRW in hypertensive models beyond SHR is expected in the future study.

Levels of circulating RAS components could be modulated by a 7-day IRW oral administration in SHRs. Specifically, levels of ACE2 and Ang (1-7) were increased, coupled with a decreased level of Ang II, showing a direct *in vivo* ACE2-activating evidence of IRW. As infusion of Ang (1-7) contributed to mitigating blood pressure in SHRs (Benter, Ferrario, Morris, & Diz, 1995), the reduction of blood pressure observed in this study was thus associated with the modulated circulating RAS. Casein-derived tripeptide Ile-Pro-Pro (IPP) was previously reported with the activity of increasing circulating Ang (1-7) in SHR (Ehlers, Nurmi, Turpeinen, Korpela, & Vapaatalo, 2011), but the contribution of the increased Ang (1-7) to blood pressure-reducing

effect of IPP was unknown. Results from this study reported for the first time about the *in vivo* ACE2-activating activity of a food protein-derived bioactive peptide. These results also suggest that targeting ACE2 represents a novel strategy to identify antihypertensive peptides from various food protein sources.

Interestingly, the circulating ACE level was unaffected upon IRW treatment, despite IRW was initially characterized as an ACE-inhibitory peptide (Majumder & Wu, 2011). The inconsistence between the results from the *in vitro* ACE-inhibitory assay and this *in vivo* study further supported that ACE2 activation is the main mechanism of observed blood pressure reduction by IRW administration. It was reported previously that ACE-inhibitory peptide identified based on the *in vitro* assay might not show ACE-inhibitory activity *in vivo* (Wu et al., 2017), this study further reinforce this notion.

Further experiments were performed to elucidate the mechanisms of IRW as an ACE2 activator *in vivo*. Endothelial dysfunction manifests a common feature of hypertension cases (Giannotti & Landmesser, 2007). Previous study showed that IRW could not only modulate tumor necrosis factor (TNF)-stimulated endothelial dysfunction in vascular endothelial cells (Majumder, Chakrabarti, Davidge, et al., 2013), but also enhance endothelium-dependent vasorelaxation in SHRs (Majumder, Chakrabarti, Morton, et al., 2013). Here, it was further found improvement of endothelial dysfunction in SHRs by IRW treatment was mediated by the ACE2/Ang (1-7)/MasR axis, which is consistent with the literature documenting that enhancement of endothelium-dependent vasorelaxation is among one of major beneficial effects of increased Ang (1-7) (Faria-Silva, Duarte, & Santos, 2005; Lemos et al., 2005; Li et al., 2017; Sampaio et al., 2007).

Amelioration of vascular inflammation is another *in vivo* mechanism of IRW in reducing blood pressure in SHRs as we reported previously (Majumder, Chakrabarti, Morton, et al., 2013). It

was also found that the activated ACE2/Ang (1-7)/MasR axis by IRW treatment contributed to mitigating inflammatory response in Ang II-stimulated vascular smooth muscle cells (Chapter 4). Results from this study supported the relationship between the IRW-activated ACE2/Ang (1-7)/MasR axis and reduced vascular inflammation in SHRs. Moreover, such effect was found through NF-κB signalling. Anti-inflammatory effect of Ang (1-7) was reported (Simões e Silva, Silveira, Ferreira, & Teixeira, 2013; Simões e Silva & Teixeira, 2016). However, specific targets mediating such effect is unknown.

As documented, chronic infusion of Ang (1-7) resulted in down-regulated MMP9 expression via ERK1/2 MAPK signalling, which was implicated in inhibiting atherosclerotic lesion formation and enhancing plaque stability (Yang, et al., 2013; Dong et al., 2008). However, the increased Ang (1-7) by IRW treatment did not show effect in inhibiting ERK1/2 MAPK phosphorylation or down-regulating MMP9 expression in aorta of SHRs, which might be due to the short treatment period (7 days) we did in this study. Besides, differential responses among endothelium-dependent vasorelaxation, vascular inflammation and MMP9 expression towards the increased Ang (1-7) by IRW treatment indicated selective roles of IRW as an ACE2 activator in SHRs.

In conclusion, IRW reduces blood pressure in SHRs via the ACE2/Ang (1-7)/MasR axis, demonstrating the role of IRW as an ACE2 activator *in vivo*; activation of ACE2 contributes to enhanced endothelium-dependent vasorelaxation and reduced vascular inflammation. Blood pressure lowering activity of numerous ACE inhibitory peptides is not due to the inhibition of ACE; the establishment of a role of the ACE2/Ang (1-7)/MasR axis in the study thus sheds new lights into the molecular targets of food protein-derived antihypertensive peptides. While, only male SHR was used in this study, it would be of interest to investigate if there is gender

difference, as female SHRs appear to be more dependent on Ang (1-7) to mediate effects of antihypertensive agents than males (Zimmerman, Harris, & Sullivan, 2014).

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Figure 5.1: IRW decreases blood pressure of SHRs via the ACE2/Ang (1-7)/MasR axis. A779 (48 µg/kg BW/h) or saline was infused 7 prior to starting IRW treatment. The co-treatment period of A779 and IRW was 7 days. Systolic pressure (A), diastolic pressure (B), mean arterial pressure (C) and heart rate (D) were recorded over a 24-hour period. NS indicated no significant. difference.

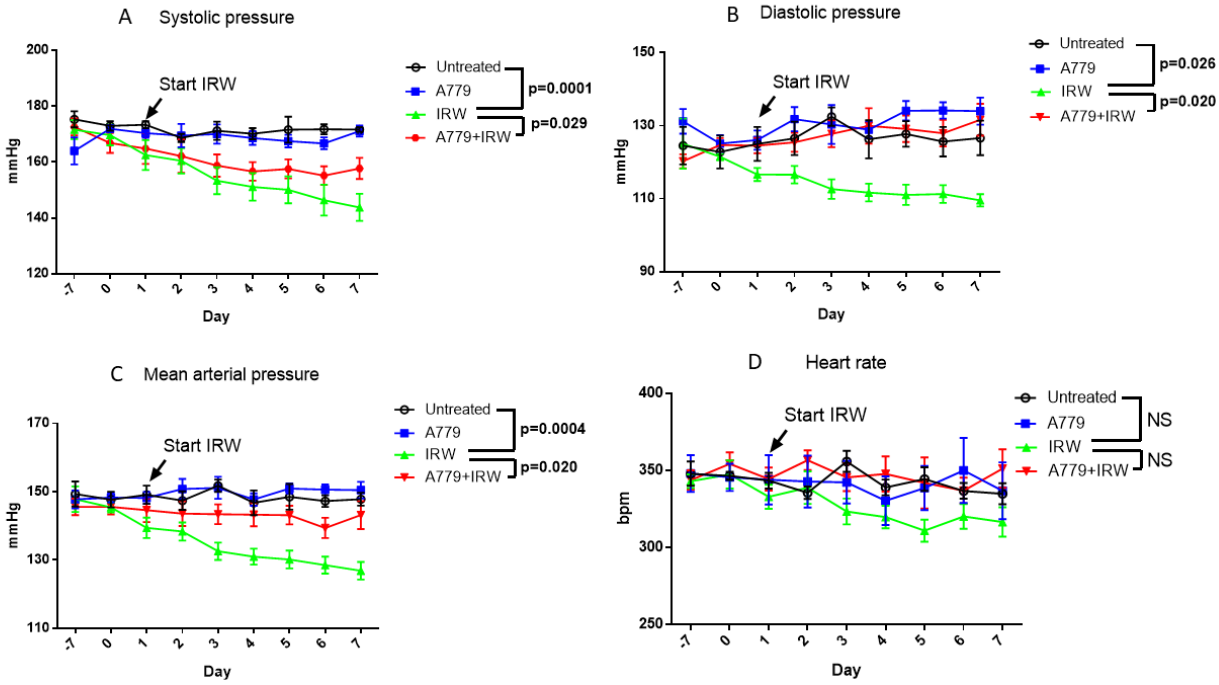


Figure 5.2: IRW modulates levels of circulating RAS components. Plasma from the rats were collected at the end point to evaluate the levels of circulating Ang II (A), ACE2 (B), Ang (1-7) (C) and ACE (D). * indicated $p<0.05$, as compared with the untreated control.

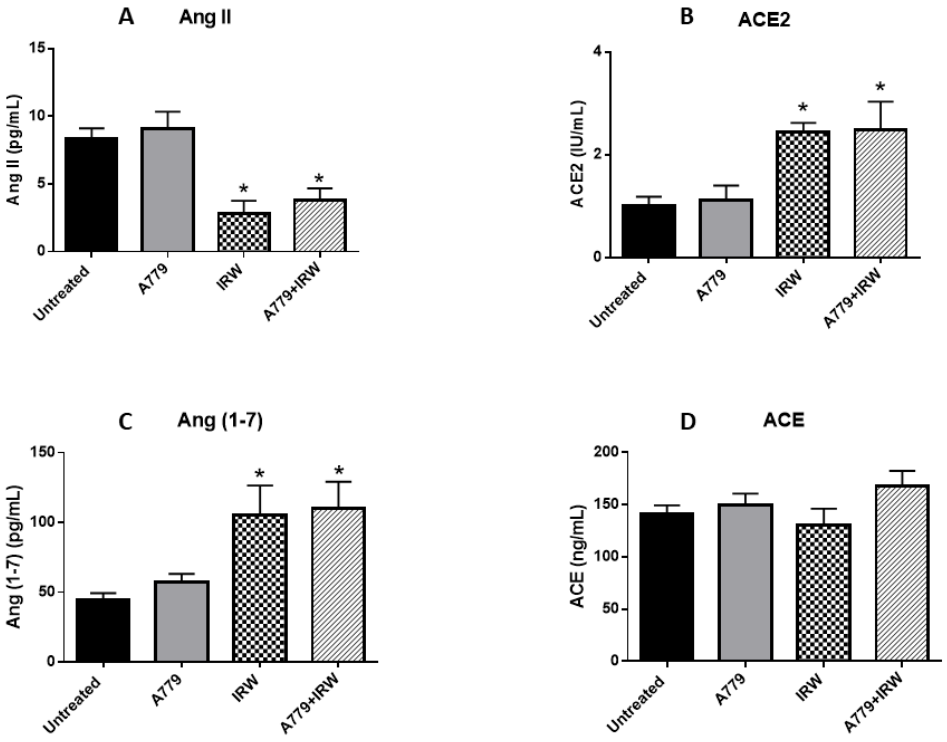


Figure 5.3: Activation of the ACE2/Ang (1-7)/MasR axis by IRW contributes to improving endothelium-dependent vasorelaxation. *Ex vivo* cumulative response curve to MCh in isolated mesenteric arteries were performed (A). Tissues were collected at the end point. Total proteins were extracted from aorta of SHR. Expressions of p-Akt (B) and p-eNOS (C) were normalized to the corresponding total forms.

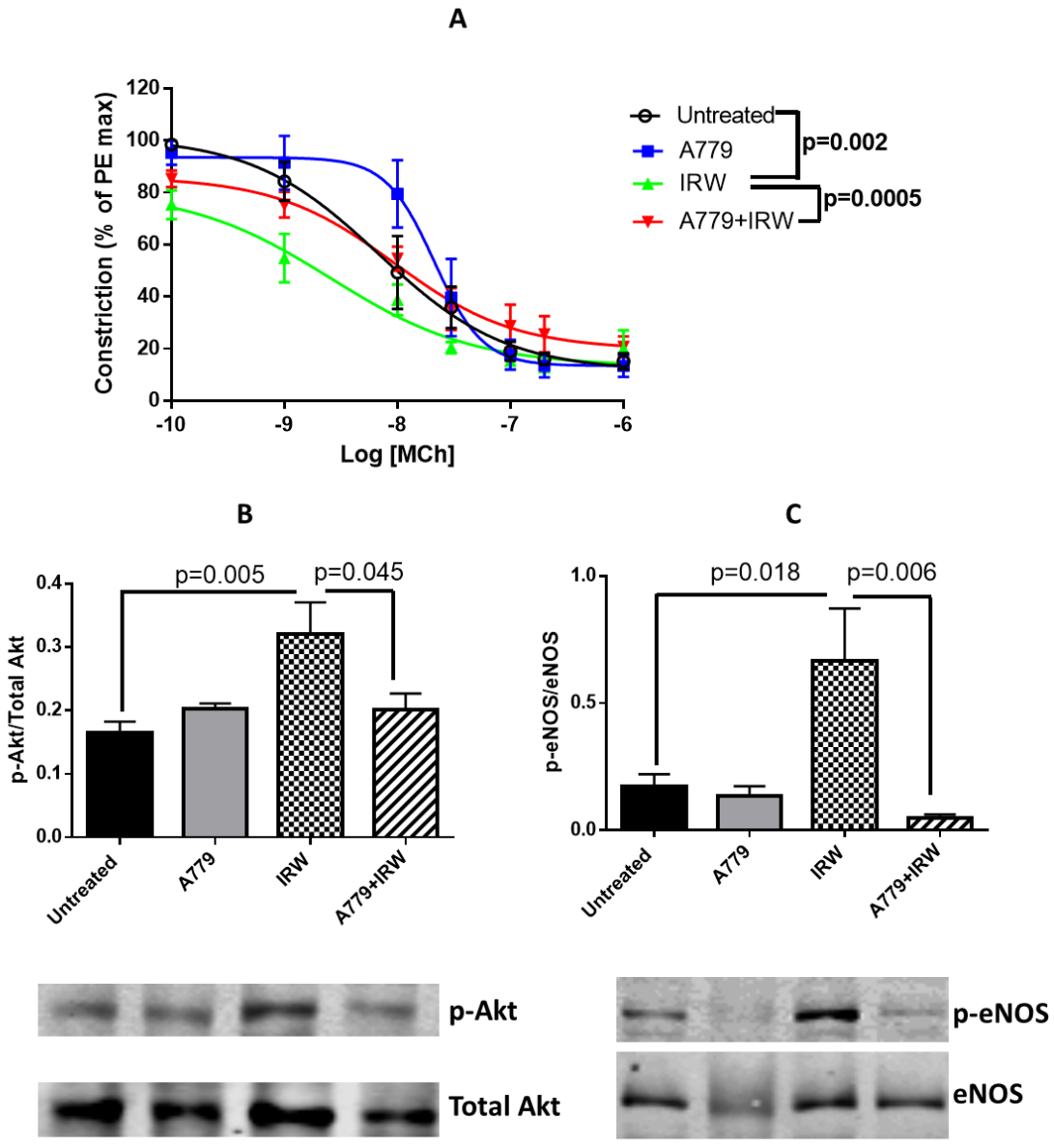


Figure 5.4: Activation of the ACE2/Ang (1-7)/MasR axis by IRW contributes to attenuating vascular inflammation. Plasma from the rats were collected at the end point to evaluate the levels of circulating IL-6 (A) and MCP-1 (B) The level of IL-6 or MCP-1 in each treatment group was normalized to the untreated. Tissues were collected at the end point. Total proteins were extracted from aorta. Expressions of IκBα (C) and COX2 (D) were normalized to GAPDH.

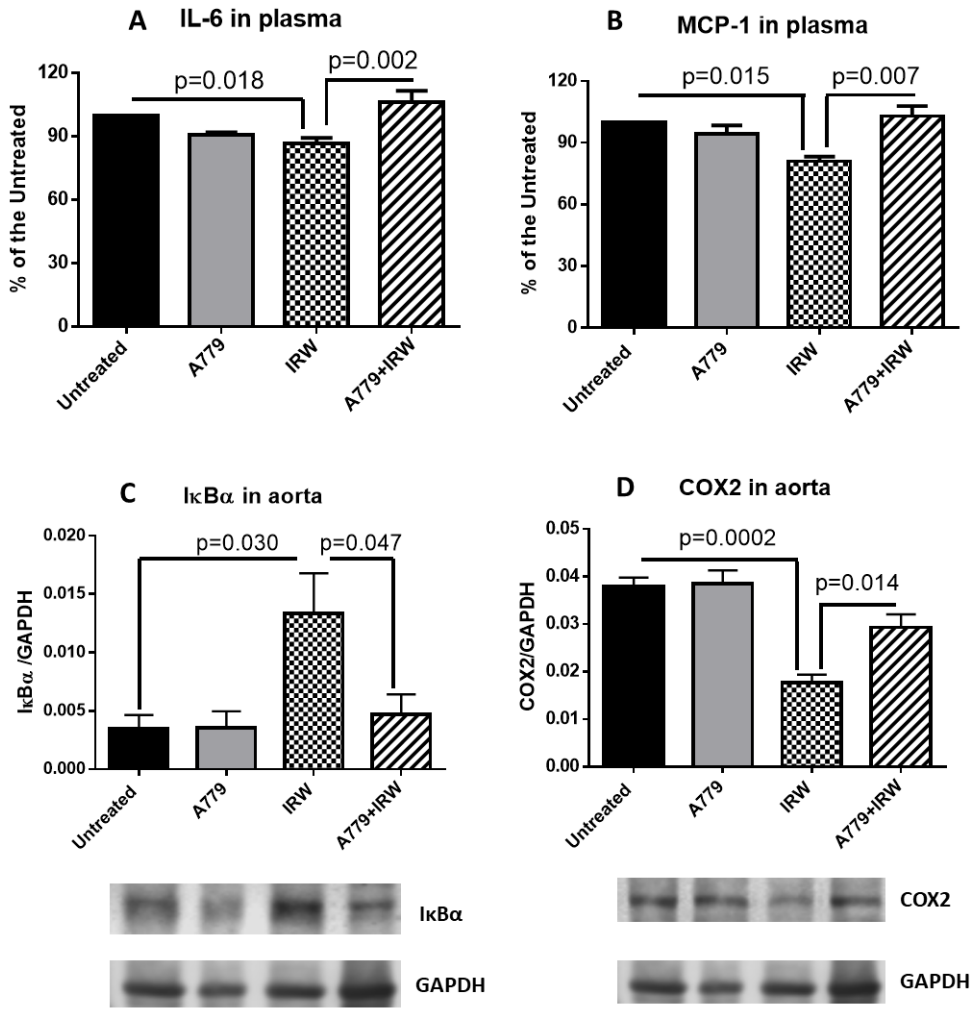
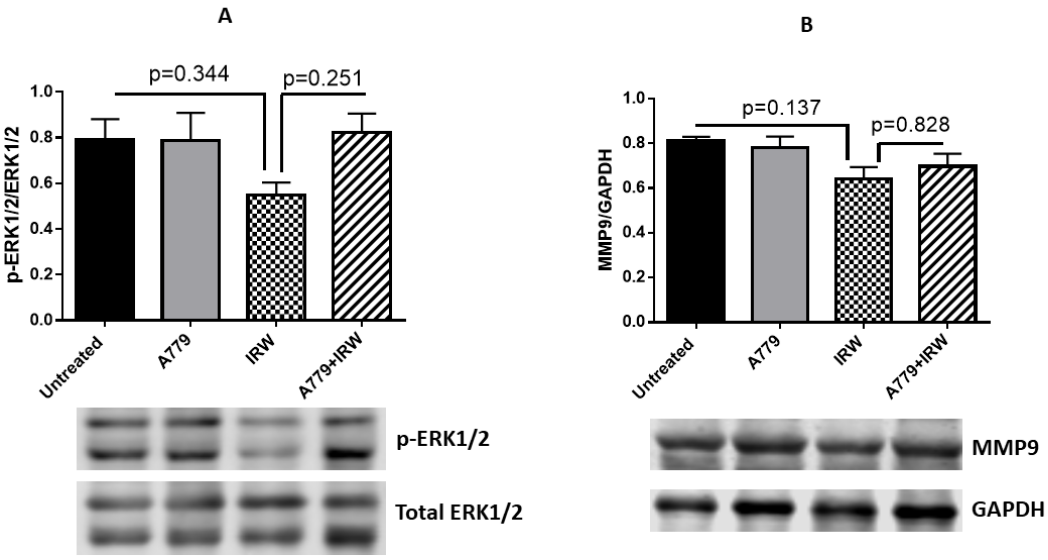


Figure 5.5: IRW treatment does not affect ERK1/2 MAPK or MMP9 expression in aorta of SHRs. Tissues were collected at the end point. Total proteins were extracted from aorta of SHR. Expression of p-ERK1/2 was normalized to total ERK1/2 (A); Expression of MMP9 was normalized to GAPDH.



CHAPTER 6[5] - Identification of Angiotensin Converting Enzyme 2 Up-regulating Peptide from Pea Protein Hydrolysate

[5] A version of this chapter will be submitted to "*Food Chemistry*" for publication.

6.1 Introduction

Food protein-derived bioactive peptides, in particular antihypertensive peptides, have been considered as a potent candidate for nutraceutical and functional food development (Li-Chan, 2015; Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012; Wu, Liao, & Udenigwe, 2017). Most antihypertensive peptides were initially characterized as inhibitors of angiotensin I converting enzyme (ACE), given the essential role of ACE in regulating blood pressure. Interestingly, results from mechanistic study indicated that *in vivo* antihypertensive mechanisms of ACE-inhibitory peptides actually was beyond ACE inhibition, which also included amelioration of endothelial dysfunction, down-regulation of angiotensin type I receptor (AT1R) expression, attenuation of vascular inflammation, and retarding vascular remodelling (Fan, Liao, & Wu, 2018; Jahandideh et al., 2016; Majumder & Wu, 2015; Wu et al., 2017). Recently, using a transcriptomics study we found that mRNA level of angiotensin converting enzyme 2 (ACE2) in spontaneously hypertensive rats (SHRs) could be up-regulated by IRW (Ile-Arg-Trp) (Majumder et al., 2015), an antihypertensive peptide we previously identified from egg white ovotransferrin (Majumder & Wu, 2011).

In contrast to ACE, which is responsible for generating vasoconstricting angiotensin II (Ang II), ACE2 degrades Ang II into angiotensin (1-7) (Ang (1-7)) to exert the counterbalancing effects of Ang II via mas receptor (MasR) (Santos et al., 2003). It was documented that in SHRs, increased ACE2 expression could contribute to reduced blood pressure (Díez-Freire et al., 2006). Indeed, up-regulation of ACE2 expression was also associated with attenuated hypertension-linked cardiac remodelling (Patel, Zhong, Grant, & Oudit, 2016). On the contrary, ACE2 knockout (ACE2KO) mice showed enhanced Ang II-induced fibrosis, superoxide production, inflammatory cytokine level and hypertrophic cardiomyopathy (Alghamri et al., 2012). In

addition, supplementation of human recombinant ACE2 could blunt Ang II-mediated hypertrophic response and superoxide production in ACE2KO mice (Zhong et al., 2010), which further confirmed the significant roles of ACE2 in cardiovascular functions. Therefore, ACE2 has been considered as a novel target for cardiovascular therapy (Ferreira et al., 2010). The discovery of IRW's potential in targeting ACE2 indicated a possible new mechanism of antihypertensive peptides, which represents a new strategy for identification of antihypertensive peptides from food protein sources.

Pea (*Pisum sativum L.*) is among the world's oldest crops (Roy, Boye, & Simpson, 2010). It is grown worldwide for human and animal consumption (Dahl, Foster, & Tyler, 2012). Pea is considered as an important agricultural commodity as it is high in fibre and protein, low in sodium and fat, and it is also an excellent source of complex carbohydrates, vitamin B and minerals (Roy et al., 2010). Given its high nutrient density, pea is recognized as a valuable food commodity, which is capable to meet the nutritious needs of undernourished people (Dahl et al., 2012). It is noteworthy that health benefits of pea consumption, beyond meeting nutrient requirement, have been reported (Curran, 2012; Dahl et al., 2012; Mudryj, Yu, & Aukema, 2014). Bioactive peptides including antihypertensive peptides, antidiabetic peptides and antioxidant peptides have been characterized from pea protein with heath promoting properties (Li & Aluko, 2010; Nongonierma & FitzGerald, 2015; Pownall, Udenigwe, & Aluko, 2010; Roy et al., 2010). However, previous study pertaining to identification of antihypertensive peptides from pea protein was based on the strategy of ACE inhibition (Roy et al., 2010). Notably, results from preliminary experiments suggested that thermoase and pepsin-digested pea protein hydrolysate showed a significant effect in up-regulating ACE2 expression in vascular smooth muscle cells

(VSMCs) (Supplementary Figure 3.1), indicating the presence of ACE2 up-regulating peptides in pea protein hydrolysate.

Overall, the objective of this study was to identify ACE2 up-regulating peptide from pea protein hydrolysate based on activity-guided purification. Besides, ACE2-mediated anti-inflammatory effect of the identified peptide was investigated to further explore the beneficial effects of the ACE2 up-regulating peptide in vasculature.

6.2 Materials and Methods

6.2.1 Materials

Propulse™, a commercial pea protein isolate (~ 82% protein content) was purchased from Nutri-Pea Ltd. (Portage la Prairie, Manitoba, Canada). Thermoase PC10F (from *Bacillus thermoproteolyticus Var. Rokko*, food grade alternative to thermolysin) was purchased from Amano Enzyme Inc. (Nagoya, Japan). Pepsin (from porcine gastric mucosa, 3200 U/mg), pancreatin, trifluoroacetic acid (TFA) and Ang II were purchased from Sigma-Aldrich (MO, USA). HPLC grade acetonitrile (ACN) was purchased from Fisher Scientific (Ottawa, ON, Canada). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) were purchased from Gibco/ Invitrogen (Carlsbad, CA, USA). Penicillin-Streptomycin as well as Gentamicin was from Life Technologies (Carlsbad, CA, USA).

6.2.2 Preparation of protein hydrolysate

Pea protein isolate was suspended in ddH_2O at a concentration of 5% (w/v). Thermoase (4%) was used for the hydrolysis at pH 8.0, 65 ℃, 3 h, followed by pepsin (2%) digestion at pH 2.0, 37 ℃, 3 h. The hydrolysate was heated to 100 ℃ for enzyme inactivation then adjusted to pH 7.0. Supernatant was collected after centrifugation (45 min, 10,000 g, 4 ℃). Hydrolysate was

desalted with a C18 cartridge column (Sep-Pak® Vac, 35 cc, C18 cartridges, Waters Corporation, Milford, Massachusetts, USA), and then lyophilized by freeze dry.

6.2.3 Purification of pea protein hydrolysate

Pea protein hydrolysate was first ultra-filtrated by membrane with a 3 kDa molecular weight cut-off in an Amicon 8400 Ultrafiltration cell (Millipore Corp. Bedford, MA). The permeate was then fractionated using C18 cartridges (Sep-Pak® Vac, 35 cc, C18 cartridges, Waters Corporation) and divided into five fractions eluted with the different concentrations of acetonitrile (ACN): 10% ACN (F1), 20% ACN (F2), 30% ACN (F3), 50% ACN (F4) and 80% ACN (F5); and all these solutions contain 0.1% TFA.

The most active fraction was then loaded to a Waters X-bridge C18 column (10 × 150 mm, 5 μm, Waters Corporation). The buffer A1 (0.1% TFA in Milli-Q water) and buffer B1 (0.1% TFA in ACN) were used as the mobile phase. Flow rate was 3.0 mL/min and absorbance was measured at 215 nm. The samples were eluted by gradients up to 99% B1. Fractions were collected based on peak separation. The fraction determined as the most active one was then purified by a cation High-Prep 16/10 column (SP Sepharose FF, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) coupled with FPLC system (AKTA explorer 10XT system). The sample was dissolved in buffer A2 (10 mM ammonium acetate, pH 4.0 adjusted with acetic acid) for the separation at a flow rate of 3 mL/min. The linear gradient elution was up to 10% buffer B2 (0.5 M ammonium carbonate solution at pH 8.8). The elution was monitored at 215 nm and collected based on the chromatography profile. Each fraction was desalted by Waters Sep-Pak C18 cartridge (Waters Corporation). The most active elution was finally loaded to a Waters Xbridge C18 column (3 ×

250 mm, 5 μm) and eluted with H_2O-ACN solvents, which was the same as buffer A1 and buffer B1. Fractions were collected based on peak separation. All the fractions were freeze-dried.

6.2.4 Identification of peptides by liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Identification of peptides in the fraction of F-2-3-4-3 was carried out by an online Q-TOF premier mass spectrometer (Waters Corporation, Milford, MA, USA) coupled with a nano-Acquity UPLC system (Waters Corporation), which was described in previous study (Wang, Liao, Nimalaratne, Chakrabarti, & Wu, 2018; Yu, Field, & Wu, 2018). A Waters Atlantis d C18 UPLC column (150 mm × 75 μm, 3 μm, Waters Corporation) was applied to separate sample by using solvent A (0.1% formic acid in Milli-Q water) and solvent B (0.1% formic acid in ACN). Briefly, 5 μL of sample was loaded onto a 5 μm trapping column to be trapped for 2 min at a flow rate of 10 μL/min. Afterwards, all the compounds were eluted using gradients from 1% to 15% B over 5 min, 15% to 45% B over 40 min, 45% to 95% B over 5 min at 0.35 μL/min. The flow entered directly into the mass spectrometer via a nano-Lockspray ionization source in a positive ion mode (capillary voltage of 3.40 kV and source temperature of 100 °C). Spectra were recorded over the mass/charge (m/z) ranges of 200-1200 in MS mode and 50-1990 in MS/MS mode. Database search was performed by Mascot search engine (www.matrixscience.com) within NCBI non-redundant (NCBInr) database. Software MassLynx V4.1 (Micromass UK, Ltd., Wythenshawe, Manchester, UK) was used in combination with manual *de novo* sequencing to process the MS/MS data and to perform peptide sequencing. The identified peptides were synthesized by 'GenScript' (Piscatway, NJ, USA) with a purity of at least 98%. Peptide sequence and purity were validated by HPLC-MS/MS.

6.2.5 Cell culture

The same as our previous study (Chakrabarti, Liao, Davidge, & Wu, 2017; Liao, Chakrabarti, Davidge, & Wu, 2016; Lin, Liao, Bai, Wu, & Wu, 2017), A7r5 cells, a VSMC cell line, was used in this study. The cells between passage 3 and 10 after received were grown in DMEM supplemented with 10% FBS and antibiotics (Penicillin-Streptomycin and Gentamicin) until reached confluence. For activity-guided purification, the confluent cells were placed in a quiescing medium (DMEM + 1% FBS + antibiotics) and then treated with hydrolysate fraction/peptide for 24 hours. For Ang II-stimulated cell experiments, cells were treated with 50 μM of peptide with/without 1 μM MasR antagonist (A779) for 1h, followed by the addition of 1 μM Ang II for a 23h-co-treatment.

6.2.6 Western blotting

After the co-treatment, A7r5 cells were lysed in boiling hot Laemmle's buffer containing 50 μM DTT (a reducing agent) and 0.2% Triton-X-100 to prepare samples for western blotting. These cell lysates were then run in 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and immunoblotted with anti-ACE2 antibody (Abcam, Cambridge, MA, USA), anti-MasR antibody (NOVUS Biologicals, Littleton, CO, USA), or anti-cyclooxygenase 2 antibody (COX2, NOVUS Biologicals). Goat anti-rabbit IRDye 680RD or Donkey anti-mouse 800CW from Licor Biosciences (Lincoln, NE, USA) was used as the secondary antibody. Protein bands were detected by Licor Odyssey BioImager (Licor Biosciences). Quantification of protein bands were performed using Image Studio Lite 5.2 (Licor Biosciences). Each band was normalized to the house-keeping protein α-tubulin (Abcam). Cell lysates from untreated cells were loaded on every gel and all data were expressed as % of the corresponding untreated.

6.2.7 Statistics

Data were presented as mean ± SEM (standard error of mean) of 4 to 6 independent experiments. One way analysis of variance (ANOVA) with the Turkey's post-hoc test was used for determination of statistical significance by PRISM 5 statistical software (GraphPad Software) was used for all analyses. $P<0.05$ was considered to be significant.

6.3 Results

6.3.1 Purification of pea protein hydrolysate

Pea protein hydrolysate was purified based on activity-guided fractionation via four steps. As cellular assay is considered to be more biologically relevant, the effect of each fraction on ACE2 was evaluated by accessing the protein expression of ACE2 in A7r5 cells.

After passing through an ultrafiltration membrane with a 3 kDa cut-off, pea protein peptides enriched in 20 % of ACN (F2) was the most active in up-regulating ACE2 expression in A7r5 cells (Figure 6.1A), which was then subjected to purification by reverse phase high performance liquid chromatography (RP-HPLC). F2 was further separated to 8 fractions and the fraction of F2-3 showed the highest activity (Figure 6.1B). In the next step, cation exchange chromatography was used to separate F2-3 based on the differences in the number of positive charge of different peptides. Among the 6 fractions obtained from this step, F2-3-4 was the most active (Figure 6.1C). Finally, F2-3-4 was fractionated by RP-HPLC to further enrich active peptides. As shown by Figure 4, F2-3-4-3 exhibited the highest activity (Figure 6.1D).

6.3.2 Identification of peptides by LC-MS/MS

F2-3-4-3 was subsequently subjected to LC-MS/MS for peptide identification. There were 3 peptides identified from F2-3-4-3 (Figure 6.2A) with the sequence of AKSLSDRFSY, VRASSNLNL and VPHYNL, respectively (Figure 6.2B). AKSLSDRFSY and VPHYNL were mapped to legumin; VRASSNLNL was mapped to provicilin. All of these three peptides were firstly reported.

6.3.3 Activity validation of pea protein-derived peptides in up-regulating ACE2 expression in A7r5 cells

Synthesized peptides identified from pea protein hydrolysate were used to validate their activity in up-regulating ACE2 expression in A7r5 cells. Only AKSLSDRFSY showed a significant effect (Figure 6.3A), indicating this peptide is the predominant component present in pea protein hydrolysate in up-regulating ACE2. Interestingly, AKSLSDRFSY could also up-regulate ACE2 and MasR expressions in Ang II-stimulated A7r5 cells (Figure 6.3B), which suggested that the up-regulated ACE2 expression by the peptide treatment could be further transited to the receptor of the ACE2/Ang (1-7) axis. More importantly, as the ACE2/Ang (1-7) axis could counterbalance harmful effects of Ang II (Santos, Ferreira, Verano-Braga, & Bader, 2013), AKSLSDRFSY might have other beneficial attributes against Ang II stimulation in VSMCs.

6.3.4 Up-regulation of ACE2 by AKSLSDRFSY mediated anti-inflammatory activity of the peptide in Ang II-stimulated A7r5 cells

Since the up-regulation of ACE2 was associated with attenuated inflammation (Simões e Silva, Silveira, Ferreira, & Teixeira, 2013), we then examined anti-inflammatory activity of AKSLSDRFSY in Ang II-stimulated A7r5 cells. It was found that Ang II-stimulated up-

regulation of COX2 could be reduced by the peptide treatment (Figure 6.4A), suggesting the anti-inflammatory activity of AKSLSDRFSY in Ang II-stimulated A7r5 cells. Surprisingly, the addition of MasR antagonist (A779) abolished the anti-inflammatory effect of the peptide (Figure 6.4B), which indicated that the anti-inflammatory activity of AKSLSDRFSY was, at least partially, contributed by ACE2 up-regulation in A7r5 cells.

6.4 Discussion

Food protein-derived antihypertensive peptides have long been considered as a safer alternative to antihypertensive drugs (Martínez-Maqueda et al., 2012). Given the essential role in regulating blood pressure, ACE is a conventional target for identification of antihypertensive peptides from various food proteins (Wu et al., 2017). However, we previously found that egg white ovotransferrin-derived antihypertensive peptides IRW could up-regulate mRNA level of ACE2 in mesenteric artery of SHRs (Majumder et al., 2015), which provided a new opportunity for characterizing antihypertensive peptides from food proteins. In other words, ACE2 may be a novel target for identification of food protein-derived antihypertensive peptides.

In the present study, thermoase and pepsin-digested pea protein hydrolysate was fractionated based on the strategy of ACE2 up-regulation in A7r5 cells. Peptides with the sequence of AKSLSDRFSY, VRASSNLNL and VPHYNL were characterized. While, only AKSLSDRFSY showed activity in up-regulating ACE2 expression in VSMCs, indicating this peptide plays a predominant role in pea protein hydrolysate to up-regulate ACE2 expression.

Bioactivities of pea protein hydrolysates have been reported including anti-hypertensive activity, anti-inflammatory activity and anti-adhesive activity (Li et al., 2011; Ndiaye, Vuong, Duarte, Aluko, & Matar, 2012; Niehues et al., 2010); the identification of peptide from pea protein

hydrolysate with the potential to up-regulate ACE2 further confirmed that pea protein is an excellent commodity to identify bioactive peptides. It is also striking that although ACE-inhibitors from pea proteins have been documented (Aluko et al., 2015; Li & Aluko, 2010), AKSLSDRFSY reported from this study is the first pea protein-derived peptide with the activity to up-regulate ACE2. Database searching result suggested that legumin is the parent protein of AKSLSDRFSY. Legumin is one of the major types of globulins, a major type of protein accounting for approximately 70% of the total protein in pulses and (Roy et al., 2010). Legumin hydrolysate was previously reported to show ACE-inhibitory activity (Yust et al., 2003). The identification of AKSLSDRFSY as an up-regulator of ACE2 from the present study raises the feasibility to use legumin, an abundant pulse protein as the starting material, to search bioactive peptides with benefits for improving cardiovascular health.

The activity of AKSLSDRFSY as an ACE2 up-regulator was further validated in Ang II-stimulated VSMCs. In addition, expression of MasR was up-regulated by the peptide treatment consistently. Stimulation of Ang II in VSMC induces inflammation, an intracellular event in developing vascular remodelling, which will ultimately lead to increasing blood pressure (Touyz & Schiffrin, 2000). It is worthwhile to note that AKSLSDRFSY also showed anti-inflammatory activity in VSMCs as it down-regulated COX2 expression in Ang II-stimulated VSMCs. More interestingly, the addition of MasR antagonist A779 could abolish the anti-inflammatory activity of AKSLSDRFSY, which indicated the anti-inflammatory activity of this peptide was at least partially, mediated by the up-regulations of ACE2/MasR. This finding was consistent with the existing literatures documenting the protective effects of ACE2 up-regulation in vasculature (Wang, Tikellis, Thomas, & Golledge, 2013; Simões e Silva, et al, 2013).

In conclusion, AKSLSDRFSY as a novel ACE2 up-regulator was characterized from thermoase and pepsin-digested pea protein hydrolysate in this study. Up-regulation of ACE2 by AKSLSDRFSY showed anti-inflammatory activity in Ang II-stimulated VSMCs. Findings from this study confirmed the feasibility to identify ACE2 up-regulating peptides from food proteins, especially pea protein. More work is warranted to further explore protective effects of ACE2 up-regulation by this bioactive peptide in vascular cells as well as *in vivo*.

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Figure 6.1A: Fractionation of pea protein hydrolysate by C18 cartridge. 2.5 mg/mL of each fraction of pea protein hydrolysate from a C18 column was treated to A7r5 cells for 24 h. F2 showed the best activity in up-regulating ACE2 expression in A7r5 cells. Expression of ACE2 was analysed by western blot. Protein bands were quantified by densitometry and normalized to their respective loading control (α-tubulin). Data are expressed as mean ± SEM of 5 independent experiments. * indicates $p<0.05$ as compared with the control group.

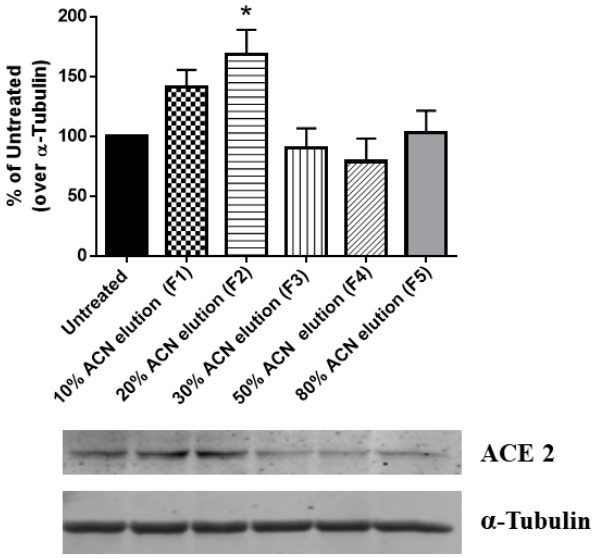


Figure 6.1B: Fractionation of F2 by RP-HPLC. Eight fractions were collected. 1 mg/mL of each fraction was treated to A7r5 cells for 24 h. F2-3 showed the best activity in up-regulating ACE2 expression in A7r5 cells. Expression of ACE2 was analysed by western blot. Protein bands were quantified by densitometry and normalized to their respective loading control (α-tubulin). Data are expressed as mean ± SEM of 5 independent experiments. * indicates $p<0.05$ as compared with the control group.

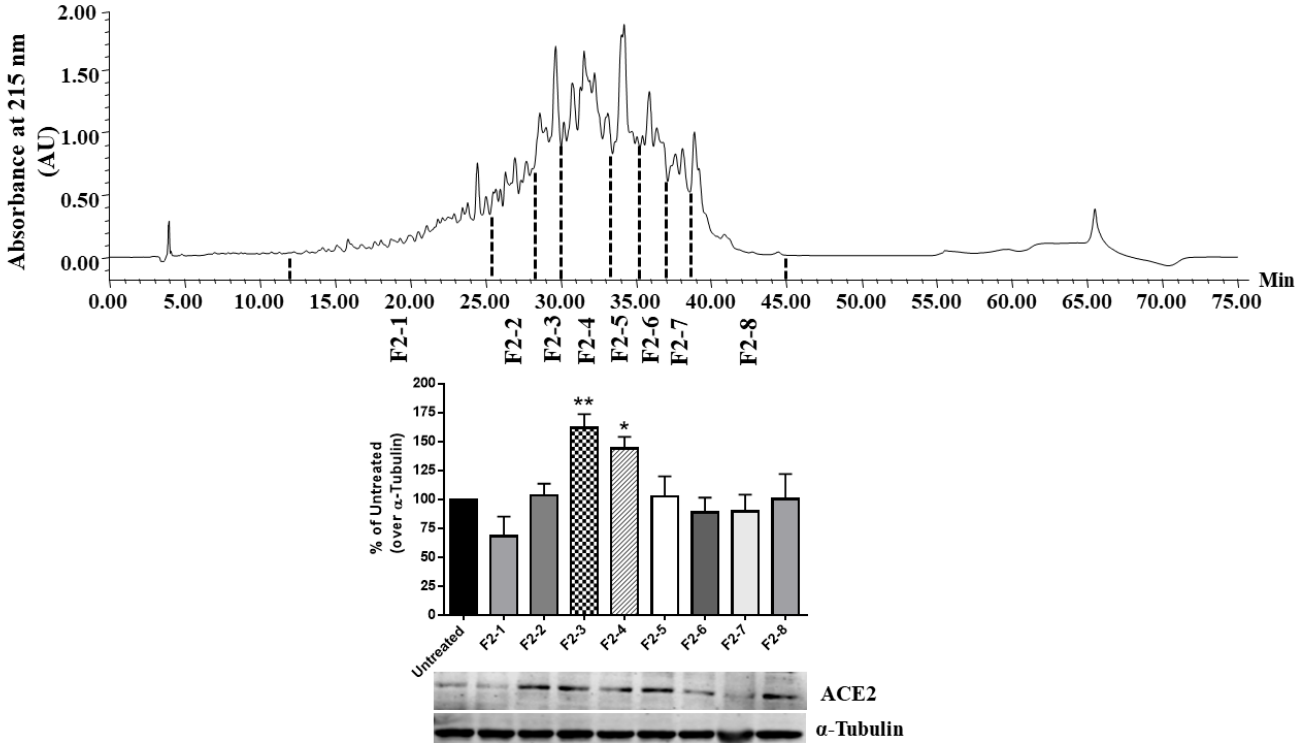


Figure 6.1C: Fractionation of F2-3 by cation exchange chromatography. Six fractions were collected. 0.5 mg/mL of each fraction was treated to A7r5 cells for 24 h. F2-3-4 showed the best activity in up-regulating ACE2 expression in A7r5 cells. Expression of ACE2 was analysed by western blot. Protein bands were quantified by densitometry and normalized to their respective loading control (α-tubulin). Data are expressed as mean ± SEM of 4 independent experiments. * indicates $p<0.05$ as compared with the control group.

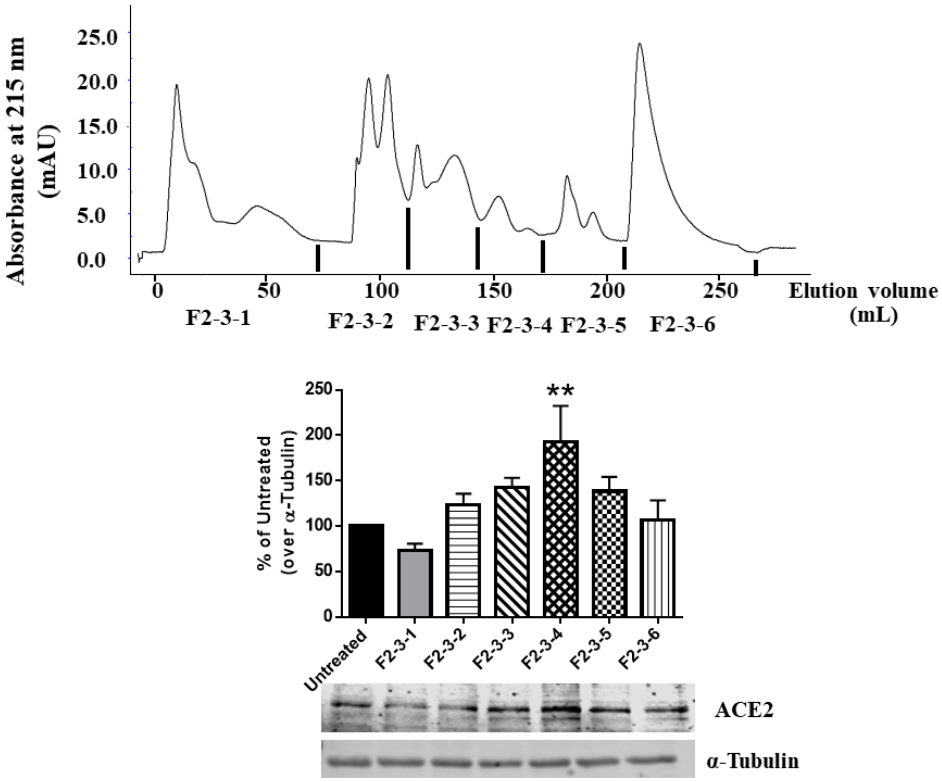


Figure 6.1D: Fractionation of F2-3-4 by RP-HPLC. Three fractions were collected. 0.1 mg/mL of each fraction was treated to A7r5 cells for 24 h. F2-3-4-3 showed the best activity in up-regulating ACE2 expression in A7r5 cells. Expression of ACE2 was analysed by western blot. Protein bands were quantified by densitometry and normalized to their respective loading control (α-tubulin). Data are expressed as mean ± SEM of 4 independent experiments. * indicates $p<0.05$ as compared with the control group.

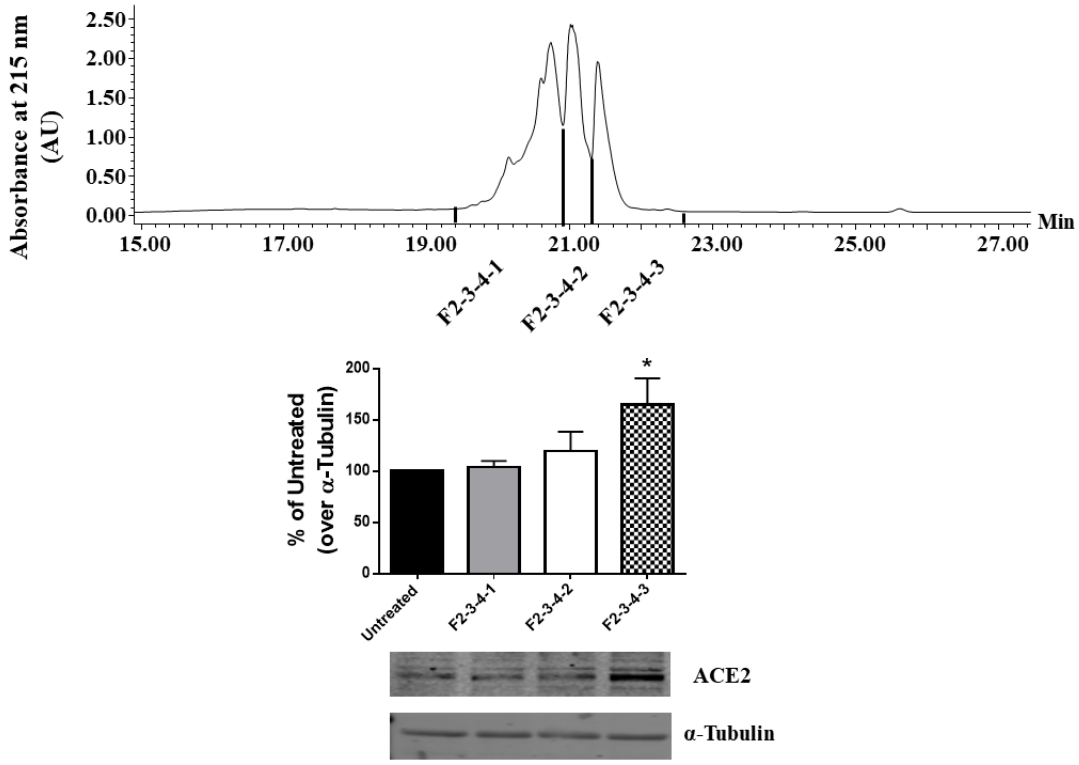


Figure 6.2A: Mass spectrum of F2-3-4-3. Peptides in F2-3-4-3 were profiled by LC-MS/MS.

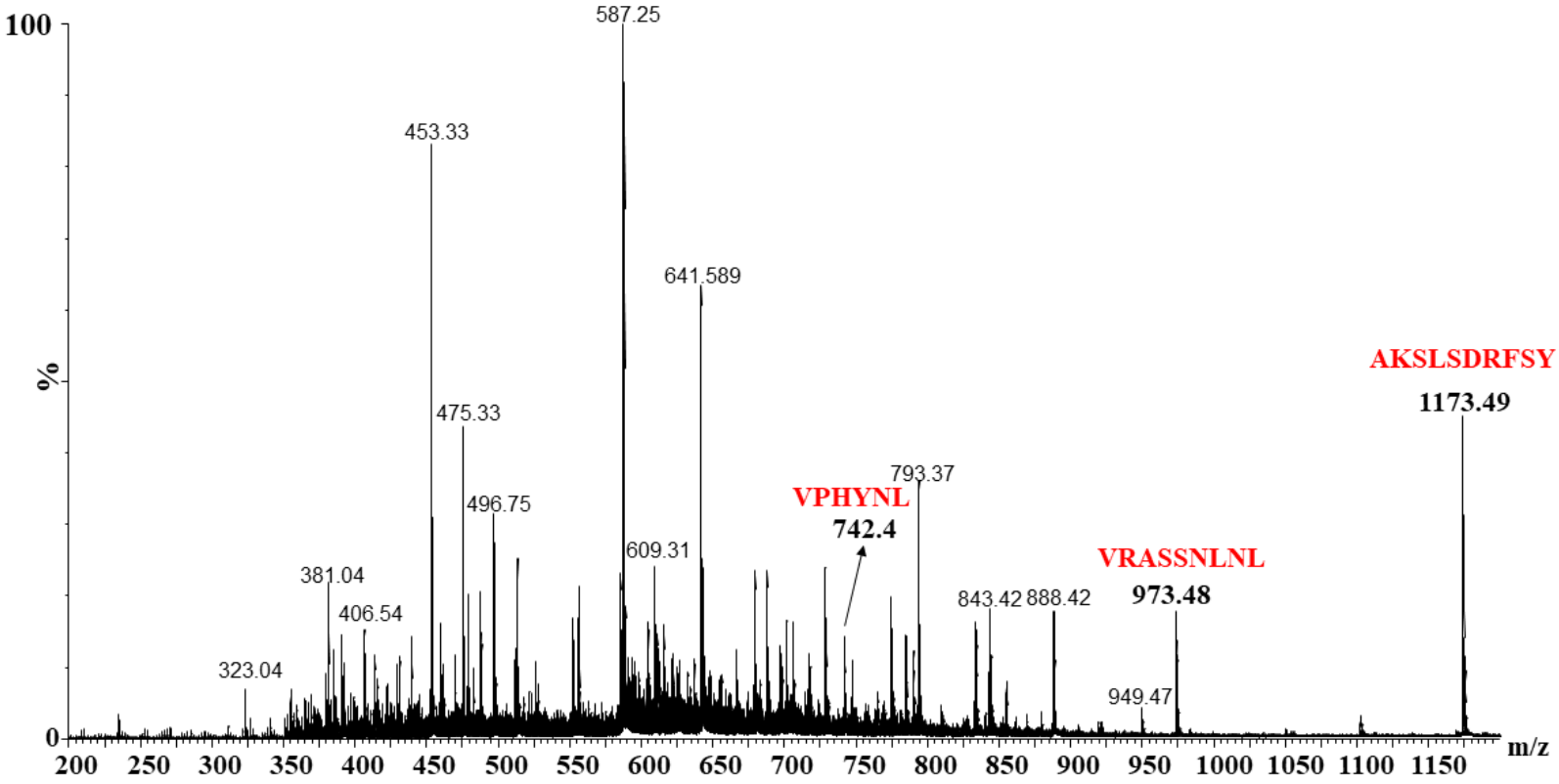


Figure 6.2B: Tandem mass spectrum of each peptide identified from pea protein hydrolysate.

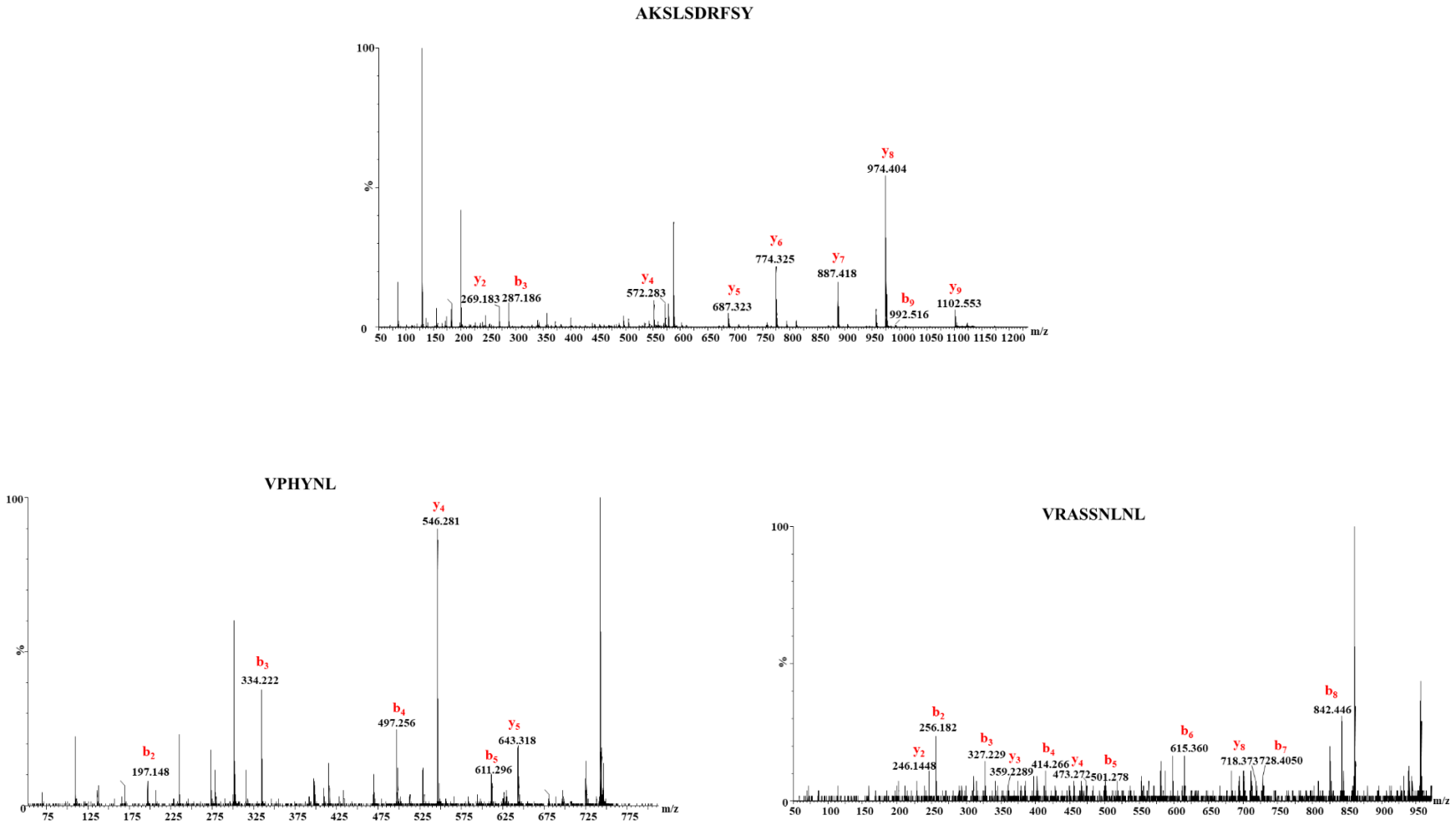


Figure 6.3A: AKSLSDRFSY showed a significant effect in up-regulating ACE2 expression in A7r5 cells. 50 μM of each identified peptide was treated to A7r5 cells for 24 h. Expression of ACE2 was analysed by western blot. Protein bands were quantified by densitometry and normalized to their respective loading control (α-tubulin). Data are expressed as mean ± SEM of 4 independent experiments.

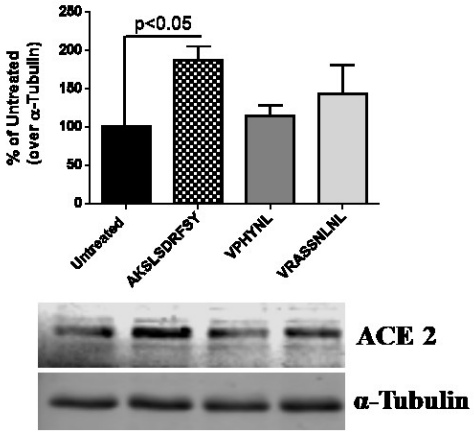


Figure 6.3B: AKSLSDRFSY up-regulated ACE2 and MasR expressions in Ang II-stimulated A7r5 cells. A7r5 cells were pre-treated with 50 μM of the peptide for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for ACE2 and MasR. Protein bands were quantified by densitometry and normalized to their respective loading control (α-tubulin). Data are expressed as mean ± SEM of 5 independent experiments.

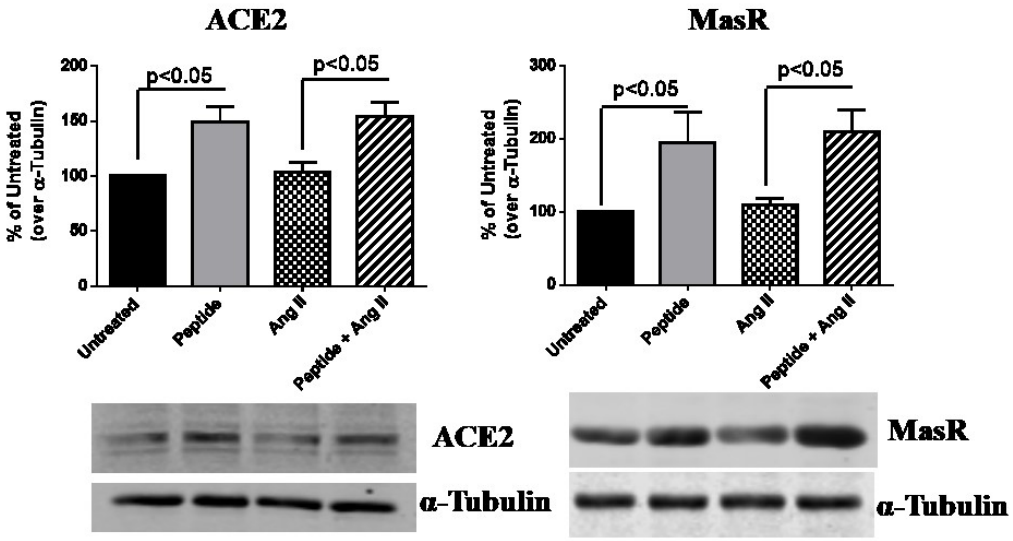


Figure 6.4A: AKSLSDRFSY down-regulated COX2 expression in Ang II-stimulated A7r5 cells. A7r5 cells were pre-treated with 50 μM of the peptide for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for COX2. Protein bands were quantified by densitometry and normalized to their respective loading control (α-tubulin). Data are expressed as mean ± SEM of 4 independent experiments.

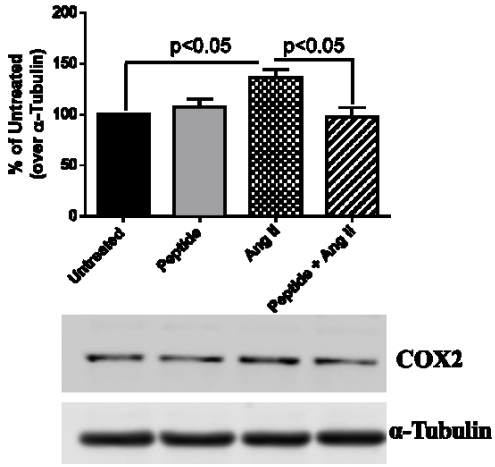
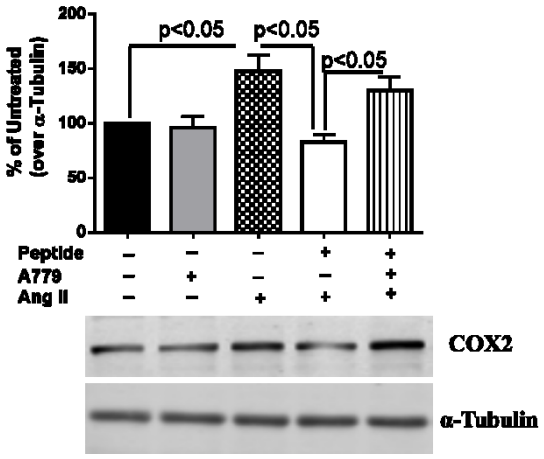
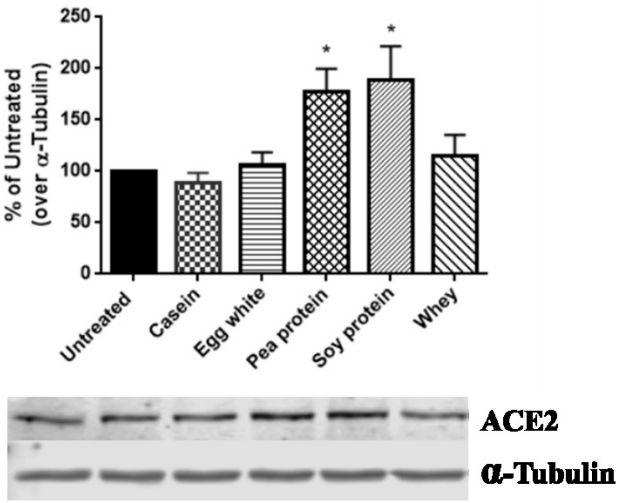


Figure 6.4B: Up-regulations of ACE2 and MasR mediated down-regulation of COX2 by AKSLSDRFSY in Ang II-stimulated A7r5 cells. A7r5 cells were pre-treated with 50 μM of the peptide and/or 1 μM of MasR antagonist (A779) for 1 h prior to 23 h stimulation with 1 μM of Ang II. Cells were lysed and immunoblotted for COX2. Protein bands were quantified by densitometry and normalized to their respective loading control (α-tubulin). Data are expressed as mean ± SEM of 6 independent experiments.



Supplementary Figure 6.1: Effects of different food protein hydrolysates on ACE2 expression in A7r5 cells. Different food proteins including casein, egg white, pea protein, soy protein and whey were digested by thermoase and pepsin. The hydrolysates were then used to screen their activity in up-regulating ACE2 expression in A7r5 cells. Different protein hydrolysates (2.5 mg/mL) was treated to A7r5 cells for 24 h. Expression of ACE2 was analysed by western blot. Protein bands were quantified by densitometry and normalized to their respective loading control (α-tubulin). Data are expressed as mean ± SEM of 5 independent experiments. * indicates $p<0.05$ as compared with the control group.



CHAPTER 7 - General Discussion and Future Directions

7.1 Foundation of the present research

Hypertension, a risk factor of cardiovascular diseases, is a global health challenge (Perkovic, Huxley, Wu, Prabhakaran, & MacMahon, 2007). Essential hypertension, which is also known as primary hypertension, accounts for 95% of all the hypertensive cases (Carretero & Oparil, 2000). Etiology of essential hypertension is complex, while, an over-activated renin angiotensin system (RAS) is considered to play a chief role (Beevers, Lip, & O'Brien, 2001). In the RAS, angiotensin I is catalyzed by angiotensin converting enzyme (ACE) into angiotensin II (Ang II). Potent vasoconstricting effect of Ang II is exerted via angiotensin type I receptor (AT1R) (Hunyady, Balla, & Catt, 1996). Indeed, detrimental effects of Ang II in vasculature is beyond vasoconstriction, it also induces vascular remodelling which further increases resistance of blood vessels (Touyz, 2005). Hence, to ameliorate the effects of Ang II by either inhibiting ACE or blocking AT1R is classical therapeutic strategy for essential hypertension.

Angiotensin converting enzyme 2 (ACE2), an analogous of ACE degrades Ang II into Ang (1-7) and further binds to mas receptor (MasR) (Donoghue et al., 2000; Santos et al., 2003; Vickers et al., 2002). Abundant literatures have documented protective effects of the activated ACE2/Ang (1-7)/MasR axis to counterbalance the harmful effects of ACE/Ang II/AT1R axis within or beyond cardiovascular functions. ACE2 has thus been considered as a novel target for cardiovascular therapy (Der Sarkissian, Huentelman, Stewart, Katovich, & Raizada, 2006; Ferrario, Trask, & Jessup, 2005; Shenoy, Qi, Katovich, & Raizada, 2011).

Food protein-derived antihypertensive peptides have gained substantial interests during the past decades as they provide food-derived alternatives to antihypertensive drugs (Martínez-Maqueda,

Miralles, Recio, & Hernández-Ledesma, 2012). Given Ang II is in the central of the vicious circle, to reduce the generation of Ang II by characterizing ACE-inhibitory peptides via *in vitro* chemical assay is a common approach in the identification of antihypertensive peptides. Previous studies suggest there are mechanisms other than ACE inhibition for antihypertensive peptides (Wu, Liao, & Udenigwe, 2017). For example, our previous research found egg white ovotransferrin-derived peptide IRW (Ile-Arg-Trp) could up-regulate mRNA level of ACE2 in spontaneously hypertensive rat (SHR) (Majumder et al., 2015). This finding also indicated the potential of IRW in targeting ACE2. Under such circumstances, the main aim of this research was to evaluate the capability of IRW as an ACE2 activator. This work also contributed to characterizing food protein-derived peptides which can up-regulate ACE2 expression.

7.2 Key findings of the present research

7.2.1 Egg white ovotransferrin-derived tripeptide IRW is an activator of ACE2

Given the potential of IRW in targeting ACE2, the objective of the first study (Chapter 3) was to evaluate effects of IRW on activity and protein expression of ACE2. To achieve the objective, human recombinant ACE2 (hrACE2) was used for enzymatic activity assay. A commercially available cell line of vascular smooth muscle cells (VSMCs), A7r5, was used to evaluate the effects of IRW on activity and expression of cellular ACE2. In addition, protein expressions of ACE2 in aorta and kidney tissues from SHR upon 18-day oral administration of IRW (15mg/kg body weight) were tested. Results indicated that IRW activated hrACE2 with an EC_{50} value (the concentration of IRW to elicit 50% of maximal response) of 7.24×10^{-5} M. This finding demonstrated a direct ACE2-activating effect of IRW. The EC_{50} value of IRW is within the same magnitude of XNT (1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyloxy]-9H-xanthene-9-one), an synthetic ACE2 activator (Hernández Prada et al., 2008),

indicating the potency of IRW as an ACE2 activator at least in the *in vitro* system. A molecular docking study found activation of ACE2 by IRW was proposed through binding to the amino acid residues in the subdomain I of ACE2 through hydrogen bonding. However, the behaviour features of IRW in interacting with ACE2 remain ambiguous. The ACE2-activating effect of IRW was also observed in A7r5 cells. While, IRW treatment (50 μM) could also significantly increase the expressions of ACE2 both at mRNA and protein levels in A7r5 cells. Findings in this cell study suggested dual roles of IRW in targeting ACE2 as IRW could directly activate ACE2 as well as up-regulate ACE2 expression in A7r5 cells. Oral administration of IRW to SHRs up-regulated ACE2 protein levels in kidney and aorta, which further confirmed that IRW could up-regulate ACE2 at protein expression level in SHRs, followed by our previous study showing the effect of IRW in up-regulating mRNA of ACE2 (Majumder et al., 2015).

Although ACE2 has been considered as a novel target for hypertension therapy, development of ACE2-activting agent, in particular naturally derived ACE2-acitivating compounds, is at infancy. In addition to IRW, only a casein-derived tripeptide IPP was reported to improve Ang (1-7) level in mesenteric artery of SHRs, but the specific role of ACE2 was not elaborated (Ehlers, Nurmi, Turpeinen, Korpela, & Vapaatalo, 2011). Results from Chapter 3 established IRW as the first food derived ACE2-activating peptide.

7.2.2 Modulatory effects of IRW on Ang II-stimulated intracellular events in VSMCs

VSMC is a highly specialized cell in vasculature. Transformation from quiescent phenotype to synthetic phenotype in response to stimulus of VSMC is a factor triggering vascular remodelling, which is a common feature among major cases of hypertension (Owens, Kumar, & Wamhoff, 2004). Ang II, an active component of the RAS, is more than a potent vasoconstrictor, which also causes phenotype transformations in VSMCs. Excessive proliferation, inflammation,

oxidative stress and migration are typical intracellular events in VSMCs during vascular remodelling (Touyz & Schiffrin, 2000). While, up-regulation of ACE2 contributes to protective effects in VSMCs against Ang II stimulation (Zhang et al., 2010). As IRW was shown to up-regulate ACE2 expression in A7r5 cells, a VSMC cell line, in Chapter 4, IRW was also applied to evaluate modulatory effects on intracellular events in Ang II-stimulated A7r5 cells, as well as the relationship between IRW-mediated ACE2 up-regulation and modulatory effects against Ang II stimulation. Results indicated that IRW treatment could attenuate Ang II-stimulated proliferation, superoxide production, inflammation and migration in A7r5 cells (Chapter 4). Our previous study found that IRW showed antioxidant and anti-inflammatory activities in vascular endothelial cells (Huang et al., 2010), results from this study revealed broader beneficial roles of IRW in different vascular components. p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor κB (NF-κB) pathways were involved in the modulatory effects of IRW in Ang II-stimulated A7r5 cells (Chapter 4). However, IRW did not affect phosphorylation of extracellular signal-regulated kinases 1/2 MAPK (ERK1/2 MAPK) or c-Jun N-terminal kinases 1/2 MAPK (JNK1/2 MAPK), which is different from our previous finding that soy protein-derived tripeptide LSW (Leu-Ser-Trp) could inhibit phosphorylation of ERK1/2 MAPK in Ang II-stimulated A7r5 cells (Lin, Liao, Bai, Wu, & Wu, 2017). The inconsistent roles of IRW and LSW in regulating MAPKs in VSMCs suggested bioactive peptides with different structures could alter their effects in intracellular pathways.

It was also shown that IRW could up-regulate expressions of ACE2 and MasR in Ang II-stimulated A7r5 cells (Chapter 4), indicating beneficial effects of IRW against Ang II in A7r5 cells might be contributed by the up-regulations of ACE2 and MasR. A further study using MasR antagonist and ACE2 knockdown demonstrated the antioxidant and anti-inflammatory activities

of IRW were dependent on ACE2/MasR up-regulations, which confirmed the IRW-mediated up-regulation of ACE2 could further contribute to beneficial effects at least in cellular level. However, ACE2 is a transmembrane containing an extracellular domain, a transmembrane region and an intracellular tail (Jiang, et al, 2014). The region of ACE2 in response to IRW treatment, which further mediates the up-regulation, needs to be further characterized.

7.2.3 IRW-mediated ACE2 activation contributes to decreasing blood pressure in SHRs

SHR is an animal model mimetic to human essential hypertension (Okamato & Aoki, 1963). The adult male SHR is a universally used model for research on antihypertensive peptides. The blood pressure-reducing effect of IRW was previously demonstrated in adult male SHRs (Majumder et al., 2013). Up-regulation of ACE2 is considered as an antihypertensive mechanism of IRW (Majumder et al., 2015). In addition, IRW could activate ACE2 *in vitro* as well as in VSMCs (Chapter 3). Up-regulation of ACE2 contributes to antioxidant and anti-inflammatory activities of IRW in VSMCs (Chapter 4). Hence, the aim of the study in Chapter 5 was to evaluate the contribution of ACE2 activation by IRW to reducing blood pressure in SHRs.

Adult male SHRs (14-16 weeks) were infused with MasR antagonist A779 (48 μg/kg body weight/h) or saline for one week, followed by co-treatment of A779 and IRW (orally administrated, 15 mg/kg body weight) for 7 days. Results indicated that blocking of MasR abolished blood pressure-reducing effect of IRW in SHRs. Circulating ACE2 and Ang (1-7) levels were increased by IRW treatment; Ang II level was decreased. However, circulating ACE level was unaffected by the treatment (Chapter 5). The above results demonstrated IRW could activate ACE2 *in vivo*, which plays a predominant role in lowering blood pressure of SHRs. Although IRW was originally characterized as an ACE-inhibitory peptide by an *in vitro* chemical assay (Majumder & Wu, 2011), conflicting results from the *in vitro* experiments and this *in vivo*

study further facilitate the notion that ACE-inhibitory peptide identified by *in vitro* assay may not target ACE *in vivo* (Wu et al., 2017).

Results from the *ex vivo* experiments suggested that infusion of A779 blunt the IRW-enhanced methacholine-induced vasorelaxation. In aorta of SHR, enhanced phosphorylation of protein kinase B (Akt) and endothelial nitric oxide synthase by IRW treatment were also reduced by A779 infusion (Chapter 5). These results indicated enhanced endothelium-dependent vasorelaxation is a mechanism of IRW as an ACE2 activator to reduce blood pressure in SHRs. Although various antihypertensive peptides have been reported with their activities in improving endothelium-dependent vasorelaxation (Majumder & Wu, 2015; Wu et al., 2017), pathways associated with such activity have not been characterized. Results from Chapter 5 provided a more in-depth view on IRW-modulated endothelial dysfunction in SHRs, which is through the ACE2/Ang (1-7)/MasR axis; it is consistent with the literature documenting endothelium-dependent vasorelaxation is among one of major beneficial effects of increased Ang (1-7) (Faria-Silva, Duarte, & Santos, 2005; Li et al., 2017; Sampaio et al., 2007).

Pro-inflammatory cytokine levels including interleukin 6 and monocyte chemoattractant protein 1 were restored with A779 infusion, as compared with the IRW alone group. Expressions of inhibitory κB α and cyclooxygenase 2 in aorta of SHRs was also affected significantly by A779 infusion (Chapter 5), suggesting IRW-ameliorated vascular inflammation was dependent on the ACE2/Ang (1-7)/MasR axis. As introduced in Chapter 2, vascular inflammation is underlying pathogenesis and persistence of essential hypertension. Findings from Chapter 5 supported that activation of ACE2 by IRW oral administration in SHRs could reduce blood pressure, at least partially, through mitigation of vascular inflammation. Indeed, this finding is consistent with the finding in Chapter 4, in which, IRW-mediated ACE2 up-regulation was found to have anti-

inflammatory effect in VSMCs. Given the essential role of VSMC in altering lumen diameter and resistance of the blood vessel, the anti-inflammatory effect of IRW-mediated ACE2 up-regulation in VSMC might also contribute to the blood pressure-reducing effect of IRW in SHRs. However, a direct evidence is needed to support this notion by using primary cultured cells isolated from the untreated and IRW-treated SHRs.

Although increased Ang (1-7) was reported to down-regulate expression of matrix metalloproteinase 9 (MMP9) via deactivating ERK1/2 MAPK (Rodrigues Prestes, Rocha, Miranda, Teixeira, & Simoes e Silva, 2017), IRW failed to affect phosphorylation of ERK1/2 or expression of MMP9, which might be due to the short treatment period in the study (Chapter 5).

Collectively, IRW decreased blood pressure of SHRs via the ACE2/Ang (1-7)/MasR axis. However, the unique roles of ACE2 in mediating the blood pressure-reducing effect of IRW need to be further explored using ACE2 knockout (KO) mice model. Whereas, the antihypertensive activity of IRW needs to be demonstrated in Ang II-induced hypertensive mice model in prior to proceeding to the ACE2 KO model.

7.2.4 Identification of ACE2 up-regulating peptides from pea protein hydrolysate

The capability of egg white ovotransferrin-derived IRW as an ACE2 activator as well as associated down-stream beneficial effects were demonstrated by Chapters 3, 4, and 5. However, it is uncertain if more peptides with the ability to target ACE2 can be identified from food protein sources. The objective of Chapter 6 was to identify peptides which can up-regulate ACE2 from food proteins. Casein, egg white, pea protein, soy protein and whey were used in the preliminary experiments. It was found that thermoase and pepsin-digested pea protein hydrolysate showed a significant effect in up-regulating ACE2 expression in A7r5 cells. Thus,

this pea protein hydrolysate was further purified based on activity-guided fractionation. A peptide with the sequence of AKSLSDRFSY was identified and its activity in up-regulating ACE2 expression in A7r5 cells was validated (Chapter 6). Bioactive peptides including antihypertensive peptides have been characterized from pea proteins (Roy, Boye, & Simpson, 2010). Identification of the peptide which can up-regulate ACE2 from pea protein hydrolysate further confirmed that pea protein is an ideal starting material to search for bioactive peptides. More importantly, the identification of AKSLSDRFSY facilitated the possibility to identify ACE2 up-regulating peptides from food protein sources. However, assessment of ACE2 protein level in VSMCs was used for activity-guided fractionation in this study to identify ACE2 up-regulating peptides. This method did not consider the effect on ACE2 activity. Future *in vivo* study is suggested to test the efficacy of the identified pea protein-derived peptide.

AKSLSDRFSY also up-regulated ACE2 and MasR expressions but down-regulated COX2 expression in Ang II-stimulated A7r5 cells (Chapter 6), showing the anti-inflammatory potential of this peptide in VSMCs. While, the addition of MasR antagonist restored the COX2 expression, indicating the anti-inflammatory activity of this pea protein-derived peptide was attributed by up-regulations of ACE2 and MasR in VSMCs. This finding was comparable as the finding from IRW that ACE2 up-regulating peptide also has the anti-inflammatory attribute in VSMC. While, signaling pathways mediating the anti-inflammatory effect upon ACE2 up-regulation need to be further characterized.

In conclusion, the research from this thesis demonstrated egg white ovotransferrin-derived antihypertensive peptide IRW could activate and up-regulate ACE2, which is associated with beneficial effects in vasculature. In VSMCs, IRW mediated ACE2 up-regulation contributes to antioxidant and anti-inflammatory effects. In SHRs, IRW activates the circulating ACE2/Ang (1-

7) axis, which further enhance endothelium-dependent vasorelaxation and mitigate vascular inflammation. All of these protective effects of IRW are implicated to lower blood pressure in SHRs. Beneficial effects of IRW in relation with ACE2 up-regulation or activation are presented in Figure 7.1. In addition, this thesis also identified a peptide with the sequence of AKSLSDRFSY from pea protein, which could up-regulate ACE2 expression in VSMCs.

7.3 Recommendations for future research

Given the findings and limitations of this thesis, future studies are recommended as follows:

1. Only adult male SHRs were used, which limited the scope of the study. It is interesting that female SHRs appear to be more dependent on Ang (1-7) to mediate effects of antihypertensive agents than males (Zimmerman, Harris, & Sullivan, 2014). Thus, it will be of interest to investigate the antihypertensive activity in particular the effect on ACE2 of IRW treatment in female SHRs. In addition, it is warranted to further explore the effect of IRW on hypertensive model beyond SHR (i.e. Ang II-induced hypertensive mice model) to broaden our horizon on the antihypertensive activity of IRW.

2. As activation of the ACE2/Ang (1-7)/MasR is associated with beneficial effects beyond cardiovascular functions (introduced in Chapter 2), it is suggested to perform more activity studies to identify if IRW-activated ACE2 could contribute to other functions in addition to hypotension, which can expand the potential applications of IRW.

3. The ability of pea protein-derived AKSLSDRFSY in up-regulating ACE2 was demonstrated in VSMCs. While, it is necessary to do some follow-up studies on the stability and *in vivo* efficacy of this peptide. On the other hand, AKSLSDRFSY was identified from thermoase and pepsin-digested pea protein hydrolysate, it is worthwhile

to try to characterize peptides which can up-regulate ACE2 from different enzymatic digests of pea protein.

4. The identification of AKSLSDRFSY from pea protein hydrolysate suggests that it is feasible to identify peptide targeting ACE2 from food proteins. Thus, it is worthwhile to further explore various food proteins with the aim to identify peptides targeting on ACE2, and if any, structure and activity study of these peptides are also suggested.

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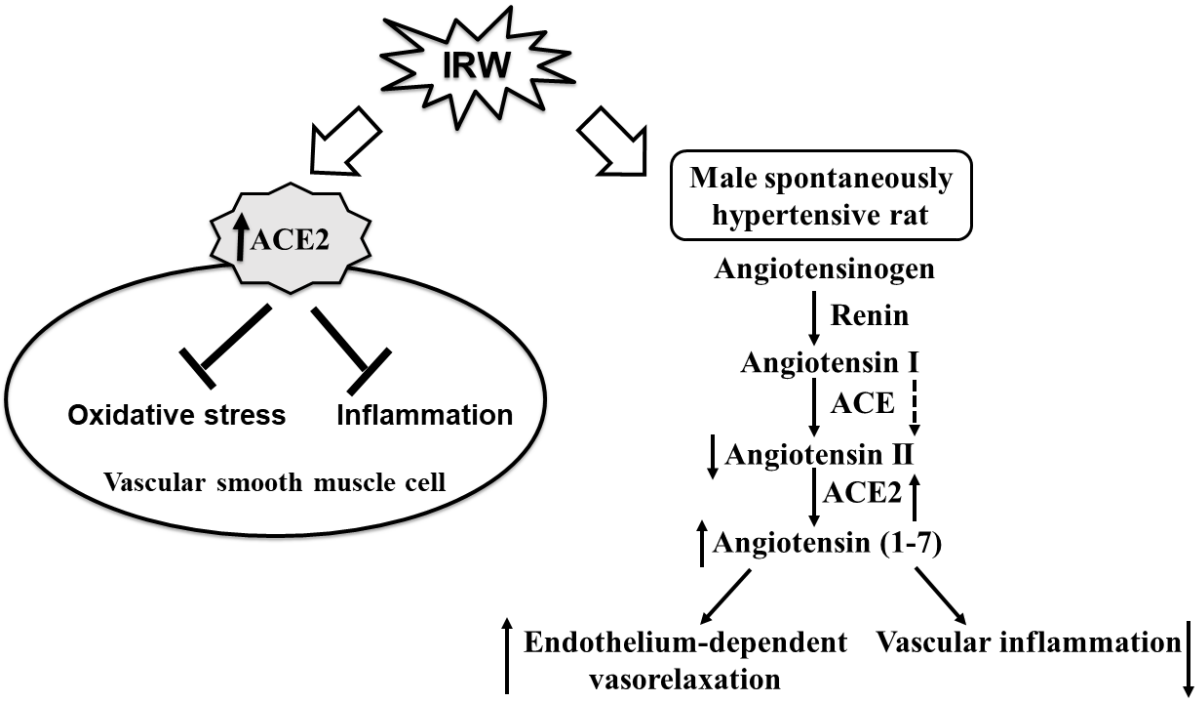
Figure 7.1: Beneficial effects of IRW in relation with ACE2 up-regulation or ACE2 activation. In VSMCs, IRW up-regulates ACE2, which contributes to antioxidant and anti-inflammatory effects of the peptide. In SHR, IRW activates the circulating ACE2/Ang (1-7) axis and then enhances endothelium-dependent vasorelaxation effect as well as decreases vascular inflammation. Although IRW was identified as an ACE-inhibitory peptide, its inhibitory effect in circulating ACE needs to be further verified. All of the protective effects of IRW-mediated ACE2 up-regulation or activation are implicated to lower blood pressure in SHR.



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