

Antihypertensive Activity of Egg White Protein Ovotransferrin-Derived Peptides

By

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ABSTRACT

Hypertension afflicts ~22% of Canadian adults and 80% of the Canadian population over the age of 65. Due to the inevitable side effects associated with drug use, there is increasing interest in developing functional foods or natural health products as an alternative for the prevention and management of hypertension. Bioactive peptides with inhibitory activity against angiotensin converting enzyme (ACE), a key enzyme for regulating blood pressure, may reduce blood pressure.

Our previous study has identified three potent ACE inhibitory peptides, IRW (Ile-Arg-Trp), IQW (Ile-Gln-Trp) and LKP (Leu-Lys-Pro) from egg white protein ovotransferrin. The specific objectives of this thesis were to 1) evaluate the anti-inflammatory and anti-oxidant activities of these peptides on endothelial cells and to understand their structural requirements for these activities, 2) test their *in vivo* anti-hypertensive activities using spontaneously hypertensive rats (SHRs), and 3) explore the possible molecular mechanism of action of egg peptide through gene expression analysis.

The anti-oxidant and anti-inflammatory activities were studied using human umbilical vein endothelial cells (HUVECs). IRW significantly inhibited tumor necrosis factor (TNF) stimulated expression of intercellular cell adhesion molecule-I (ICAM-1) and vascular cell adhesion molecule-I (VCAM-1). IQW and LKP, however, only inhibited the expression of ICAM-1 or VCAM-1, respectively. The anti-inflammatory activities of IRW and IQW were mediated differentially through transcription factor nuclear-kappa B (NF- κ B). Both IRW and IQW exhibited anti-oxidant activity by reducing superoxide generation, whereas LKP did not show any effect. The structural integrity of these peptides (IRW and IQW) was essential for their

activities, since dipeptides or the combination of their constituent amino acids did not exhibit the same effect.

In vivo antihypertensive activity was studied in SHR_s using the telemetric method. Oral administration of IRW, IQW, and LKP (15 mg/kg BW) significantly lowered mean arterial pressure (MAP) by 40, 19, and 30 mmHg, respectively, over a period of 18 days. IRW treatment led to decreased angiotensin II (Ang II) levels, restored nitric oxide (NO) dependent vasorelaxation, and decreased expression of inflammatory markers such as ICAM-1 and VCAM-1 as well as tissue fibrosis. Therefore, the antihypertensive effect of IRW was likely mediated through ACE inhibition, endothelial nitric oxide synthase, and anti-inflammatory properties. IQW and LKP treatments also reduced plasma Ang II level and restored NO-dependent vasorelaxation; however, only IQW treatment reduced ICAM-1 expression and nitrotyrosine levels in the arteries. The antihypertensive activities of IQW and LKP were likely mediated through similar pathways involving increased NO-mediated vasorelaxation and ACE inhibition, but only IQW reduced vascular inflammation and oxidative stress.

Since IRW (at 15 mg/kg BW) exhibited the most pronounced anti-hypertensive, anti-inflammatory, and anti-oxidant activities compared to other two peptides, further transcriptomics study was used to delineate the molecular mechanism. The study revealed that IRW treatment could significantly modulate the gene expression in mesenteric artery. Interestingly, IRW treatment could significantly up-regulated the expression of angiotensin converting enzyme-2 (ACE-2), a vasodilatory enzyme of the renin-angiotensin system (RAS). Upregulation of ACE-2 reduces the hyperactivity of the RAS and reduces blood pressure. In addition, IRW could reduce

the gene expression of pro-inflammatory adhesion molecules, ICAM-1 and VCAM-1 that further supported the anti-inflammatory activity of egg peptide.

Thus the present study confirmed the anti-hypertensive activity of these three egg protein derived peptides. ACE inhibitory peptides were thought to function through the inhibition of ACE. Our study added new insights that the antihypertensive activity of these peptides could be due to anti-inflammation and antioxidant activities, restored NO-dependent vasodilation, and up-regulation of ACE-2. Findings from this study support the potential of egg protein derived peptides for uses in the prevention and management of hypertension.

PREFACE

This thesis is an original work done by Mr. Kaustav Majumder. The use of human umbilical endothelial cells, which is a part of this thesis, was approved by the University of Alberta Ethics Committee (Protocol # 00000944) and the investigation also conformed to the principles outlined in the Declaration of Helsinki and also Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All subjects provided informed consent before inclusion into this study. The experimental protocol for the animal study, another part of this thesis, was also approved by the University of Alberta Animal Care and Use 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.

The thesis is consisted of seven chapters: Chapter 1 gave a general introduction on the context and the objectives of the thesis; Chapter 2 was literature review (a revision of chapter 2 was submitted to *International Journal of Molecular Sciences* as an invited review article); Chapter 3 has been published as “Structure and Activity Study of Egg Protein Ovotransferrin Derived Peptides (IRW and IQW) on Endothelial Inflammatory Response and Oxidative Stress” in *Journal of Agricultural and Food Chemistry*; Chapter 4 has been published as “Egg-derived Tri-peptide IRW Exerts Antihypertensive Effects in Spontaneously Hypertensive Rats” in *PLoS One*; Chapter 5 entitled as “Egg-derived ACE-inhibitory peptides IQW and LKP Reduce Blood Pressure in Spontaneously Hypertensive Rats” has been submitted for consideration to *Journal of Functional Foods*; Chapter 6 entitled as “Oral Administration of Egg-derived Tri-peptide IRW Affects the Gene Expression of Spontaneously Hypertensive Rats” will be submitted to

Molecular Nutrition and Food Research; the last Chapter 7 gave some concluding remarks and future research directions. The concept of the thesis arose mainly from my supervisor's grant applications that funded this research; Drs. Sandra T. Davidge and Susan Jacobs were the co-applicants for the application. Drs. Jianping Wu, Sandra T. Davidge and Susan Jacobs contributed to experimental design, data interpretation, manuscript preparation and edits. I was responsible for literature search required for the study, experimental designs, performing experiments, data collection and analysis, and drafting the manuscripts. The cell culture study (Chapter 3), Dr. S Chakrabarti, as a co-first author, has assisted me in the experimental design, data collection and contributed to manuscript edits; I used that published manuscript in this thesis with his permission. Mrs. Sareh Panahi provided substantial technical support in animal study including animal husbandry, animal surgery, animal care, and isolation of mesenteric arteries etc., while Dr. Jude S. Morton provided technical support in vessel function study and data interpretation. Dr. Leluo Guan contributed to experimental design and technical support in gene expression analysis and helped data interpretation. Mr. Xu Sun provided the technical support for RNA extraction from animal tissues; Ms. Yanhong Chen provided the methodology for polymerase chain reaction and Mr. Guanxiang Liang has assisted me in the computational analysis of gene expression.

DEDICATION

This thesis is dedicated to the memory of my loving brother Mr. Biswadeep Ghosh.

Wherever you are stay happy and keep smiling.

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There have been countless of supports both academically and socially which I cherished a lot during this journey of becoming a philosopher.

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Abbreviations used:

ABCB-1: ATP-binding cassette-sub family

ACE 2: Angiotensin converting enzyme 2

ACE: Angiotensin I converting enzyme

Ang I: Angiotensin I

Ang II: Angiotensin II

APoE: Apolipoprotein E

ATP: Adenosine tri-phosphate

BP: Blood pressure

CDH-1: E-Cadherin

cGMP: cyclic guanosine-monophosphate

CVDs: Cardiovascular diseases

DBP: Diastolic blood pressure

DE: Differentially expressed

DNA: Deoxyribonucleic acid

ECE: endothelin converting enzyme

ECs: Endothelial cells

EGFs: Epidermal growth factors

ELISA: Enzyme-linked immunosorbent assay

eNOS: endothelial nitric oxide synthase

ET-1: Endothelin

GPCR: g-protein coupled receptor

HR: Heart rate

HUVEC: Human umbilical vein endothelial cells

ICAM-1: Intercellular adhesion molecule 1

IL: interleukin

iNOS: inducible nitric oxide synthase

IRF-8: Interferon Regulatory factor 8

I κ B: inhibitor kappaB

KKS: Kallikrein kinin-system

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

MAP: Mean arterial pressure

MCh: Methacholine

MCP-1: Monocyte chemotactic protein-1

MMP: metalloproteinases

mRNA: Messenger RNA

NADPH: nicotinamide adenine dinucleotide phosphate

NEP: neutral endopeptidase

NF- κ B: Nuclear factor kappaB

NO: Nitric oxide

NOS: Nitric oxide synthase

PCR: Polymerase chain reaction

PE: Phenylephrine

PGI₂: Prostacyclin

qRT-PCR: Quantitative Real Time PCR

RAS: Renin angiotensin system

RNA: Ribonucleic acid

RNAseq: RNA sequencing

ROS: Reactive oxygen species

SBP: Systolic blood pressure

sGC: soluble guanylyl cyclase

SHR: Spontaneously hypertensive rats

SOD: Superoxide dismutase

TNF: Tumor necrosis factor

VCAM-1: Vascular adhesion molecule-1

VSM: Vascular smooth muscle

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 health crisis

Cardiovascular diseases (CVD) are the leading cause of morbidity and mortality worldwide (WHO, 2011). In North America, CVD afflict 83 million individuals, of 68 million with hypertension (Centers for Disease Control and Prevention, 2011). Hypertension, a medical term for persistent high blood pressure, is one of the major risk factors for CVD and is defined as persistent elevation of systolic blood pressure (SBP) greater than 140 mmHg and/or a diastolic blood pressure (DBP) greater than 90 mmHg (WHO, 2012).

There are two main types of hypertension. Primary or essential hypertension represents 90–95% of all cases, but the specific medical etiology is not yet known. It has been suggested that various factors including obesity, lack of physical activity, unhealthy diet, salt sensitivity, genetics, hyperactivity of the renin-angiotensin system (RAS), insulin resistance, and sleep apnea might lead to the occurrence of primary hypertension (Carretero & Oparil, 2000). On the other hand, secondary hypertension resultants from specific pathology in different organs, such as those associated with kidney diseases, adrenal disorders, or thyroid disorders (Hall et al., 2012; Viera & Neutze, 2010).

Primary hypertension is regarded as one of the major lifestyle-related diseases, and therefore, lifestyle modifications, such as regular physical activities, weight loss, moderate alcohol consumption, and intervention of healthy diet may reduce the risk of development of this condition (Beilin, 1999). It is well known that diet plays a critical role in the onset and progress of hypertension. For example, a DASH (Dietary Approaches to Stop Hypertension) diet rich in fruits and vegetables has been proven to be effective in treating hypertension (Craddick, Elmer,

Obarzanek, Vollmer, Svetkey & Swain, 2003; Rouse, Armstrong, Beilin & Vandongen, 1983). Subsequent research has found that some active compounds present in food are largely responsible for the observed antihypertensive effects of DASH diet (Batchu, Chaudhary, Wiebe & Seubert, 2013; Fernández-Mar, Mateos, García-Parrilla, Puertas & Cantos-Villar, 2012; Kris-Etherton et al., 2002; Shukla & Kumar, 2013). Hence, many bioactive food compounds have been characterized for their potential roles in the prevention and management of hypertension (Craddick, Elmer, Obarzanek, Vollmer, Svetkey & Swain, 2003; Thornburg & Challis, 2014). Thus, there is increasing interest in exploring the bioactive food components or functional foods as alternative therapy in the prevention and management of hypertension.

1.2 Overview of functional foods and bioactive peptides

The concept of functional food was first introduced in Japan in 1984, as Foods for Specified Health Uses (FOSHU, Siro, Kapolna, Kapolna & Lugasi, 2008). Rising awareness among consumers towards health benefits of foods and their potential for disease prevention and health enhancement is the major driving force for the global growth of functional foods, estimated at least \$30 billion in USA, EUR 6.4 billion in Europe, \$20 billion in Japan and \$11 billion in Canada (Agricultural Ministry of Japan, 2013; Agriculture and Agri-Food Canada, 2011; Price Waterhouse Coopers, 2011; Stein & Rodriguez-Cerezo, 2008). According to Health Canada, “*A functional food is similar in appearance to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions*” whereas “*A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated*

with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease” (Health Canada, 2013).

Food protein-derived biologically active peptides are important constituents of functional foods. Bioactive peptides are generally latent in their parent protein sequences but can exert various biological functions beyond their expected nutritional value, after release through enzymatic digestion, fermentation, or food processing (Hartmann & Meisel, 2007). Bioactive peptides have been reported to have mineral binding, immunomodulatory, anti-microbial, anti-oxidant, anti-thrombotic, and anti-hypertensive properties (Erdmann, Cheung & Schröder, 2008; Korhonen & Pihlanto, 2003; Meisel, 1997; Shahidi & Zhong, 2008; Yoshikawa et al., 2000). Because of their potential health-promoting effects, they may also be used as ingredients for the development of novel functional foods and nutraceuticals. Many of these bioactive peptides are multifunctional, and therefore can exert more than one physiologically beneficial activity (Erdmann, Cheung & Schröder, 2008; Meisel, 2004). Protein rich foods such as milk, egg, meat, fish and pulses are good candidates for the production of bioactive peptides. Anti-hypertensive peptides have gained extensive attention due to the prevalence of hypertension (Erdmann, Cheung & Schröder, 2008; Miguel & Aleixandre, 2006; Phelan & Kerins, 2011). Antihypertensive peptides derived from food proteins are considered as safer alternatives to the pharmacological drugs; although effective in controlling high blood pressure, use of antihypertensive drugs is associated with various side effects (Agyei & Danquah, 2011; Khanna, Lefkowitz & White, 2008; Moser & Franklin, 2007). Antihypertensive peptides from milk proteins have been extensively studied as potential anti-hypertensive functional foods (Carey et al., 2005; Cicero, Gerocarni, Laghi & Borghi, 2011; Geleijnse & Engberink, 2010). Eggs are also a good source of bioactive peptides including antihypertensive peptides (Hartmann & Meisel, 2007; Korhonen & Pihlanto, 2003;

Kovacs-Nolan, Phillips & Mine, 2005). We have previously identified three potent antihypertensive peptides (Ile-Arg-Trp: IRW; Ile-Glu-Trp: IQW; Leu-Lys-Pro: LKP) from the egg white protein ovotransferrin through *in vitro* analysis (Majumder & Wu, 2010). These newly identified peptides could potentially exert biological activity and provide health benefits after oral administration.

1.3 Hypotheses and objective

In addition to *in vitro* antihypertensive activity, these peptides (IRW, IQW, and LKP) also exhibited *in vitro* anti-oxidant effect (Huang, Shen, Nimalaratne, Li, Majumder & Wu, 2012), but their *in vivo* efficacies have not been evaluated. The underlying molecular mechanisms of action of these peptides are not known. Development of hypertension is a complex process involving interplay among various pathways (as described in Chapter 2). These bioactive peptides with ACE-inhibitory and anti-oxidant properties might potentially exert anti-hypertensive effects through different mechanisms. We hypothesized that ovotransferrin derived peptides (IRW, IQW and LKP) could exert antihypertensive activity *in vivo* after oral administration of peptides in an animal model. The specific objectives of this study were to:

1. Evaluate the anti-inflammatory and anti-oxidant activities of ovotransferrin-derived peptides (IRW, IQW, and LKP) on cultured endothelial cells and to understand their structural requirements for these activities.
2. Test the *in vivo* anti-hypertensive activities of these peptides (IRW, IQW, and LKP) through a suitable animal model of hypertension.
3. Explore the possible molecular mechanism of action of egg peptide through gene expression analysis.

To achieve these specific objectives, human umbilical vein endothelial cells (HUVECs), a widely used model for studying the vascular endothelium (Narumiya, Zhang, Fernandez-Patron, Guilbert & Davidge, 2001) were used for the *in vitro* cell culture studies, while spontaneously hypertensive rats (SHRs), a suitable animal model of essential hypertension (Trippodo & Frohlich, 1981; Zicha & Kunes, 1999) were used for *in vivo* studies. Brief descriptions of each experimental chapter are given below.

Chapter 2 provides a literature review encompassing several different topics relevant to this thesis, including the pathogenesis of hypertension and the significance and potential of food-derived bioactive peptides. The underlying mechanism of action of different food derived bioactive peptides was also discussed in detail.

Chapter 3 studied the anti-inflammatory and anti-oxidant properties of these three peptides (IRW, IQW, and LKP) on tumor necrosis factor (TNF, a pro-inflammatory cytokine) induced HUVEC monolayers. The results indicated that IRW significantly inhibited TNF-induced up-regulation of 2 distinct pro-inflammatory leukocyte adhesion molecules, namely, intercellular cell adhesion molecule-I (ICAM-1) and vascular cell adhesion molecule-I (VCAM-1). In contrast, IQW and LKP only inhibited the up-regulation of ICAM-1 and VCAM-1 respectively. The anti-inflammatory mechanism of action of IRW and IQW appeared to be mediated through differential regulation of transcription factor nuclear-kappa B (NF- κ B). In addition, both IRW and IQW exhibited anti-oxidant effects as shown by reduction of TNF-induced superoxide generation, but LKP did not show any such effect. Further study revealed that the structural integrity of these peptides (IRW and IQW) was essential for their activities, since dipeptides or the combination of constituent amino acids did not exhibit the same effect.

Chapter 4 tested the *in vivo* anti-hypertensive effect of IRW through oral administration in adult male SHR. The results showed that IRW (at 15 mg/kg BW) treatment over a period of 18 days significantly reduced mean arterial pressure (MAP) by 40 mmHg compared to the untreated animals. The reduction of blood pressure by IRW treatment was also accompanied by restoration of diurnal variations in blood pressure, nitric oxide dependent vasorelaxation, reduction of plasma angiotensin II (Ang-II), and decrease in inflammatory markers such as ICAM-1 and VCAM-1 as well as tissue fibrosis. Our results demonstrated that the anti-hypertensive effect of IRW was mediated by several different pathways.

Chapter 5 tested the *in vivo* anti-hypertensive effect of two other peptides, IQW and LKP in the same animal model. IQW and LKP treatment (15 mg/kg BW) decreased MAP by 19 and 30 mmHg respectively compared to the untreated SHRs. Plasma Ang II level was also significantly reduced after treatment by both peptides. However, only IQW treatment reduced ICAM-1 expression and nitrotyrosine levels in arteries, suggesting protective effects of IQW (but not LKP) against the accompanying inflammation and oxidative/nitrosative stress.

Chapter 6 further investigated the anti-hypertensive mechanisms through gene expression study in SHRs. Since IRW demonstrated the greatest effects compare to IQW and LKP on blood pressure reduction and activated multiple pathways, only this peptide was chosen for further study. To investigate the gene expression profile, mRNA was extracted from the tissues (kidney and mesenteric arteries) of both IRW-treated (at 15 mg/Kg BW for 18 days) and untreated SHR. The differentially expressed (DE) genes were identified by comparison of gene expression (mRNA fold change) in tissues from both untreated and IRW treated animals. Our results revealed that genes from mesenteric arteries showed greater changes in response to IRW

treatment (compared to those from the kidney), and many of the affected genes could modulate cardiovascular functions. Interestingly, IRW treatment also upregulated angiotensin converting enzyme (ACE-2) which can breakdown Ang II into an inactive metabolite (which no longer has pressor effect on the blood vessels) and consequently reduce blood pressure. In addition, the study also revealed that IRW reduced the gene expression of inflammatory adhesion molecules such as ICAM-1 and VCAM-1 that further validates observations from our previous animal and cell culture studies.

Findings from this thesis suggest the potential of egg protein derived peptides as important ingredients for the functional foods or nutraceuticals with potential applications in the prevention and management of hypertension and/or other associated cardiovascular diseases.

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CHAPTER 2 - Literature Review¹

It is well recognized that diet plays an important role in human health. A great deal of epidemiological evidences have suggested that food habit or dietary choice can affect the prevalence of chronic disease such as cardiovascular disease, obesity, and diabetes (Miguel, Maria, Lluís, Denis, Ramón & Antonia, 2009; Mozaffarian, Hao, Rimm, Willett & Hu, 2011; Stephanie, Suhad & Peter, 2009). A healthy diet contains many bioactive compounds that might exert medicinal benefits in addition to their nutritional value (Cha, Lee & Song, 2010; Hamer, Owen & Kloek, 2005). These bioactive compounds can either present naturally in the food or can be produced during food processing. Dietary proteins are a rich source of such bioactive compounds. Indeed, many food proteins contain active peptide fragments encrypted within their structure that can exert beneficial effects on human health above and beyond their expected nutritional value. These active peptide fragments, known as bioactive peptides, can be released from their parent proteins by gastrointestinal digestion, fermentation, or food processing (Hartmann & Meisel, 2007). Food derived bioactive peptides have vast potential for applications as functional foods and nutraceuticals.

2.1 Bioactive peptides from food proteins

Bioactive peptides from various food proteins have attracted extensive attention due to their biological effects such as inhibiting the growth of pathogenic bacteria and/or fungus, modulating the immune system, enhancing mineral absorption, scavenging harmful free radicals, and inhibiting the activity of regulatory enzymes. Bioactive peptides can modulate physiological functions through binding to specific receptors or action on specific sites on target cells (Meisel,

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1997; Shahidi & Zhong, 2008). Bioactive peptides derived from various food proteins and their respective activities are listed in Table 2.1. A brief overview about the various bioactive properties of bioactive peptides is given in the following subsections (2.1.1 to 2.1.6). The details about food derived antihypertensive peptides, the complex pathophysiology of hypertension, and the underlying mechanisms of action of these peptides are discussed in Section 2.2.

2.1.1 Anti-microbial peptides

Antimicrobial peptides from food sources represent a broad spectrum of activity against bacteria, viruses, and fungi. These peptides are usually positively charged and enriched with both hydrophobic and hydrophilic side chains to maintain solubility in aqueous environment as well as to penetrate through the lipid-rich cell membranes (Izadpanah & Gallo, 2005). Peptides from milk proteins are extensively studied for their antimicrobial activity. Lactoferricins, derived from whey protein lactoferrin upon pepsin hydrolysis, are a well-known example (Tomita, Takase, Bellamy & Shimamura, 1994; Tomita, Wakabayashi, Yamauchi, Teraguchi & Hayasawa, 2002). Lactoferricins can inhibit the growth of different Gram-positive and Gram-negative bacteria (i.e., *Escherichia*, *Helicobacter*, *Listeria*, *Salmonella*, and *Staphylococcus*), yeasts, and filamentous fungi (Dionysius & Milne, 1997; Fernández-Musoles, Salom, Martínez-Maqueda, López-Díez, Recio & Manzanares, 2013; Tomita, Wakabayashi, Yamauchi, Teraguchi & Hayasawa, 2002). The disruption of normal membrane permeability is one of the mechanisms that are partly responsible for the antibacterial activity of lactoferricins (Centeno et al., 2006; Fernández-Musoles, Castello-Ruiz, Arce, Manzanares, Ivorra & Salom, 2013; Orsi, 2004; Tomita, Wakabayashi, Yamauchi, Teraguchi & Hayasawa, 2002). Two other peptides were also isolated from the N-terminal of lactoferrin which exerted antimicrobial activities against a number of

pathogenic and food spoilage microorganisms (Fernandez-Musoles, Castello-Ruiz, Arce, Manzanares, Ivorra & Salom, 2013; Orsi, 2004). The antimicrobial action of lactoferrin-derived peptides is also partially due to their iron-binding ability, which makes this essential mineral unavailable for normal growth of microorganisms (Madureira, Tavares, Gomes, Pintado & Malcata, 2010; Simões, Simões & Vieira, 2010). In addition, the presence of α -helix structure and positively charged groups in antimicrobial peptides enables binding with anionic phospholipids-rich membrane of the microbes, which facilitates cell lysis and increases cell permeability (Orsi, 2004). Some other antimicrobial peptides have also been identified from the milk proteins α_{s1} and α_{s2} caseins (Florence & Rehault, 2007; Izadpanah & Gallo, 2005; Meisel, 2004; Pellegrini, 2003).

Two well-known antimicrobial proteins from egg are lysozyme and ovotransferrin (Kovacs-Nolan, Phillips & Mine, 2005). A 92-amino-acid-long peptide (OTAP-92) isolated from ovotransferrin was reported to exhibit antimicrobial activity through a membrane damage mechanism (Ibrahim, Sugimoto & Aoki, 2000). Another peptide (IVSDGDGMNAW), isolated from egg lysozyme, showed antimicrobial activity against Gram negative bacteria *Escherichia Coli* (Kovacs-Nolan, Phillips & Mine, 2005). Antimicrobial peptides were also reported from barley, oats, and wheat (Coutinho, Lobo, Bezerra & Lobo, 2008; Pellegrini, 2003).

2.1.2 Immunomodulatory peptides

Some specific peptides from food proteins are reported to modulate the functions of the immune system including cytokine regulation, modulating the activity of natural killer cells (NK cells), lymphocyte proliferation, and antibody production (Hartmann & Meisel, 2007). Furthermore, immunomodulatory peptides can enhance mucosal immunity in the gastrointestinal tract and may

also decrease allergic reactions in humans (Korhonen & Pihlanto, 2003; Korhonen & Pihlanto, 2007; Takahashi, Moriguchi, Yoshikawa & Sasaki, 1994). Several immunomodulatory peptides have been identified in bovine and human milk proteins (Chatterton, Rasmussen, Heegaard, Sørensen & Petersen, 2004; Cross & Gill, 2000; Kitts & Weiler, 2003). β -casein (f54-f59) and α -lactalbumin (f51-f53) are two immunomodulatory peptides isolated from human milk protein that enhance the phagocytic activity of macrophages in both animal and human subjects (Sandré et al., 2001). Peptides derived from α_{s1} -casein have been shown to have a wide range of immunomodulatory activities, including suppression mitogen-stimulated human peripheral blood mononuclear cell proliferation (Brück, Graverholt & Gibson, 2003), enhancement of phagocytosis, *in vivo* promotion of antibody formation, and protection against *Klebsiella pneumoniae* infection in mice (Brück, Graverholt & Gibson, 2003; Clare & Swaisgood, 2000). Peptides isolated from rice and soybean proteins after trypsin digestion have been found to stimulate non-specific immune defense systems (Kitts & Weiler, 2003). Peptides derived from egg also showed immunostimulating activities and were used to increase immune functions during cancer immunotherapy (Kovacs-Nolan, Phillips & Mine, 2005; Mine, 2007). Though the main mechanism of action of these immunomodulatory peptides has not been fully elucidated, promotion of proliferation and maturation of immune system cells could be responsible for much of their effects.

2.1.3 Anti-inflammatory peptides

Inflammation is the body's response to non-lethal injury, involving increased endothelial permeability, expression of adhesion molecules and chemokines, and infiltration of leukocytes into the extravascular tissues. Bioactive peptides from milk proteins have been studied for their

anti-inflammatory effects. VPP and IPP from sour milk appear to exhibit beneficial effect in an animal model of intestinal enterocolitis through their anti-inflammatory effects (Chatterton, Nguyen, Bering & Sangild, 2013). VPP also decreased leukocyte-endothelial interactions in an *in vitro* study, through inhibition of pro-inflammatory c-Jun N-terminal kinase (JNK) pathway (Aihara, Ishii & Yoshida, 2009). Recent study from our lab also demonstrated anti-inflammatory properties of two egg peptides (IRW and IQW). Both of these peptides attenuate cytokine; tumor necrosis factor (TNF) induced inflammatory protein expression in vascular endothelium, through the modulation of the NF- κ B (nuclear factor-kappa B) pathway (Majumder, Chakrabarti, Davidge & Wu, 2013a). The anti-inflammatory activity of IRW was further observed in spontaneously hypertensive rats (Majumder et al., 2013b). Similarly, a soy protein derived peptide, lunasin exhibited anti-inflammatory effects by suppressing the activity of NF- κ B, reducing cytokine expression and cyclooxygenase-2 (COX-2) levels, decreasing the production of reactive oxygen species (ROS), and down regulating prostaglandin-E₂ (PGE₂) synthesis in activated macrophages (de Mejia & Dia, 2009; Hernandez-Ledesma, Hsieh & de Lumen, 2009). Furthermore, administration of chicken collagen hydrolysate significantly reduced plasma levels of inflammatory cytokines in ApoE-deficient mice (Zhang, Kouguchi, Shimizu, Sato, Takahata & Morimatsu, 2010).

2.1.4 Opioid-like peptides

Opioid-like peptides are opioid receptor ligands, exhibiting agonist or antagonist effects. Opioid-like activity of food protein-derived peptides was first discovered in the late 1970s (Zioudrou, Streaty & Klee, 1979). Peptides with opioid activity mainly affect appetite, gastrointestinal motility, and behavior (Zioudrou, Streaty & Klee, 1979). Bovine milk α -casein derived peptide,

also known as casomorphins are involved in regulating gut functions, enhancing water and electrolyte absorption, and acting as an antidiarrheal compound (Hartmann & Meisel, 2007, Meisel, 2001). Peptides with opioid-like activity can also exhibit antihypertensive effect upon oral administration, which is discussed in Section 2.2.2.4.

2.1.5 Anticancer peptides

Peptides with anticancer activity have also been reported. Lunasin, a peptide derived from soy protein has exhibited anticancer properties in chemical and viral oncogene-induced cancers, through the modulation of epigenetic effects such as histone (H) modifications, specifically by inhibiting histone acetyl transferase (HAT). The inhibition of acetylation of H3 and H4 resulted in, repression of cell cycle progression (arrest at G1/S phase) and apoptosis in cancer cells (Hernández-Ledesma, Hsieh & de Lumen, 2009). Peptides prepared from enzymatic hydrolysis of different varieties of soybean could also inhibit the viability of cultured leukemia cells (Wang, Bringe, Berhow & Gonzalez de Mejia, 2008). A lunasin-containing fraction from tryptic digest of *Amaranthus hypochondriacus* induced 30% and 38% apoptosis in HeLa cells at 1 and 5 µg/ml, respectively (Silva-Sanchez, de la Rosa, Leon-Galvan, de Lumen, de Leon-Rodriguez & de Mejia, 2008). Future studies involving animal models and human volunteers are required for better understanding of food derived anticancer peptides (Korhonen & Pihlanto, 2003).

2.1.6 Antioxidant peptides

Free radicals are generated during normal physiological processes such as aerobic respiration. These free radicals can exert diverse functions in signaling pathways as well as providing defense against infections (Sarmadi & Ismail, 2010). But excessive generation of free radicals can result in cellular damage including DNA damage and/or oxidation of lipids, which can

contribute to diseases including atherosclerosis, arthritis, diabetes, and cancer (Cavalca, Cighetti & Bamonti, 2001; Mujumdar, Aru & Tyagi, 2001; Pennathur & Heinecke, 2007). Under normal physiological conditions, the antioxidant defense system in human body can remove excessive free radicals through various endogenous enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and non-enzymatic factors such as vitamins (Mohazzab, Kaminski & Wolin, 1994; Paravicini & Touyz, 2006). However, under conditions of oxidative stress, there is excessive production of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which may overwhelm the endogenous oxidant defense system (Muller & Morawietz, 2009; Valko, Leibfritz, Moncol, Cronin, Mazur & Telser, 2007).

Food protein-derived antioxidant peptides have been widely studied. The antioxidant capacity of amino acids and short peptides was first described in the 1960s (Marcuse, 1960). These peptides exert antioxidant effects by scavenging reactive radicals by donating electrons or abstracting hydrogen atom. The antioxidant capacity of many bioactive peptides is measured based on either an electron donation reaction such as ferric ion reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), cupric ion reducing antioxidant capacity (CUPRAC), and total phenol assay by Folin-Ciocalteu reagent or hydrogen atom transfer reactions such as, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), oxygen radical absorbance capacity (ORAC) and total radical-trapping antioxidant potential (TRAP) (Carocho & Ferreira, 2013, Huang, Ou & Prior, 2005, Niki, 2010). But bioactive peptides from food proteins identified from *in vitro* studies often failed to exhibit the same effect in a more physiologically relevant cellular or animal model study. The molecular mechanisms of antioxidant activity of food derived bioactive peptides have not been fully elucidated, but various studies have categorized

antioxidant peptides as inhibitors of lipid peroxidation (Sarmadi & Ismail, 2010; Valko, Leibfritz, Moncol, Cronin, Mazur & Telser, 2007; Wang, Li, Chi, Ma, Luo & Xu, 2013), scavenger of free radicals (Qian, Jung & Kim, 2008; Rajapakse, Mendis, Jung, Je & Kim, 2005), and chelating metal ions (Rajapakse, Mendis, Jung, Je & Kim, 2005). In addition, previous reports also suggested that antioxidant peptides can ameliorate ROS damage by inducing gene expression of protective proteins like heme oxygenase-1 (HO-1) and ferritin in endothelial cells (Erdmann, Grosser, Schipporeit & Schroder, 2006). Another study revealed that a peptide from skin gelatin hydrolysate of hoki fish could enhance the activities of GSH-Px and SOD and exhibited antioxidant effects in human hepatoma cells (Sarmadi & Ismail, 2010). Our recent study also demonstrated that the antioxidant effect of egg derived tripeptides, IRW and IQW on human endothelial cells (Majumder, Chakrabarti, Davidge & Wu, 2013a). Oral administration of egg white hydrolysate also exerted antioxidant effect in adult spontaneously hypertensive rats (SHR) by reducing malondialdehyde (MDA) concentration in aortic tissues (Manso, Miguel, Even, Hernández, Aleixandre & López-Fandiño, 2008). Due to a close relationship between excessive oxidative stress and disease progression, bioactive peptides that directly or indirectly reduce oxidative stress can play a crucial role in preventing of chronic diseases. The beneficial role of different food derived antioxidant peptides that has a role in reducing blood pressure have been described in Section 2.2.2.2.

2.2 Antihypertensive peptides from food proteins

Among various food derived bioactive peptides, those with antihypertensive activity have attracted significant attention due to the prevalence and global burden of hypertension, as well as the inevitable side effects of pharmacological interventions. Recent studies have identified a

number of food protein derived peptides with blood pressure lowering activity mainly by inhibiting angiotensin-converting enzyme (ACE; Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012; Miguel & Aleixandre, 2006; Torruco-Uco, Dominguez-Magana, Davila-Ortiz, Martinez-Ayala, Chel-Guerrero & Betancur-Ancona, 2008). ACE plays a crucial role in regulating blood pressure and electrolyte balance through renin-angiotensin system (RAS) (Peah, 1977; Perazella & Setaro, 2003; Zaman, Oparil & Calhoun, 2002). A number of ACE inhibitory peptides have been identified from food proteins (Cheung, Nakayama, Hsu, Samaranayaka & Li-Chan, 2009; Davalos, Miguel, Bartolome & Lopez-Fandino, 2004; Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012; Miguel, Recio, Gomez-Ruiz, Ramos & Lopez-Fandino, 2004; Akpaffiong & Taylor, 1998; Hideaki et al., 1990; Maeno, Yamamoto & Takano, 1996; Nakamura, Naramoto & Koyama, 2013; Nakamura, Yamamoto, Sakai, Okubo, Yamazaki & Takano, 1995; Nakamura, Yamamoto, Sakai & Takano, 1995). Apart from ACE inhibitory properties, peptides with renin inhibitory properties, anti-oxidant activity, anti-inflammatory, and, opioid receptor binding activity were also reported to exhibit *in vivo* anti-hypertensive effects in animal models of hypertension (Akpaffiong & Taylor, 1998; De Ciuceis et al., 2009). However a correlation between *in vitro* ACE inhibition and *in vivo* blood pressure lowering has not been observed. This lack of correlation might be due to the complex multifactorial nature of the development of hypertension. Therefore, it is important to understand the complex pathophysiology of hypertension for further elucidating the mechanisms of action of food derived antihypertensive peptides.

2.2.1 Pathophysiology of hypertension

Hypertension develops from a complex interaction of genetic and environmental factors although more than 90% of cases do not have a clear etiology (Carretero & Oparil, 2000; Viera & Neutze, 2010). Previous research has identified several contributing factors: (i) increased sympathetic nervous system activity, (ii) increased levels of long term high sodium intake, inadequate dietary intake of potassium and calcium, (iii) altered renin secretion related to over activity of RAS, (iv) increased activity of ACE resulting over production of angiotensin II (Ang-II) and deactivation of kallikrein kinin–system (KKS), (v) endothelial dysfunction and deficiency of vasodilators including reduced nitric oxide (NO) bioavailability, and (vi) abnormalities in resistant vessels due to vascular inflammation, increased activity of vascular growth factors and altered cellular ion channel (Cuzzocrea, Mazzon, Dugo, Di Paola, Caputi & Salvemini, 2004; Hall et al., 2012; Intengan & Schiffrin, 2001; Paravicini & Touyz, 2006; Schulz, Gori & Munzel, 2011). Although all of the above factors clearly contribute to the pathogenesis of hypertension, the hyperactivity of the RAS, endothelial dysfunction, enhanced activation of sympathetic nervous system and structural abnormalities in resistant vessels play critical roles in the development and progression of this disease (Beavers, Y. H. Lip & O'Brien, 2001; Foëx & Sear, 2004; Hall et al., 2012).

2.2.1.1 Renin Angiotensin System (RAS)

Physiologically, RAS is the primary pathway for controlling blood pressure and vascular tone in the human body (Oparil & Haber, 1974; Zhuo, Ferrao, Zheng & Li, 2013). In the kidney, renin converts angiotensinogen into angiotensin I (Ang I). Ang I is an inactive decapeptide that is later converted into a vasoconstrictive octapeptide, angiotensin II (Ang II), by the action of angiotensin converting enzyme (ACE). Ang II can be further cleaved by angiotensin converting

enzyme-2 (ACE-2), to form angiotensin 1-7 (Ang₁₋₇), a vasodilatory peptide (Bader & Ganten, 2008; Perazella & Setaro, 2003). In addition, ACE-2 can also cleave a single amino acid from Ang I, producing inactive angiotensin 1-9 (Ang₁₋₉). Ang II is an important regulator of fluid and sodium and also participates in cellular growth and remodeling (Michel, 2004; Oparil & Haber, 1974).

Ang II acts through two main receptors, angiotensin type 1 (AT₁) and type 2 (AT₂) receptors (Peah, 1977; Zhuo, Ferrao, Zheng & Li, 2013; Figure 2.2). Acting on AT₁ receptor causes vasoconstriction in vascular smooth muscle cells (VSMC). It also stimulates release of aldosterone to increase water and salt retention in the kidney, hypertrophic growth of cardiomyocytes, and collagen synthesis of cardiac fibroblasts resulting in cardiac remodeling. In pathogenic conditions involving tissue remodeling and vascular inflammation, AT₂ receptor is up-regulated (Liu, 2009, Millatt, Abdel-Rahman & Siragy, 1999; Sriramula, Cardinale, Lazartigues & Francis, 2011). It mediates vasodilation upon activation and releases nitric oxide (NO) and inhibits cell growth (Stankevicius, Kevelaitis, Vainorius & Simonsen, 2003). Therefore, AT₁ receptor mediates actions with potentially harmful consequences, whereas AT₂ receptor, mainly presented in the surface of vascular smooth muscle cells, mediated action exhibits protective effects against hypertension (Danyel, Schmerler, Paulis, Unger & Steckelings, 2013; Zhuo, Ferrao, Zheng & Li, 2013; Figure 2.2).

Alternatively, ACE also actively participates in the kallikrein kinin system (KKS). Activation of ACE inactivates bradykinin, a potent vasodilator. Bradykinin acts through two different receptors, type 1 (B₁) and type 2 (B₂). Both receptors induce NO generation in endothelial cells (Nicholls, 1985; Reaves, Beck, Wang, Raizada & Katovich, 2003; Figure 2.2). In addition, B₂

receptors also activate phospholipase A₂ that releases arachidonic acid, which leads to the formation of several vasodilators such as prostacyclins (Peah, 1977, Zaman, Oparil & Calhoun, 2002, Zhuo, Ferrao, Zheng & Li, 2013).

Though RAS is widespread in the body, the main source of renin is the juxtaglomerular apparatus of the kidney, while that of ACE is in the kidney and vascular endothelial cells (Behrendt & Ganz, 2002; Pober, Min & Bradley, 2009). However, there is increasing evidence supporting an important role of local RAS, such as in the microvasculature of kidney, heart, and arterial tree, in the regulation of blood pressure (Aroor et al., 2013; Michel, 2004; Peah, 1977).

2.2.1.2 Endothelial Dysfunction:

Endothelial cells (EC) play important physiological functions in regulation of the vascular homeostasis or vascular balance under normal conditions. Failure of EC results in endothelial dysfunction. Endothelial dysfunction often disturbs the vascular function and creates a vascular imbalance that is responsible for various cardiovascular diseases including hypertension. Endothelial cells produce a number of vasoactive substances, including nitric oxide (NO) and endothelin (ET-1). NO, a key vasodilators, and ET-1, a potent vasoconstrictor, are the vital mediators of endothelial functions. An imbalance between these two factors is a feature of endothelial dysfunction.

NO is a relatively stable gas and can easily diffuse through cell membrane. It also has a capability to react with various substances present within the cell (Stankevicius, Kevelaitis, Vainorius & Simonsen, 2003). NO is generated in endothelial cells by nitric oxide synthase (NOS) in a two-step five-electron oxidation of the terminal guanidine nitrogen of L-arginine, generating L-citrulline as a by-product. Three isoforms of NOS have been characterized:

endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Zhang et al., 2009). Both eNOS and nNOS are present in the normal vascular endothelium (Capettini, Cortes & Lemos, 2010; Forstermann & Sessa, 2012). After diffusion from endothelial to vascular smooth muscle cells, NO causes vasodilation (Forstermann & Sessa, 2012) primarily by activating soluble guanylyl cyclase (sGC) and increasing intracellular concentration of cyclic guanosine-monophosphate (cGMP; Cohen & Vanhoutte, 1995; Figure 2.3). Acute NOS inhibition results in vasoconstriction and reduction in peripheral blood flow (Behrendt & Ganz, 2002). These hemodynamic alterations are entirely reversible with administration of NO donors, such as glyceryl trinitrate or sodium nitroprusside (Sudano et al., 2006), suggesting that the continuous presence of NO is required to prevent vasoconstriction. In addition, NO also affects cell metabolism and inhibits mitochondrial respiration and ATP synthesis (Stankevicius, Kevelaitis, Vainorius & Simonsen, 2003).

It has been suggested that NO bioavailability can be reduced in the presence of excessive ROS such as superoxide anion (O_2^-). Ang II enhances the formation of superoxide in endothelial cells by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Superoxide readily reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$). Peroxynitrite is a strong oxidant, causing damage to cell membrane while leads to cell death and/or inflammation (Pennathur & Heinecke, 2007; Figure 2.3). Excessive formation of superoxide (O_2^-) also modifies the catalytic subunit of NOS, named as tetrahydrobiopterin (BH_4 ; Behrendt & Ganz, 2002). In the absence of this cofactor, NOS can become uncoupled and paradoxically generated superoxide (O_2^-) instead of NO (Millatt, Abdel-Rahman & Siragy, 1999). Decreased bioactivity of NO could switch the cellular signaling from NO-mediated cellular processes to oxidant-

mediated redox signaling, stimulating pro-inflammatory pathways, and ultimately leading to vascular remodeling and resulting in increased blood pressure (Sudano et al., 2006).

In contrast to NO, circulating endothelins have vasoconstrictory properties. Three isoforms of endothelins (ET) has been characterized: ET-1, ET-2, and ET-3. ET-1 present predominantly in endothelial cells and actively modulates vascular functions; however all other isoforms can also modulate vascular functions (Barton & Yanagisawa, 2008). ET-1 is synthesized predominantly in endothelial cells and also in vascular smooth muscle cells (Barton & Yanagisawa, 2008). Its precursor, preproET-1 (ppET-1) is a functionally inactive peptide which is sequentially cleaved by cellular enzymes and ultimately produces the vasoactive ET-1. Furin-like proteases cleave ppET-1 to generate a 39 amino acid peptide (38 amino acids in humans) called big-ET-1 (bET-1). Under normal physiological conditions, endothelin-converting enzyme (ECE) converts big endothelin (bET-1) to ET-1, whereas current evidence suggests ET-1 can be produced from bET-1 through several other proteolytic digestions involving matrix metalloproteinases (MMPs), and neutral endopeptidase (NEP). ET-1 exerts its functions by binding to G protein-coupled ET receptors, endothelin receptor A (ET_A) and endothelin receptor B (ET_B; Bourque, Davidge & Adams, 2011). ET_A receptors are located within the vascular smooth muscle cells (VSMC), whereas ET_B receptors are located both on vascular endothelium, as well as, on VSMC. Binding with ET_A and ET_B receptors in vascular smooth muscle ET-1 exerts vasoconstriction. Alternatively ET-1 binding to ET_B receptors in the endothelium results vasodilation through increased NO and prostacyclin synthesis (Barton & Yanagisawa, 2008; Figure 2.3). The interplay between NO and ET-1 is important in numerous pathophysiological conditions (Alonso & Radomski, 2003). The reduction in NO bioavailability is associated with increased ET-1

expression. Similarly, NO antagonizes the ET-1 pathway via several different mechanisms (as reviewed in Bourque, Davidge & Adams, 2011). These relationships suggest an intimate link between these two mediators to maintain a delicate balance in endothelial function (Barton & Yanagisawa, 2008). ET-1 also stimulates the release of pro-inflammatory cytokine such as interleukin (IL)-1, and IL-8. Factors like Ang-II, thrombin and inflammatory cytokines (tumor necrosis factor- α , IL-1, IL-2) can modulate the expression of ET-1 in endothelial cells by enhancing the gene expression of ppET-1. Therefore reduced bioavailability of NO and excessive production of Ang II can directly induce endothelial dysfunction and subsequent increase blood pressure.

2.2.1.3 Sympathetic nervous system

The sympathetic nervous system is a part of the autonomic nervous response system that can be activated by environmental stress. Increased sympathetic nervous system activity can cause both arteriolar constriction and arteriolar dilation (Mark, 1996). Thus, the autonomous nervous system contributes to the development and maintenance of hypertension through stimulation of cardiac output in heart, fluid retention in kidney and increased vascular resistance in peripheral vasculature (Mark, 1996).

Sympathetic nervous system stimulates the release of catecholamines (norepinephrine and epinephrine) from post-ganglionic neurons (Tsuru, Tanimitsu & Hirai, 2002). The release of catecholamines activates the hypertrophic growth of cardiomyocytes (Currie, Freel, Perry & Dominiczak, 2012). Simultaneously, catecholamine release increases the activity of β -adrenoceptors while decreases the activity of α -adrenoceptors, which in turn results in the conversion of prorenin to the active form of renin (Currie, Freel, Perry & Dominiczak, 2012).

The release of renin subsequently activates RAS and results in increased blood pressure through the production of Ang II (Figure 2.4). Ang II also amplifies the response of the sympathetic nervous system by a peripheral mechanism, that is, pre-synaptic facilitatory modulation of norepinephrine release (Mark, 1996; Tsuru, Tanimitsu & Hirai, 2002). Additionally, ROS and endothelin (ET-1) may also stimulate the sympathetic activity and its effects on the vasculature (Prabhakar, Peng, Kumar, Nanduri, Di Giulio & Lahiri, 2009; Tsuru, Tanimitsu & Hirai, 2002; Figure 2.4). Thus increased sympathetic activity is associated with the development of hypertension.

2.2.1.4 Vascular remodeling

Vascular remodeling contributes to increased peripheral resistance, alterations in vessel structures, development of hypertension, and the consequent end organ damage during hypertension (de las Heras et al., 2006; Intengan & Schiffrin, 2001). Hypertension associated with structural changes in the vessels has been called vascular remodeling. Vascular remodeling is an active process and involves changes in cellular processes, such as cell growth, cell migration, cell death, and degradation/synthesis of extracellular matrix (Intengan & Schiffrin, 2001; Renna, de Las Heras & Miatello, 2013). Remodeling of vessels increases peripheral resistance in precapillary vessels including arterioles and small arteries (lumen diameters < 300 μM). These structural changes of the vessels reduce the lumen diameter and increase the media-to-lumen ratio (M/L) and ultimately, increase both vascular reactivity and peripheral resistance (Intengan & Schiffrin, 2001).

Vascular inflammation can induce endothelial dysfunction, which ultimately results in vascular remodeling. Under resting conditions, EC prevent leukocyte adhesion but over production of

Ang II can initiate the expression of adhesion molecules on endothelial cells which results in adhesion of leukocyte to the inner arterial wall in a stepwise manner known as leukocyte recruitment (Giannotti & Landmesser, 2007). Subsequently Ang II can also induce oxidative stress resulting in excessive production of reactive oxygen species (ROS). ROS, in turn, stimulates the production of cytokines such as tumor necrosis factor- α (TNF), IL-1 β etc. (Cheng, Vapaatalo & Mervaala, 2005). TNF activates the phosphorylation of nuclear transcription factor- κ B (NF- κ B), which leads to the expression of adhesion molecules (ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1), and the release of monocyte chemoattractant protein-1 (MCP-1). The over-expressions of these molecules then recruit the leukocytes (monocytes and macrophages) to the site of inflammation (Giannotti & Landmesser, 2007). Over-expressed inflammatory response together with the oxidized lipid molecules forms plaques in the interstitial space between endothelial and vascular smooth muscle. Formation of plaque directly contributes to vascular remodeling, increases blood pressure and initiates atherosclerosis.

Matrix metalloproteinases (MMPs) are zinc (Zn) and calcium (Ca) dependent proteolytic enzymes that degrade extracellular matrix proteins (Visse & Nagase, 2003). Several different MMPs are present in the vasculature and their synthesis is induced by cytokines and cell-to-cell matrix interactions. An increasing body of scientific evidence demonstrates that uncontrolled proteolytic process is one of the key mechanisms for the development of hypertension and MMP play a crucial role in this process (Schmid-Schönbein, 2011; Visse & Nagase, 2003). In Ang II-induced hypertension, MMP are responsible for elevated blood pressure and tissue fibrosis (Schmid-Schönbein, 2011). Acute release of MMP2 cleaves the sarcomere proteins (titin,

troponin I and myosin light chain-I) that can impair cardiomyocyte contractility (Cheung, Sawicki, Wozniak, Wang, Radomski & Schulz, 2000; Yasmin, et al., 2005). Similarly, MMP-7 is one of the major inducing factors for endothelial dysfunction. MMP-7 also promotes G-protein coupled receptor (GPCR) agonist (i.e: Ang-II) induced vasoconstriction through epidermal growth factors (EGF) and subsequently increases the blood pressure and cardiovascular hypertrophy (Hao, Du, Lopez-Campistrous & Fernandez-Patron, 2004). Thus MMP with accessory signaling molecules can modulate cell-cell interaction, release of cytokine and chemokines, which ultimately propagate the vascular inflammatory response.

Apart from the pathways described above, there are other factors such as sodium/water excretion, adrenal steroids, etc. that also contribute to the development of high blood pressure. Interestingly, one or more component of each distinctive pathway can modulate or activate another pathway and thus create a complex cycle for the development of hypertension (Figure 2.5). The pathophysiologic mechanisms that lead to blood pressure elevation are so complex that anti-hypertensive treatment based on a single pathway is rarely feasible (ACE inhibitors may be an exception, although Ang II involves both RAS and pro-inflammatory pathways); (Neutel & Smith, 2013). Current pharmacological treatment approaches for treating hypertension are very much selective, which may explain the inadequacy in palliation of hypertension and adverse side effects. Food protein derived peptides have been widely studied for controlling elevated blood pressure, but it is essential to understand their effects on the pathogenetic mechanisms and the interplay between different molecules to develop these as novel therapeutic agents.

2.2.2 Antihypertensive peptides from food proteins-mechanisms of action

Food protein-derived peptides have been shown to exhibit antihypertensive effects through various mechanisms. Most of these peptides have been identified as ACE inhibitors using *in vitro* methods. Peptides with ACE inhibitory properties were isolated first from snake (*Bothrops jararaca*) venom (Ferreira, Bartelt & Greene, 1970; Ondetti, Williams, Sabo, Pluscec, Weaver & Kocy, 1971). This work encouraged several subsequent investigations to look for food protein-derived peptides as antihypertensive alternatives. Earlier studies have identified several ACE-inhibiting peptides from both plant (especially soybean) and animal sources (milk, fish, and egg proteins) (Gouda, Gowda, Rao & Prakash, 2006; Hideaki et al., 1990; Miguel & Aleixandre, 2006; Miguel, Manso, Aleixandre, Alonso, Salaiques & Lopez-Fandino, 2007; Shin et al., 2001). Given the complexity of blood pressure regulation, it is important to understand the mechanism of action of a peptide in order to develop functional foods or nutraceuticals for the prevention and management of hypertension.

2.2.2.1 Antihypertensive activity of peptides modulating RAS function

ACE inhibition is the main mechanism by which peptides can modulate RAS function and exert antihypertensive effects. A number of *in vivo* studies performed in animals and humans have demonstrated that various food-derived peptides significantly reduced blood pressure through ACE inhibition upon either intravenous or oral administration (Balti, Bougatef, Guillochon, Dhulster, Nasri & Nedjar-Arroume, 2012; Boelsma & Kloek, 2010; Majumder et al., 2013b; Masuda, Nakamura & Takano, 1996; Miguel, Gomez-Ruiz, Recio & Aleixandre, 2010; Shin et al., 2001; Xu, Qin, Wang, Li & Chang, 2008). Milk protein-derived peptides are known for their antihypertensive activity. The release of antihypertensive peptides from milk protein has been

achieved through two different approaches: hydrolysis of milk protein by proteolytic enzymes and fermentation of milk. One of the first peptides identified from tryptic digestion of α_{s1} -casein, FFVAPFPGVFGK, significantly reduced both SBP (by 34 mmHg) and plasma ACE activity at a dose of 100 mg/kg BW in spontaneously hypertensive rats (SHR; Hideaki et al., 1990). MKP, another peptide identified from the tryptic digest of bovine casein has also shown antihypertensive effect in SHR. The crude hydrolysate containing only 0.053% of MKP significantly reduced the SBP by 40 mmHg at a dose of 100 mg/kg BW 2 hrs after administration, whereas the purified peptide MKP exhibited a maximum SBP reduction of 45 mmHg 8 hrs after administration in SHR animals. Both preparations also exhibited ACE inhibitory properties (Yamada, Sakurai, Ochi, Mitsuyama, Yamauchi & Abe, 2013). Three peptides, IAK, YAKPVA and WQVLPNAVPAK from α_{s1} -casein produced by combined action of pepsin, chymotrypsin and trypsin showed a significant decrease in both SBP and diastolic blood pressure (DBP) in SHR with doses of 4, 6 and 7 mg/kg BW, respectively (Miguel, Gomez-Ruiz, Recio & Aleixandre, 2010). The authors determined that the ACE inhibitory property of these peptides was responsible for the observed antihypertensive effect (Miguel, Gomez-Ruiz, Recio & Aleixandre, 2010). Two tripeptides, VPP and IPP, produced from milk fermentation with a combination of *Lactobacillus helveticus* and *Saccharomyces cerevisiae*, were the well-known antihypertensive peptides from milk (Nakamura, Yamamoto, Sakai, Okubo, Yamazaki & Takano, 1995; Nakamura, Yamamoto, Sakai & Takano, 1995). Single oral administration of VPP and IPP at a dose of 5 mg/Kg BW significantly reduced SBP by 32 and 28 mmHg, respectively (Nakamura, Yamamoto, Sakai & Takano, 1995). SHR fed with fermented milk containing these peptides demonstrated significant decreases in serum ACE activity and BP (Sipola, Finckenberg, Korpela, Vapaatalo & Nurminen, 2002).

Apart from milk, fish protein-derived peptides have also been shown to exhibit antihypertensive effect through ACE inhibition. Three peptides, LKP, IKP, and IWH identified from hydrolysate of dried bonito have been shown to significantly reduce SBP in SHR animals (Fujita & Yoshikawa, 1999; Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012). Another peptide LKPNM, also identified from bonito hydrolysate, was found to exert a longer-term effect on SHRs than LKP. The authors identified LKPNM as a 'pro-drug' type ACE inhibitor, which could serve as a precursor to the actual ACE inhibitor released upon gastrointestinal proteolysis (Fujita & Yoshikawa, 1999).

Egg protein ovalbumin-derived peptide YPI reduced blood pressure by 30 mm Hg after a single oral administration to SHRs and its actions were likely mediated through ACE inhibition (Miguel, Alvarez, López-Fandiño, Alonso & Salaices, 2007). Four other peptides, YRGGLEPI, YR, ESI, and NF from egg protein ovalbumin have also demonstrated *in vivo* antihypertensive effect in SHR (Miguel, Alvarez, López-Fandiño, Alonso & Salaices, 2007); their actual mechanisms of action remain unknown. However, it was evident that these antihypertensive effects were independent of ACE inhibition, as in an isolated mesenteric artery experiment vasorelaxation activity of these peptides was blocked by the treatment of nitric oxide (NO) inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) and cyclooxygenase inhibitor indomethacin (Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012; Miguel, Alvarez, López-Fandiño, Alonso & Salaices, 2007). A study from our own research group identified a potent ACE inhibitory tri-peptide IRW from thermolysin-pepsin hydrolysate of egg white protein ovotransferrin (Majumder & Wu, 2010). In SHRs, IRW significantly reduced SBP by 40 mmHg

after 18 days of continuous treatment of 15 mg/kg BW, concomitantly decreased plasma Ang II levels, likely through ACE inhibition (Majumder et al., 2013b).

AT₁, the Ang II receptor, is one of the targets to modulate increased RAS activity. In addition to inhibition of ACE, AT₁ receptor blockade is an effective therapy for hypertensive patients (Contreras, de la Parte, Cabrera, Ospino, Israili & Velasco, 2003). Moreover, it is a useful alternative approach for the patients sensitive to side-effects of ACE inhibitors (Contreras, de la Parte, Cabrera, Ospino, Israili & Velasco, 2003). Similarly, renin is a key regulator of RAS; therefore inhibition of renin could also alter RAS activation. Milk lactoferrin-derived peptides RRWQWR, LIWKL, and RPYL significantly reduced blood pressure in SHR and were also found to reduce Ang-II induced vasoconstriction in isolated rabbit carotid arterial segments (Fernandez-Musoles, Castello-Ruiz, Arce, Manzanares, Ivorra & Salom, 2013). Among these three peptides, RPYL showed the maximum effect, and demonstrated inhibition of Ang-II binding to AT₁ receptors (Fernandez-Musoles, Castello-Ruiz, Arce, Manzanares, Ivorra & Salom, 2013). Recently, egg protein-derived peptide RVPSL has been shown to significantly decrease SBP by 25 mmHg after 4 weeks of continuous treatment at a dose of 50 mg/Kg BW. The mRNA levels of renin, ACE, and AT₁ receptor in kidney and serum level of AngII and renin were all significantly decreased by RVPSL treatment (Yu, Yin, Zhao, Chen & Liu, 2014). Results from these studies suggest that food-derived bioactive peptides can indeed act upon the AT₁ receptor and/or act as a renin or ACE inhibitors to exert their *in vivo* antihypertensive effects (Yu, Yin, Zhao, Chen & Liu, 2014).

Interestingly in a different study, Ehlers et al. had demonstrated that the vasorelaxation effect of IPP may be mediated through ACE-2, Ang₁₋₇, and Mas axis. In an *ex vivo* experiment the authors

demonstrated that the administration of IPP can produce more Ang₁₋₇ and exert vasorelaxation activity on Mas receptor possibly through modulation of ACE-2 (Ehlers, Nurmi, Turpeinen, Korpela & Vapaatalo, 2011). Similarly, study from our group has found that IRW treatment increased the gene expression of ACE-2 in mesenteric artery (*unpublished data*), which may further convert Ang II to Ang₁₋₇ and exerts vasodilation. Thus activation of ACE-2 through peptide treatment could exert beneficial effect for the prevention of hypertension.

2.2.2.2 Antihypertensive peptides ameliorating endothelial dysfunction

Increased production of vasoconstrictory/pro-inflammatory mediators like ET-1 and superoxide (O₂⁻) decreases the bioactivity of vasodilatory nitric oxide (NO) resulting in endothelial dysfunction. Food protein-derived antihypertensive peptides have been shown to improve endothelial dysfunction. The three main mechanisms by which food derived antihypertensive peptides modulate endothelial function are increased production of vasodilatory factors (i.e.: NO and prostaglandins), reduced production of vasoconstriction factors (i.e: ET-1), and increased anti-oxidant activity.

Egg protein ovalbumin-derived peptide RADHP could significantly reduce blood pressure by 28 mmHg after a single oral administration in SHR animals (Yamada, Matoba, Usui, Onishi & Yoshikawa, 2002). RADHP also exhibited a dose-dependent relaxation in an isolated SHR mesenteric artery. However, the removal of endothelium from the mesenteric artery was associated with disappearance of the relaxation effect, suggesting endothelium-dependent vasodilator activity of RADHP (Yamada, Matoba, Usui, Onishi & Yoshikawa, 2002). Moreover, pretreatment with L-NAME, a NOS inhibitor, inhibited the RADHP mediated vasodilation; while pretreatment with superoxide dismutase (SOD), a radical scavenger, did not alter

RADHPF induced vasodilation, suggesting that the vasodilatory effect was unlikely to be caused by scavenging (O_2^-), but possibly resultant from stimulating NO production (Miguel, Alvarez, López-Fandiño, Alonso & Salaices, 2007). In addition to their ACE inhibitory effects, VPP and IPP also demonstrate the capability to improve vascular endothelial dysfunction. Yamaguchi et al. demonstrated that VPP and IPP administration could significantly increase the mRNA expression of eNOS in SHR; (Yamaguchi, Kawaguchi & Yamamoto, 2009). Increased expression of eNOS directly correlates with the production of NO and reduction of BP. A later study also showed that administration of VPP and IPP to cultured endothelial cells could significantly increase NO production (Hirota et al., 2011). Results from these studies clearly indicate that antihypertensive peptides VPP and IPP could induce vasodilation through NO, independently of ACE inhibition. Results from our previous study also demonstrate that egg protein ovotransferrin-derived peptide IRW treatment increased the NO mediated vasodilation in mesenteric arteries of SHR animals, probably through increasing eNOS expression (Majumder et al., 2013b).

Increased bioavailability of NO can also improve vasodilation and reduce BP. Therefore, antioxidant and free radical scavenging activities are the key factors for altering endothelial dysfunction. Milk-derived peptides RYLGY and AYFYPEL, obtained from bovine casein hydrolysate, have shown *in vivo* ACE inhibitory and anti-oxidant effects (Sanchez et al., 2011). Oral administration of these peptides significantly reduced BP in SHR animals at a dosage of 5 mg/kg BW. In the same experiment, oral intake of casein hydrolysate containing these peptides in SHR attenuated the development of hypertension, prevented left ventricular hypertrophy, and significantly decreased interstitial fibrosis (Sanchez et al., 2011). Therefore, the vasculo-

protective activity of these peptides could reduce blood pressure and potentially ameliorate vascular fibrosis. Additionally, another peptide MY, derived from sardine muscle, exhibited antihypertensive effect by suppressing ROS generation in endothelial cells via induction of hemeoxygenase-1 (HO-1) and ferritin (Erdmann, Grosser, Schipporeit & Schroder, 2006). A study from our group has shown that the egg derived peptides IRW and IQW significantly reduced the tumor necrosis factor- α (TNF- α) induced oxidative stress in cultured endothelial cells (Majumder, Chakrabarti, Davidge & Wu, 2013a). Moreover, oral administration of IRW reduced oxidative stress in aorta and kidneys in intact SHR animals (Majumder et al., 2013b). Thus, by acting as an anti-oxidant, these peptides play a crucial role to improve NO bioavailability and consequently modulate endothelial function and BP.

Apart from increased bioavailability of NO, treatment of bioactive peptide can also release various other vasodilatory factors such as prostaglandins (PGI₂). Zhao et al. identified an antihypertensive peptide MRW from pepsin-pancreatin digest of Rubisco (Ribulose bisphosphate carboxylase/oxygenase), a spinach protein. Oral administration of MRW at a dose of 5 mg/Kg BW significantly reduced the SBP by 20 mmHg in 25 wk old male SHR (Zhao, Usui, Ohinata & Yoshikawa, 2008). MRW also exhibited a dose-dependent vasodilation in an *ex-vivo* study on isolated mesenteric arteries of SHR; the relaxation effect of MRW was not NO-dependent, and was mediated by upregulation of PGI₂ possibly through B2 receptor activation (Zhao, Usui, Ohinata & Yoshikawa, 2008). A Similar effect was observed with RIY, a peptide derived from the rapeseed protein napin (Marczak et al., 2003). The study by Yamada et al. suggested that the antihypertensive effects of RIY in SHRs are induced mainly by the production of PGI₂ (Yamada, Ohinata, Lipkowski & Yoshikawa, 2011).

The interplay between NO and ET-1 is well established in the context of endothelial dysfunction. The ECE plays an important role in converting inactive bET-1 to vasoactive ET-1, which subsequently binds to ET receptors and induces vasoconstriction. Therefore, ECE inhibitors or ET receptor agonists are the key targets for the antihypertensive therapy (Lüscher & Barton, 2000). Bovine β -lactoglobulin derived peptide ALPMHIR was found to suppress the ET-1 activity in porcine aortic endothelial cells (Maes et al., 2004), possibly through ECE inhibition. In another study, eight peptides derived from lactoferricin B those were previously characterized as ACE inhibitors have showed significant inhibition of ECE activity when vasoconstriction was induced by big ET-1. Lfcin₁₇₋₂₅ (FKCRRWQWR), LfcinB₁₇₋₃₁ (FKCRRWQWRMKKLGGA), LfcinB₁₇₋₃₂ (FKCRRWQWRMKKLGAP), and Lfcin₁₉₋₂₅ (CRRWQWR), were the most potent among them, these peptides were shown to inhibit ECE in isolated aortic segments from rabbits (Fernández-Musoles et al., 2010). Furthermore, in a follow up study, the same group identified two more ECE inhibitory peptides, GILRPY and REPYFGY, from bovine lactoferin hydrolysate (Fernández-Musoles, Salom, Martínez-Maqueda, López-Díez, Recio & Manzanares, 2013). Interestingly, these studies also suggested that these peptides may act either as dual vasopeptidase inhibitors (ACE/ECE), or as specific ECE inhibitors to produce their vasorelaxant effects (Fernández-Musoles, Salom, Martínez-Maqueda, López-Díez, Recio & Manzanares, 2013). So far there is little evidence to prove the relationship between ACE and ECE inhibition (Fernández-Musoles, Salom, Martínez-Maqueda, López-Díez, Recio & Manzanares, 2013); therefore these peptides may have dual enzyme inhibitory effect, which might result in pronounced blood pressure reducing effects.

2.2.2.3 Antihypertensive effect of peptides by blocking the calcium channel in vascular smooth muscle (VSM)

Endothelial dysfunction leads to influx of Calcium (Ca^{2+}) in vascular smooth muscle cells (VSMC) and increases vasoconstriction by activation of AT_1 and ET_A/ET_B receptors (Figure 2.3). Therefore, blocking of calcium channel reduces influx of Ca^{2+} and results in vasodilation. Peptides derived from fish protein hydrolysate have been shown antihypertensive effects by blocking the Ca^{2+} channels (Tanaka, Matsui, Ushida & Matsumoto, 2006). VY, a peptide derived from sardine muscle, exhibited an antihypertensive effect in SHR as well as in Tsukuba-Hypertensive Mouse (THM) at doses of 10 and 0.1 mg/g BW respectively (Matsui et al., 2003; Matsui et al., 2004). A study by Tanaka et al. showed that the antihypertensive effect of VY is actually mediated upon the VSM and it acts as an L-type Ca^{2+} channel blocker (Tanaka, Matsui, Ushida & Matsumoto, 2006). Similar mechanism has also been proposed for the sardine-derived peptide WH which suppresses the extracellular Ca^{2+} influx by blocking the L-type Ca^{2+} channel blocker in human VSM cells (Tanaka, Tokuyasu, Matsui & Matsumoto, 2008; Wang, Watanabe, Kobayashi, Tanaka & Matsui, 2010).

2.2.2.4 Antihypertensive peptides modulating blood pressure by opioid-like activity

Opioid receptors are present in the central nervous system and they are involved in the regulation of blood pressure through increasing the activity of the sympathetic nervous system (Feuerstein & Siren, 1987). A peptide (YGLF) derived from pepsin/trypsin digestion of α -lactorphin has been shown to reduce blood pressure in SHR by binding to opioid receptors (Nurminen et al., 2000). The vasodilatory effect of YGLF is endothelial dependent and can be inhibited by

selected eNOS inhibitors (Sipola et al., 2002). Therefore, a novel mechanism of binding to endothelial opioid receptors and subsequent NO release might be responsible for the vasodilatory effects of this peptide.

2.2.2.5 Peptides modulating vascular inflammation

Vascular inflammation-induced peripheral resistance is a contributor to elevated blood pressure and associated vascular pathologies (Renna, de Las Heras & Miatello, 2013). Milk-derived peptide tri-peptide VPP pretreatment significantly decreased phorbol 12-myristate 13-acetate (PMA) induced monocyte adhesion to human endothelial cells (Aihara, Ishii & Yoshida, 2009). In addition, treatment with the both VPP and IPP offered protection against the development of atherosclerotic plaques in the apolipoprotein E (ApoE) knockout mice through a combined action involving the modulation of inflammatory as well as hypertensive pathways (Nakamura et al., 2013). In our laboratory, the egg-derived tri-peptide IRW has demonstrated antihypertensive and anti-inflammatory properties by controlling both the hyperactive RAS pathway as well as the inflated pro-inflammatory cytokine levels in SHR (Majumder et al., 2013b). Increased circulating levels of proinflammatory cytokines during the hypertension could further contribute to endothelial dysfunction and up-regulation of leukocyte adhesion in the vasculature, which ultimately results in vascular remodeling. Therefore, peptides that control the inflammatory pathways could potentially modulate vascular pathogenesis, and hence, control vascular remodeling and reduce elevated blood pressure.

To date, several food protein-derived antihypertensive peptides have been reported with a significant antihypertensive activity in animal studies, mostly with SHR (Table 2.2). Based on current available scientific evidence, it can be concluded that food protein-derived peptides may

exert antihypertensive activity through multiple mechanistic pathways as follows: ACE inhibition, renin inhibition, ACE 2 activation, AT₁ receptor blocking, increase NO production, ECE inhibition, PGI₂ activation, blocking of Ca²⁺ channel, opioid activity, anti-oxidant activity and anti-inflammatory activity (Figure 2.6). Different peptide sequences have different modes of action that may be mediated through their structural features. Various vasodilatory mechanisms of different peptides are summarized in Table 2.2.

2.2.3 Antihypertensive effects of food derived peptides-clinical studies

Clinical trials are necessary to evaluate the efficacy of food protein-derived bioactive peptides in the context of human populations. It is also important to study the pharmacokinetics for the development of nutraceuticals and/or functional foods from food protein derived bioactive antihypertensive peptides. Two well-known peptides, VPP and IPP have been shown efficacy as antihypertensive alternatives in clinical studies (Geleijnse & Engberink, 2010). Oral administration of VPP and IPP incorporated in different food formulas (fermented milk, fruit juice) demonstrated significant decreases in blood pressure (SBP and DBP) in Japanese and Finnish hypertensive volunteers (Mizushima et al., 2004; Seppo, Jauhiainen, Poussa & Korpela, 2003; Table 2.3). However, the oral intake of these same peptides failed to reduce BP in Dutch and Danish hypertensive subjects, suggesting possible variation in sensitivity among different human populations (Engberink, Schouten, Kok, van Mierlo, Brouwer & Geleijnse, 2008). A meta-analysis of 18 clinical trials has shown that oral administration of these peptides (VPP and IPP) reduces BP in hypertensive subjects but the beneficial effect is more prominent in Asian subjects (Cicero, Gerocarni, Laghi & Borghi, 2011). The controversial results obtained by different studies about the effect of lactopeptides in Caucasian populations was addressed by

Boelsma et al., in a double-blinded placebo control trial with 70 Caucasian pre-hypertensive or stage-1 hypertensive subjects. The result from this study reveals that oral administration of IPP exerts clinically relevant BP-lowering effects in Caucasian subjects with stage-1 hypertension (Boelsma & Kloek, 2010). Furthermore, another study has demonstrated that administration of milk tri-peptides along with plant sterols can exhibit a clinically significant reduction in SBP as well as serum total and low density lipoprotein (LDL)-cholesterol without adverse effects in hypertensive, hypercholesterolemic subjects in a randomized, placebo-controlled double-blind intervention (Turpeinen, Ikonen, Kivimaki, Kautiainen, Vapaatalo & Korpela, 2012). Results from all these studies suggested that the reduction of blood pressure was observed after at least 1-2 weeks of treatment with maximum effect of 13 mm Hg for SBP and 8 mm Hg for DBP with a dose range of approximately 3-55 mg/day. A recent meta-analysis showed that small doses (2.0-10.2 mg/day) of milk casein-derived tri-peptides (VPP and IPP) exhibited an overall reduction of SBP by 4.0 mm Hg and DBP by 1.9 mm Hg, respectively in mildly hypertensive subjects (Turpeinen, Jarvenpaa, Kautiainen, Korpela & Vapaatalo, 2013). In addition to VPP and IPP, another study showed that consumption of yogurt enriched with casein-derived antihypertensive peptides (RYLGY and AYFYPEL) reduced significantly SBP by 12 mmHg after 6 weeks of intake in a normalized placebo control trial (Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012). Human clinical trials with pea protein hydrolysate also showed significant decrease in SBP by 5-7 mm Hg after 2 weeks of treatment, but the smaller number (n=7) used in this study is obviously not enough to judge the efficacy of the hydrolysate (Li et al., 2011). Similarly, sardine muscle derived di-peptide (VY) had used for human clinical trials. A randomized double-blind placebo control trials with 29 hypertensive subjects demonstrated a decrease in SBP and DBP by 9.3 and 5.2 mmHg, respectively, after 4 weeks of treatment

(Kawasaki et al., 2000; Table 2.3). However the clinical impact is controversial due to the small number of heterogeneous subjects in the trials (Marques et al., 2012). Another trial involving 63-hypertensive subjects showed that consumption of a vegetable drink containing VY significantly reduced the blood pressure in high and mild hypertensive subjects, without any adverse side effects (Kawasaki et al., 2002). The third clinical study showed that a single oral administration of VY significantly increased plasma VY level, indicating the absorption of peptide in the blood stream through gastrointestinal tract. However no marked decrease in blood pressure was observed with the increase plasma VY level, indicating that VY did not exhibit an acute anti-hypertensive effect after oral ingestion and a longer-term treatment was likely necessary for the clinical benefits (Matsui, Tamaya, Seki, Osajima, Matsumo & Kawasaki, 2002). Results from these studies would increase the scientific knowledge about food-derived peptides and may facilitate the development of peptide-based functional foods or nutraceuticals for the management of hypertension. However, more clinical studies engaging volunteers from various ethnicities are required to establish a worldwide acceptability of food-derived bioactive peptides as clinically relevant antihypertensive agents.

2.3 Current market scenario of bioactive peptides

In the past few decades, increasing interest in bioactive peptides with health benefits has significantly contributed to the expanding market of functional foods. Unlike pharmaceutical drugs, functional foods enriched with bioactive peptides have attracted consumer attention due to their food-derived origins, oral route of intake, low price, and perceived lack of adverse side effects. Specific amino acid sequences can exhibit multifunctional effects and can reduce the risk of chronic disease. A number of approved functional foods and nutraceuticals enriched with

biologically active peptides with different health claims are now commercially available as over the counter products for direct purchase by consumers. Japan is one of the largest markets for functional foods containing bioactive peptides, especially with antihypertensive activity, with a total market sales of 15 trillion ¥ in 2009 (Toshio, 2012). Milk protein casein-derived peptides VPP and IPP are the first peptides that launched in the market in 1997, with a product name of ‘Ameal S’ by Calpis Co. Ltd, Japan. Currently it is one of the top brands with a 70% market share of functional foods in Japan (Calpis, 2012). A Finish company, Valio, launched a similar product called ‘Evolus’, containing VPP and IPP with blood pressure-lowering properties and plant sterols with cholesterol lowering properties, available in the European market (Turpeinen, Ikonen, Kivimaki, Kautiainen, Vapaatalo & Korpela, 2012; Valio, 2013). Some of the bioactive rich products are launched in the market as health drink/beverages, such as; ‘C12 peption’ by DMV, Netherlands, ‘Peptio’ Kanebo, Japan. ‘Capolac’ and ‘CE90PP’ are casein phosphopeptide-rich products that have been launched by Arla Foods, Sweden and DMV, Netherlands respectively. Food-derived bioactive peptides are also commercialized by pharmaceutical industries; for example VY, a peptide extracted from sardine muscle is commercialized by Sato pharmaceuticals, while LKPNM, a bonito peptide launched as Vasotensin by Metagenics, USA, in the form of blood pressure lowering tablets (Toshio, 2012). Similar to casein phosphopeptides, egg protein phosphovitin-derived phosphopeptides are also commercialized by Evalupharma, Japan. The product is launched as ‘Bonepep’ and claimed to reduce osteoporosis with a worldwide sales of around \$2 million USD in 2011 (Evaluate, 2012). In addition to the oral administration for the prevention and management of chronic diseases, bioactive peptides have a wide range of applications in the cosmetic and skin care industries. Therefore, bioactive peptides have a diverse range of applications with a wide commercial

market. Table 2.4 summarizes some of the current commercially available functional foods enriched with bioactive peptides.

2.4 General Conclusion

There is a tremendous global interest in promoting the use of food proteins/peptides as novel alternatives for present pharmaceutical therapeutics. Bioactive peptides derived from both plant and animal food proteins have been shown health-promoting effects in animal and human studies. Bioactive peptides can be assimilated in the form of active ingredients in functional foods or in dietary supplements with the purpose of delivering specific health benefits. The translation of food-derived bioactive peptides for human health improvements is an exciting scientific challenge, but simultaneously offers the potential for successful commercial applications.

- Further research is required to identify the molecular targets of peptide action which will establish the health promoting effects of these bioactive peptides.
- It is also important to explore the physical and chemical properties and the structural attributes of the peptide sequences, which will be helpful to develop model foods that contain active peptides and able to retain their activity at the time of consumption.
- Detailed studies are required to determine the molecular mechanisms of action of these bioactive peptides. In this context, the use of emerging technologies such as proteomics, RNA sequencing, computational study with molecular docking and gene functional analysis are important to unlock the molecular mechanisms.
- Further clinical studies with human subjects are also required to evaluate the ultimate efficacy and to support the health claims.

- Furthermore, the safety of these bioactive peptides should also be evaluated before commercialization. Concomitant long-term research is also needed to study the potential adverse effects associated with long term use of these peptides.

The present study tries to address some of these issues to bridge the knowledge gap between *in vitro* and *in vivo* activities of food derived bioactive peptides. These studies may provide new insights, which could justify the use of bioactive peptides for the treatment and management of various chronic diseases in the days to come.

2.5 References

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Table 2.1: Representative bioactive peptides derived from various food proteins

Activity	Food product	Protein	Peptide name/ sequence	Observed effect	Reference
Anti-microbial	Milk	Lactoferrin	Lactoferricin	↓ Gram positive and negative bacteria	(Gifford, Hunter & Vogel, 2005)
		α 1-Casein	Casecidins	↓ Gram negative bacteria	(Lahov, Edelsten, Sode-Mongensen & Sofer, 1971)
		κ -Casein	Kappacin A	↓ Gram positive and negative bacteria	(Malkoski et al., 2001)
	Egg	Ovotransferrin	OTAP-92	↓ Gram positive bacteria	(Ibrahim, Sugimoto & Aoki, 2000)
		Lysozyme	Lzp	↓ Gram positive and negative bacteria	(Thammasirak et al., 2010)
Immunomodulatory	Rice	Rice albumin	Oryzatensin	↑ Phagocytosis	(Takahashi, Moriguchi, Yoshikawa & Sasaki, 1994)
	Milk	β -casomorphin	YPPFGPIPNSL	↓ Lymphocyte proliferation	(Gill, Doull, Rutherford & Cross, 2000)
		Para- κ -casein	FFSNK	↑ Antibody formation and Phagocytosis	(Gill, Doull, Rutherford & Cross, 2000)
	Wheat	Gluten	Immunopeptides	↑ Natural killer cell activity	(Cavazos & Gonzalez, 2013)
Anti-inflammatory	Milk	Casein	VPP, IPP	↓ Leukocyte recruitment ↓ Cytokine expression	(Nielsen, Theil, Larsen & Purup, 2012)

		Lactoferrin	Lactoferricin	↓ Adhesion molecule expression	(Haversen, Ohlsson, Hahn-Zoric, Hanson & Mattsby-Baltzer, 2002)
	Egg	Ovotransferrin	IRW, IQW	↓ Adhesion molecule expression ↓ Cytokine expression	(Huang, Chakrabarti, Majumder, Jiang, Davidge & Wu, 2010, Majumder, Chakrabarti, Davidge & Wu, 2013)
	Soybean	Soy protein	Lunasin VPY	↓ Cytokine expression	(de Mejia & Dia, 2009, Hernandez-Ledesma, Hsieh & de Lumen, 2009)
Opioid	Milk	Lactoferrin	Lactoferroxins Casoxins	Opioid agonist	(Teschemacher, Koch & Brantl, 1997)
		α -Casein			
Anti-cancer	Milk	α - β casein	HIQKED YPFPGPI	Triggered apoptosis	(Kaminski, Cieslinska & Kostyra, 2007)
Anti-oxidant	Hempseed	Heampseed protein isolate	HVRETALV	↓ Oxidative apoptosis	(Lu et al., 2010)
	Rice	Defatted rice endosperm	FRDEHKK	Scavenge intercellular ROS	(Zhang, Zhang, Wang, Guo, Wang & Yao, 2010)
	Egg	Ovotransferrin	IRW, IQW	Reduced cellular superoxide	(Majumder, Chakrabarti, Davidge & Wu, 2013)

Table 2.2: Antihypertensive activity and vasodilatory mechanism of food derived bioactive peptides in spontaneously hypertensive rats

Food	Protein source	Peptide sequence	Dose mg/kg BW	SBP decrease (mm Hg)	Vasodilatory mechanism	References
Milk	α -casein	MKP	0.5	-30.0	ACE inhibition	(Yamada, Sakurai, Ochi, Mitsuyama, Yamauchi & Abe, 2013)
		RYLGY	5.0	-32.0	Anti-oxidant	(Contreras, Carrón, Montero, Ramos & Recio, 2009)
		AYFYPEL	5.0	-22.5	Anti-oxidant	(Contreras, Carrón, Montero, Ramos & Recio, 2009)
	κ -casein	IAK	4.0	-20.7	ACE inhibition	(Miguel, Gomez-Ruiz, Recio & Aleixandre, 2010)
		YAKPVA	6.0	-23.1	ACE inhibition	(Miguel, Gomez-Ruiz, Recio & Aleixandre, 2010)
	β -casein	IPP		0.3	-28.3	ACE inhibition ACE 2 activation eNOS up-regulation Anti-inflammatory
VPP			0.6	-32.1	ACE inhibition eNOS up-regulation Anti-inflammatory	(Hirota et al., 2011; Nakamura et al., 2013; Nakamura, Yamamoto, Sakai, Okubo, Yamazaki & Takano, 1995)
	α -lactorphin	YGLF	1.0	-23.7	Opioid-agonist	(Nurminen et al., 2000)

Egg	Ovalbumin	RADHP	2.0	-28.3	Increased NO Activate B ₁ receptor	(Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012)
	Ovotransferrin	IRW	15.0	-40.0	ACE inhibition ACE 2 Up-regulation eNOS Up-regulation Anti-oxidant Anti-inflammatory	(Huang, Chakrabarti, Majumder, Jiang, Davidge & Wu, 2010; Majumder & Wu, 2010; Majumder et al., 2013)
	Egg white protein	RVPSL	50.0	-25.0	ACE inhibition Renin inhibition AT ₁ blocker	(Yu, Yin, Zhao, Chen & Liu, 2014)
Fish	Sardine muscle	MY	10.0	-19.4	Anti-oxidant	(Erdmann, Grosser, Schipcoreit & Schroder, 2006; Marques et al., 2012)
		VY	10.0	-18.5	Ca ²⁺ channel blocker	(Tanaka, Matsui, Ushida & Matsumoto, 2006)
	Bonito muscle	LKP	2.25	-5.0	ACE inhibition	(Fujita & Yoshikawa, 1999)
Rapeseed	Napin	RIY	7.5	-28.0	PGI ₂ activator	(Marczak et al., 2003)
Spinach	Rubisco	MRW	5.0	-20.0	PGI ₂ activator	(Zhao, Usui, Ohinata & Yoshikawa, 2008)

Table 2.3: Human clinical trials of food protein derived antihypertensive peptides

Active peptide	Administered product	Study description	Dose/ day	Duration (Weeks)	SBP decrease (mmHg)	References
VPP and IPP	Fermented milk	Double-blinded placebo-controlled randomized trial, 46 men with high-normal blood pressure.	160 g (2.0 mg VPP and 1.2 mg IPP)	8	-5.2 mm Hg	(Seppo, Jauhiainen, Poussa, & Korpela, 2003)
	Evolus [®] (fermented milk flavored with fruit juice)	Placebo-controlled randomized trial, 42 subjects with mild hypertension.	150 mL (3.0 mg VPP and 2.25 mg IPP)	21	-6.7 mm Hg	(Mizushima et al., 2004)
	Low-fat yoghurt drinks	Randomized double-blind placebo-controlled trial, 135 hypertensive subjects (male/female: 88/47)	200 mL (5.8 mg VPP and 5.4 mg IPP)	8	No significant difference in blood pressure between the treatment and placebo controlled group	(Engberink, Schouten, Kok, van Mierlo, Brouwer & Geleijnse, 2008)
	Milk protein hydrolysate	Placebo control, double blinded, crossover including 70 Caucasian subjects	2- tablets/day (each tablet contains 7.5mg IPP)	4	-4.0 mm Hg in SBP (significant reduction) No change in DBP	(Boelsma & Kloek, 2010)
	Fruit Juice fortified with Lacto tri-peptides	Randomized double blinded, 52 (men:women=29:21) mildly hypertensive patients	25 ml/day (3.0 mg of VPP and IPP)	6	-5.0 mm Hg in SBP	(Cicero et al., 2011)
	A lacto spread contained VPP, IPP and plant sterols	Randomised, placebo-controlled double-blind intervention, 104 hypertensive, hypercholesterolemic subjects	20 g/day (containing 4.2mg of VPP and IPP; 2 g of plant sterols)	10	-4.1 mm Hg in SBP, No change in DBP and significantly reduce plasma LDL cholesterol	(Turpeinen, Ikonen, Kivimaki, Kautiainen, Vapaatalo & Korpela, 2012)

RYLGY and AYFYPEL)	Casein hydrolysate	Normalized placebo control trial	20 (5.5mg RYLGY and AYFYPEL)	ml/day 6		-12 mm Hg in SBP	(Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012).
VY	A beverage enriched with sardine muscle hydrolysate	Randomized placebo-controlled trial, 29 subjects with mild hypertension.	2 x 100 mg VY)	mL (6 4		-9.3 mm Hg	(Kawasaki et al., 2000)
	A vegetable drink	Randomized placebo-controlled trial, 63 subjects (male/female: 51/12) with mild hypertension.	195 g VY)	(0.4 mg 13		-7.6 mm Hg	(Kawasaki et al., 2002)

Table 2.4: Commercially available functional foods and nutraceuticals enriched with bioactive peptides and their health claims

Health claim	Product name	Manufacturer	Type of food product	Bioactive peptides
Hypotensive	Ameal S 120	Calpis Co. Japan	Sour milk	VPP, IPP
	Ameal S handy		Tablet	
	Ameal S vegetable	Nippon Supplement	Juice	LKPNM
	Katsubushi		Soup	β -lactoglobulin fragments
	BioZate	Davisco, USA	Whey protein supplement	
	C12 Peption	DMV, Netherlands	Protein bar, Beverage	FFVAPFPEVFGK
	Peptio	Kanebo, Japan	Beverage	FFVAPFPEVFGK
	PeptACE	Natural factors, Canada	Capsules	Peptide mixture from bonito
	Vasotensin	Metagenics, USA	Capsules	LKPNM
	Sato Marine SuperP	Sato Pharmaceuticals, Japan	Capsules	VY
Bunaharitake	Yakul Health Foods Co. Ltd, Japan	Drink powder	IY	
Controls blood pressure Reduce sleep apnea	Bioactive milk peptides	LifeExtension, USA	Capsules	Peptides from milk protein
	Vivinal Alpha	BDI, Netherlands	Ingredient	α -lactalbumin rich whey protein hydrolysate
Controls blood pressure and lowers cholesterol	Evolus	Valio, Finland	Fermented milk	VPP, IPP and plant sterols
Mineral absorption	Capolac	Arla Foods, Sweden	Ingredient	CPP (casein phosphopeptides)
	Tekkotsu Inryou	Suntory, Japan	Beverage, soft drink	CPP
	Kotsu kotsu	Asahi, Japan	Beverage, soft drink	CPP
	CE90CPP	DMV, Netherlands	Ingredient	CPP
Hypocholesteromic	CholesterBlock	Kyowa Hakko, Japan	Drink	Soy peptides bound phospholipids
Reduce stress	ProDiet F200	Ingredia, France	Beverage	YLGYLEQLLR
Immunomodulatory	Glutamine Peption	DMV, Netherlands	Milk	Glutamine-rich peptides

Anti-carcinogenic Anti-microbial	BioPURE-GMP	Davisco, USA	Whey protein supplement	Glycomacropptides
Reduce Osteoporosis	Bonepep	EvaluatePharma, Japan	Beverage	Egg phosvitin- phosphopeptides
Delays skin ageing	Peptan Sontal	Rousselot, USA Lifestyle Nutrition, South Africa	Capsules Capsules	Collagen peptides Collagen peptides
Reduce Acne	Praventin	DMV, Netherlands	Capsules	Lactoferrin enriched whey protein hydrolysate

Table information gathered from (Hartmann & Meisel, 2007; Korhonen, 2009; Toshio, 2012)

Figure 2.1 Different bioactivity of food derived peptides. Bioactive peptides derived from food proteins can exert various biological activities.

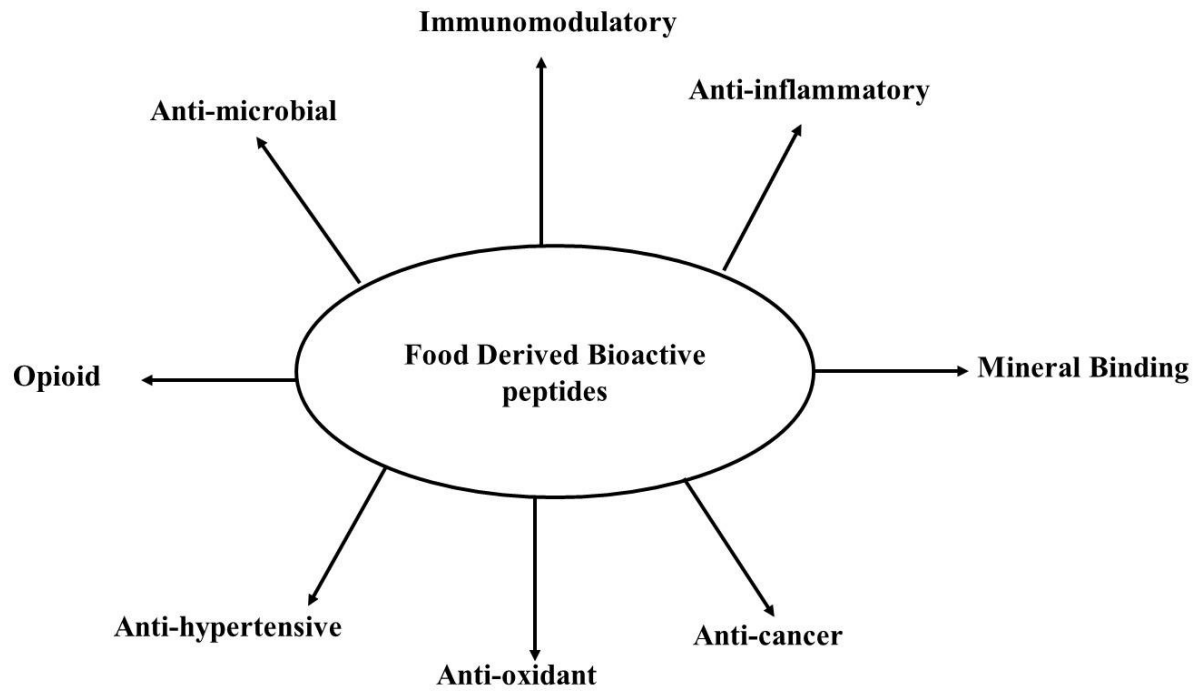


Figure 2.2: Renin-angiotensin system and kallikrein kinin system in regulation of blood pressure. Ang I (angiotensin I), Ang II (angiotensin II), ACE (angiotensin converting enzyme), ACE 2(angiotensin converting enzyme 2), AT₁ (angiotensin receptor 1), AT₂ (angiotensin receptor 2), B₁ (bradykinin receptor 1), B₂ (bradykinin receptor 2), NO (nitric oxide), PGI₂ (prostaglandins 2).

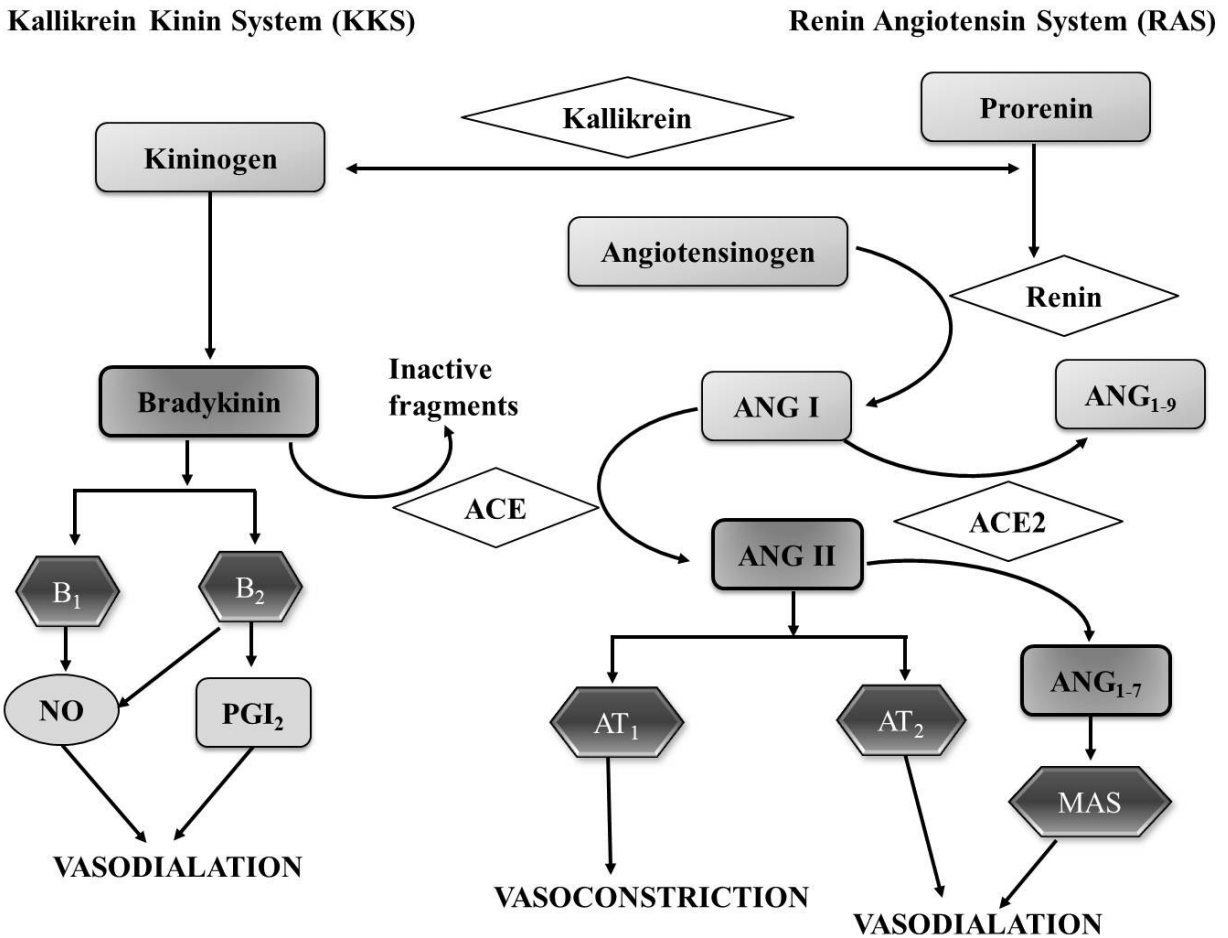


Figure modified from (Oparil & Haber, 1974; Peah, 1977; Stankevicius, Kevelaitis, Vainorius & Simonsen, 2003; Zhuo, Ferrao, Zheng & Li, 2013).

Figure 2.3: Endothelial dysfunction and blood pressure regulation. ACE (angiotensin converting enzyme) converts Ang I (angiotensin I) to Ang II (angiotensin II), Ang II binds with AT₁ receptor (angiotensin 1 receptor) on endothelium cells as well as vascular smooth muscle cells, then AT₁ receptor increases calcium ion (Ca²⁺) concentration in vascular smooth muscle cells (VSMC) and exerts vasoconstriction. In endothelium cells activation of AT₁ receptor increases the production of bET-1 (big endothelin-1). ECE (Endothelin-Converting Enzyme) converts bET-1 to ET-1 (endothelin-1) and exerts vasoconstriction by activating ET_{A/B} receptors in the VSMC. In contrast, activation of ET_B receptor in endothelium cells mediates vasodilatory effects via release of NO (nitric oxide) by activating eNOS (nitric oxide synthase) in endothelium. ACE also converts Bk (Bradykinin) into inactive peptides. Bk binds with bradykinin receptor (B_{1/2}) and activates eNOS, which converts L-Arg to L-citrulline and produces NO. NO exerts vasodilation by activating cyclic guanosine monophosphate (cGMP) by inhibiting the concentration of Ca²⁺ in VSM. In endothelium cells Ang II produces superoxide (O₂⁻) which scavenges NO and produces peroxynitrite (ONOO⁻), exerts vasoconstriction effect by limiting the supply of NO to the VSM. Endothelin-Converting Enzyme (ECE) converts big-endothelin (bET-1) to endothelin-1 (ET-1). Signaling pathways illustrated with solid line arrows (⇓) are representing vasoconstriction and with compound line arrows (⇩) are representation vasodilation network.

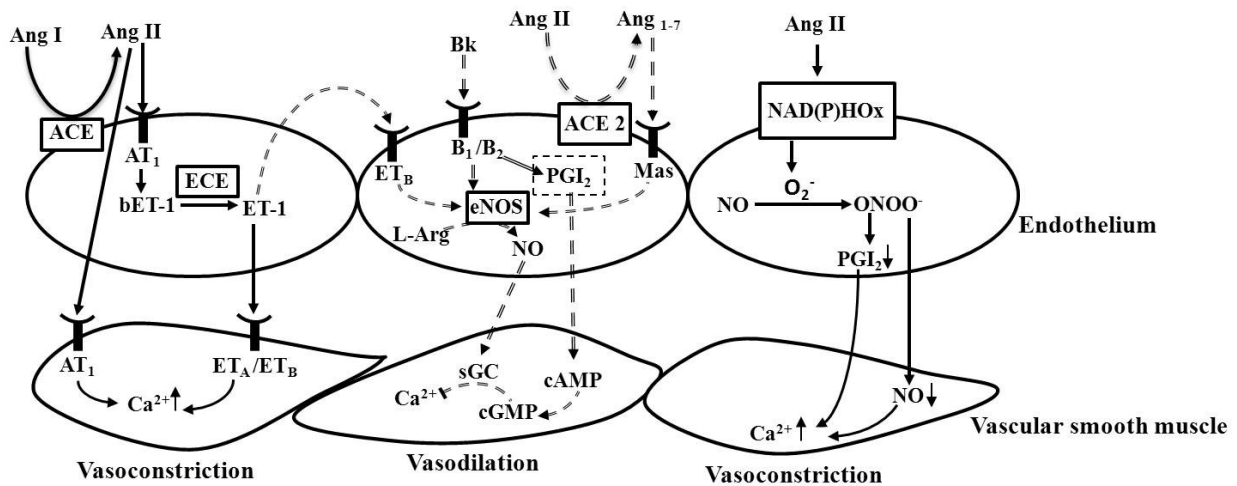


Figure modified from (Sudano et al., 2006; Barton & Yanagisawa, 2008)

Figure 2.4: Regulation of blood pressure through autonomic nervous system. Increased sympathetic nervous system stimulates the release of catecholamines from post ganglionic neurons. Catecholamines increase the hypertrophic growth of cardiomyocytes and release more renin in adrenal cortex. Increased production of renin activates RAS (renin angiotensin system) and produces more Ang II (Angiotensin II). Hypertrophic growth of cardiomyocytes and increase production of Ang II result in vasoconstriction. In addition, Ang II production increases ET-1 (Endothelin-1) and ROS (reactive oxygen species) production and directly affect the over activity of sympathetic nervous system.

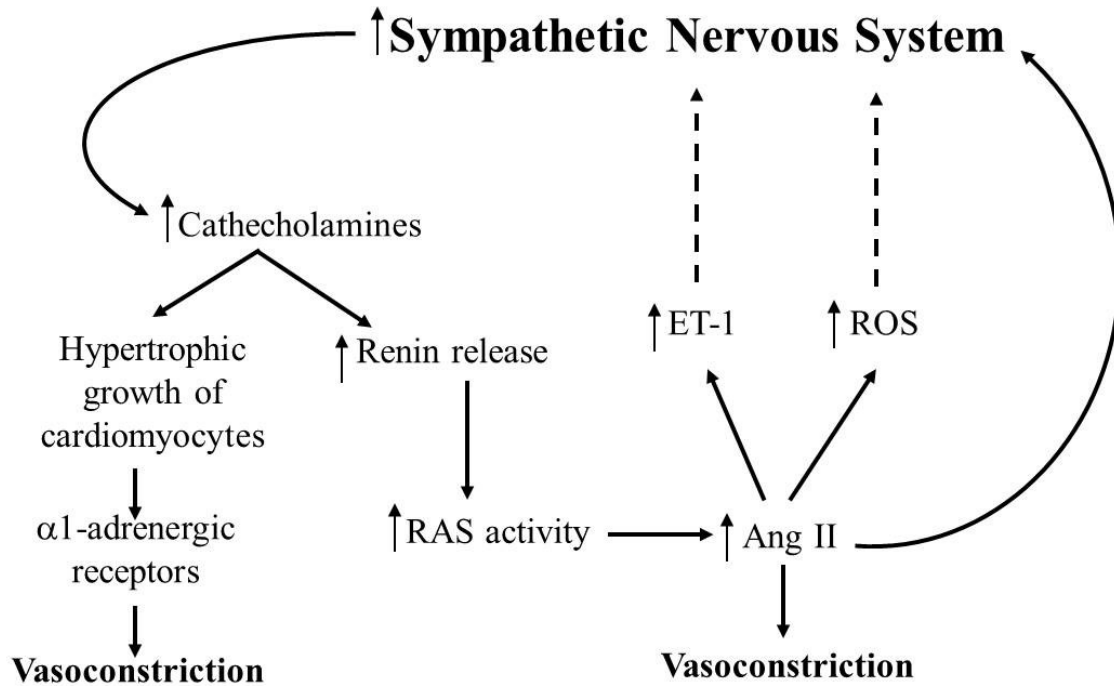


Figure 2.5: Pathophysiology of hypertension – a vicious cycle. Renin angiotensin system (RAS), endothelial dysfunction, vascular remodeling, and activity of sympathetic nervous system are correlated with each other. Enhanced RAS activity leads to over production of angiotensin II (Ang II) which accelerates endothelial dysfunction. Ang II induced endothelial dysfunction results in vasoconstriction as well up regulates the activity of transcription factors (such as NF- κ B, nuclear factor κ B), promoting vascular inflammation. Vascular inflammation up regulates the expression of vascular adhesion molecule ICAM-1 (Intercellular adhesion molecule 1), VCAM-1 (Vascular adhesion molecule-1) and inflammatory cytokines TNF- α (Tumor necrosis factor- α), IL-1 β (Interleukin -1 β). Similarly, during endothelial dysfunction over expression of ET-1 (Endothelin-1) and increased levels of oxidative stress such as; superoxide (O_2^-) can directly increase the sympathetic nervous system. Finally, increased sympathetic nervous system increases renin production which eventually activates RAS.

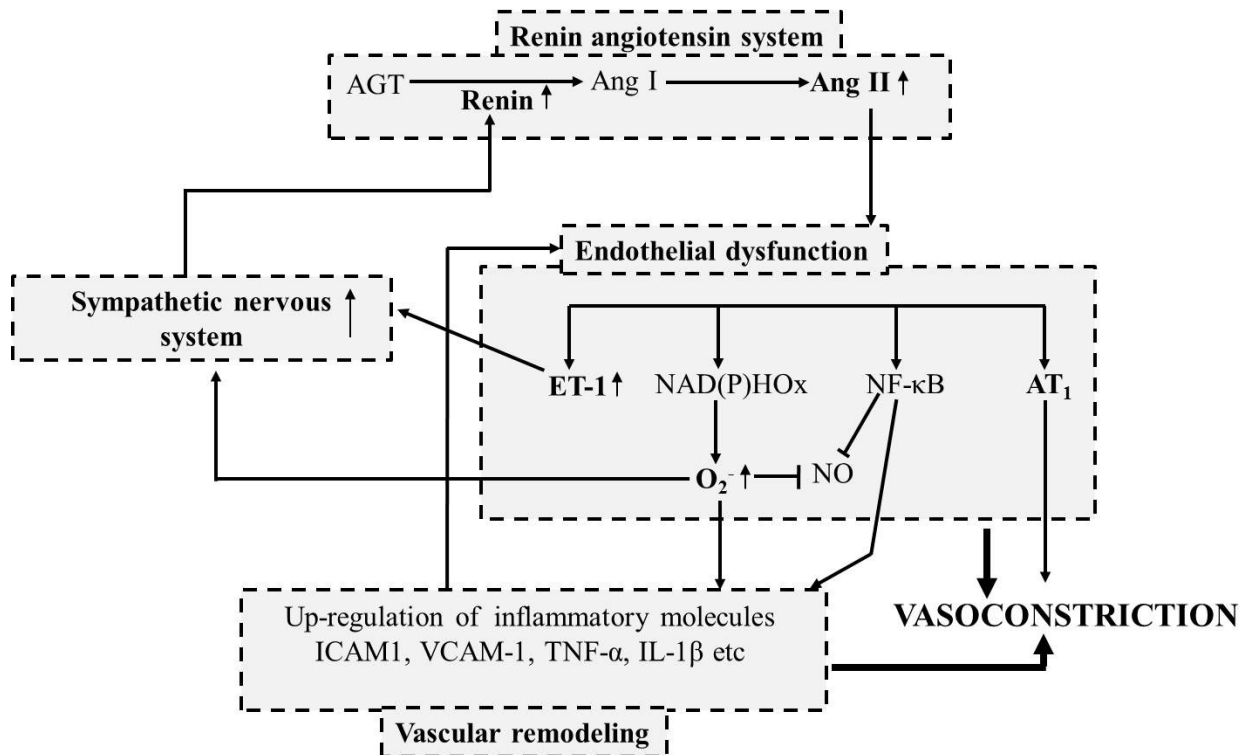
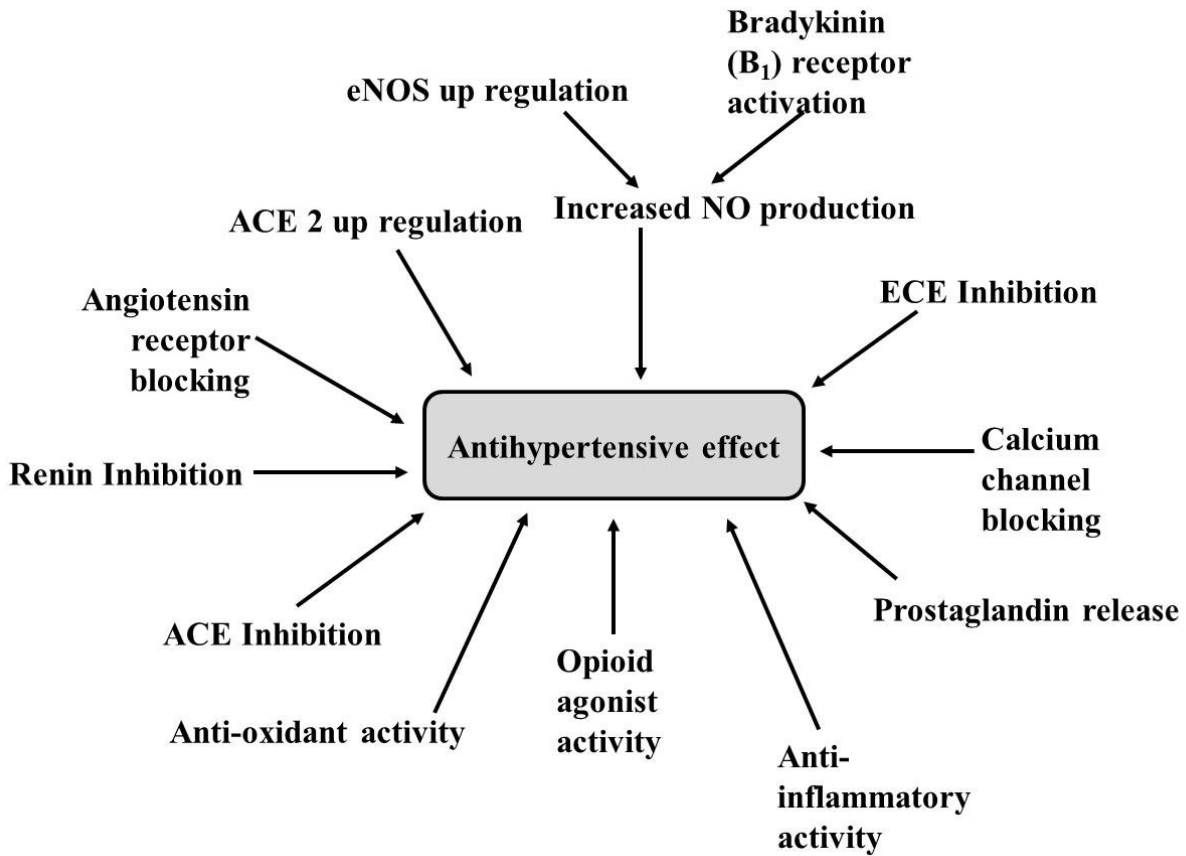


Figure 2.6: A schematic diagram representing the antihypertensive mechanisms of food derived peptides. ACE (Angiotensin-I converting enzyme), ACE 2 (Angiotensin converting enzyme 2), eNOS (Endothelial nitric oxide synthase), NO (Nitric oxide), ECE (Endothelin converting enzyme)



CHAPTER 3² - Structure and Activity Study of Egg Protein Ovotransferrin Derived Peptides (IRW and IQW) on Endothelial Inflammatory Response and Oxidative Stress

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3.1 Introduction

Endothelial cells are the major regulator of vascular tone. Endothelial dysfunction manifests one of the common features of cardiovascular diseases (CVDs), the leading cause of morbidity and mortality worldwide (Behrendt & Ganz, 2002; Chockalingam, 2008; Giannotti & Landmesser, 2007). Vascular inflammation and oxidative stress are two key factors that lead to endothelial dysfunction.

Tumor necrosis factor (TNF), a pro-inflammatory cytokine, participates in the inflammatory response and plays an important role in the development of atherosclerotic lesions (Sprague & Khalil, 2009; Zhang et al., 2009). TNF-activated endothelial cells up-regulate the expression of adhesion molecules intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), which is important in the onset and progression of vascular inflammation (McEver, 1992). TNF-induced up-regulation of adhesion molecules is mediated through the transcription factor NF- κ B (Nuclear factor- κ B) pathway (Sprague & Khalil, 2009, Zhang et al., 2009). TNF also increases the production of superoxide (O_2^-) through activation of NADPH oxidase (Pennathur & Heinecke, 2007). Increased O_2^- production is responsible for impaired bioavailability of nitric oxide (NO) and endothelial vasodilator dysfunction, which may lead to hypertension (Muller & Morawietz, 2009). Subsequently, O_2^- can also increase cytoplasmic level of H_2O_2 that can activate NF- κ B (Rahman, Gilmour, Jimenez & MacNee, 2002), resulting in a pro-inflammatory shift in the endothelial gene expression, endothelial activation, and increased leukocyte recruitment to the endothelium that accelerates the development of atherosclerotic lesions (Guzik & Harrison, 2006; Pober, Min & Bradley, 2009; Rahman, Gilmour, Jimenez & MacNee, 2002). Therefore, the targeting of TNF-induced

inflammation and oxidative stress provides a strategy for controlling vascular diseases such as atherosclerosis and hypertension.

Due to the unavoidable side effects of synthetic drugs, there is an increasing interest in searching novel bioactive food components, such as angiotensin converting enzyme (ACE) inhibitory peptides, for the prevention and treatment of CVDs (Torruco-Uco, Dominguez-Magana, Davila-Ortiz, Martinez-Ayala, Chel-Guerrero & Betancur-Ancona, 2008). Many food-derived compounds, such as bioactive peptides, are known to possess a wide range of bioactivities including anti-microbial, anti-carcinogenic, anti-inflammatory, anti-oxidant, and anti-hypertensive effects (Korhonen & Pihlanto, 2003, Meisel, 2004). IRW, characterized through an integrated quantitative structure and activity relationship (QSAR) and bioinformatics approach from egg white protein ovotransferrin (Majumder & Wu, 2010), was found to exhibit anti-inflammatory effect through NF- κ B pathway by blocking the nuclear translocation of p65 (Huang, Chakrabarti, Majumder, Jiang, Davidge & Wu, 2010). IQW, another potent ACE inhibitory peptide differing only one amino acid residue from IRW, was also derived from ovotransferrin. However, the anti-oxidant and anti-inflammatory effects and the underlying mechanism of IQW have not been studied. In addition, the structural requirements of these peptides for the anti-inflammatory and anti-oxidant properties are not known. Therefore, the objectives of the present study were to investigate the structure and activity relationships of IRW and IQW and to examine the underlying molecular mechanisms of their anti-oxidant and anti-inflammatory activities.

3.2 Materials and Methods

3.2.1 Reagents and antibodies

Dulbecco's phosphate buffered saline (PBS), M199 medium with phenol red, porcine gelatin, dithiothreitol (DTT), catalase and polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) were bought from Sigma Chemical Co. (St Louis, MO). Oligofectamine, Optimem1, M199 medium without phenol red, and fetal bovine serum (FBS) were obtained from Gibco/Invitrogen (Carlsbad, CA). Type 1 Collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Triton-X-100 and endothelial cell growth supplement (ECGS) were obtained from VWR International (West Chester, PA). Both IRW and IQW were synthesized and supplied by GenScript Corporation (Piscataway, NJ), and their purity (>95%) was verified by HPLC-MS/MS. All other chemicals and reagents were of the analytical grade.

3.2.2 Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords obtained from the Royal Alexandra Hospital (Edmonton, AB, Canada) (Arenas, Xu, Lopez-Jaramillo & Davidge, 2004; Chakrabarti & Davidge, 2009; Merchant, Narumiya, Zhang, Guilbert & Davidge, 2004; Narumiya, Zhang, Fernandez-Patron, Guilbert & Davidge, 2001; Sankaralingam, Xu, Sawamura & Davidge, 2009). HUVECs are a widely used model for studying the vascular endothelium (Narumiya, Zhang, Fernandez-Patron, Guilbert & Davidge, 2001). The protocol was approved by the University of Alberta's Research Ethics Board and the investigation also conformed to the principles outlined in the Declaration of Helsinki and also Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All subjects provided informed consent

before inclusion into this study. Following collection of umbilical cords, the umbilical vein was first flushed with PBS to remove blood clots and then HUVECs were isolated out using a type 1 collagenase-containing buffer. The cells were grown in a humidified atmosphere at 37°C with 5% CO₂/95% air in M199 medium with phenol red supplemented by 20% FBS as well as L-Glutamine (Gibco/ Invitrogen, Carlsbad, CA), penicillin-streptomycin (Life Technologies, Carlsbad, CA) and 1% endothelial cell growth supplement (ECGS, from VWR International, West Chester, PA). We have previously confirmed the endothelial nature of these cells by staining for the endothelium-specific marker, von Willebrand's factor (vWF) (Chakrabarti, Chan, Jiang & Davidge, 2012).

3.2.3 Experimental protocols and treatments

Second passage confluent HUVEC monolayers were used in this study. Cells grown in 48 well plates (80-100 K cells/well) were treated with bioactive tripeptides (IRW, IQW), their respective dipeptides (IR, RW, IQ and QW) or amino acids (I, R, Q and W, individually or in combination) for 20 h. Cells were then treated with TNF (5 ng/mL) for different time periods for different experimental paradigms as described in the subsequent sections.

3.2.4 Adhesion molecule expression

Expression of the adhesion molecules (ICAM-1 and VCAM-1) was determined through the western blot technique. After pretreatment with peptides/amino acid, cells were stimulated for 4 h with TNF. The cells were lysed using boiling hot Laemmli's buffer containing 0.2% Triton-X-100 and DTT as a reducing agent. Samples were then run in a 9% SDS-PAGE and the protein bands of interest were detected by specific antibodies. Bands for VCAM-1 (rabbit polyclonal antibody from Santa Cruz Biotechnologies, Santa Cruz, CA) and ICAM-1 (mouse monoclonal

antibody from Santa Cruz Biotechnologies, Santa Cruz, CA) were normalized to α -Tubulin (rabbit polyclonal antibody from Abcam, Cambridge, MA). Anti-tubulin was used at 0.4 $\mu\text{g}/\text{mL}$, while all others were used at 1 $\mu\text{g}/\text{mL}$. Goat anti-rabbit and donkey anti-mouse fluorochrome-conjugated secondary antibodies were purchased from Licor (Licor Biosciences, Lincoln, NB). The protein bands were detected by a Li-cor Odyssey Bio-Imager and analyzed by densitometry using corresponding software (Li-cor Biosciences, Lincoln, NB). Samples generated from one particular umbilical cord were run on the same gel. Cell lysates from untreated cells were loaded on every gel and all data were expressed as fold change over the corresponding untreated control.

3.2.5 Superoxide detection

Endothelial superoxide generation was measured by staining with dihydroethidium (DHE) similar the previous work (Chakrabarti & Davidge, 2009). Cells were pre-treated with peptides/amino acids and then followed by a 1h TNF stimulation. DHE is cell-permeable and reacts with superoxide to yield ethidium, which binds to nuclear DNA and generates nuclear fluorescence (Peshavariya, Dusting & Selemidis, 2007). Following stimulation with TNF, with or without pre-treatment with the peptide, HUVEC monolayers were washed once and incubated for 30 min at room temperature with 10 $\mu\text{mol}/\text{L}$ of DHE in Q-medium (phenol red free M199 with 1% FBS). After a 30 min incubation period, cells were washed once and fluorescence was visualized in an Olympus IX81 fluorescent microscope (Carson Scientific Imaging Group; Ontario, Canada) using the Slidebook 2D, 3D Timelapse Imaging Software (Intelligent Imaging Innovations Inc.; CO, USA). For each data point, images from three randomly chosen fields were taken. The total fluorescence intensity and the number of cells in each field were noted and the

mean fluorescence intensity per cell (MFI/Cell) was determined as previously described. Superoxide generation was measured as fold increase in MFI/Cell over the untreated control.

3.2.6 NF- κ B activity detection

NF- κ B activity was determined by nuclear translocation of p65 and p50, in the presence of the bioactive peptides [IRW and IQW (50 μ mol/L)]. HUVEC monolayers were pre-treated with bioactive peptides for 20 h, followed by a 30 min TNF stimulation. Then the cells were fixed in 4% formalin, permeabilized with 0.1% Triton-X-100 and immunostained using overnight incubation with antibodies against p50 (rabbit polyclonal antibody from Santa Cruz Biotechnologies, Santa Cruz, CA) and p65 (mouse monoclonal antibody from Santa Cruz Biotechnologies, Santa Cruz, CA). Cells were treated with fluorescent-labeled anti-rabbit and/or anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) for 30 min in the dark. Nuclei were stained with the Hoechst33342 nuclear dye from Molecular Probes (Eugene, OR). Cells were visualized under an Olympus IX81 fluorescent microscope (Carson Scientific Imaging Group; Ontario, Canada) using the Slidebook 2D, 3D Timelapse Imaging Software (Intelligent Imaging Innovations Inc.; CO, USA). All images have been presented in (X100) magnification.

3.2.7 Statistical analysis

All data presented were mean value \pm SEM of four-eight independent experiments using HUVECs isolated from different umbilical cords for all experiments. Data were expressed as fold change over the untreated control. One-way analysis of variance (One way-ANOVA) with Tukey's post-hoc test was performed for multiple comparisons. A repeated measure test was used whenever applicable. Differences were considered to be significant with a P value of <0.05.

3.3 Results

3.3.1 Effects of peptides and respective amino acids on ICAM-1 expression

Time-course study showed that pre-treatment of either tripeptide for 20 h, but not for 4 and 8 h, could significantly suppressed TNF mediated ICAM-1 and VCAM-1 expression; therefore, pre-treatment of peptide for 20 h was determined. TNF stimulation increased ICAM-1 expression that was inhibited by both IRW and IQW (50 $\mu\text{mol/L}$) (*Figure 3.1A*). Interestingly, when cells were pretreated with respective 50 $\mu\text{mol/L}$ dipeptides (IR and RW) of IRW, it was observed that both dipeptides had no effect on TNF simulated ICAM-1 expression, but the parent tripeptide IRW (50 $\mu\text{mol/L}$) could significantly inhibit TNF-induced ICAM-1 expression (*Figure 3.1B*). Similarly, pretreatment of dipeptides (IQ and QW) at 50 $\mu\text{mol/L}$ had no effect on TNF simulated ICAM-1 expression, but the intact IQW (50 $\mu\text{mol/L}$) could significantly inhibit the TNF simulated ICAM-1 expression (*Figure 3.1C*). When the HUVEC monolayers were pretreated with 50 $\mu\text{mol/L}$ of constituent amino acids individually or in combination, then amino acid arginine (R) and its combination of (I+R+W) showed a minor decrease, but the remaining did not exert any effect on TNF-induced increased expression of ICAM-1 (*Figure 3.1D*).

3.3.2 Effects of peptides and respective amino acids on VCAM-1 expression

Similar to ICAM-1, TNF-stimulation significantly increased the expression of VCAM-1 in endothelial cells. Pretreatment with IRW (50 $\mu\text{mol/L}$) significantly reduced the TNF-stimulated increased expression of VCAM-1 but surprisingly IQW did not exert any effect in contrast to the findings with ICAM-1 (*Figure 3.2A*). In addition, pretreatment with 50 $\mu\text{mol/L}$ dipeptides (IR, RW, IQ and QW) and 50 $\mu\text{mol/L}$ amino acids individually or in combination did not exert any

effect on TNF-stimulated VCAM-1 expression (*Figure 3.2B and C*). But similar to ICAM-1, arginine (R) and its combination of (I+R+W) showed a minor decrease in TNF-induced increased expression of VCAM-1 (*Figure 3.2D*).

3.3.3 Effects of peptides and respective amino acids on TNF-induced superoxide generation

Inflammatory responses are often associated with increased levels of oxidative stress and vice versa. Therefore the effects of ovotransferrin-derived bioactive peptides (IRW, IQW), their respective dipeptides (IR, RW, IQ and QW) and amino acids (I, R, Q, W) on TNF-stimulated superoxide production were studied. Our results showed that both IRW and IQW at 50 $\mu\text{mol/L}$ significantly reduced TNF-stimulated superoxide generation (*Figure 3.3A*). However, the pretreatment with 50 $\mu\text{mol/L}$ of dipeptides (IR, RW, IQ, and QW) did not exhibit significant effect on TNF-induced superoxide production (*Figure 3.3B*). On the other hand, respective amino acids (I, R, Q, W) treated individually or in combination also did not exert any effect on TNF-induced superoxide generation except for arginine (R) and its combination (I+R+W) exerted a minor effect, which indicates that the presence of free arginine could exhibit a minor reduction in TNF-induced superoxide production (*Figure 3.3C*).

3.3.4 Effects of arginine on ICAM-1 and VCAM-1 expression

Since arginine alone showed a minor but significant anti-inflammatory effect (*Figure 1D and 2D*), we further examined the effect of increasing concentrations of arginine on TNF-induced inflammatory molecule expression. We found that none of the concentrations (50, 100 and 200 $\mu\text{mol/L}$) of arginine used could elicit the same extent of anti-inflammatory responses as that of

IRW (50 $\mu\text{mol/L}$), suggesting that the significance of the integrity of the tripeptide (IRW) for the full extent of the anti-inflammatory effect observed (*Figure 3.4A and B*).

3.3.5 Effects of superoxide dismutase on adhesion molecule expression

A previous study showed that inhibition of superoxide could prevent adhesion molecule expression on human aortic endothelial cells.(Lin et al., 2005) We used two different concentrations (100U/mL and 200U/mL) of cell-permeable superoxide dismutase (SOD) to pretreat the HUVEC monolayers prior to TNF-stimulation. Interestingly SOD treatment had no effect on ICAM-1 and VCAM-1 expression (*Figure 3.5A and B*). However, the same concentrations of SOD significantly reduced TNF-stimulated superoxide (*Figure 3.5C*). SOD possibly generates H_2O_2 from superoxide; a combination of catalase with SOD was used to assess the contribution of H_2O_2 . Our results showed that a combination of these two enzymes had no effect on ICAM-1 and VCAM-1 (data not shown). These data suggest that the tripeptides (IRW and IQW) exerted their anti-inflammatory effects independent of their anti-oxidant properties.

3.3.6 Effect of TNF stimulation on NF- κ B translocation and ICAM-1 and VCAM-1 expression

TNF is able to induce a range of cellular responses via modulating a number of gene expressions through activation of various nuclear transcription factors, such as NF- κ B, AP-1 etc. Cells pretreated with NF- κ B inhibitor (BAY11-7085) almost abolished the TNF simulated up-regulation of ICAM-1 and VCAM-1 (*Figure 3.6A*). Therefore, TNF-induced increased expression of ICAM-1 and VCAM-1 is primarily NF- κ B dependent.

On activation of the NF- κ B pathway, the p65 and p50 homo-dimer and/or hetero-dimer proteins are released from the cytosol and then migrate into the cell nucleus, where they interact with the promoter regions of various proteins and up-regulate the expression of inflammatory adhesion molecules like ICAM-1 and VCAM-1. Thus, nuclear translocation of p65 and p50 is widely used as an index of NF- κ B activation. As expected, TNF stimulation caused rapid nuclear translocation of p65 and p50. Pretreatment with IRW, however abolished the TNF-induced translocation of both p65 and p50 (*Figure 3.6B and C*). Surprisingly, pretreatment with IQW restricted only the translocation of p50 but did not affect p65 translocation (*Figure 3.6B and C*), possibly accounting for the differential effects observed with these two peptides.

3.4 Discussion

CVD are the number one killer worldwide (WHO, 2012). In 2008, an estimated 17.3 million people died of CVD and this number is expected to reach 23.6 million by 2030 (WHO, 2012). Occurrences of CVDs are often linked to diet, leading to an increased interest in using food bioactives as a strategy to reduce the risk of CVD. Many food components exhibit beneficial effects towards cardiovascular health, such as fruits, vegetable, legumes, cereals, and tea (Korhonen & Pihlanto, 2003). Peptides of food origin are also found to be beneficial against CVDs, such as peptides with blood pressure lowering (ACE inhibitory), cholesterol lowering, anti-thrombotic and anti-oxidant activities. Moreover, some peptides are multifunctional (Meisel, 2004). Such peptides can be released during fermentation or digestion of food proteins by proteolytic enzymes, thus exhibiting relevant biological activities. Therefore ‘food-derived bioactive peptides’ refers to different peptides of plant or animal origin that can exhibit regulatory functions in the human system beyond its nutritional value. As an economically and

nutritionally important food commodity, egg is a well-known rich source of many bioactive peptides (Kovacs-Nolan, Phillips & Mine, 2005; Miguel, Recio, Gomez-Ruiz, Ramos & Lopez-Fandino, 2004; Miguel, Manso, Aleixandre, Alonso, Salaices & Lopez-Fandino, 2007; Yoshikawa et al., 2000). IRW and IQW are two ACE inhibitory peptides characterized previously from egg white protein ovotransferrin (Majumder & Wu, 2010).

Although the beneficial effects of biologically active amino acids or peptides have been suggested, their mechanisms of action have not been fully elucidated (Phelan & Kerins, 2011; Quazi, Palaniswamy & Frishman, 2009). It is essential to understand the roles of bioactive peptides in cell-mediated pathways. Pro-inflammatory cytokines such as TNF can mediate vascular inflammation and thus play a pivotal role in the pathogenesis of atherosclerosis and its complications. The leukocyte adhesion molecules ICAM-1 and VCAM-1 are the key players of inflammatory responses in leukocyte adhesion and cell signal transduction (Bradley, 2008). Both ICAM-1 and VCAM-1 are up-regulated after exposure of vascular endothelial cells to TNF (Sitia et al., 2010). The results showed that TNF treatment significantly increased the expression of both ICAM-1 and VCAM-1 in HUVECs. Our previous study demonstrated that pretreatment with IRW (50 $\mu\text{mol/L}$) significantly inhibited TNF-induced increased expression of both ICAM and VCAM-1 (Huang, Chakrabarti, Majumder, Jiang, Davidge & Wu, 2010). Interestingly, pretreatment with IQW (50 $\mu\text{mol/L}$), differing only amino acid residue from that of IRW, significantly blocked the TNF-induced increased expression of only ICAM-1 but not VCAM-1. Since both tripeptides have the same N terminal Ile (I) and the C-terminal Trp (W), this difference was possibly due to the presence of the positively charged amino acid Arg (R) in IRW but not in IQW. In comparison to tripeptides, neither their respective dipeptides (IR, RW, IQ and

QW) nor amino acids (individually or in combination) with the exception of arginine (R) and amino acid combination (I+R+W) affected TNF-induced increased expression of ICAM-1 and VCAM-1. Therefore, our study showed that the integrity of both peptides is essential for inhibiting the TNF-induced increased expression of ICAM-1 and VCAM-1.

The inhibitory effect observed in the TNF-induced increased expression of ICAM-1 and VCAM-1 with various concentrations of R and amino acid combination (I+R+W) treatments may be mediated through the nitric oxide synthase (NOS) pathway. L-arginine is a known substrate for NOS present in the endothelial cells, which can convert L-arginine to L-citrulline and produced NO (Luiking, Engelen & Deutz, 2010; Stechmiller, Childress & Cowan, 2005). The increased production of NO has previously been shown to attenuate TNF-induced expression of ICAM-1 and VCAM-1 (Waldow, Witt, Weber & Matschke, 2006). In our study, pretreatment of various concentrations of arginine (R) showed only a marginal decrease in TNF-induced expression of ICAM-1 and VCAM-1 compared to almost complete prevention with IRW. This result suggests that the tripeptide IRW possess a different mechanism of action from R alone. While R alone contributes to increase NO, IRW appears to act through NF- κ B modulation and reduction in oxidative stress.

This study examined whether IQW could also ameliorate TNF-induced oxidative stress (Huang, Chakrabarti, Majumder, Jiang, Davidge & Wu, 2010). In the present study IQW demonstrated anti-oxidant effects by inhibiting TNF-induced superoxide generation in endothelial cells. Furthermore our results demonstrated that both IRW and IQW, but not their respective dipeptides and amino acids with the exception of arginine (R), could significantly reduce the TNF-induced superoxide generation in endothelial cells, similar to the trend observed in the TNF-induced inflammatory response. While oxidative stress can activate pro-inflammatory

pathways in endothelial cells, our findings showed that the presence of SOD did not inhibit TNF-induced ICAM-1 and VCAM-1 expression, which indicated that these inflammatory changes are independent of the concomitant increase of superoxide generation. Increased oxidative stress such as generation of superoxide was reported to contribute to the inflammatory changes in the endothelium (Bradley, 2008; Chen & Goeddel, 2002; Luiking, Engelen & Deutz, 2010, Sitia et al., 2010; Stechmiller, Childress & Cowan, 2005; Waldow, Witt, Weber & Matschke, 2006). However TNF-mediated inflammatory changes are mediated through activation of pro-inflammatory pathways of NF- κ B. Both peptides inhibited TNF induced activation of NF- κ B as well as superoxide generation. The differential regulation of NF- κ B may explain the different responses observed with the two peptides. It appears that both tripeptides (IRW and IQW) exert anti-inflammatory and anti-oxidative effects in our model system.

Increased TNF signaling can activate various transcription factors such as NF- κ B, AP-1, IRF-1, etc (Bradley, 2008; Chen & Goeddel, 2002; Luiking, Engelen & Deutz, 2010; Sitia et al., 2010; Stechmiller, Childress & Cowan, 2005; Waldow, Witt, Weber & Matschke, 2006). NF- κ B activation causes enhanced expression of genes whose protein products mediate monocyte binding, monocyte chemotaxis into the subendothelial space, and conversion into macrophages; the monocyte binding is largely mediated through the over activation of adhesion molecules (e.g., ICAM-1 and VCAM-1) (Giannotti & Landmesser, 2007; Pober, Min & Bradley, 2009). Anti-inflammatory activity of several food bioactive components was also reported to be associated with the NF- κ B pathway. Lunasin, a bioactive peptide isolated from soy protein, was reported to inhibit inflammation in LPS-induced RAW 264.7 macrophage by suppressing NF- κ B pathway (de Mejia & Dia, 2009). Curcumin was also shown to inhibit the TNF induced nuclear

translocation of p65 subunit of NF- κ B in human myelomonoblastic leukemia cell line (Al-Shalmani et al., 2011). A widely known flavonoid, quercetin had also shown anti-inflammatory effects in endothelial cells through inhibiting the NF- κ B signaling pathway (Al-Shalmani et al., 2011). In the previous study we revealed that IRW exhibit the anti-inflammatory effect through NF- κ B pathway by blocking the nuclear translocation of p65 (Huang, Chakrabarti, Majumder, Jiang, Davidge & Wu, 2010); in the present study we further revealed that IRW also inhibit the nuclear translocation of p50, indicating that IRW can completely inhibit TNF-induced NF- κ B activation and thus exhibit anti-inflammatory effect. On the other hand, IQW can partially affect the NF- κ B activity by blocking the translocation of only p50. These data suggest that VCAM-1 expression might be more dependent on p65 translocation as IQW did not affect p65 translocation and also did not have any effect on VCAM-1 levels.

Our study demonstrates the anti-inflammatory and anti-oxidant activities of IRW and IQW, indicating their potential as functional food ingredients or nutraceuticals for the prevention of endothelial dysfunction, a key factor for the development of CVDs. Given the role of inflammatory processes in many other diseases, these peptides may also find usage in the treatment of diverse conditions such as asthma, arthritis, and inflammatory bowel disease. Our study further confirms that the anti-inflammatory effects of IRW and IQW were independent of their anti-oxidant properties and the anti-oxidant effect was not mediated through endothelial NOS pathway. The structural integrities are essential to exert their activities. Although the underlying anti-inflammatory mechanisms of both peptides were associated with NF- κ B signaling pathway, IRW can inhibit the translocation of both p50 and p65 whereas IQW can only suppress the translocation of p50. It should be noted that the results were derived from *in vitro*

cell experiment, which may deviate from *in vivo* activity as effect of digestion and absorption *in vivo* could also affect their bioavailability.

3.5 References

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Figure 3.1: Effects of bioactive peptides and their derivatives on TNF-induced ICAM-1 expression. (A) Bioactive peptides (IRW and IQW), (B) respective dipeptides of IRW (IR and RW), (C) respective dipeptides of IQW (IQ and QW), (D) individual amino acids (I, R, Q and W); peptide sequence combination of respective amino acids (I+R+W and I+Q+W). Confluent HUVEC monolayers were pretreated for 20 h with peptides and amino acids prior to 4 h of incubation with 5 ng/mL TNF. ICAM-1 protein levels are expressed as fold increase over the untreated control. Bars represent mean values (mean \pm SEM, n=8 independent experiments). Representative Western blots are shown below. #### Indicates P<0.001, ## indicates P<0.01 and # indicates P<0.05 as compared to untreated control, *** indicates P<0.001, ** indicates P<0.01 and * indicates P<0.05 compared to TNF alone. Bar graph having lighter color represents that a particular treatment has a significant effect from TNF alone.

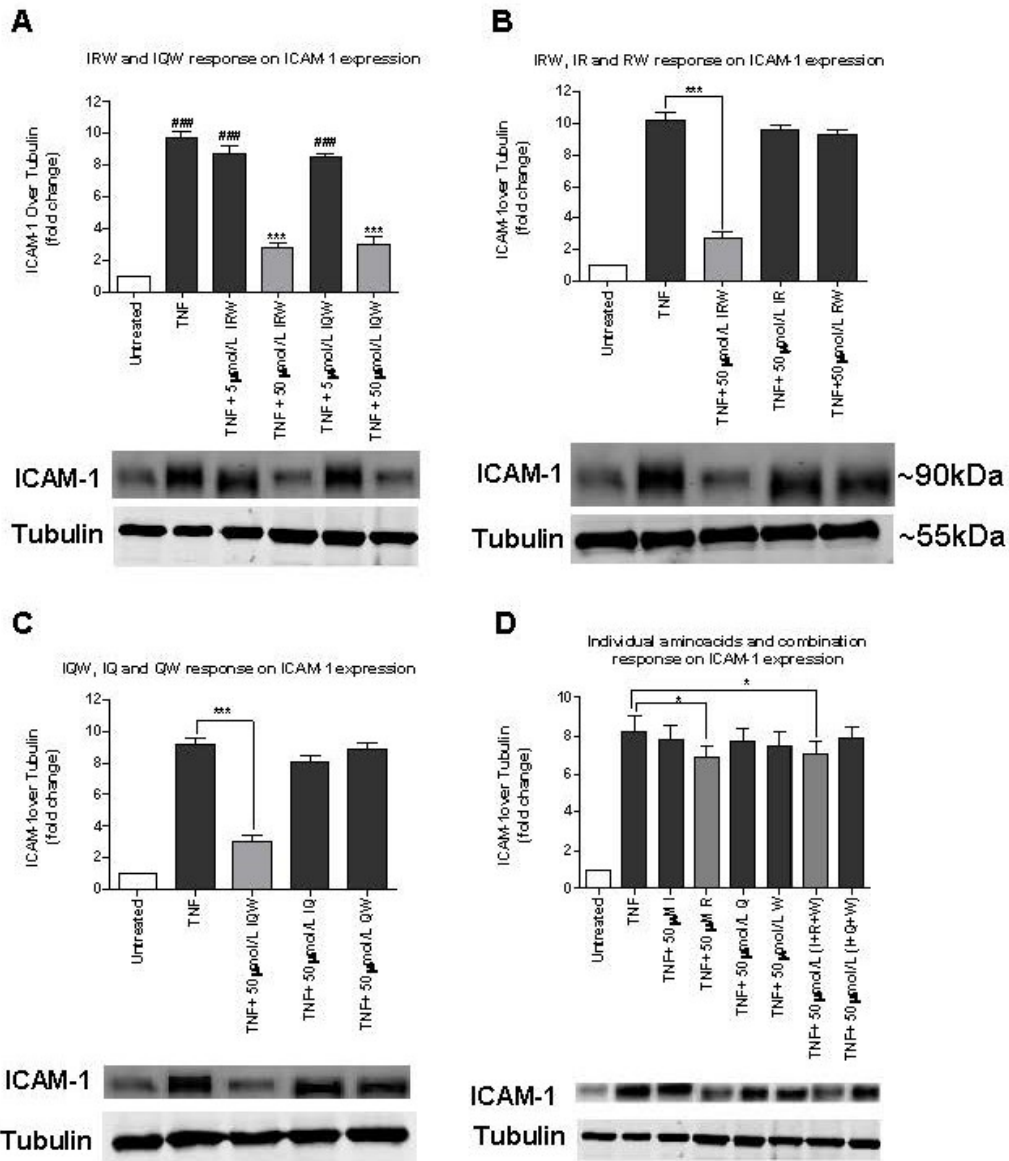


Figure 3.2: Effects of bioactive peptides and their derivatives on TNF-induced VCAM-1 expression. (A) Bioactive peptides (IRW and IQW), (B) respective dipeptides of IRW (IR and RW), (C) respective dipeptides of IQW (IQ and QW), (D) individual amino acids (I, R, Q and W); peptide sequence combination of respective amino acids (I+R+W and I+Q+W). Confluent HUVEC monolayers were pretreated for 20 h with peptides and amino acids prior to 4 h of incubation with 5 ng/mL TNF. VCAM-1 protein levels are expressed as fold increase over the untreated control. Bars represent mean values (mean \pm SEM, n = 8 independent experiments). Representative Western blots are shown below. #### Indicates P<0.001, ## indicates P<0.01 and # indicates P<0.05 as compared to untreated control, *** indicates P<0.001, ** indicates P<0.01 and * indicates P<0.05 compared to TNF alone. Bar graph having lighter color represents that a particular treatment has a significant effect from TNF alone.

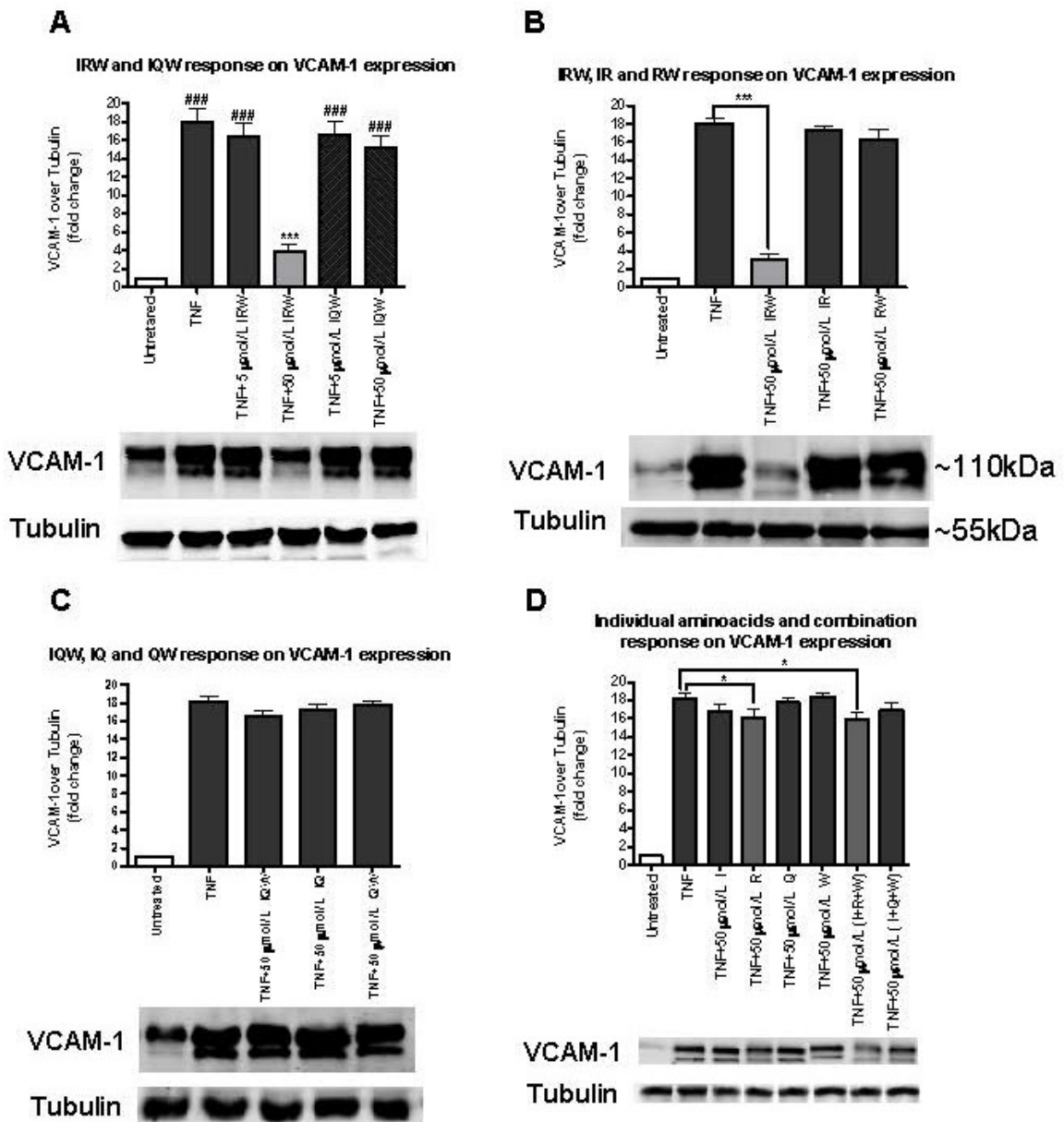


Figure 3.3: Effect of bioactive peptides and their derivatives on TNF-induced endothelial superoxide generation. (A) Bioactive peptides (IRW and IQW), (B) respective dipeptides of IRW (IR and RW) and IQW (IQ and QW), (C) individual amino acids (I, R, Q and W); peptide sequence combination of constituent amino acids (I+R+W and I+Q+W). Confluent HUVEC monolayers were pretreated for 20 h with 50 mol/L peptide and amino acids prior to 1 h of incubation with 5 ng/mL TNF. A representative set of images are shown. Data were calculated as MFI/Cell and expressed as fold increase over the untreated control. Bars represent mean values (mean \pm SEM, n = 8 independent experiments). ### Indicates P<0.001 and # indicates P<0.05 as compared to untreated control, ** indicates P<0.01 and * indicates P<0.05 compared to TNF alone. Bar graph having lighter color represents that a particular treatment has a significant effect from TNF alone.

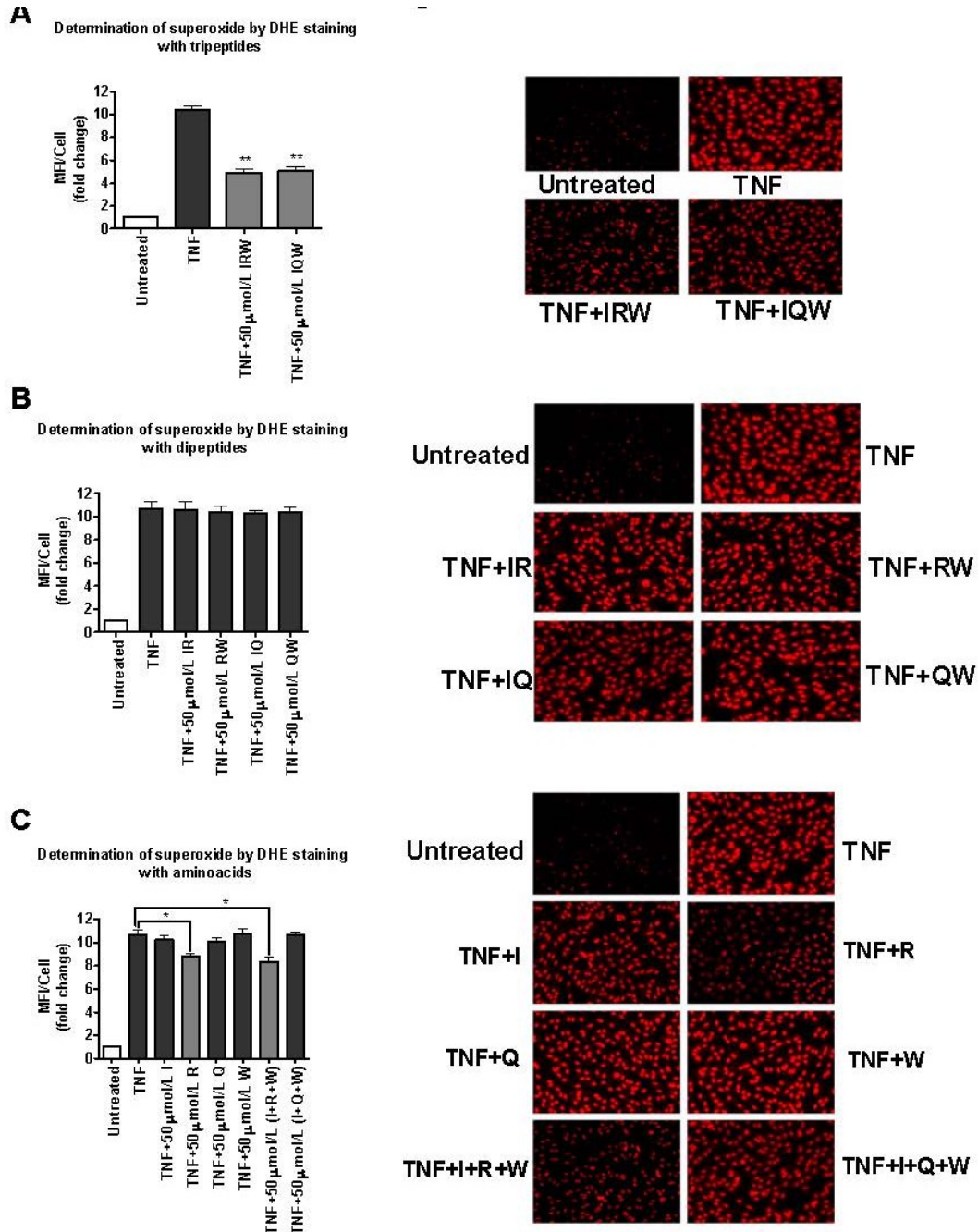


Figure 3.4: Effect of various concentrations of arginine on TNF-induced ICAM-1 and VCAM-1 expression. (A) Various concentrations (50mol/L, 100mol/L and 200mol/L) of arginine (R) and IRW (50mol/L) on ICAM-1 expression, (B) Various concentrations (50mol/L, 100mol/L and 200mol/L) of arginine (R) and IRW (50mol/L) on VCAM-1 expression. ICAM-1 and VCAM-1 protein levels are expressed as fold increase over the untreated control. Bars represent mean values (mean \pm SEM, n = 8 independent experiments). Representative Western blots are shown below. *** indicates P<0.001,

** indicates $P < 0.01$ and * indicates $P < 0.05$ compared to TNF alone. Bar graph having lighter color represents that a particular treatment has a significant effect from TNF alone.

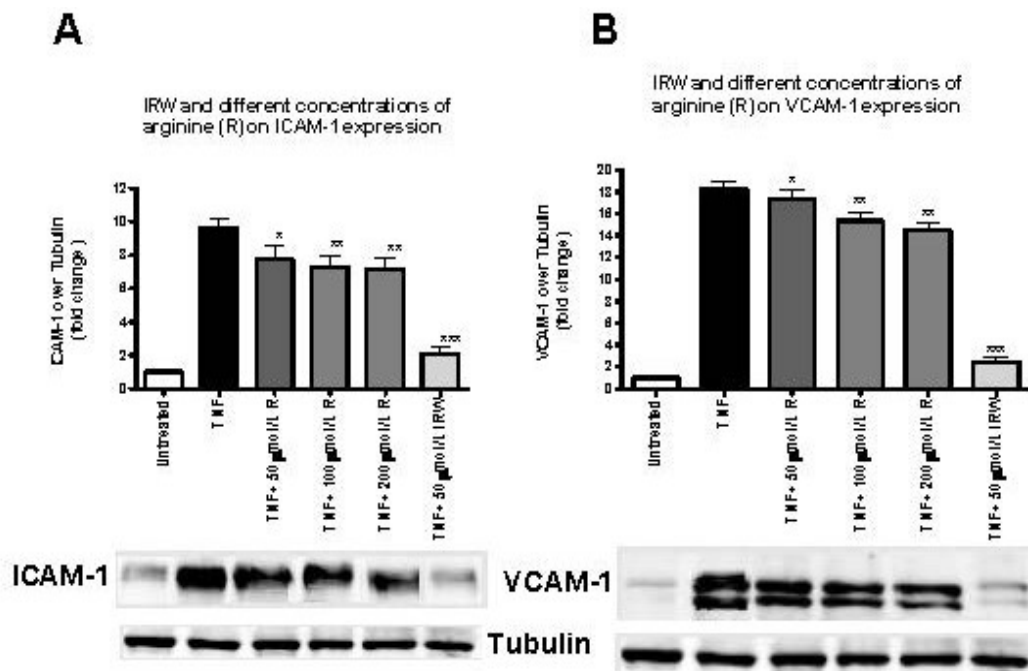


Figure 3.5: Effect of various concentrations of superoxide dismutase (SOD) on TNF-induced adhesion molecules expression and superoxide generation. (A) Various concentrations of SOD (100U/mL and 200U/mL), IRW and IQW (50mol/L) on ICAM-1 expression, (B) Various concentrations of SOD (100U/mL and 200U/mL), IRW and IQW (50mol/L) on VCAM-1 expression. Confluent HUVEC monolayers were pretreated with peptides for 20 h and then treated with SOD (100U/mL and 200U/mL) for 1 h prior to 4 h of incubation with 5 ng/mL TNF. ICAM-1 and VCAM-1 protein levels are expressed as fold increase over the untreated control. Bars represent mean values (mean \pm SEM, n = 4 independent experiments). Representative Western blots are shown below. *** indicates $P < 0.001$, ** indicates $P < 0.01$ and * indicates $P < 0.05$ compared to TNF alone. (C) Various concentrations of SOD (100U/mL and 200U/mL) on superoxide generation. Confluent HUVEC monolayers were pretreated for 1 h with SOD (100U/mL and 200U/mL) prior to 1 h incubation with 5 ng/mL TNF. A representative set of images are shown. Bars represent mean values (mean \pm SEM, n = 4 independent experiments). # indicates $P < 0.05$ compared to untreated control. Bar graph having lighter color represents that a particular treatment has a significant effect from TNF alone.

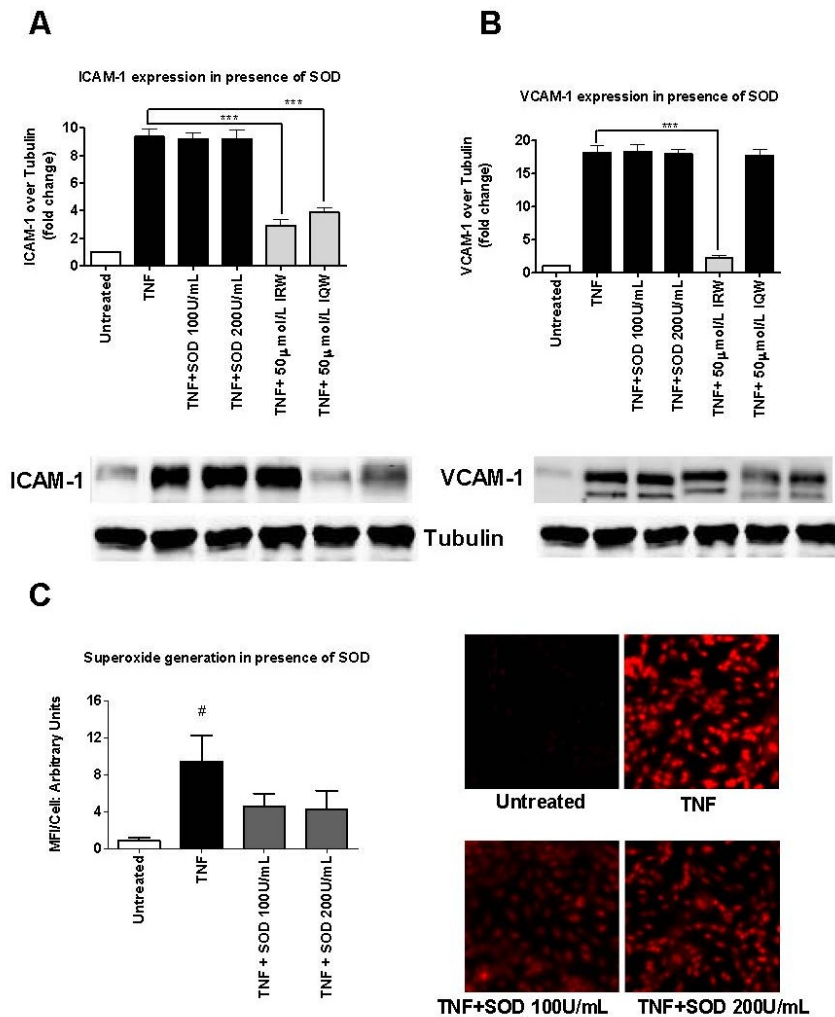
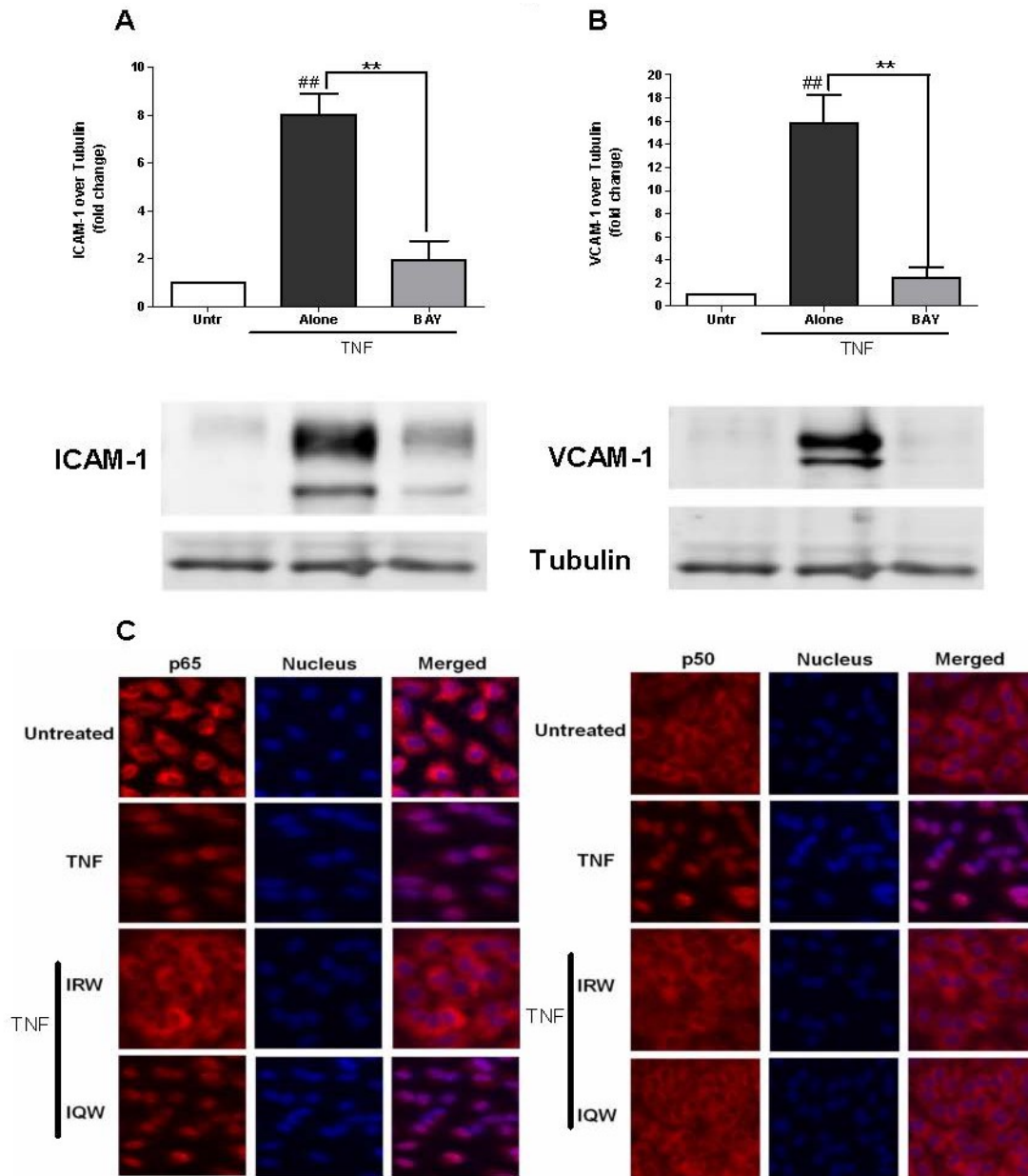


Figure 3.6: Role of IRW and IQW on TNF induced NF- κ B activation. (A) Confluent HUVEC monolayers were treated with BAY 11-7085 (NF- κ B inhibitor) for 15 min before 4 h with 5 ng/mL TNF. ICAM-1 and VCAM-1 protein levels are expressed as fold increase over the untreated control. Bars represent mean values (mean \pm SEM, n = 4 independent experiments). Representative Western blots are shown below. ## Indicates P<0.01 as compared to untreated control, ** indicates P<0.01 compared to TNF alone. (B and C) Confluent HUVEC monolayers were pretreated for 20 h with 50 mol/L IRW and IQW prior to 30 min of incubation with 5 ng/mL TNF. Cells were fixed, permeabilized and immunostained for p65 (B) and p50 (C). Representative sets of images from six independent experiments were shown. Bar graph having lighter color represents that a particular treatment has a significant effect from TNF alone.



3.6 Appendix A: Supplemental Information

The previous chapter addressed the activity of IRW and IQW on TNF induced vascular inflammation and oxidative stress on endothelial cells. Another ovotransferrin derived bioactive peptide was also tested on endothelial cells and the results were illustrated below:

3.6.1 Effects of LKP on TNF induced ICAM-1 expression

Different concentrations (5 and 50 μ M) peptide was pretreated for 20 hrs followed by a 4 hrs of stimulation with TNF. TNF stimulation increased ICAM-1 expression that was not inhibited by the LKP treatments (*Supplementary Figure 3.1*).

3.6.2 Effects of LKP on TNF induced VCAM-1 expression

Different concentrations (5 and 50 μ M) peptide was pretreated for 20 hrs followed by a 4 hrs of stimulation with TNF. TNF stimulation increased VCAM-1 expression that was not altered by 5 μ M LKP treatment. However the expression level of VCAM-1 was down regulated by 50 μ M LKP treatment (*Supplementary Figure 3.2*).

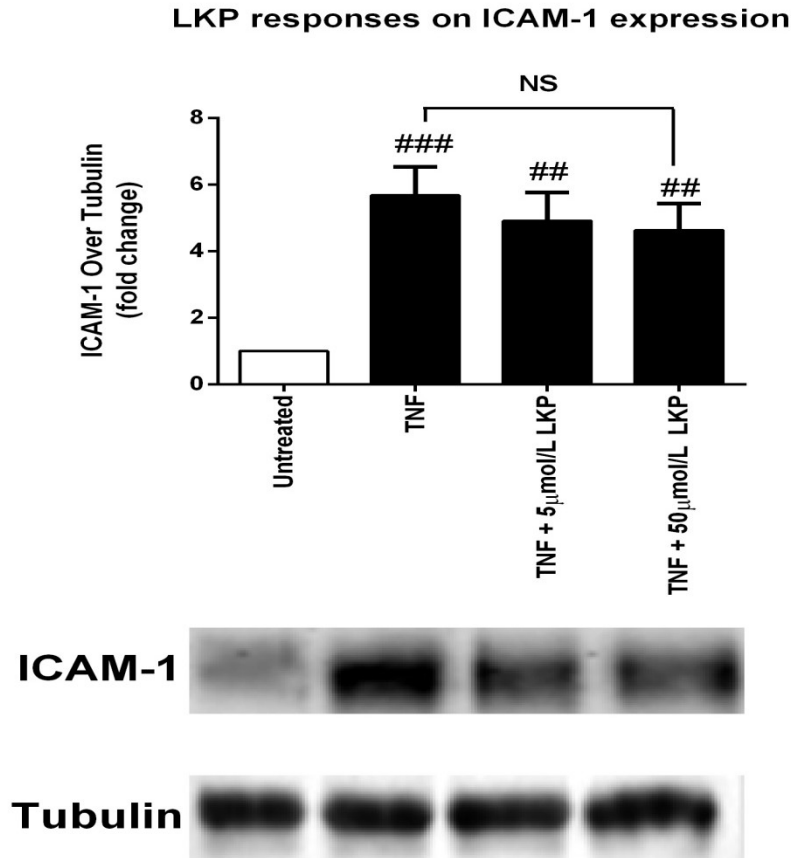
3.6.3 Effects of LKP on TNF-induced superoxide generation

Potency of LKP (50 μ M) as an anti-oxidant agent was evaluated after 20 hrs pretreatment followed by a 1h TNF simulation. Superoxide production was detected by DHE staining as described in chapter 3. The results showed that LKP could not able to inhibit TNF induced superoxide production in endothelial cells (*Supplementary Figure 3.3*).

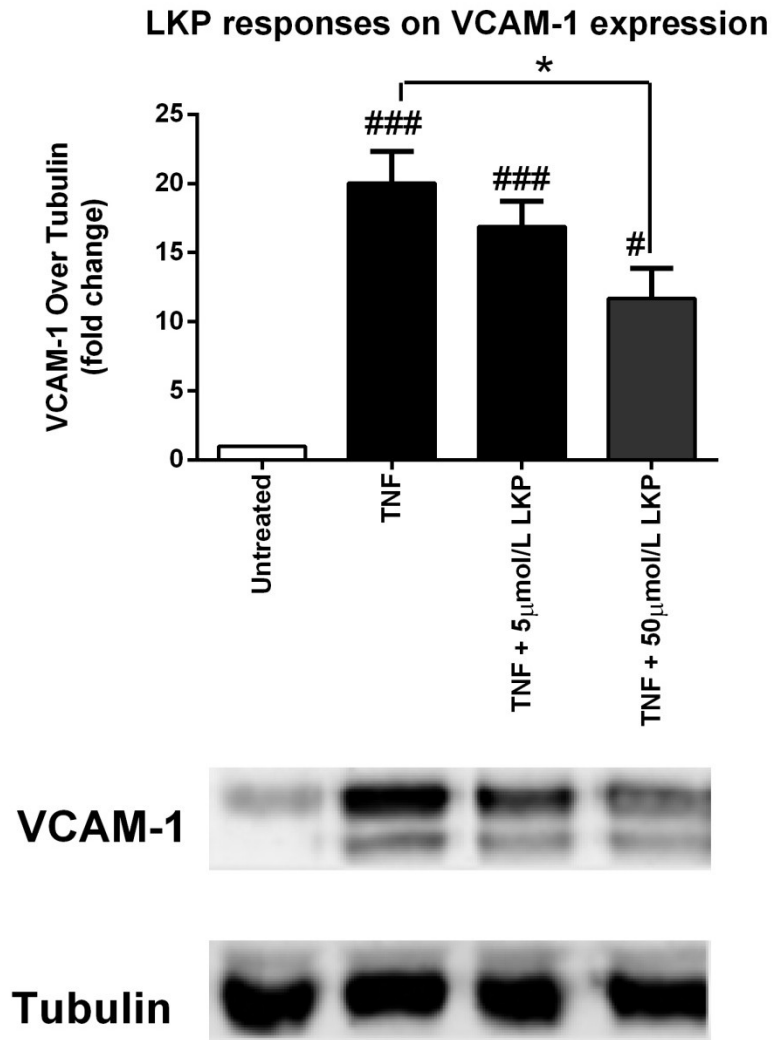
The results from this part of the study revealed that LKP treatment could down regulate the expression of adhesion molecule VCAM-1 on TNF induced endothelial cells, but have no effect

on ICAM-1 expression. Similarly, LKP does not show any inhibitory effect on TNF induced production of oxidative stress in endothelial cells.

Supplementary Figure 3.1: Effects of LKP on TNF-induced ICAM-1 expression. ICAM-1 protein levels are expressed as fold increase over the untreated control. Bars represent mean values (mean \pm SEM, n=6 independent experiments). Representative Western blots are shown below. ### Indicates P<0.001, ## indicates P<0.01 and # indicates P<0.05 as compared to untreated control, *** indicates P<0.001, ** indicates P<0.01 and * indicates P<0.05 compared to TNF alone where NS represents no significant change.

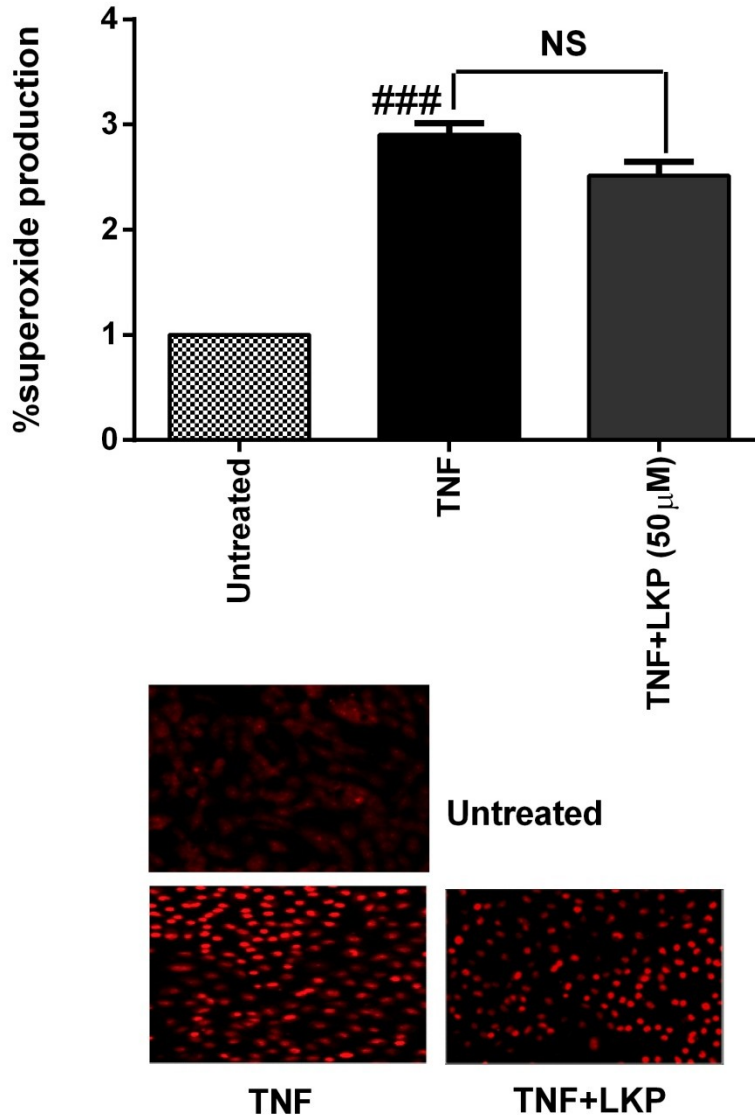


Supplementary Figure 3.2: Effects of LKP on TNF-induced VCAM-1 expression. VCAM-1 protein levels are expressed as fold increase over the untreated control. Bars represent mean values (mean \pm SEM, n=6 independent experiments). Representative Western blots are shown below. ### Indicates P<0.001, ## indicates P<0.01 and # indicates P<0.05 as compared to untreated control, *** indicates P<0.001, ** indicates P<0.01 and * indicates P<0.05 compared to TNF alone.



Supplementary Figure 3.3: Effects of LKP on TNF-induced Superoxide Generation. Confluent HUVEC monolayers were pretreated for 20 h with 50 mol/L peptide and amino acids prior to 1 h of incubation with 5 ng/mL TNF. A representative set of images are shown. Data were calculated as MFI/Cell and expressed as fold increase over the untreated control. Bars represent mean values (mean \pm SEM, n = 6 independent experiments). ### Indicates P<0.001 and # indicates P<0.05 as compared to untreated control, ** indicates P<0.01 and * indicates P<0.05 compared to TNF alone. Bar graph having lighter color represents that a particular treatment has a significant effect from TNF alone whereas 'NS' represents as no significant change.

Determination of superoxide by DHE staining



CHAPTER 4³ - Egg-derived Tri-peptide IRW Exerts Antihypertensive Effects in Spontaneously Hypertensive Rats

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4.1 Introduction

Cardiovascular diseases (CVD) are the single greatest cause of morbidity and mortality worldwide (WHO, 2012). Hypertension, the persistent increase of blood pressure (BP) at or above 140/90 mmHg, is a major predisposing factor for CVD (Chockalingam, 2008). Uncontrolled hypertension leads to widespread chronic damage to the vasculature and contributes to myocardial infarctions; cerebrovascular insufficiency and chronic renal disease (do Carmo Pinho Franco et al., 2003, Feihl, Liaudet, Levy & Waeber, 2008). The aetiology of hypertension is complex, although important roles for the renin-angiotensin system (RAS), oxidative stress, and inflammation have been identified (Koh, Oh & Quon, 2009, Paravicini & Touyz, 2006, Sriramula, Cardinale, Lazartigues & Francis, 2011). Different therapies exist, yet many patients still have poorly controlled hypertension and remain at increased risk for its complications (Brown, 2006, Khanna, Lefkowitz & White, 2008, Moser & Franklin, 2007). In addition, use of anti-hypertensive drugs is associated with significant adverse effects (Atkinson & Robertson, 1979). Nutraceuticals or food-derived therapies have been explored for many disease conditions as safer alternatives to traditional pharmacological agents (Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012, Torruco-Uco, Dominguez-Magana, Davila-Ortiz, Martinez-Ayala, Chel-Guerrero & Betancur-Ancona, 2008). Given the significance of hypertension to the global burden of CVD, there is increasing interest in developing alternative strategies for the management of hypertension.

In addition to their well-known nutritional role, eggs are a rich source of numerous bioactive proteins and peptides with anti-oxidant, anti-microbial, and anti-inflammatory properties which may have potential applications as nutraceuticals and/or functional foods (Korhonen & Pihlanto,

2003, Meisel, 2004, Yoshikawa et al., 2000). We have previously identified a tri-peptide IRW (Ile-Arg-Trp) from enzymatic digestion of the egg white protein ovotransferrin with demonstrated angiotensin converting enzyme (ACE) inhibitory activity *in vitro* (Majumder & Wu, 2010). As uncontrolled RAS activity contributes to the pathogenesis of hypertension, this peptide could be of potential therapeutic interest. Recent work from our group has further characterized its anti-oxidant and anti-inflammatory effects on cultured endothelial cells, a site of inflammatory changes that lead to atherosclerosis and consequently, CVD (Huang, Chakrabarti, Majumder, Jiang, Davidge & Wu, 2010, Majumder, Chakrabarti, Davidge & Wu, 2013). However, the *in vivo* cardiovascular effects and oral bioavailability of IRW remain unknown, precluding its further development as a viable therapeutic option.

The spontaneously hypertensive rat (SHR) is a well-characterized rodent model for hypertension. SHR develop persistent hypertension at an early age (~12-14 weeks) and remain hypertensive throughout their lives (Trippodo & Frohlich, 1981, Zicha & Kunes, 1999). These animals show increased RAS activity together with increased oxidative stress in the vasculature and a pronounced pro-inflammatory phenotype (Liu, 2009, Reaves, Beck, Wang, Raizada & Katovich, 2003, Sriramula, Cardinale, Lazartigues & Francis, 2011, Wu & Juurlink, 2002). Thus, SHR have been widely used to study the pathophysiology of hypertension (Dornas & Silva, 2011, Dornas & Silva, 2011, Hultstrom, 2012, Reaves, Beck, Wang, Raizada & Katovich, 2003, Wu & Juurlink, 2002). Various food-derived peptides with known ACE inhibitory properties *in vitro* have been used to test for *in vivo* antihypertensive effects in SHR with varying degrees of success (Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012, Matoba, Usui, Fujita & Yoshikawa, 1999, Nurminen et al., 2000). Given the anti-oxidant, anti-inflammatory, and

ACE inhibitory properties of IRW, we used SHR to study its efficacy as a novel anti-hypertensive agent and the potential mechanisms of its actions.

4.2 Materials and Methods

4.2.1 Animal model and ethics statement

Fourteen-to-fifteen-week old male SHRs (290.0±10.5 g) were obtained from Charles River (Senneville, QC, Canada). These animals were kept at the University of Alberta animal facility for a week for acclimatization. Rats were exposed to a 12:12 hour, light:dark cycle, in a humidity and temperature-controlled (23°C) environment. All rats were given standard rat chow (0.3% NaCl) and water *ad libitum*. The University of Alberta Animal Care approved the experimental procedures and Use 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.

4.2.2 Experimental Design

Fifteen-to-sixteen-week old animals were surgically implanted with telemetry transmitters for blood pressure monitoring. A one-week period of recovery was allowed following surgery. The animals were then randomly assigned to three treatment groups (n=6): untreated (control), low dose IRW (3 mg/Kg BW) and high dose IRW (15 mg/Kg BW). The doses were selected based on previously published *in vivo* studies on bioactive peptides (Iroyukifujita, Eiichiyokoyama & Yoshikawa, 2000, Sipola, Finckenberg, Santisteban, Korpela, Vapaatalo & Nurminen, 2001). IRW was dissolved in 20 mL of Ensure (Abbott Nutrition, QC, Canada) and administered once per day for 18 days. Untreated animals were given Ensure alone. Blood Pressure (BP) was

recorded for a 24 h period (10 sec of every 1 min) on days 0 (baseline), 3, 6, 9, 12, 15 and 18 under the conditions described above. At the end of the recording period, the animals were sacrificed by decapitation; the blood was collected in EDTA coated tubes (BD vacutainer, NJ, USA), tissues were collected for further analysis and the mesenteric arteries were isolated for vascular function studies.

4.2.3 Anesthesia and surgical procedure

A telemetry transmitter was implanted to measure the BP and heart rate (HR) as follows. Anesthesia was induced using O₂ and 3% isoflurane, and was maintained throughout the surgical procedure by the administration of O₂ and 1.5-2% isoflurane. During surgery, body temperature was maintained at 37°C (Homothermic Blanket, Harvard Apparatus, Canada). Animals were then chronically instrumented with a pressure transmitter (PA-C40; Data Sciences International, Minneapolis, MN, USA) according to the manufacturer's manual. When the animal had reached a surgical plane, an approximately 3-4 cm vertical incision was made on the left side of the spine just above the hip area. Then using Metzanbaum scissors a small pocket was created just under the skin, large enough to fit the transmitter. The transmitter was placed in the pocket and anchored with 4/0 silk suture. The left femoral artery was exposed by an approximate 2 cm long incision on the rat's left groin area and the cannula (polyethylene, 0.58 mm ID, 0.97 mm OD) of the telemetry probe was then inserted into the femoral artery and advanced up to the aorta. The catheter was then secured at the point of entry to the vessel by using a 4/0 silk suture. All procedures were done under a Zeiss dissecting microscope (Carl Zeiss, Toronto, ON, Canada). After surgery, the rats were caged individually and allowed to recover for one week. During the week of recovery, animals were fed with 50 mL of Ensure (Abbott Nutrition, QC, Canada) to

regain post-operative weight, along with rodent chow and water *ad libitum* (Schwartz, Salorio, Skoglund & Moran, 1999). For pain management, the rats received one dose (0.05 mg/kg BW) of buprenorphine (0.3 mg/mL) (Animal Resources Centre, McGill University, Montreal, QC, Canada) just after the surgery; this was repeated during the next 2-3 days to a maximum of twice a day, based on the condition of the individual animal.

4.2.4 Telemetry recording and signal processing

Chronic measurement of mean arterial pressure (MAP) and HR of the animals were performed in a quiet room with minimal electrical interference. Each individual rat cage was placed on top of a receiver (Model RPC-1, ADI instruments, CO, USA) and the signals were recorded through a pressure output adaptor (Model R11CPA, ADI instruments) for measurement of various cardiovascular parameters. Using the data acquisition software LabChart version 7.3 (ADI instruments), the experimental data were recorded continuously in real time. An atmospheric-pressure monitor (Model APR-1, ADI instruments) was also installed to normalize the pressure values received from the transmitters; this provided the actual BP values irrespective of changes in atmospheric pressure. From the MAP signal, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were extracted and HR was calculated between two successive points and expressed in beats per minute (BPM).

4.2.5 Vascular function

Second order branches of the mesenteric artery were carefully isolated after animal euthanasia, cleaned of all surrounding adipose and connective tissues and placed in ice-cold HEPES-PSS (in mmol/L: NaCl 142, KCl 4.7, MgSO₄ 1.17, CaCl₂ 4.7, K₂PO₄ 1.18, HEPES 10 and glucose 5.5; pH 7.4). Arteries with internal diameters ranging 150-250 μ m and ~2 mm in length were

mounted on two 40 μm tungsten wires (Fine Wire Company, California, USA) and attached to a wire-myograph (DMT, Copenhagen, Denmark) to allow isometric tension recordings. Vessels were normalized through a series of stepwise increases in diameter to determine their optimal resting tension, set to $0.8 \times \text{IC}_{100}$ (the internal circumference (IC) equivalent to 100 mmHg). Following a 30 min equilibration, mesenteric arteries were exposed to a single dose of phenylephrine (PE; 10 $\mu\text{mol/L}$; Sigma Aldrich, Oakville, Canada) twice, followed by a single dose of methacholine (MCh; 3 $\mu\text{mol/L}$; Sigma) to assess the functional integrity of the endothelium and smooth muscle. A cumulative concentration response curve to PE (10^{-8} to 10^{-4} mol/L) was performed to determine constrictor responses. To assess endothelial-dependent relaxation, a cumulative concentration response curve to MCh (10^{-10} to 10^{-4} mol/L) was performed. To study the role of nitric oxide (NO) in MCh-dependent relaxation, vessels were studied in presence or absence of the nitric oxide synthase (NOS) inhibitor *N*₆-nitro-L-arginine methyl ester (L-NAME, 100 $\mu\text{mol/L}$, Sigma). After L-NAME incubation, the vessels were pre-constricted (80% of maximum) using PE until it reached a plateau response. Cumulative doses of MCh were then added to the bath to assess vascular relaxation. At the end of the experiment, the vessels were exposed to high K^+ buffer to confirm viability.

4.2.6 Plasma biomarker analysis

After animal sacrifice, blood samples were collected and kept on ice. The samples were centrifuged (10,000 \times g for 20 min at 4°C) and the plasma was stored at -80°C until analysis. Both angiotensin II (Ang II) and bradykinin were quantified by respective ELISA kits (Ang II ELISA, Cayman Chemical, Ann Arbor, MI, USA; Bradykinin ELISA, Phoenix Pharmaceuticals, Burlingame, CA, USA) as per the manufactures' instructions. Commercially available rat

cytokine ELISA strips (Signosis Sunnyvale, CA) were used for estimation of cytokines/chemokines in the plasma samples (Alcendor, Charest, Zhu, Vigil & Knobel, 2012).

4.2.7 Endothelial cell culture with ethics statement

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as previously described (Arenas, Xu, Lopez-Jaramillo & Davidge, 2004); (Chakrabarti & Davidge, 2009, Merchant, Narumiya, Zhang, Guilbert & Davidge, 2004) . Following collection of umbilical cords, the umbilical vein was first flushed with PBS to remove blood clots and then HUVECs were isolated out using a type 1 collagenase containing buffer. The cells were grown in a humidified atmosphere at 37°C with 5% CO₂/95% air in M199 medium with phenol red supplemented by 20% FBS as well as L-Glutamine (Gibco/ Invitrogen, Carlsbad, CA), Penicillin-Streptomycin (Life Technologies, Carlsbad, CA) and 1% endothelial cell growth supplement (ECGS, from VWR International, West Chester, PA). We have previously confirmed the endothelial nature of these cells by staining for the endothelium-specific marker, von Willebrand's factor (vWF; Chakrabarti, Chan, Jiang & Davidge, 2012). The protocol was approved by the University of Alberta's Research Ethics Board and the investigation also conformed to the principles outlined in the Declaration of Helsinki and also Title 45, US Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001. All subjects provided written informed consent before inclusion into this study. Second passage HUVEC were grown to confluence and treated with 10% SHR plasma (untreated or high dose IRW treated) for 4 hours. Cell lysates were prepared and used for western blotting to measure leukocyte adhesion molecule expression.

4.2.8 Western blotting

Effect of the IRW treatment on vascular protein expression was determined using western blotting. Frozen (-80°C) aortas and mesenteric arteries from the SHR animals were thawed on ice and homogenized in a protein extraction buffer (20 mmol/L Tris, 5 mmol/L EDTA, 10 mmol/L Na₄P₂O₇, 100 mmol/L sodium fluoride and 1% NP-40) containing 1% (v/v) protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 10,000xg for 10 min at 4°C. Protein concentration in the supernatants was determined by bicinchoninic acid (BCA) assay, using bovine serum albumin as a standard. Samples were stored at -80°C until western blotting.

Bands for intercellular cell adhesion molecule-1/ICAM-1 (mouse monoclonal antibody, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), vascular cell adhesion molecule-1/VCAM-1 (rabbit polyclonal antibody, Santa Cruz Biotechnologies) and endothelial nitric oxide synthase/eNOS (mouse monoclonal antibody, BD Biosciences, San Jose, CA, USA) were normalized to β-actin (rabbit polyclonal antibody, Abcam Inc., Toronto, ON, Canada) or α-tubulin (rabbit polyclonal antibody, Abcam Inc., Toronto, ON, Canada) and expressed as fold change compared to untreated samples run on the same gel. Anti- β-actin was used at 0.5 μg/mL, anti α-tubulin was used at 0.4 μg/mL, while eNOS, ICAM-1, and VCAM-1 antibodies were used at 1 μg/mL. Goat-anti-rabbit and Donkey-anti-mouse conjugated secondary antibodies (Li-Cor Biosciences, Lincoln, NB, USA) were used to visualize the bands in a Li-Cor Odyssey BioImager and quantified by densitometry with corresponding software (Odyssey V3.0, Li-cor Biosciences).

4.2.9 Immunofluorescence

Kidney and aorta specimens were embedded in Tissue-Tek O.C.T Compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) and frozen immediately in liquid nitrogen for subsequent

analysis. 10 µm tissue sections were prepared, mounted on glass slides at -20 °C and stored at -80 °C. On the day of the experiments, tissue sections were first fixed in acetone and incubated with blocking buffer (1% bovine serum albumin in phosphate-buffer saline) for 30 min. The sections were then immunostained for 2 h at room temperature with rabbit polyclonal antibodies against nitrotyrosine (Dilution 1:200; Chemicon, Temecula, CA, USA) or type I collagen (Dilution 1:200; Novus Biologicals, Littleton, CO, USA). Incubation with the secondary antibody (Dilution 1: 150; Alexa Fluor 546 (red), Invitrogen, Burlington, ON, Canada) was done for 30 min in the dark. Glass cover-slips were mounted with a Vectashield H-1200 Mounting Kit, containing nuclear stain, DAPI (Vector Laboratories, Burlington, ON, Canada), and immediately visualised under an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). Images were obtained using SlideBook imaging software (Olympus) and presented at 100x magnification. A control image with secondary antibody alone was used to detect any nonspecific binding (data not shown). The images were then quantified by subtracting the background fluorescence in the control image, so only the fluorescence from specific immunostaining was visible.

4.2.10 Statistics

All data presented are mean ± SEM of 3-6 animals from each treatment group. For BP data, one-way ANOVA was used to determine the effect of IRW (*Figure 4.1*) and a two-way ANOVA was used to determine the interaction between two factors (IRW dose and circadian rhythm of BP, *Figure 2*), with a Bonferroni's post-test to compare among groups. MCh curves were fitted using nonlinear regression, and E_{\max} values were compared using one-way ANOVA followed by Bonferroni's post-test or unpaired t-test as appropriate. ICAM-1, VCAM-1 and eNOS bands,

plasma levels of Ang II and bradykinin as well as quantifications for nitrotyrosine and type I collagen immunostaining were analysed by unpaired *t*-tests. Data from endothelial cell experiments were analyzed by one way ANOVA with Tukey's post test. A *p* value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 IRW treatment attenuates BP in SHRs

The SHR demonstrated already established hypertension at the beginning of the study (day 0). IRW treatment caused attenuation of BP as early as day 3 (*Figure 4.1A*). After 18 days of IRW treatment, SBP was significantly decreased in both low and high dose groups to 191.4±2.0 mmHg and 172.1±4.7 mmHg respectively, compared to the untreated group (*Figure 4.1A*). Similar effects were observed in MAP and DBP with high dose IRW treatment (*Figure 4.1B and C*). Although the same trends were observed in the low dose group, they failed to reach significance. Despite the changes in BP, no changes were observed in HR (*Figure 4.1D*).

4.3.2 IRW treatment restores circadian rhythm of BP

Circadian variations or nocturnal dipping in blood pressure (MAP, SBP, and DBP) were measured during the treatment period. The mean BP during each 12 h light cycle (light/dark) was calculated. The circadian variation of BP was disturbed in the untreated animals, there being little difference in blood pressure during the light and dark cycles. After treatment with both low and high doses of IRW, the impaired circadian variation in MAP, SBP, and DBP were restored. Indeed, the animals in both low and high dose IRW treatment groups had the circadian variations in MAP, SBP and DBP restored (*Figure 4.2A, B, C, and D*).

4.3.3 IRW restores NO sensitive vasorelaxation

Vascular responses to PE constriction in the mesenteric arteries were unaffected in the IRW treatment groups compared to the untreated SHR (*Data not shown*). As illustrated in *Figure 4.3A*, vasodilation to MCh was significantly enhanced at high dose IRW. MCh mediated vasodilation is multifactorial, involving multiple vascular pathways such as NO, prostaglandins and endothelial derived hyperpolarizing factor (EDHF) (Morton, Rueda-Clausen & Davidge, 2010). While incubation with the NOS inhibitor L-NAME did not alter vasodilation in the untreated and low dose IRW groups, vasorelaxation in the high dose animals was significantly decreased (*Figure 4.3B, C and D*), suggesting restoration of NO-dependent vasorelaxation after IRW treatment. Given that clearly defined changes were only observed in the high dose group, all further experiments were performed only in the untreated and high dose IRW treated groups.

4.3.4 IRW appears to inhibit ACE-I *in vivo*

The effect of high dose IRW on the SBP, MAP, and DBP of SHR was associated with concomitant changes in circulating levels of Ang II and bradykinin. IRW treatment decreased Ang II levels from 35.3 ± 5.4 pg/mL in the untreated group to 14.2 ± 2.1 pg/mL in the high dose treated group (*Figure 4.4A*). The treatment also increased the circulating levels of bradykinin (a molecule metabolized by ACE), which increased from 1.5 ± 0.2 ng/mL in the untreated group to 3.0 ± 0.6 ng/mL in the high dose treated group (*Figure 4.4B*). These results suggest that IRW can act as an ACE inhibitor and thus decrease the production of Ang II as well as inhibit the degradation of bradykinin

4.3.5 IRW ameliorates inflammation

High dose IRW treatment significantly decreased the expression of inflammatory biomarkers in plasma such as interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), compared to the untreated animals (*Figure 4.5A and B*). Similarly, high dose IRW also decreased the expression of inflammatory adhesion molecules, namely, ICAM-1 and VCAM-1 in mesenteric arteries (*Figure 4.5C and D*).

Plasma from both untreated and high dose IRW treated SHR up-regulated the expression of leukocyte adhesion molecules such as ICAM-1 and VCAM-1 in cultured human endothelial cells. However the effects with untreated plasma were much higher than that observed in the IRW treated group, suggesting at least a partial amelioration of the pro- inflammatory phenotype (*Figure 4.6A and B*).

4.3.6 IRW restores vascular eNOS expression

High dose IRW treatment significantly increased the expression of eNOS in the mesenteric arteries (*Figure 4.7A*), compared to the untreated animals. Similar effects were also observed in aortic lysates (*Figure 4.7B*). These results suggest that IRW enhanced the expression of eNOS in the vasculature, which could potentially explain the increase in NO-mediated vasorelaxation

4.3.7 IRW ameliorates oxidative/nitrosative stress and fibrosis *in vivo*

High dose IRW treatment reduced oxidative/nitrosative stress in SHR as demonstrated by a significant decrease in nitrotyrosine staining in aortas and kidneys of IRW treated animals (*Figure 4.8A and B*). In addition, IRW treatment also reduced immunostaining for type I collagen in both aortas and kidneys, suggesting decreased tissue fibrosis (*Figure 4.8C and D*).

4.4 Discussion

The major findings of this study were: (i) Egg derived bioactive tri-peptide IRW significantly attenuated established hypertension in adult male SHR, (ii) IRW treatment increased eNOS expression and increased NO-mediated vasorelaxation, (iii) IRW treatment attenuated plasma Ang II levels and ameliorated markers of inflammation, oxidative/nitrosative stress, and fibrosis in SHR animals.

Data from this study may provide a natural health option for treating hypertensive complications leading to CVD. Occurrence of CVD is often linked to diet. This has led to an increased interest in using bioactive foods as a strategy to reduce the risk of CVDs. Thus, several active compounds have been identified in the past two decades from different food components and with various cardio-protective benefits (Davalos, Miguel, Bartolome & Lopez-Fandino, 2004; Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012; Theuwissen & Mensink, 2007). Specifically, food-derived peptides with blood pressure lowering (ACE inhibitory), cholesterol lowering, anti-thrombotic and anti-oxidant activities have been proven beneficial against CVDs (Aleixandre, Miguel & Muguera, 2008; Cheung, Nakayama, Hsu, Samaranayaka & Li-Chan, 2009, Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012). Moreover, some of these peptides exhibit multiple bioactive functionalities (Meisel, 2004). Therefore food derived bioactive peptides, irrespective of their plant or animal origin, may exert regulatory functions on human health beyond their nutritional value. In addition to being an economically and nutritionally important food commodity, egg is a well-known source of many bioactive peptides (Davalos, Miguel, Bartolome & Lopez-Fandino, 2004; Hasler, 2000; Huang, Majumder & Wu, 2010; Miguel & Aleixandre, 2006). IRW, a positively charged bioactive tri-peptide with

ACE inhibitory activity, was previously identified and characterized from egg white protein ovotransferrin (Majumder & Wu, 2010). IRW also exhibits anti-inflammatory and anti-oxidant properties in cultured endothelial cells (Huang, Chakrabarti, Majumder, Jiang, Davidge & Wu, 2010; Majumder, Chakrabarti, Davidge & Wu, 2013). Hence the next logical step was to test the *in vivo* efficacy of IRW in an animal model of hypertension.

SHR is a well-established model of human essential hypertension. Various studies performed to determine the anti-hypertensive effects of food derived bioactive peptides have used SHR animals as a model system (Dornas & Silva, 2011; Trippodo & Frohlich, 1981). The development of high blood pressure in these animals is similar to that in human subjects. Hypertension appears at 12-14 weeks of age in SHR and they develop established hypertension by early adulthood (Zicha & Kunes, 1999). Along with elevated BP, studies have shown that SHR animals have blunted diurnal (light-dark cycle) variations of BP compared to normotensive rats (Henry, Casto & Printz, 1990; Lemmer, Mattes, Bohm & Ganten, 1993). The underlying pathological mechanisms include increased activity of the renin-angiotensin system (RAS) as well as increases in vascular inflammation and oxidative stress (Hultstrom, 2012; Reaves, Beck, Wang, Raizada & Katovich, 2003; Wu & Juurlink, 2002). Hence SHR is a suitable model to study the *in vivo* efficacy of IRW.

While IRW treatment significantly reduced the elevated BP in SHR, no significant change was observed in HR, suggesting that IRW treatment did not affect the cardiac functions in this study. This is beneficial from a therapeutic point of view since the preservation of normal cardiac responses would presumably minimize the risk of side-effects such as arrhythmias and related complications. IRW also restored the attenuated circadian variation in BP which is

characteristically observed in SHR. Given that loss of “nocturnal dipping” can contribute to clinical events like myocardial ischemia, acute myocardial infarct and sudden cardiac death (COCA, 1994; Portaluppi, Tiseo, Smolensky, Hermida, Ayala & Fabbian, 2012; Shimamura, Nakajima, Iwasaki, Hayasaki, Yonetani & Iwaki, 1999), the restoration of impaired nocturnal dipping in blood pressure by IRW may help to prevent target organ damage in hypertension.

IRW appears to act through multiple pathways leading to lower BP in SHR. It is known that loss of vascular eNOS activity causes endothelial dysfunction and contributes to the pathogenesis of hypertension and atherosclerosis (Gielis, Lin, Wingler, Van Schil, Schmidt & Moens, 2011; Kietadisorn, Juni & Moens, 2012). In our study, no significant difference in vascular relaxation was observed after L-NAME treatment in the mesenteric arteries of untreated SHR, suggesting that SHR may have impaired NO dependent vasorelaxation. Indeed, a study by Bagnost *et al.* has demonstrated the loss of NO dependent vasorelaxation in SHRs compare to their wild type (WKY) controls (Bagnost et al., 2010). We also found that IRW upregulated eNOS and enhanced NO mediated vasorelaxation in SHR, suggesting the rectification of endothelial dysfunction as seen in the untreated animals. Based on our previous study we postulated that IRW would also exhibit ACE inhibitory activity *in vivo* (Majumder & Wu, 2010). Indeed IRW treatment reduced the plasma Ang II levels with a corresponding increase in bradykinin, suggesting an ACE inhibitory effect.

We have previously demonstrated that IRW reduces TNF-induced upregulation of MCP-1, ICAM-1 and VCAM-1 in an endothelial cell culture system (Huang, Chakrabarti, Majumder, Jiang, Davidge & Wu, 2010; Majumder, Chakrabarti, Davidge & Wu, 2013). A similar effect was observed *in vivo* in the current study; IRW decreased levels of the inflammatory

cytokines/chemokines IL-6 and MCP-1. This phenomenon was accompanied by decreases in expression of inflammatory adhesion molecules (ICAM-1 and VCAM-1) in vascular tissues, suggesting generalized anti-inflammatory effects of IRW *in vivo*. In addition, plasma from IRW treated SHR induced lower levels of ICAM-1 and VCAM-1 expression in cultured endothelial cells, further supporting a reduction in proinflammatory properties. Given that SHR have increased circulating levels of pro-inflammatory cytokines, which may contribute to the endothelial dysfunction, and upregulate leukocyte adhesion molecules in the vasculature, we propose that controlling the inflammatory pathways with IRW could potentially ameliorate the vascular pathologies.

Increased oxidative stress contributes to the pathology of hypertension (Cuzzocrea, Mazzon, Dugo, Di Paola, Caputi & Salvemini, 2004). Reactive oxygen species (ROS) such as superoxide can interact with NO to generate peroxynitrite (-ONOO), a highly reactive species that contributes to oxidative/nitrosative stress (Salvemini, Ischiropoulos & Cuzzocrea, 2003). Peroxynitrite leads to tyrosine nitration of various proteins and contributes to a pro-inflammatory phenotype. Studies have demonstrated that reductions in ROS and –ONOO levels in SHR can reduce blood pressure, suggesting a role for these species on the pathology (Cabassi et al., 2001; Hong, Hsiao, Cheng & Yen, 2001). Our study showed that high dose IRW could reduce nitrotyrosine levels in both aorta and kidneys, suggesting an anti-oxidant effect of IRW on these tissues. These findings are in accordance with our previous study showing anti-oxidant effects of IRW on the human endothelium (Majumder, Chakrabarti, Davidge & Wu, 2013). Inflammatory and oxidative processes can also lead to increased fibrosis and consequent remodeling in various tissues. SHR animals are prone to fibrotic changes in renal and aortic tissues, which may further

contribute to the complications of hypertension (de las Heras et al., 2006). IRW treatment significantly attenuated type I collagen levels both in kidney and aorta, suggesting a reduction in hypertension induced tissue remodeling.

The biological activity of orally administered peptides is highly dependent on their ability to reach the target site without being degraded and/or inactivated by intestinal or plasma peptidases. A previous study on milk-derived bioactive tripeptides has shown evidence for oral absorption without degradation (Masuda, Nakamura & Takano, 1996). Our previous study in cells has shown the importance of integrity of IRW in exerting anti-inflammatory and antioxidant activities (Majumder, Chakrabarti, Davidge & Wu, 2013) ; the current study has now conclusively demonstrated the *in vivo* efficacy of IRW, further supporting its oral bioavailability. This finding on oral bioavailability of IRW is in accordance with our previous findings using an intestinal epithelial cell culture system where the peptide was observed to cross the epithelial barrier (Bejjani & Wu, 2013), although metabolism of IRW *in vivo* has not been studied yet.

In this study, we used only male animals and had a limited study period of 18 days. Future studies may incorporate animals of both sexes, a longer treatment period and observation of the effects of treatment withdrawal on BP regulation to avoid the limitations of the present study. Future studies could also involve elucidation of the molecular mechanisms underlying the anti-inflammatory and NO generating effects observed *in vivo*.

In summary, the *in vivo* anti-hypertensive effects of orally given IRW appear to be mediated through several different pathways, such as, increased NO mediated vasodilation, regulating RAS through ACE inhibition and reducing vascular inflammation. Additionally, IRW could restore the disturbed circadian variations of blood pressure in these animals. These findings

might support the use of egg derived peptide as a functional food or nutraceutical ingredient with potential applications in the prevention and management of hypertension.

4.5 References

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Figure 4.1: IRW administration lowers BP in SHR. (A, B and C) SBP, DBP and MAP (mmHg) values from SHRs left untreated (Untr) or treated with a low dose (3mg/Kg BW) or high dose (15mg/Kg BW) of IRW over period of 18 days. BP values for each time point represent the mean BP recorded over a 24 hr period. (D) Heart rate (bpm) of SHRs in the 3 treatment groups over a period of 18 days. Data represented as mean \pm SEM from n=6 animals per treatment group. * and *** indicate $P < 0.05$ and $P < 0.001$ respectively, as compared to the untreated group. 'ns' indicates not significant compared to the untreated group.

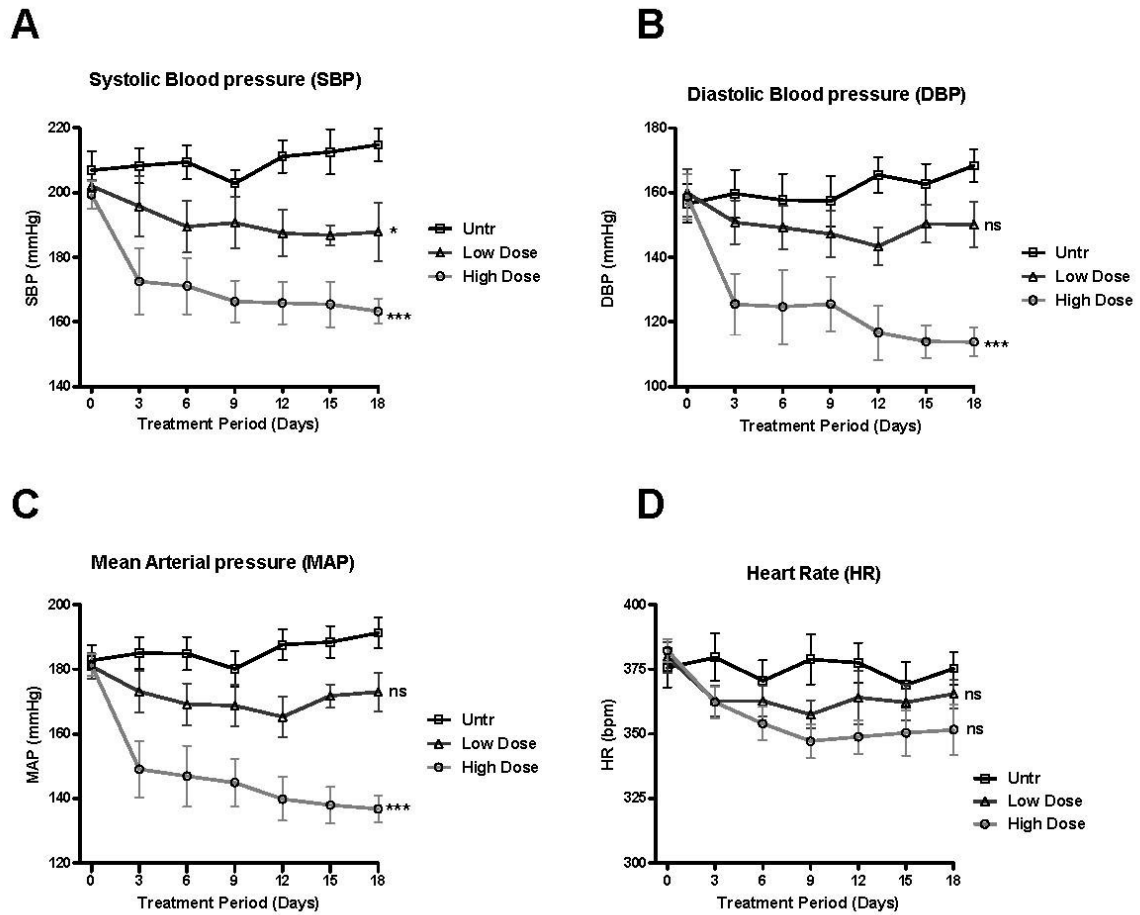


Figure 4.2: IRW treatment restores the circadian rhythms of BP in SHR. (A, B and C) SBP, DBP and MAP (mmHg) values from SHR left untreated or treated with a low dose (3mg/Kg BW) or high dose (15mg/Kg BW) of IRW were recorded during light and dark cycles over a period of 18 days. (D) 2way ANOVA to demonstrate the effects of IRW (low and high dose) on circadian rhythm in MAP. Data represented as mean \pm SEM from n=6 animals per treatment group.

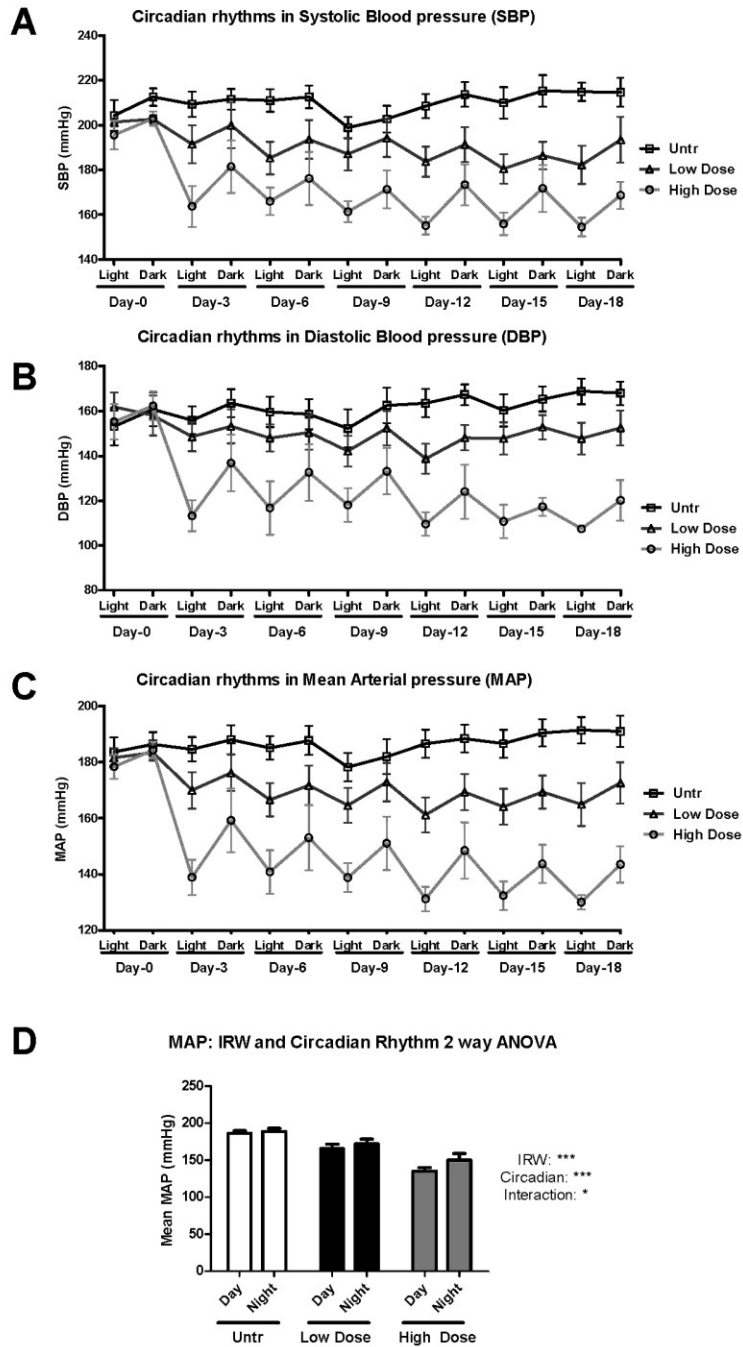


Figure 4.3: IRW treatment restores the nitric oxide contribution to vasodilatation in mesenteric arteries of SHRs. (A) IRW at the high dose (15mg/Kg BW) but not at the low dose (3mg/Kg BW) significantly increased maximal vasorelaxation in response to MCh. (B, C and D) Addition of L-NAME (100 μ M) prior to MCh treatment attenuated vasorelaxation in the high dose (D) but not in the low dose (C) or the untreated (B) groups. Data represented as mean \pm SEM from n=6 animals per treatment group. * indicates P<0.05 compared to the untreated group.

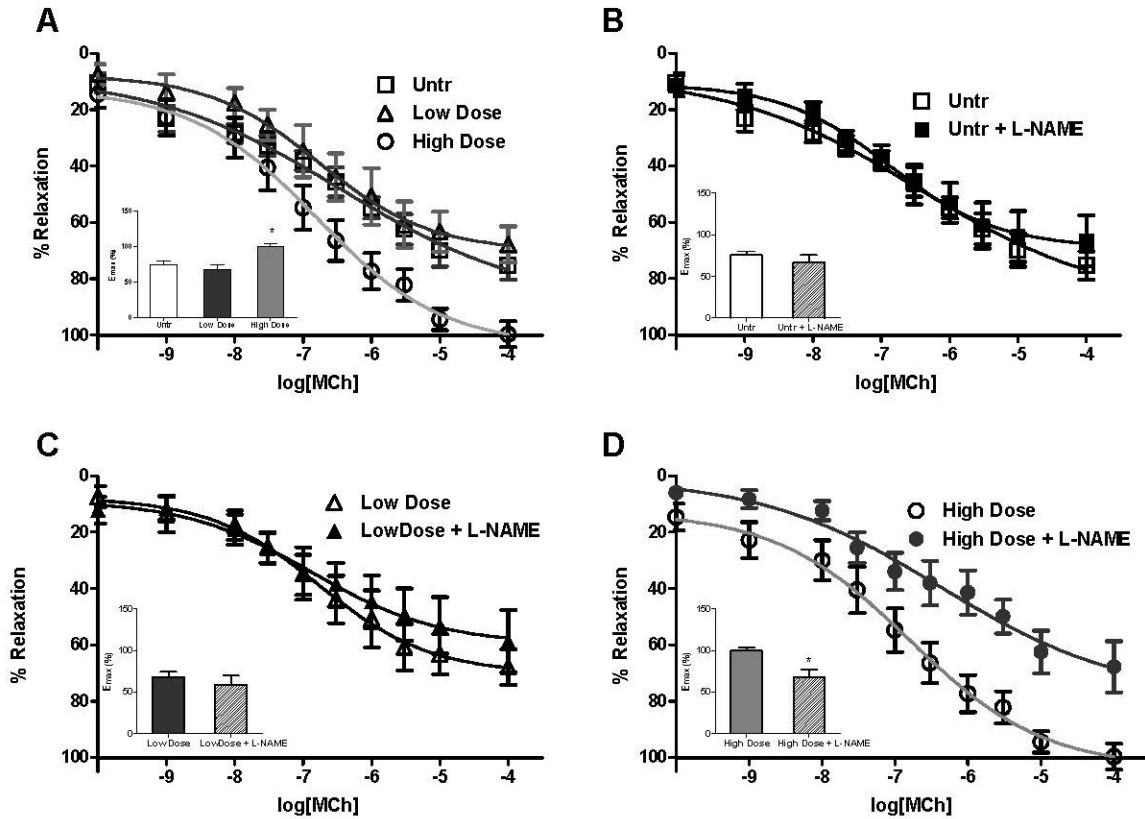
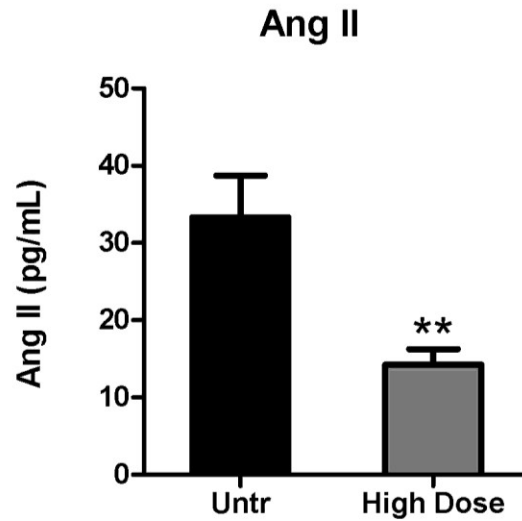


Figure 4.4: IRW treatment attenuates plasma Ang II levels through possible ACE inhibitory effects. (A) Plasma Ang II (pg/mL) levels from untreated and high dose (15mg/Kg BW) IRW treated SHR are shown. (B) Plasma bradykinin (ng/mL) levels from untreated and high dose (15mg/Kg BW) IRW treated SHR. Data represented as mean \pm SEM from n=6 animals per treatment group. * and ** indicate P<0.05 and P<0.01 respectively, as compared to the untreated group.

A



B

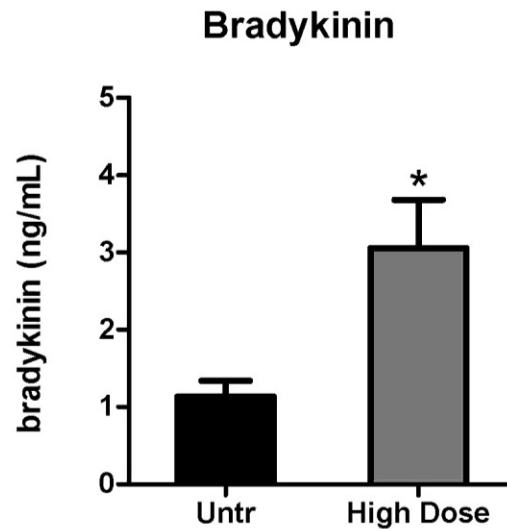


Figure 4.5: IRW treatment attenuates inflammatory markers in SHRs. (A and B) Relative changes in plasma IL-6 and MCP-1 levels in untreated and high dose (15mg/Kg BW) IRW treated SHRs. (C and D) ICAM-1 and VCAM-1 expression, normalized to β actin in mesenteric artery lysates from untreated and high dose (15mg/Kg BW) IRW treated animals. Data represented as mean \pm SEM from n= 4-6 animals per treatment group. *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively, as compared to the untreated group.

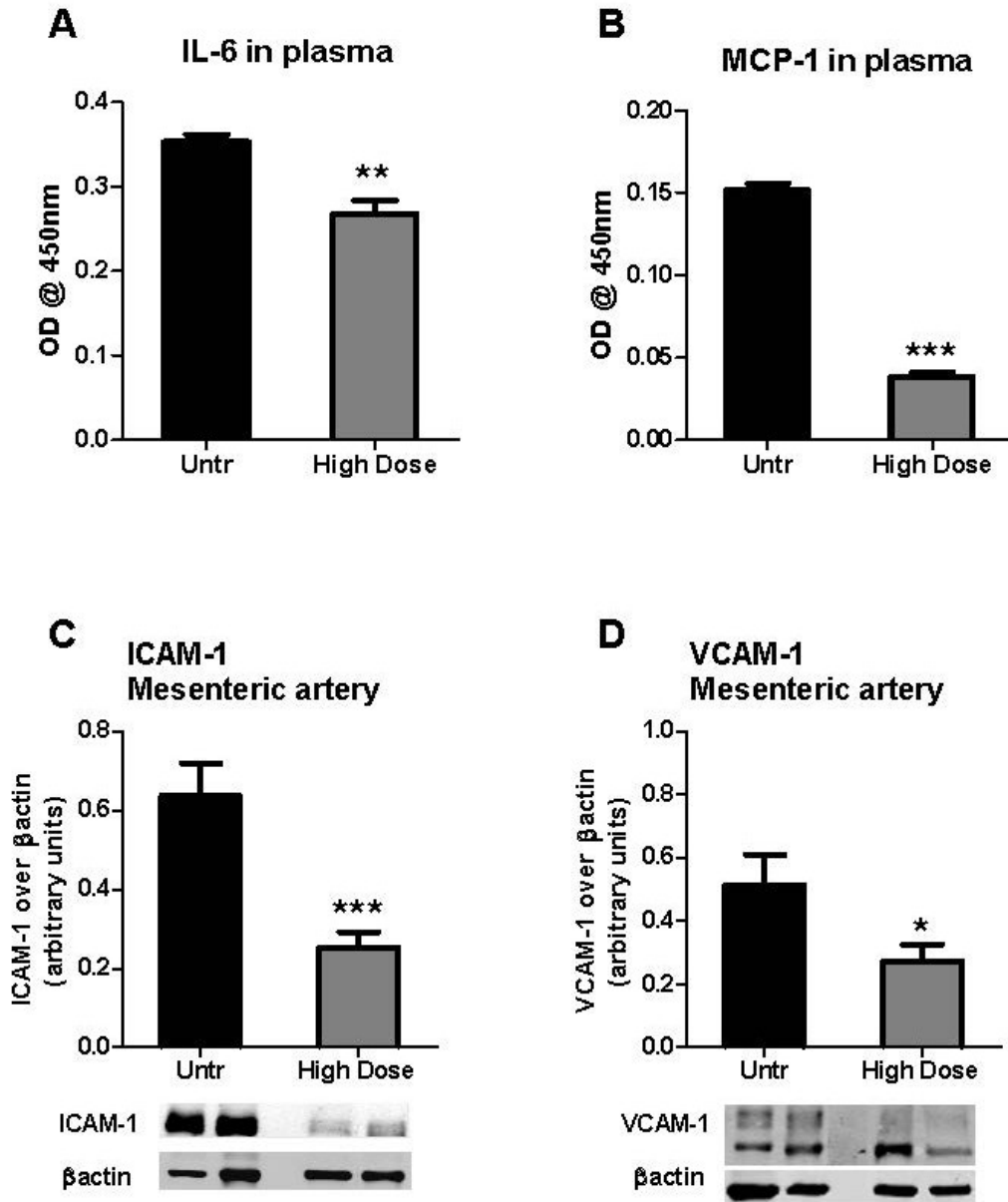


Figure 4.6: IRW treatment reduces the inflammatory potential of SHR plasma. (A and B) Confluent HUVEC monolayers were treated with 10% plasma from untreated or high dose IRW treated SHRs for 4 hours. Cells were lysed and immunoblotted for ICAM-1 and VCAM-1 levels. Data from 3-4 different experiments are summarized as mean \pm SEM. A representative set of images are shown. *** indicates $P < 0.001$ as compared to the No plasma group.

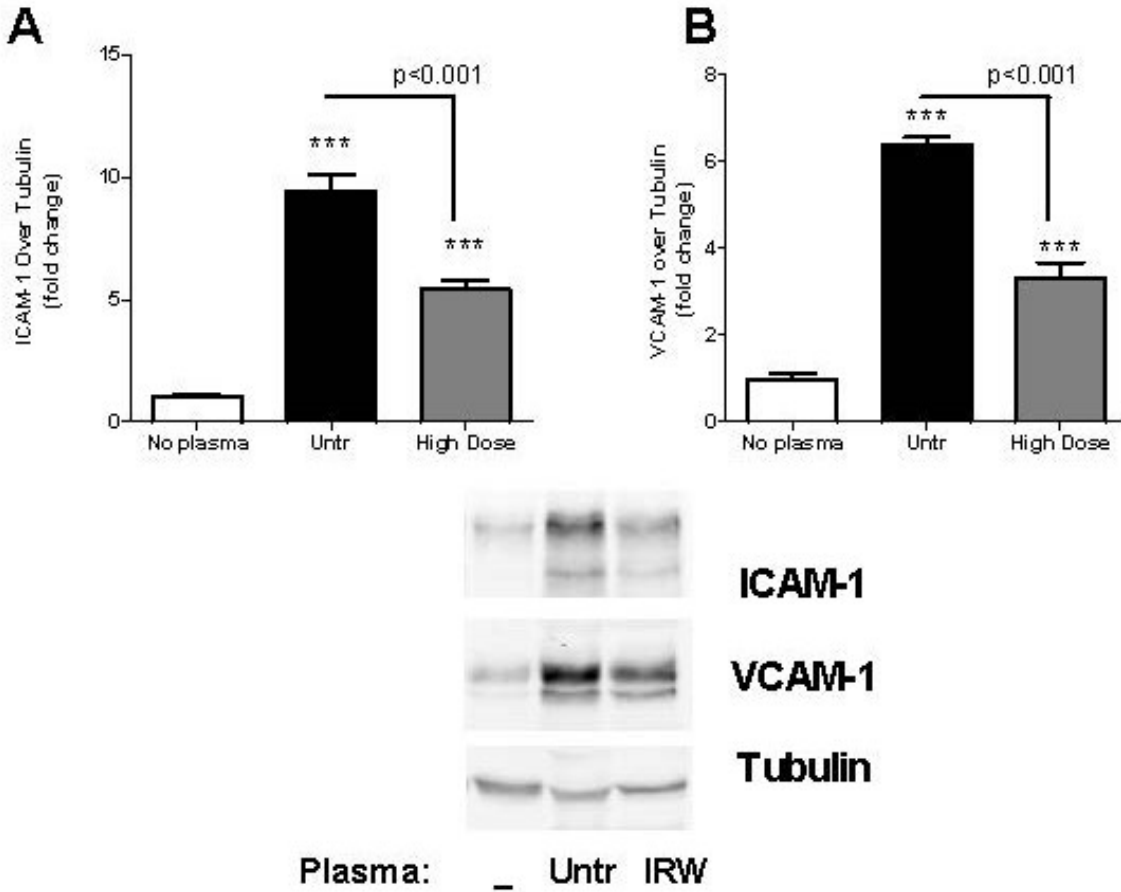


Figure 4.7: IRW treatment restores eNOS expression in SHR vasculature. Expression of eNOS, normalized to β actin in mesenteric artery (A) and aortic (B) lysates from untreated and high dose (15mg/Kg BW) IRW treated animals. Data represented as mean \pm SEM from n=6 animals per treatment group. * indicates $P < 0.05$ compared to the untreated group.

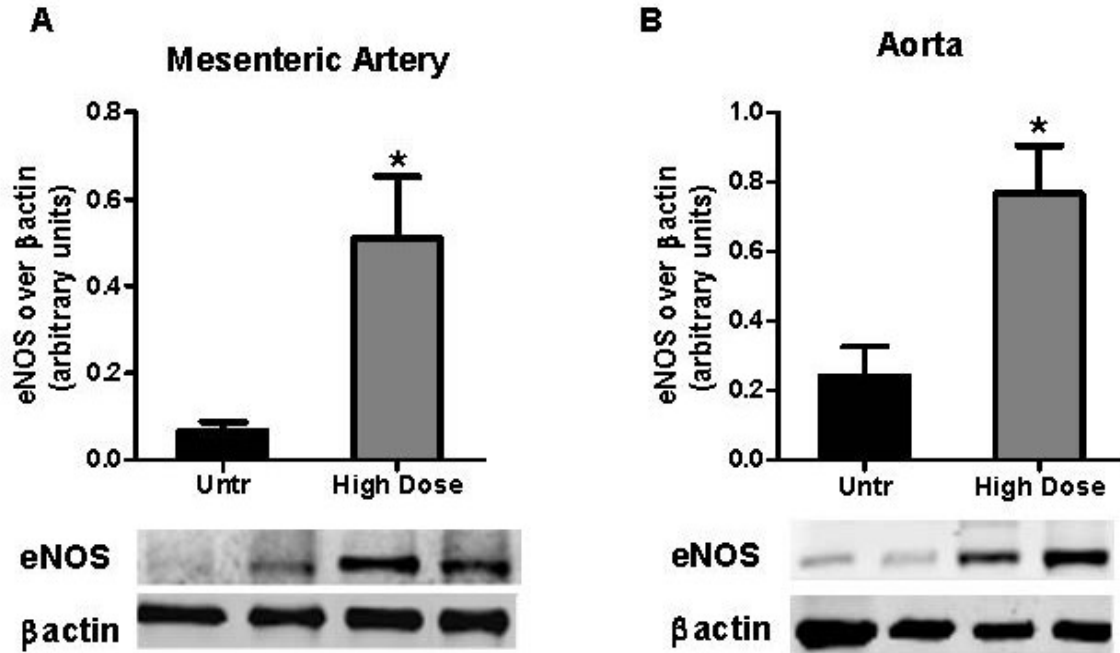
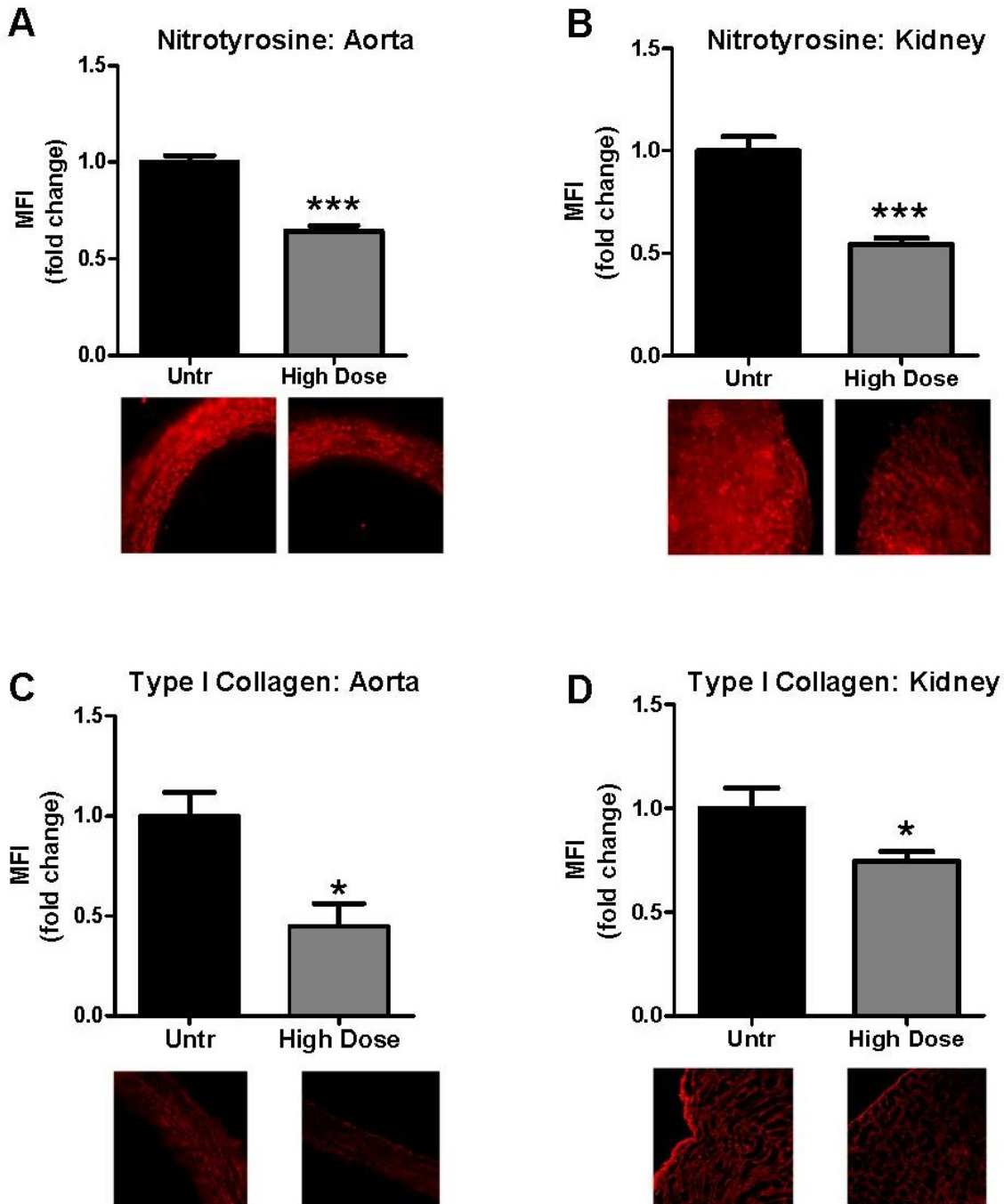


Figure 4.8: IRW treatment attenuates tissue nitrotyrosine and fibrosis in SHR. (A and B) Immunostaining for nitrotyrosine in aortic and kidney sections from untreated and high dose (15mg/Kg BW) IRW treated SHR. (C and D) Immunostaining for type I collagen in aortic and kidney sections from untreated and high dose (15mg/Kg BW) IRW treated SHR. Data represented as mean \pm SEM from n= 3-4 animals per treatment group. * and *** indicate $P < 0.05$ and $P < 0.001$ respectively, as compared to the untreated group.



CHAPTER 5⁴ - Egg-derived ACE-inhibitory peptides IQW and LKP Reduce Blood Pressure in Spontaneously Hypertensive Rats

⁴ A version of this chapter has been submitted to the *Journal of Functional Foods* for publication

5.1 Introduction

Functional foods and nutraceuticals have gained considerable attention due to increasing evidence of their beneficial effects on treatment and management of chronic diseases above and beyond their nutritional value. Food derived bioactive peptides with potential health benefits are one of the major areas of recent research (reviewed in Shahidi & Zhong, 2008)). These peptides are generally latent in their parent proteins but exhibit physiological benefits over and above their expected nutritional value only after release through enzymatic digestion, fermentation, or food processing (Kitts & Weiler, 2003; Rutherfurd-Markwick & Moughan, 2005). Among various food-derived bioactive peptides, antihypertensive peptides with angiotensin converting enzyme (ACE) inhibitory properties are of research interest due to the high prevalence of hypertension (Tibazarwa & Damasceno, 2014). Hypertension is a major risk factor for developing cardiovascular diseases (CVDs) which is a key cause of global morbidity and mortality (Danaei, et al., 2013). Chronically elevated blood pressure (BP) level at or above 140/90 mmHg is defined as hypertension (Chockalingam, 2008). A number of pharmacological drugs have been used in the management of hypertension. Many of these drugs require lifelong adherence to therapy; yet many patients still have poorly controlled BP and suffer adverse side effects (Khanna, Lefkowitz, & White, 2008; Moser & Franklin, 2007). Functional foods and/or nutraceuticals are derived from natural sources and generally considered safe; hence these have become potential alternatives to synthetic pharmacological drugs.

Multiple food-derived factors can significantly reduce high BP (Hermansen, 2000; Zarraga & Schwarz, 2006). It has been suggested that partially replacing dietary carbohydrate with protein may also help in the prevention and treatment of hypertension (Rebholz, et al., 2012). The

pathophysiology of hypertension is complex, although the crucial roles of renin-angiotensin system (RAS), oxidative stress, and vascular inflammation in the development and the persistence of hypertension have been determined (Oparil & Haber, 1974; Paravicini & Touyz, 2006; Rahman, Gilmour, Jimenez, & MacNee, 2002; Salvemini, Ischiropoulos, & Cuzzocrea, 2003; Stauffer, Westby, & DeSouza, 2008). Bioactive peptides from food protein hydrolysis have been known to exhibit anti-oxidant, anti-inflammatory, and anti-thrombotic activities along with ACE inhibitory effects (Balti, et al., 2012; Fujita, Sasaki, & Yoshikawa, 1995; Ichimura, Yamanaka, Otsuka, Yamashita, & Maruyama, 2009; Miguel & Aleixandre, 2006). Therefore, a multifunctional peptide with ACE-inhibitor, anti-oxidant and anti-inflammatory activities could be an ideal candidate for the prevention and management of hypertension.

Eggs are the most economic source of dietary protein worldwide. In addition to their well-known nutritional value, the egg is also a rich source of bioactive peptides. Ovotransferrin, an iron-binding glycoprotein, contributes ~13% of the total protein in egg white (Williams, Elleman, Kingston, Wilkins, & Kuhn, 1982). Our earlier study had identified three tri-peptides IRW (Ile-Arg-Trp, molecular weight: 473.58 Da), IQW (Ile-Gln-Trp, molecular weight: 445.51 Da) and LKP (Leu-Lys-Pro, molecular weight 356.46 Da) from enzymatic digestion of the ovotransferrin with *in vitro* angiotensin converting enzyme (ACE) inhibitory properties (Majumder & Wu, 2010). Further studies also demonstrated that some of these peptides have significant anti-oxidant and anti-inflammatory properties in cultured vascular endothelial cells (Majumder, Chakrabarti, Davidge, & Wu, 2013). Another publication from our group has further characterized the *in vivo* antihypertensive effect of IRW and its possible mechanisms in a rat model of hypertension (Majumder, Chakrabarti, Morton, et al., 2013). Spontaneously

hypertensive rat (SHR) is a well-recognised animal model for hypertension that develops hypertension at an early age (~12-14 weeks) and remains hypertensive throughout their lives (Dornas & Silva, 2011; Trippodo & Frohlich, 1981). To test novel anti-hypertensive therapies and to study the pathophysiology of hypertension SHRs have been widely used in scientific research (Bagnost, et al., 2010; Cutts & Kim, 2010; Katayama, et al., 2008; Nakamura, Naramoto, & Koyama, 2013). Despite the *in vitro* evidence, a lack of *in vivo* data may impede the further development of IQW and LKP as viable anti-hypertensive options. Therefore, the present study evaluates the *in vivo* efficacy and likely mechanisms of action of IQW and LKP in a well-characterized animal model of hypertension.

5.2 Materials and Methods

5.2.1 Animal model

The animal experimental procedures were similar to those used in our previously published study on the effects of ovotransferrin derived tri-peptide IRW in SHRs (Majumder, Chakrabarti, Morton, et al., 2013). Briefly, 12 to 14 week old male SHR animals (270.0 ± 10.5 g) were obtained from Charles River (Senneville, QC, Canada). They were kept in the University of Alberta animal facility for at least 1 week to undergo acclimatization. During the acclimatization and experimental phases SHR animals were exposed to a 12 hour (light:dark) cycle, in a humidity and temperature-controlled (23 °C) quiet room. Standard rat chow (0.3% NaCl) and water *ad libitum* were given to all the rats. The University of Alberta Animal Care approved all procedures and Use Committee (Protocol # 611/09/10/D) in accordance with the guidelines issued by the Canada Council on Animal Care.

5.2.2 Experimental Design

After acclimatization, 13-15 week old SHR animals were surgically implanted with telemetry transmitters (PA-C40; Data Science International Minneapolis, MN) for BP monitoring in live animals. Following 7-10 days of recovery period after surgery, the animals were randomly assigned to 3-treatment groups- untreated (control), IQW (15 mg /Kg BW) and LKP (15mg /Kg BW). The doses were selected based on our previously published *in vivo* studies on the egg-derived peptide IRW (Majumder, Chakrabarti, Morton, et al., 2013; Majumder, Panahi, Kaufman, & Wu, 2013). On the first day of recording (day 0) all the animals received 20mL of Ensure (Abbott Nutrition, QC, Canada). From day 1 onwards, the peptides (IQW and LKP) were dissolved in Ensure for palatability and administered once per day continuously up to 18 days. Untreated animals were given Ensure alone as a vehicle control. BP was recorded for a 24 h period (10 sec of every 1 min) on days 0 (baseline), 3, 6, 9, 12, 15 and 18 under the conditions described above. The animals were then sacrificed by exsanguination via excision of the heart under inhaled isoflurane anesthesia, at the end of the experimental period. The blood was collected from the heart and transferred immediately into EDTA coated tubes (BD vacutainer, NJ, USA) and centrifuged (1,000Xg for 20 min at 4°C) to obtain plasma, while other tissues were collected, rinsed with ice-cold saline, weighed, flash frozen in liquid nitrogen and stored at -80°C for further analysis. Mesenteric arteries were also isolated and used immediately afterwards for vascular function studies.

5.2.3 Anesthesia and surgery

Blood pressure measurements were carried out by telemetry using surgically implanted transmitters in the left femoral artery of the rats, as described previously (Majumder,

Chakrabarti, Morton, et al., 2013). Briefly, the animals were operated under isoflurane (1.5-2%) anesthesia using a Zeiss dissecting microscope (Carl Zeiss, Toronto, ON, Canada). After reaching the surgical plane, an approximately 3-4 cm vertical incision was made a small pocket was created under the skin on the left side of the spine just above the hip. The transmitter was placed and anchored in the pocket. Approximate 2 cm long incision was then made on the groin area to expose the femoral artery and polyethylene cannula (0.58 mm ID, 0.97 mm OD) of the telemetry probe was inserted and advanced up to the aorta and securely sutured with the surrounding tissues. The rats were then caged individually and allowed to recover for 7-10 days, receiving (0.05 mg/kg BW) of buprenorphine (0.3mg/mL, Animal Resources Centre, McGill University, Montreal, QC, Canada) for pain management. During the recovery period, animals were fed 50 mL of Ensure (Abbott Nutrition, QC, Canada) to provide essential nutrients to regain the pre-operative weight (Schwartz, Salorio, Skoglund, & Moran, 1999) along with standard rodent chow and water *ad libitum*.

5.2.4 Telemetric blood pressure recording

Individually caged rats were placed in a temperature, humidity and light phase (12 hour; light:dark) controlled quiet room with least electrical interference to record the mean arterial blood pressure (MAP) and heart rate (HR), according to established procedures similar to our previous work (Majumder, Chakrabarti, Morton, et al., 2013; Majumder, Panahi, et al., 2013). Real time data collection on live animals was done using DMT data quest ART software version 4.3 (Data Science International Minneapolis, MN).

5.2.5 Vascular function studies

Vascular function studies were performed on isolated second order branches of the mesenteric artery (ID: 150-250 μm and length ~ 2 mm) by using wire-myography, similar to our previously published study (Majumder, Panahi, et al., 2013). Briefly, similar to previous studies from our group cumulative concentration response curves were obtained for vessel constriction and relaxation by phenylephrine (PE 10^{-8} to 10^{-4} mol/L; vasoconstrictor; Sigma Aldrich, Oakville, Canada) and methacholine (MCh 10^{-10} to 10^{-4} mol/L; vasodilator; Sigma), respectively (Liu, et al., 2009; Majumder, Chakrabarti, Morton, et al., 2013; Majumder, Panahi, et al., 2013). The role of nitric oxide (NO) in endothelium-dependent relaxation was assessed by studying MCh-mediated vasorelaxation in presence or absence of the non-selective nitric oxide synthase (NOS) inhibitor N-nitro-L-arginine methyl ester (L- NAME, 100 $\mu\text{mol/L}$; Sigma).

5.2.6 Western immunoblotting

To determine the effect of peptides treatment on protein expression, western immunoblotting was performed on vascular tissue (mesenteric arteries and aortas) lysates similar to our previous study (Majumder, Chakrabarti, Morton, et al., 2013). A protein extraction buffer (20 mmol/L Tris, 5 mmol/L EDTA, 10 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 100 mmol/L sodium fluoride and 1% NP-40) containing 1% (v/v) protease inhibitor cocktail (Sigma) was used to extract proteins from aortas and mesenteric arteries. The protein concentration of the respective samples was determined by bicinchoninic acid (BCA) assay, using bovine serum albumin as a standard. Protein bands for intercellular cell adhesion molecule-1/ICAM-1 (Anti-ICAM-1 mouse monoclonal antibody, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), vascular cell adhesion molecule-1/VCAM-1 (Anti-VCAM-1 rabbit polyclonal antibody, Santa Cruz Biotechnologies) and endothelial nitric

oxide synthase (eNOS) (Anti-eNOS mouse monoclonal antibody, BD Biosciences, San Jose, CA, USA) were normalized to β -actin (Anti- β -Actin rabbit polyclonal antibody, Abcam Inc., Toronto, ON, Canada) and expressed as a fold change compared to untreated samples. Samples from all treatment groups were loaded on the same gel for quantitative analysis. Anti- β -actin was used at 0.5 μ g/mL, while eNOS, ICAM-1 and VACM-1 antibodies were used at 1 μ g/mL. Secondary Goat-anti-rabbit and Donkey-anti-mouse antibodies (Li-Cor Biosciences, Linclon, NB, USA) were used to visualize the bands in a Li-Cor Odyssey BioImager and quantified by densitometry with corresponding software (Odyssey V3.0, Li-cor Biosciences).

5.2.7 Plasma biomarker analysis

Blood plasma samples were collected, centrifuged (1,000Xg for 20 min at 4°C) and stored at -80°C for further analysis. Angiotensin II (Ang II) was quantified by ELISA kits (Ang II ELISA, Cayman Chemical, Ann Arbor, MI, USA) as per the manufactures' instructions.

5.2.8 Immunofluorescence

Aorta samples were frozen immediately in liquid nitrogen and stored at -80 °C after embedding in Tissue-Tek O.C.T Compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) for further analysis. For immunostaining, 10 μ m tissue sections were prepared and mounted on glass slides (3 sections/slide), tissue sections were fixed in acetone (10 min) and incubated with blocking buffer (1% bovine serum albumin in phosphate-buffer saline) for 30 min. The tissue sections were then incubated with rabbit polyclonal antibody against nitrotyrosine (Dilution 1:500; Chemicon, Temecula, CA, USA), overnight at 4°C. The secondary antibody (Dilution 1: 200; Alexa Fluor 546 (red), Invitrogen, Burlington, ON, Canada) incubation was done in the dark for 30 min. Vectashield H-1200 Mounting Kit, containing the nuclear stain DAPI (Vector

Laboratories, Burlington, ON, Canada) was used as a mounting media with glass cover-slips and the tissues sections were visualised immediately under an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan). Metamorph imaging software (Molecular Devices, Sunnyvale, CA) was used to capture the images at 200X magnification. To ensure a good representation at least three different images were taken from each tissue section. To detect any nonspecific binding, a control image from a tissue section treated with secondary antibody alone was used in each slide. The fluorescence intensity for each image was then calculated by subtracting the background fluorescence intensity from the respective control image, as described in our previous study (Majumder, Chakrabarti, Morton, et al., 2013). The mean fluorescence intensity was then calculated for quantitative analysis.

5.2.9 Statistics

All data presented are mean \pm SEM of 4-6 animals for each treatment group. Two-way ANOVA was used to determine the interaction between two factors (time and treatment), with a Bonferroni's post-hoc test was used to compare the BP differences between different groups. For vascular function (MCh curve) data, nonlinear regression method were used for curve fitting, and one-way ANOVA followed by Bonferroni's post-hoc test or unpaired *t*-test were used to determine the maximum response (E_{max}) values as necessary. ICAM-1, VCAM-1 and eNOS bands, nitrotyrosine levels in tissue as well as Ang II levels in the plasma were analysed by using one-way ANOVA. A *p* value < 0.05 was considered statistically significant.

5.3 Results

5.3.1 IQW and LKP treatments do not alter body and organ weights

No significant change in body weight was observed due to the peptide treatment (*Figure 5.1A*). The average body weights of the animals at the end of the study (day 18) in the SHR untreated group, IQW treated group and LKP treated group were 369.17 ± 4.9 g, 359.06 ± 4.1 g and 366.2 ± 4.13 g respectively. No significant differences were observed in the liver, kidney and heart weights (*Figure 5.1B, C and D*) at the end of the experiments between different treatment groups.

5.3.2 IQW and LKP administration reduce blood pressure in SHRs

After 18 days of IQW and LKP treatment, SBP was significantly decreased in both IQW and LKP groups to 161.40 ± 1.6 mmHg and 152.23 ± 0.8 mmHg respectively, compared to the untreated value of 182.43 ± 3.0 mmHg (*Figure 5.2A*). MAP and DBP with both IQW and LKP treatment also showed similar effects. The MAP and DBP for the untreated group was 168.49 ± 2.1 mmHg and 142.79 ± 2.9 mmHg, compared to 144.20 ± 2.0 and 121.13 ± 1.6 mmHg for the IQW treatment group, and 137.81 ± 1.0 mmHg and 117.32 ± 2.8 mmHg for the LKP treatment group, respectively, after 18 days of treatment (*Figure 5.2B and C*). With both peptides, significant blood pressure reduction was observed initially on day 12 and the trend continued to the end of the study period (day 18). However, no significant change was observed in HR (*Figure 5.2D*) among the groups.

Circadian variations or nocturnal dipping of blood pressure (MAP, SBP, and DBP) were calculated from the mean BP during each 12 h light cycle (light/dark). The circadian variations of BP were disturbed in the untreated animals during the light and dark cycles. Despite the

reductions in BP, the impaired circadian variation in BP (typically observed in SHRs with established disease) was not restored by IQW or LKP treatment (*Figure 5.3A, B, C and D*).

5.3.3 IQW and LKP restore nitric oxide (NO) mediate vasorelaxation

Response to phenylephrine (PE) vessel constriction in the mesenteric arteries was unaffected in the IQW and LKP treatment groups compared to the untreated group (*data not shown*). MCh induced vasodilatation was significantly enhanced in the both treatment groups (*Figure 5.4A*). Incubation with the non-selective nitric oxide synthase (NOS) inhibitor L-NAME had no effects in the untreated groups, but vasorelaxation in both the IQW and LKP treated groups were significantly decreased by L-NAME, indicating the crucial role of NO in this process (*Figure 5.4B, C and D*).

An increase in NO-mediated vasorelaxation is often associated with an upregulation of eNOS expression in the vasculature; however, neither IQW nor LKP treatment altered eNOS expression in mesenteric arteries (*Figure 5.5A*) or in aortic lysates (*Figure 5.5B*).

5.3.4 IQW and LKP decrease plasma Ang II levels

The anti-hypertensive effects of IQW and LKP were associated with changes in circulating levels of Ang II in plasma. Ang II levels in the untreated group were 23.96 ± 1.7 pg/mL compared to 12.42 ± 0.7 pg/mL and 12.62 ± 1.04 in the IQW and LKP treated groups, respectively (*Figure 6*). These results suggest that both these peptides (IQW and LKP) can inhibit ACE- *in vivo* and thus decrease the production of circulating Ang II in SHR plasma.

5.3.5 IQW and LKP treatment alters inflammatory markers and oxidative/nitrosative stress

IQW treatment significantly decreased the expression of the pro-inflammatory adhesion molecule, ICAM-1 without affecting levels of VCAM-1, whereas LKP did not alter the expression of either ICAM-1 or VCAM-1 in mesenteric arteries (*Figure 5.7A and B*).

Similarly, a significant decrease in nitrotyrosine staining (a marker of increased peroxynitrite generation, and, hence increased oxidative/nitrosative stress, Cabassi, et al., 2001; Hong, et al., 2001) in aortas of IQW treated animals were observed but nitrotyrosine levels did not alter after LKP treatment (*Figure 5.8*). These results suggest that IQW can also act as an anti-inflammatory and anti-oxidant agent and thus make an additional contribution to ameliorate cardiovascular disease conditions.

5.4 Discussion

Results from the present study demonstrated that: (i) Egg-derived bioactive tripeptides IQW and LKP significantly reduced blood pressure in adult male SHRs, (ii) Both IQW and LKP restored NO mediated vasorelaxation and reduced plasma Ang II levels and (iii) IQW but not LKP treatment attenuated the expression of pro-inflammatory adhesion molecule (ICAM-1) and oxidative/nitrosative stress.

Given the global burden of hypertension and associated CVD, results from this study may provide a food-derived option for management of hypertension and generate a template to develop anti-hypertensive nutraceuticals with these peptides. Various peptides of different food origins have proven beneficial against CVD, such as peptides with anti-hypertensive (ACE inhibitory), cholesterol lowering, anti-thrombotic, anti-inflammatory, and anti-oxidant activities (Balti, et al., 2012; Turpeinen, et al., 2009). Furthermore, food-derived peptides exhibit multiple bioactive functionalities, which can counteract the disease, process at multiple points in its

pathophysiological development (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004; Meisel, 2004). Therefore, it is apparent that food protein-derived bioactive peptides can exhibit regulatory functions above and beyond their basic nutritional value. Egg is an economically and nutritionally important food commodity and a rich source of bioactive peptides (Miguel & Aleixandre, 2006; Song & Kerver, 2000). IQW and LKP are two ACE inhibitory peptides previously characterized from the egg white protein ovotransferrin (Majumder & Wu, 2010). Moreover, IQW also exhibited anti-inflammatory and anti-oxidant properties in cultured human vascular endothelial cells (Majumder, Chakrabarti, Davidge, et al., 2013). Given this background, we evaluated the antihypertensive effects of IQW and LKP in animals after oral administration.

SHR is an animal model of human essential hypertension, which demonstrates persistent high BP along with increased RAS activity, increased oxidative stress, and pronounced inflammatory response. These features make them hypertensive for the rest of their lives. Hence, SHR are recognized as a well-established animal model to validate the efficacy of new antihypertensive agents (Trippodo & Frohlich, 1981). Results from the present study show that the BP can be significantly reduced after 18 day treatment with IQW or LKP. Both the peptides decreased BP without any significant changes in HR. Therefore; the peptides appear to preserve the normal cardiac responses that would likely minimize the risk of developing arrhythmias and other related complications. The reduction of blood pressure after IQW and LKP treatments are concomitant with the reduction in plasma Ang II levels at the end of the study. Ang II as a vasoconstrictor is a key contributor in the RAS pathway, which regulates BP and plays a key role in the pathophysiology of hypertension (Bader & Ganten, 2008; Oparil & Haber, 1974; Peach,

1977). However, this is not the sole mechanism by which these peptides modulate blood pressure. The vascular function studies revealed that both IQW and LKP restored the nitric oxide (NO) mediated vasorelaxation (Ramzy, et al., 2006; Salvemini, et al., 2003; Yang, Huang, Kaley, & Sun, 2009). This suggests that these treatments could increase the bioavailability of NO; resulting in increased relaxation. Interestingly, neither of these peptides increased eNOS expression, suggesting the restoration of NO mediated vasorelaxation may be mediated by increasing NO bioavailability. These findings are in contrast to our previous results with IRW where a combination of ACE inhibitor, anti-inflammatory and anti-oxidant effects were observed to reduce BP (by ~40 mm Hg) in the same animal model (Majumder, Chakrabarti, Morton, et al., 2013).

An increased level of reactive oxygen species (ROS) often contributes to the pathology of hypertension (Hong, Hsiao, Cheng, & Yen, 2001; Pennathur & Heinecke, 2007; Salvemini, et al., 2003). ROS such as superoxide (O_2^-) can hinder the bioavailability of NO by generation of peroxynitrite (-ONOO), a highly reactive species that leads to a pro-inflammatory phenotype through tyrosine nitration of various proteins. Earlier studies have shown that a reduction in – ONOO and tyrosine nitration in SHR, brought about by inhibiting ROS can reduce blood pressure (Cabassi, et al., 2001; Hong, et al., 2001). Similarly, our study showed that IQW but not LKP treatment can significantly reduce nitrotyrosine levels in aorta, suggesting an anti-oxidant effect of IQW. In addition, IQW also reduced the expression of the inflammatory adhesion molecule ICAM-1. The latter finding is in accordance with our previous study showing an anti-oxidant and anti-inflammatory effect of IQW on human endothelial cells (Majumder, Chakrabarti, Davidge, et al., 2013).

Our findings also showed reduced circulating Ang II levels in SHR treated with either IQW or LKP. LKP was identified as an ACE inhibitor in our previous study from egg (Majumder & Wu, 2010) and from bonito fish protein by Yokoyama et al (Yokoyama, Chiba, & Yoshikawa, 1992). The same group has also investigated the effect of LKP *in vivo* via intravenous administration where it reduced blood pressure by ~18mm Hg in 4 hrs in hypertensive animals (Fujita & Yoshikawa, 1999). Moreover, a longer lasting effect was reported for LKPNM, a pro-drug type ACE inhibitor of LKP. Similarly, our current study supports the earlier findings and further establishes LKP as an ACE inhibitor that can reduce blood pressure upon oral administration. In addition, LKP also appears to enhance NO sensitive vasorelaxation although no effects on eNOS expression and NO scavenging (by nitrotyrosine staining) could be found, suggesting a novel mechanism of action possibly through modulation of vascular reactivity at the level of endothelial derived hyperpolarizing factors (EDHF) or endothelin pathways (Jakala, et al., 2009; Maes, et al., 2004) which could be a target for future research. In contrast, IQW not only acts as an ACE inhibitor but also exerts anti-inflammatory and anti-oxidant effects, suggesting its multifactorial action on blood pressure. Moreover, our present study shows that peptides identified as ACE inhibitors (through *in vitro* studies) may also exhibit *in vivo* antihypertensive effects beyond ACE inhibition.

In conclusion, the *in vivo* anti-hypertensive effects of orally administered IQW and LKP appear to be mediated through similar pathways involving increased NO mediated vasodilatation and regulating RAS through ACE inhibition, with some additional beneficial effects mediated by IQW alone (i.e. reducing vascular inflammation and oxidative stress). While our work validates the anti-hypertensive properties of these peptides in a relevant model system there are a few

limitation associated with this study. For example we used only adult male rodents with fully developed hypertension. Future studies with a longer treatment period in younger animals and also in animals of both sexes may be performed to determine the preventive effects, if any, of these peptides. In conclusion, these findings might justify the use of egg as a source for the development of novel functional food and/or nutraceuticals for the prevention and management of hypertension and associated cardiovascular disorders.

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Figure 5.1: IQW and LKP do not alter body and organ weights in treated SHR. Whole body weight (A) over the treatment period and weights of liver (B), kidney (C) and heart (D) at the time of sacrifice following 18 days of treatment. Data represented as mean \pm SEM from n=4-6 animals per treatment group.

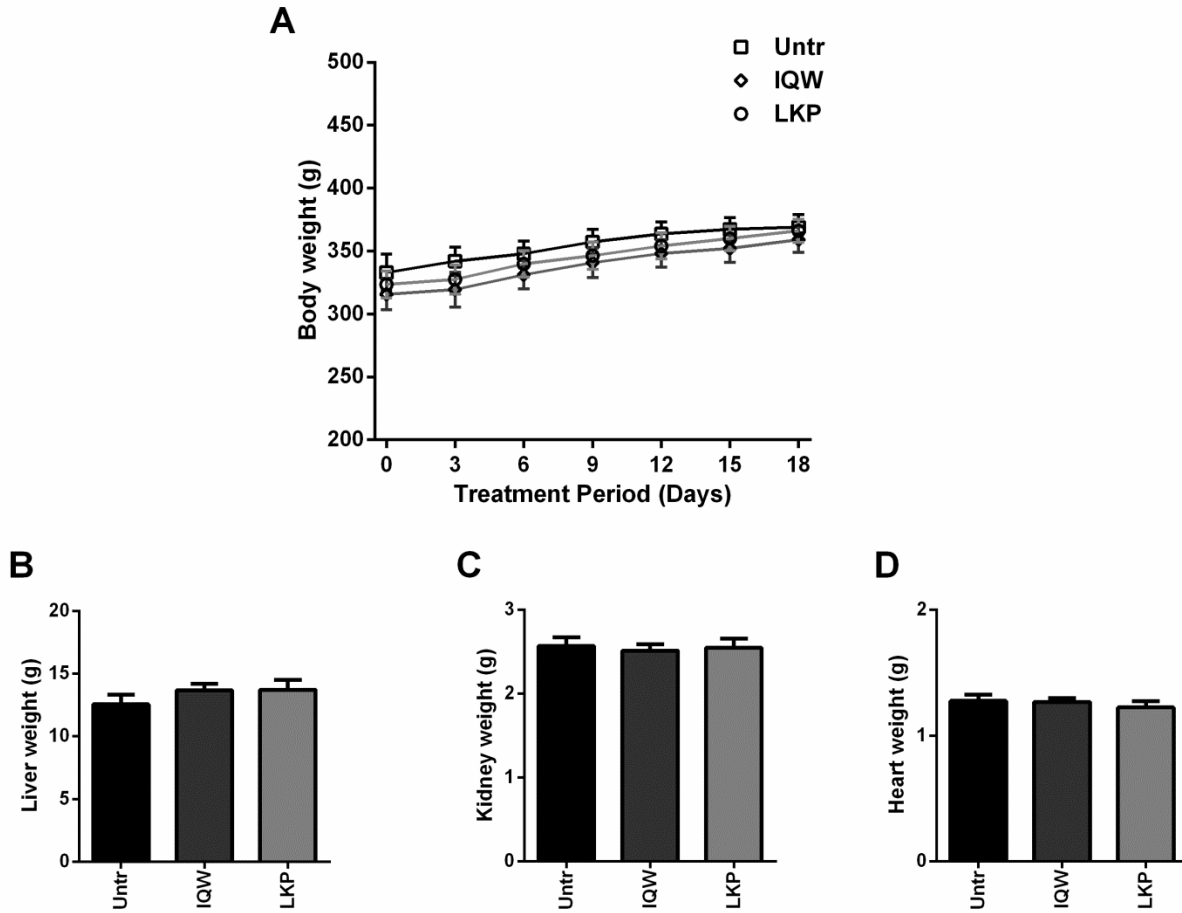


Figure 5.2: IQW and LKP administration reduces BP in SHR. (A, B and C) SBP, DBP and MAP (mmHg) values from untreated (Untr) SHRs or those treated with IQW or LKP over the treatment period. BP values for each time point represent the mean BP recorded over a 24 hr period. (D) Heart rate (bpm) of SHRs in the 3 treatment groups over 18 days. Data represented as mean \pm SEM from n=4-6 animals per treatment group. * and ** indicate $P < 0.05$, and $P < 0.01$ respectively, as compared to the untreated group. 'ns' indicates not significant compared to the untreated group.

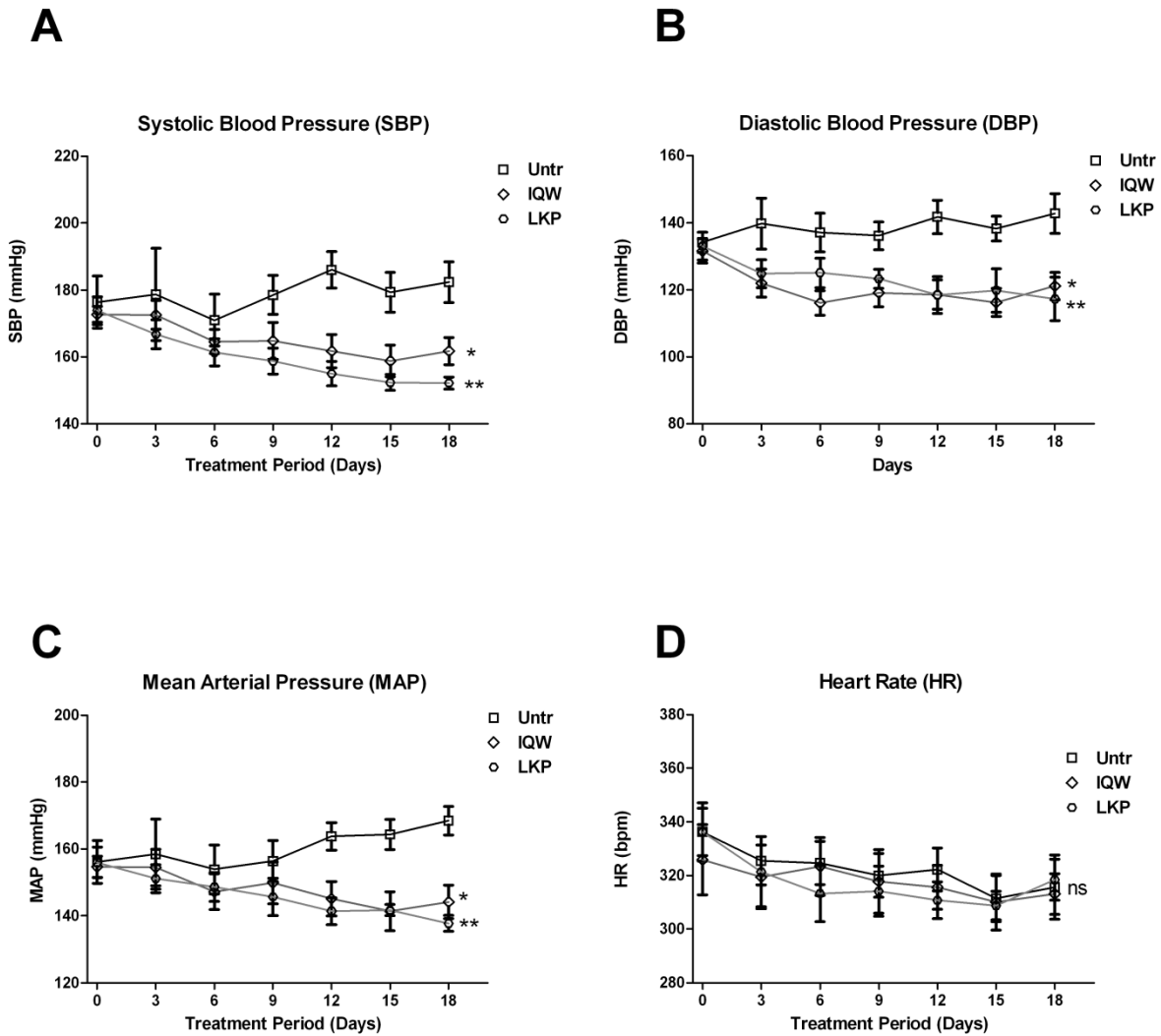


Figure 5.3: IQW and LKP cannot restore the circadian rhythms of BP in SHR. (A, B and C) SBP, DBP and MAP (mmHg) values from untreated SHRs or those treated with IQW or LKP were recorded during light and dark cycles over 18 days. (D) Summary graphs to demonstrate the effects of peptides (IQW and LKP) on circadian rhythm in MAP. Data represented as mean \pm SEM from n=4-6 animals per treatment group.

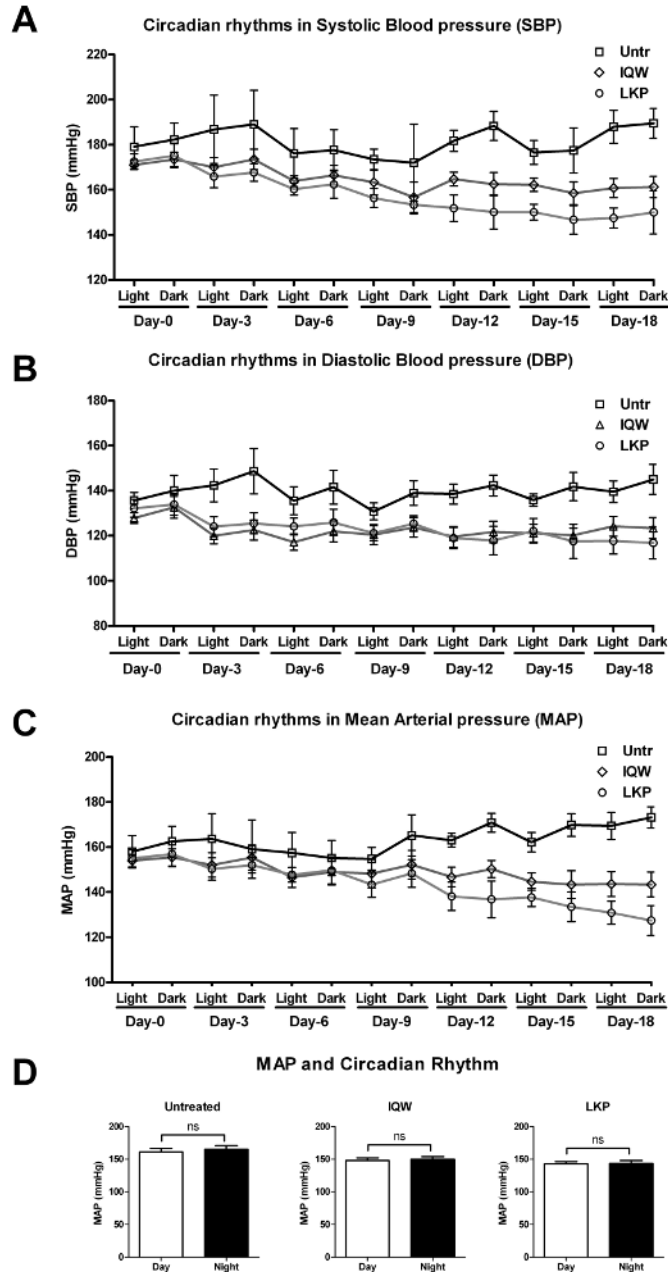


Figure 5.4: IQW and LKP administration restore the nitric oxide dependent vasodilatation in mesenteric arteries of SHRs. (A) IQW and LKP significantly increased maximal vasorelaxation to MCh. (B, C and D) Addition of L-NAME (100 μ M) prior to MCh treatment attenuated vasorelaxation in (C) IQW-treated and (D) LKP-treated but not in the (B) untreated groups. Data represented as mean \pm SEM from n=4-6 animals per treatment group. ** indicates $P < 0.01$ compared to the untreated group.

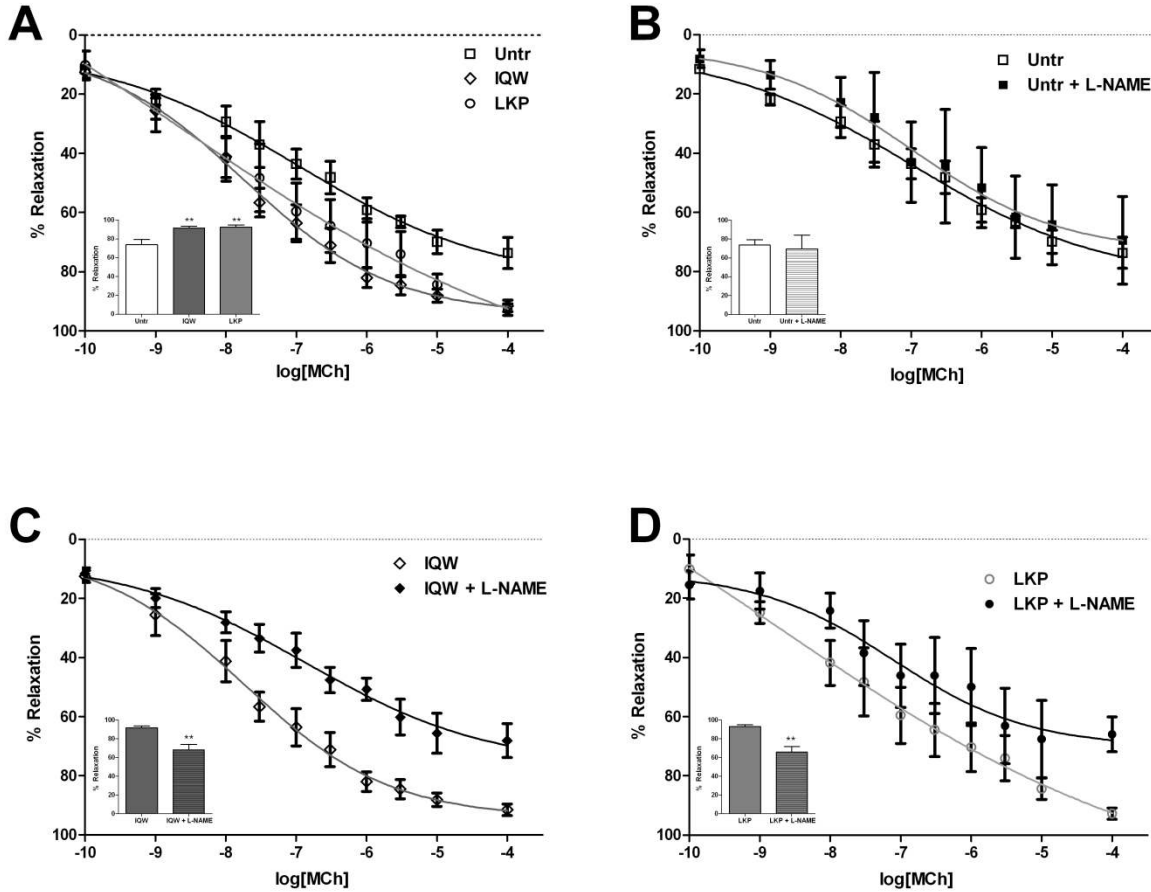


Figure 5.5: IQW and LKP treatment do not restore eNOS expression in SHR vasculature. Protein levels of eNOS, normalized to β actin in mesenteric artery (A) and aortic (B) lysates from untreated, IQW and LKP treated animals. Data represented as mean \pm SEM from n=4 animals per treatment group.

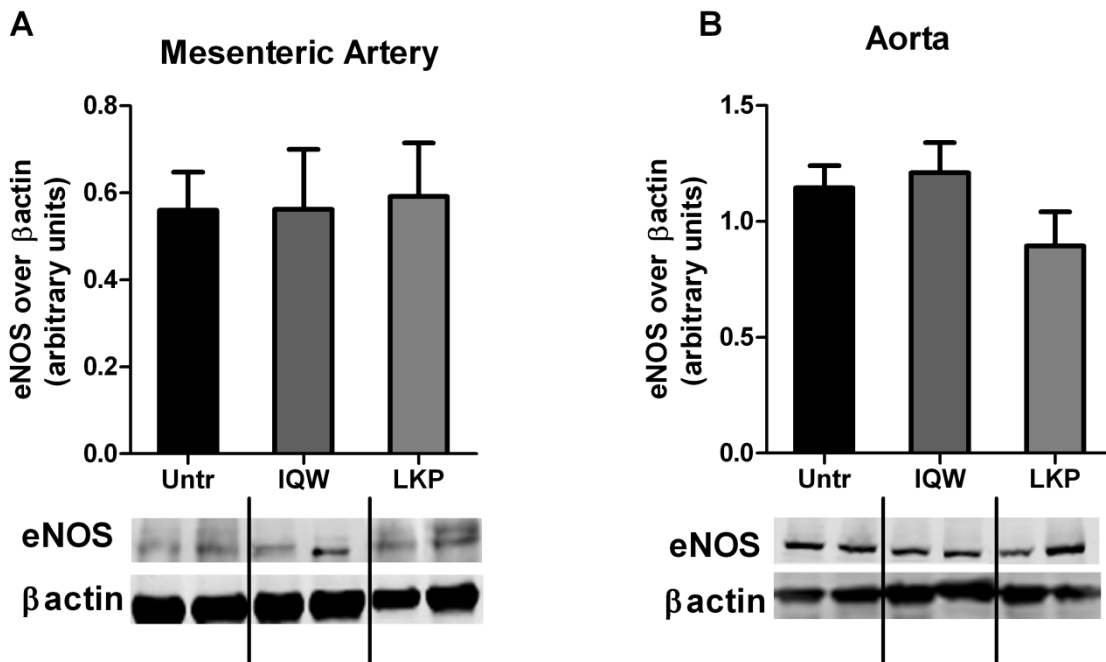


Figure 5.6: IQW and LKP treatment attenuate plasma Ang II levels. Plasma Ang II (pg/mL) levels from untreated, IQW and LKP treated SHR are shown. Data represented as mean \pm SEM from n=4 animals per treatment group. * indicate $P < 0.05$, as compared to the untreated group.

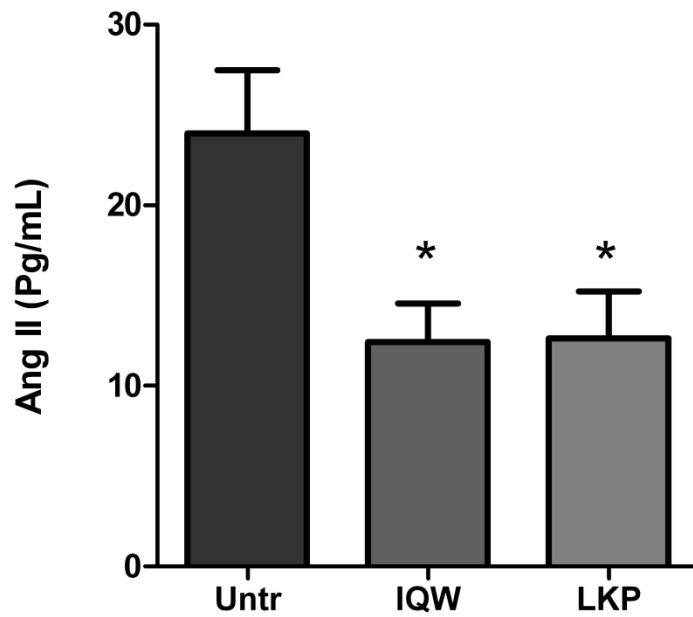


Figure 5.7: IQW but not LKP treatment alters inflammatory markers in SHRs mesenteric artery. (A and B) ICAM-1 and VCAM-1 expression, normalized to β actin in mesenteric artery lysates from untreated, IQW and LKP treated animals. Data represented as mean \pm SEM from n= 4 animals per treatment group. * indicates $P < 0.05$, as compared to the untreated group. 'ns' indicates not significant compared to the untreated group.

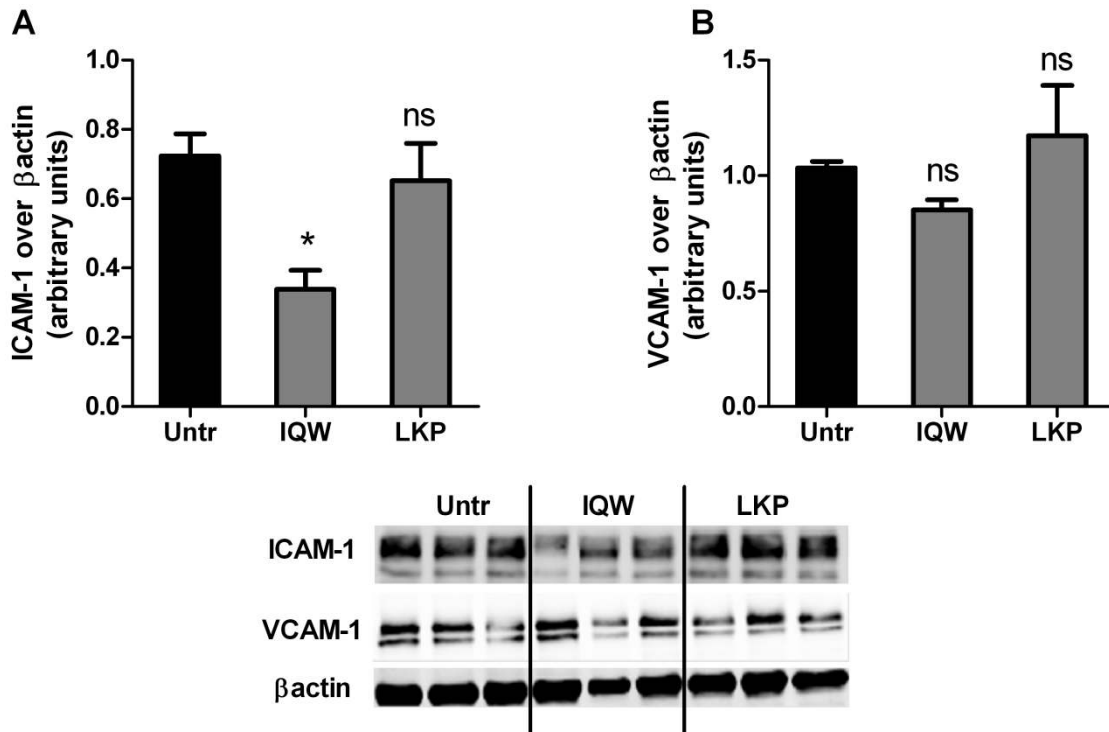
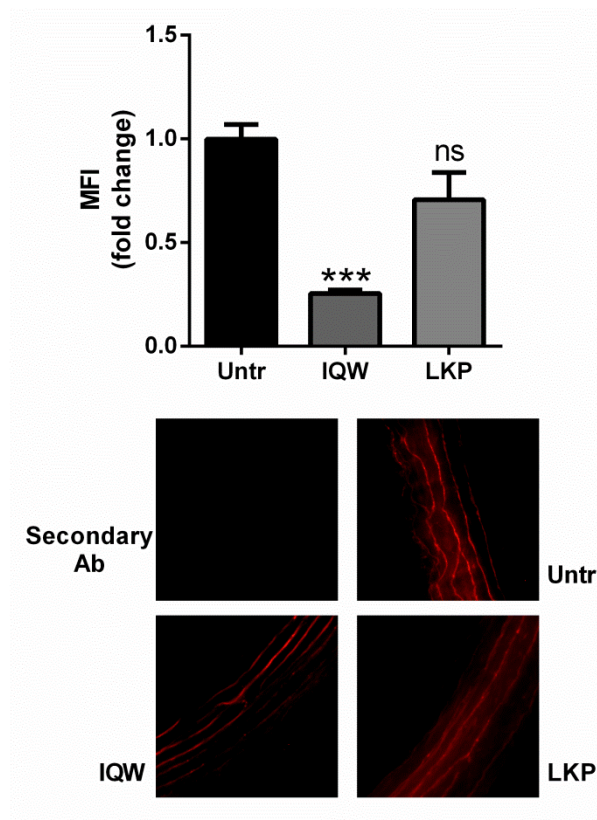


Figure 5.8: IQW but not LKP treatment alters nitrotyrosine levels in SHR vasculature. Immunostaining for nitrotyrosine in aortic sections from untreated, IQW and LKP treated SHRs. Data represented as mean \pm SEM of the mean fluorescence intensity (MFI) from n=4 animals per treatment group. *** indicates $P < 0.001$ compared to the untreated group. 'ns' indicates not significant compared to the untreated group.



CHAPTER 6⁵ - Oral Administration of Egg-derived Tri-peptide IRW Affects the Gene Expression of Spontaneously Hypertensive Rats

⁵ A version of this chapter to be submitted for publication in Molecular Nutrition and Food Research

6.1 Introduction

Hypertension, the persistent increase of systolic/diastolic blood pressure over 140/90 mm Hg, respectively, is a major health concern afflicting around 1 billion people worldwide (WHO, 2013). Hypertension is one of the risk factors for cardiovascular diseases (CVD) such as myocardial infarction and stroke that account for ~30% of global deaths (Chockalingam, 2008; Hall et al., 2012). Since pharmacological antihypertensive therapies are often associated with undesirable adverse side effects (Gavras & Gavras, 1988; Viera & Neutze, 2010), a multifunctional, safer, and easily available therapeutic alternative is urgently needed to alleviate the global burden of hypertension. Many food derived bioactive factors, including food protein derived bioactive peptides derived from milk, fish or egg proteins, may exert antihypertensive activity (Boelsma & Kloek, 2010; Cicero, Gerocarni, Laghi & Borghi, 2011; Ehlers, Nurmi, Turpeinen, Korpela & Vapaatalo, 2011; Turpeinen, Jarvenpaa, Kautiainen, Korpela & Vapaatalo, 2013). Blood pressure is regulated through multiple biological mechanisms; food derived bioactives were reported to function through action on multiple pathways. For example, the underlying mechanisms of antihypertensive action of these peptides are likely due to inhibition of angiotensin converting enzyme (ACE), inhibition of renin, interaction with opioid receptors and concomitant release of nitric oxide. Due to natural origin and perceived lack of adverse side effects, these peptides are considered as safer alternatives to current therapies (Geleijnse & Engberink, 2010; Yamaguchi, Kawaguchi & Yamamoto, 2009).

Eggs are an economical source of dietary protein. Apart from their well-known nutritional value, egg proteins are also a rich source of bioactive peptides, which can be released by enzymatic digestion or food processing (Agyei & Danquah, 2012; Miguel & Aleixandre, 2006; Wu,

Majumder & Gibbons, 2010). Many of these egg protein-derived peptides were initially identified as ACE inhibitors through *in vitro* studies but they actually exhibited multiple mechanisms of antihypertensive action upon *in vivo* administration. For example, our research group had identified an ACE inhibitory peptide, IRW, from egg white protein ovotransferrin, which also exhibited anti-oxidant effect in biochemical assays (Huang, Shen, Nimalaratne, Li, Majumder & Wu, 2012; Majumder & Wu, 2010). *In vitro* cell culture studies, as described in chapter 3 suggested that IRW had additional anti-inflammatory effects and modulated the expression of several adhesion molecules and inflammatory cytokines (Majumder, Chakrabarti, Davidge & Wu, 2013a). The *in vivo* study (chapter4) in spontaneously hypertensive rats (SHRs), a widely used animal model of essential hypertension, demonstrated pronounced antihypertensive effects of orally administered IRW which were likely mediated through several different pathways such as modulation of renin angiotensin system (RAS) through ACE inhibition, reduced vascular inflammation, increased nitric oxide (NO) mediated vasorelaxation through increased expression of endothelial nitric oxide synthase (eNOS) and NO bioavailability by reducing oxidative/nitrosative stress (Majumder et al., 2013b). Similarly, two well-known milk derived antihypertensive peptides IPP and VPP were also reported to have anti-inflammatory effects in adipose tissue of high-fat diet-induced mice (Aihara, Osaka & Yoshida, 2014; Turpeinen, Ikonen, Kivimaki, Kautiainen, Vapaatalo & Korpela, 2012). Lactoferrin derived antihypertensive peptides, GILRPY and REPYEGY, were reported to inhibit angiotensin II-induced vasoconstriction (Fernandez-Musoles, Castello-Ruiz, Arce, Manzanares, Ivorra & Salom, 2013). Based on their multifunctional properties, it is likely that many of these bioactive peptides exert health benefits through modulation of various biologically active proteins at the transcriptional and post-transcriptional levels. Therefore it is important to evaluate the functions

of the bioactive peptides on the transcriptome level to identify the alteration of gene expression that may be responsible for the biological effect.

There are few studies performed to identify the genetic level changes in an intact organism after treatment of bioactive peptides (Yamaguchi, Kawaguchi & Yamamoto, 2009; Yu, Yin, Zhao, Chen & Liu, 2014). Moreover, these studies were performed through DNA microarray or quantitative-real time polymerase chain reaction (qRT-PCR) techniques. Microarray technique is highly target specific and has technical constraints that often fail to analyze the comprehensive and precise characterization of the genome (Valdés, Simó, Ibáñez & García-Cañas, 2014). Therefore the effects of bioactive peptides on global gene expression profiles in intact organisms remain unexplored. An alternative unbiased sequencing technique, termed RNA-sequencing (RNA-Seq) is now available for genome-wide high-throughput analysis of the transcriptome (Valdés, Simó, Ibáñez & García-Cañas, 2014; Wang, Gerstein & Snyder, 2009). The transcriptome is defined as the complete set of RNA produced by the genome at a given moment in a given tissue in a selected organism; it is considered an important link between the phenotype and the information encoded in the genome (Wang, Gerstein & Snyder, 2009). RNA-seq provides opportunities to explore many different aspects of the entire transcriptome and also helpful to quantify the gene expression levels by digital analysis (Valdés, Simó, Ibáñez & García-Cañas, 2014). Thus this technique is helpful to investigate the detailed molecular mechanisms of action behind biological activity of food-derived compounds. Therefore, the main objective of the present study was to investigate the antihypertensive and anti-inflammatory mechanism of egg protein ovotransferrin derived bioactive peptide. Since IRW demonstrated the greatest effects on blood pressure and activated multiple beneficial pathways (as described in

Chapter 4), only this peptide was chosen for further study. The effect of IRW treatment on SHR animals is determined through transcriptome analysis by RNA-Seq technique.

6.2 Materials and Methods

6.2.1 Animal experimental procedures and sample collections

The animal study and experimental procedures are similar to that described in our previous publication (Majumder et al., 2013b). Briefly, 14-15 week old male SHR rats were obtained from Charles River (Senneville, QC, Canada) were surgically implanted with telemetry transmitters (PA-C40; Data Science International Minneapolis, MN) to monitor blood pressure (BP) in real time for 18 days, animals are randomly allocated to 3-treatment groups- untreated (control), IRW (3 mg /Kg BW) and IRW (15mg /Kg BW). Animals were sacrificed via decapitation after the treatment period and tissue samples were collected, weighed, and stored for further analysis. All experimental protocols were reviewed and approved by the University of Alberta Animal care and Use 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. Mesenteric arteries were cut loose from the anterior portion of the abdominal aorta and cleaned of adherent surrounding adipose and connective tissues in sterile ice-cold HEPES-PSS (in mmol/L: NaCl 142, KCl 4.7, MgSO₄ 1.17, CaCl₂ 4.7, K₂PO₄ 1.18, HEPES 10 and glucose 5.5; pH 7.4) solution in a petri dish. The mesenteric arteries were put in *RNAlater* RNA Stabilization Reagent (Qiagen, Toronto, Ontario) after cleaning, whereas kidneys were directly put into RNA Stabilization Reagent and stored in -80°C until further analysis. Our previous study has shown

that antihypertensive effect after oral administration of IRW was only observed in high dose (15 mg/kg BW) group. Similarly, the protein level expression changes were only prominent in kidney and mesenteric arteries (Majumder et al., 2013b); therefore kidney and mesenteric arteries of untreated (Untr) and high dose IRW (IRW-H) treated groups were subjected to transcriptome analysis.

6.2.2 RNA isolation

Kidney tissue samples were ground into fine powder in liquid nitrogen using oven baked (for sterilization), pre-chilled mortar and pestle prior to nucleic acid extraction, whereas mesenteric artery samples were cut into small pieces using RNAlater RNA Stabilization Reagent (Qiagen, Toronto, Ontario) by sterile surgical blades. The tissue samples (~ 80 mg) were first homogenized using a Precellys1 24 homogenizer (Bertin Technologies, Montigny, France) with two cycles of 30 s each at 5500 rpm and 10 s pause in between and the total RNA were then extracted using *mirVana*TM miRNA Isolation Kit (Ambion, Carlsbad, CA), in a phenol-free manner according to the manufacturer's instructions. The quality and quantity of the RNA were determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), respectively. Only the RNA samples with integrity number (RIN) higher than or equal to 7.0 were preceded for RNA-seq library construction.

6.2.3 RNA-seq library construction and sequencing

Total RNA (20 ng/ μ L) from each sample was used to construct RNA-seq libraries with a unique index using the TruSeq mRNA Sample Preparation kit (Illumina, San Diego, CA) and the quantification of the library was performed using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). The libraries were sent to G enome Qu ebec (Montr eal, Canada) for sequencing on a HiSeq

2000 system (Illumina). Sequencing reads were 100 bp paired-end, and they were de-multiplexed according to their index numbers with CASAVA version 1.8 (Illumina). Reads that did not pass the Illumina chastity filter test were removed for further analysis.

6.2.4 RNA-seq reads mapping and annotation

Rat genome (*Rattus norvegicus*, Rnor_5.0) was downloaded from ENSEMBL (<http://uswest.ensembl.org/>) server and RNA-seq reads were aligned to the rat genome by using Tophat 2.0.10 with default parameters (Kim, Pertea, Trapnell, Pimentel, Kelley & Salzberg, 2013). The output mapping files from the TopHat2 alignment, along with the GTF file from ENSEMBL (<http://uswest.ensembl.org/>) rat gene annotation v75.30, were used in the htseq-count (<http://www.huber.embl.de/users/anders/HTSeq/>) to determine the total number of reads mapped to each gene. The abundance of mRNAs in each library was obtained by normalizing reads number to counts per million reads (CPM): $CPM = (\text{gene reads number} / \text{total mapped reads number per library}) \times 1,000,000$. The expressed genes with $CPM > 1$ in at least 60% of the samples were used for further analysis.

6.2.5 Identification of differentially expressed genes

Differential expression of genes between Untr and IRW-H treated groups was then assessed using edgeR bioinformatics tool (Robinson, McCarthy & Smyth, 2010). Only higher abundance genes ($CPM > 5$ in at least in 60% of the samples) were used for the identification of differentially expressed (DE) genes. The significantly DE genes were determined based on Benjamini and Hochberg multiple testing correction test by calculating the false discovery rate (FDR) < 0.05 and fold change (FC) > 1.5 (Benjamini & Hochberg, 1995), which is similar to a previous study by Liang et al. (Liang et al., 2014).

6.2.6 Functional analysis of differentially expressed genes

Ingenuity pathway analysis (IPA, Ingenuity Systems, www.ingenuity.com) was used to analyze the correlation between gene expression and key functions of vascular tissues and kidney. A threshold of value of $P < 0.01$ was applied to enrich significant biological functions. The functions of significantly expressed DE genes with high abundance with presence in rat and mouse were also analyzed. The functional change according to the FC of DE genes and the direction for a given function (increase or decrease) were determined by using the z-score algorithm provided by the IPA server.

6.2.7 Quantitative real time (qRT-PCR) analysis

To verify the DE genes obtained from RNA-seq analysis, qRT-PCR was performed on some selected genes (angiotensin converting enzyme 2: ACE-2, ATP-binding cassette-sub family B:ABCB-1, interferon regulatory factor 8: IRF-8, E-Cadherin: CDH-1, intercellular adhesion molecule 1: ICAM-1 and vascular cell adhesion molecule-1: VCAM-1) using a gene specific primer pairs, designed using Primer Express software (Applied Biosystems, Foster City, CA) (Table 6.1). The total RNA was reverse transcribed to synthesize the first strand and second strand of complementary DNA (cDNA) using SuperScript® II Reverse Transcriptase (Invitrogen by Life Technologies, Foster City, CA, USA). The qRT-PCR was performed with Step OnePlus™ Real-Time PCR System (Applied Biosystems by Life Technologies, Foster City, CA, USA) using the SYBR green chemistry. Thermal cycling was conditioned at 95°C for 20 s for initial denaturation, and then 40 cycled at 95°C for 3 s followed by annealing/extension for 30 s at specific temperature for each primer pair (Table 1), followed by a final melting curve stage (95°C for 15 s – 60°C for 1 min – 95°C for 15 s), with fluorescence signal recording at every

0.3°C intervals. The expression levels were calculated at the cycle threshold values (C_T), where the fluorescent signal was detected above background intensity. The relative gene expression was calculated by using β -actin as a reference gene. Relative gene expression was calculated based on ΔCT and $\Delta\Delta CT$ values, where $\Delta CT = [\Delta CT (\text{Target gene}) - \Delta CT (\beta\text{-actin})]$; and $\Delta\Delta CT = [\Delta CT (\text{IRW-H}) - \Delta CT (\text{UN})]$. The fold changes between the untreated (Untr) and treated (IRW-H) groups were obtained using $2^{-\Delta\Delta CT}$.

The qRT-PCR data were analyzed using GraphPad Prism 5 (GraphPad software, Sandiego, CA) and subjected to unpaired, two-tailed t-test ($n=5-6/\text{group}$) to determine the significant difference, if any, between untreated (Untr) and high dose IRW (IRW-H) treated groups. A P value of <0.05 was considered statistically significant.

6.3 Results

6.3.1 IRW treatment does not alter body and organ weights

The average body weight of the animals at the end of the experimental period (day 18) in the Untr and IRW-H treated groups were 372.74 ± 5.2 g and 369.9 ± 6.1 g, respectively. No significant change in body weight was observed between Untr and IRW-H groups (*Figure 6.1A*). Similarly, no significant differences were observed in the liver, kidney and heart weights (*Figure 6.1B, C, D*) as per the proportion of the body weight at the end of the experimental period between Untr and IRW-H groups

6.3.2 Overview of RNA-seq dataset

A total of 658,605,105 high-quality 100 bp reads were obtained from 23 libraries (12 samples from kidney, 6 Untr and 6 IRW-H, and 11 samples from mesenteric artery, 5 Untr and 6 IRW-H)

with an average of $30 \text{ M} \pm 10\text{M}$ reads per sample. About 82% of reads from kidney and ~87% in mesenteric artery were mapped to the rat genome (*Rattus norvegicus*, Rnor_5.0). In total, 12,764 expressed genes were detected (CPM>1 in at least 60% of the sample) in kidney and 13,352 genes in the mesenteric artery (MA). Among these genes, 11,890 genes were expressed in both kidney and mesenteric artery, while 874 genes were exclusive to kidney and 1,462 genes to mesenteric artery (*Figure 6.2*).

6.3.3 Significantly altered gene expression profiles in response to IRW

Analysis revealed that 17 genes were DE in kidney, 2 of them were down regulated and 15 genes were regulated after IRW-H treatment at \log_2 fold change >1.5. In comparison, 151 genes were DE in mesenteric artery, out of those 37 genes were down regulated (cut off \log_2 fold change >1.5). All the DE genes identified in both tissues are listed in the *supplementary Table 6.1*. DE genes were obviously more pronounced in mesenteric artery than that of kidney in terms of fold change after IRW-H treatment (*Figure 6.3*).

6.3.4 Functions of DE genes in response to IRW

Functional analysis conducted through the IPA server revealed that DE genes after IRW-H treatment can modulate various physiological functions. Briefly, cellular movement, cell to cell signaling and interaction, molecular transport, cellular morphology, and cellular function and maintenance were the top 5 molecular and cellular functions identified by the IPA server in MA. These cellular and molecular functions modulated various diseases and disorders such as inflammatory disease, inflammatory response, metabolic disease, cardiovascular disease, and immunological disease (*Figure 6.4A*). The focus of the current study to evaluate the effect of IRW-H treatment on cardiovascular disease, therefore further analysis was performed to

delineate the functions of the DE genes that can modulate various cardiovascular functions. The results showed that the DE genes from mesenteric artery may be associated with various cardiovascular disease functions such as occlusion of artery, hypertension, and ischemic cardiomyopathy (*Figure 6.4B*). A total of 32 genes were affected in mesenteric arteries that could modulate these various cardiovascular functions, a detailed list of these DE genes and their respective functions in vascular tissues are presented in the *supplementary table 6.2*. Given these results, it is clearly observed the IRW-H treatment is much more effective in the mesenteric arteries rather than kidney; therefore all further experiments were performed only in the mRNA samples isolated from the mesenteric arteries of Untr and IRW-H treated groups.

6.3.5 Target gene validation

Functional analysis showed that DE genes in the mesenteric artery may modulate different cardiovascular functions. The six most significantly DE genes, ACE-2, ABCB-1, CDH-1, IRF-8, ICAM-1 and VCAM-1, were selected for further analysis using qRT-PCR. According to RNA-seq analysis, expression of 4 target genes, ACE-2, ABCB-1, IRF-8 and CDH-1, were significantly up-regulated, while expression of 2 target genes, ICAM-1 and VCAM-1, were significantly down-regulated in the mesenteric artery of the IRW-H group. The potential roles of these genes in modulating cardiovascular disease condition and the impact of IRW treatment are presented in Table 6.2. These 6 genes have significant roles in all of these pathways and significant fold change (increase or decrease) was observed in the mesenteric artery tissues. qPCR results are in alignment with the RNA-seq data (Table 6.2). The expression of ACE-2, ABCB-1, IRF-8, and CDH-1 are up regulated while the expressions of ICAM-1 and VCAM-1 genes are down regulated by IRW treatment (*Figure 6.5*).

6.4 Discussion

Hypertension is termed as “a silent killer” affecting 1 billion people worldwide, contributing to ~ 9 million deaths per year (WHO, 2013). Increased activity of the renin-angiotensin system (RAS) is one of the key factors for the development of hypertension. Moreover, reduced NO bioavailability, inflammation, and oxidative stress also play crucial roles in raising blood pressure and are considered key contributors towards the development of high blood pressure (Salim et al., 2011). The relationship between diet and hypertension has largely been recognized by various epidemiological studies (Batchu, Chaudhary, Wiebe & Seubert, 2013; Craddock, Elmer, Obarzanek, Vollmer, Svetkey & Swain, 2003; Turpeinen, Jarvenpaa, Kautiainen, Korpela & Vapaatalo, 2013). Recent studies have characterized various food bioactive compounds that may be responsible for the treatment and management of hypertension and associated cardiovascular disorders (Al-Shalmani et al., 2011; Batchu, Chaudhary, Wiebe & Seubert, 2013; Fernández-Mar, Mateos, García-Parrilla, Puertas & Cantos-Villar, 2012; Sudano et al., 2006). Most of these compounds are secondary metabolites of plants such as polyphenols and flavonoids (Huntley, 2009, Schramm & German, 1998); food protein derived peptides with blood pressure lowering, cholesterol lowering, anti-thrombotic and anti-oxidant activities have been proven beneficial against CVDs (Aleixandre, Miguel & Muguerza, 2008; Cheung, Nakayama, Hsu, Samaranayaka & Li-Chan, 2009; Martinez-Maqueda, Miralles, Recio & Hernandez-Ledesma, 2012). These bioactive compounds/peptides can activate signaling pathways, which may regulate the expression of specific genes and consequently their translation into the corresponding proteins (Getek et al., 2013). IPP and VPP were reported to increase significantly the expression of eNOS, connexin 40 and cyclooxygenase-1 (COX-1) gene, whereas decreased the expression of nuclear factor kappa B subunit (NF- κ B) and peroxisome

proliferator activator receptor gamma (PPAR γ) gene in SHRs using DNA microarray study (Yamaguchi, Kawaguchi & Yamamoto, 2009). Using real time polymerase chain reaction (RT-PCR) analysis, flavonoids and phenolic compounds from lingonberry and cranberry reduced the expressions of angiotensin-converting enzyme (ACE), cyclooxygenase-2 (COX-2), monocyte chemoattractant protein-1 (MCP-1) and P-selectin genes and exhibit anti-inflammatory and anti-thrombotic effect in SHR (Kivimäki, Ehlers, Siltari, Turpeinen, Vapaatalo & Korpela, 2012). Another study showed that peanut polyphenols can exert hypolipidemic effects by increasing the expression of fatty acid synthase (FAS), sterol receptor element binding protein (SREBP)-1c, acetyl-CoA carboxylase (ACC1) and PPAR γ genes in Wistar rats (Bansode, Randolph, Hurley & Ahmedna, 2012). However, targeting a few specific genes performed all of these studies. Therefore, the effects of bioactive peptides on global gene expression in a given tissue or organ have not been elucidated yet. To the best of our knowledge, this is the first study that explores the effect of oral administration of a bioactive peptide on gene expression profile of vascular tissue through transcriptome analysis.

In the present study we evaluated the effect of IRW treatment on genetic-level alterations of SHR in two different vascular tissues, i.e kidney (a solid organ rich with blood vessels), and mesenteric arteries. Our results reflect the similarities between these two particular tissues as 11890 genes are expressed in both the tissues. Interestingly all the DE genes identified in this study are identified in the common set for both the tissues but only differentially expressed in the mesenteric arteries (MA) after IRW treatment. Additional study also revealed that DE genes identified from MA could modulate functions of the vascular tissues, suggesting a pronounced

effect of IRW treatment on MA. For this reason, we chose to focus more on the DE genes from MA rather than those from the kidney for further analysis.

In a previous study, we had demonstrated that egg protein ovotransferrin derived peptide IRW significantly reduced blood pressure in SHR_s (Majumder et al., 2013b). The antihypertensive effect of IRW is accompanied by ACE inhibitory, anti-inflammatory and anti-oxidant effects, which have been validated through chemical analysis, animal experiments, and cell culture studies (Majumder, Chakrabarti, Davidge & Wu, 2013a). It has been suggested that food derived bioactive peptide can significantly alter the expression of the genes involved in the RAS pathway and exert *in vivo* antihypertensive effects. A study by Yu et al. reported that 4-week treatment of RVPSL, an egg derived peptide, significantly reduced the mRNA expressions of renin, ACE, and AT1 receptor in kidney, and exhibited antihypertensive effects in 14-16 weeks old SHR_s (Yu, Yin, Zhao, Chen & Liu, 2014). In the present study we found that IRW treatment significantly increased the gene expression of ACE-2 but had no effect on other RAS components such as renin or ACE. Earlier studies have demonstrated that ACE-2 can cleave angiotensin-II (Ang-II) to angiotensin₁₋₇ (Ang₁₋₇) and exert vasorelaxation. Moreover, ACE-2 can convert angiotensin-I (Ang-I) to angiotensin₁₋₉ (Ang₁₋₉) prior to the action of Angiotensin converting enzyme (ACE) (Castro-Chaves, Cerqueira, Pinalhao & Leite-Moreira, 2010; Kuba, Imai, Ohto-Nakanishi & Penninger, 2010). Therefore, the antihypertensive effect and the reduction of plasma angiotensin-II level after IRW treatment observed in our previous study could be mediated, at least partially, through increased expression of ACE-2, as revealed in the present study. Similarly, a study by Ehlers et al. concluded that the milk derived ACE inhibitor peptide IPP may exert additional antihypertensive effects through activation of the ACE-2-Ang-(1-7)-Mas axis of RAS system

(Ehlers, Nurmi, Turpeinen, Korpela & Vapaatalo, 2011). However, the authors were not able to demonstrate further conclusive evidence about the role of IPP on the modulation of ACE-2 activity or expression. Therefore, our study documented for the first time that a food-derived bioactive peptide IRW could increase the gene expression of ACE-2 and potentially contribute towards its antihypertensive effect.

Our present study also revealed that IRW reduced gene expression of ICAM-1 and VCAM-1 in the mesenteric arteries. This reduced gene expression indicates suppression of protein upregulation at the transcriptional level which correlates with the reduced ICAM-1 and VCAM-1 observed in our previous *in vivo* study (Majumder, Chakrabarti, Davidge & Wu, 2013, Majumder et al., 2013a). Cellular activation of the pro-inflammatory transcription factor NF- κ B causes nuclear translocation of p65-p50 dimers and thus enhances expression of genes whose protein products (such as ICAM-1 and VCAM-1) mediate leukocyte recruitment under inflammatory condition and subsequently contribute towards vascular inflammation that later leads to atherosclerosis (Giannotti & Landmesser, 2007; Pober, Min & Bradley, 2009). We also demonstrated that IRW treatment inhibited the translocation of both p65 and p50 in endothelial cells (Majumder, Chakrabarti, Davidge & Wu, 2013a) which is in agreement with the present study showing down regulation of ICAM-1 and VCAM-1 gene expression in the IRW-H group. Therefore our study suggests that IRW can inhibit the gene expression of adhesion molecules (ICAM-1 and VCAM-1) likely through inhibiting the translocation of p65/p50 dimers without modulating the gene expression of transcription factor NF- κ B.

Another 2 DE genes are the transporters, CDH-1 and ABCB-1. Expressions of these 2 genes were significantly increased after IRW-H treatment. As a member of ABC (ATP-binding

cassette) proteins, ABCB-1's primary role is to provide a first line of defense at the cellular level by inhibiting entry of potentially harmful compounds into the cell and excreting toxic metabolites out of the cell (Mori et al., 2013). Over expression of ABCB-1 also controls the production of leukocytes and thus can control the inflammatory response (Ieiri, Takane & Otsubo, 2004). It was also reported that overexpression of ABCB-1 might affect lipid homeostasis and could increase the reverse transport of cholesterol (Jeannesson et al., 2009). Therefore upregulation of ABCB-1 could modulate vascular inflammation via differential regulation of leukocytes and could be helpful in destabilization of atherosclerotic plaque through reverse transport of cholesterol (Cucuianu, Coca & Hancu, 2007). Some studies suggest that polymorphisms in this transporter gene might affect individual's susceptibility to ischemic heart disease and atherosclerosis, but the results are still inconclusive (Keskitalo, Kurkinen, Neuvonen, Backman, Neuvonen & Niemi, 2009; Pennings et al., 2006; Wang et al., 2014). Moreover, apart from CVD, increased expression of ABCB-1 gene can also reduce the risk of colitis and inflammatory bowel disease (Onnie et al., 2006), suggesting a potential role of IRW in treating several other disease conditions in addition to CVDs. Another transporter gene, CDH-1 expression was significantly up-regulated by IRW treatment. CDH-1 is a protein belongs to cadherin family whose main function is to help neighboring cells stick to one another (cell adhesion) to form organized tissues (Bex et al., 1995). Up-regulation of CDH-1 could inhibit the hyperplasia of endothelial and vascular smooth muscle cells (VSMC) (Armstrong & Bischoff, 2004; Lee, Dedhar, Kalluri & Thompson, 2006). Similarly, another target gene IRF-8 was significantly up-regulated by IRW, which could also inhibit the hyperplasia of the vascular cells (Zhang et al., 2014). These results suggest that IRW treatment can reduce vascular inflammation as well as the hyperplasia of the vascular smooth muscle cells, both of which predispose to

arteriosclerosis, a complication of long standing hypertension (Cizek, Bedri, Talusan, Silva, Lee & Stone, 2007). Increased inflammation and oxidative stress can also lead to fibrosis and remodeling in vascular tissues. Our previous study demonstrated that IRW treatment significantly attenuated type I collagen levels (Majumder et al., 2013), suggesting a reduction in hypertension induced tissue remodeling. Results from the current study are in alignment with our previous findings as the up-regulation of CDH-1 and IRF-8 genes inhibits hyperplasia, which may leads to the alteration of tissue fibrosis and vascular remodeling. Schematic representations of IRW treatment on gene expressions are illustrated in figure 6.

In conclusion, IRW treatment in SHR shows increased expression of genes for anti-hypertensive and anti-inflammatory mediators in vasculature concomitant to a down regulation in pro-inflammatory genes. These results may explain the mechanisms underlying the anti-hypertensive, anti-inflammatory and improved endothelial functions observed upon IRW treatment *in vivo*. However, further studies incorporating animals from different sex and age groups might be necessary to confirm the beneficial mechanisms and adverse side effects, if any, of this novel peptide. Future trials in humans incorporating various demographic groups are also essential to confirm the clinical efficacy of IRW as a nutraceutical in the prevention and management of hypertension and associated cardiovascular disorders.

6.5 References

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Table 6.1: PCR-primer pair for selective gene

Gene Name	Primer Pair	Temperature	Reference
ACE-2 (Angiotensin Converting Enzyme2)	Forward Primer GCTAAACATGATGGCCCACT Reverse Primer CCCACAGTCGAATTCCTGTT	64°C	Designed and verified in this study
ABCB1 (ATP- binding cassette, sub-family B)	Forward Primer CGTTGCCTACATCCAGGTTT Reverse Primer TGGAGACGTCATCTGTGAGC	62°C	Designed and verified in this study
ICAM-1 (Intercellular adhesion molecule 1)	Forward Primer GAGTCTCCAGCACCAGCAT Reverse Primer GTGCCTACCCTCCCACAACA	64°C	Designed and verified in this study
VCAM-1 (Vascular cell adhesion molecule 1)	Forward Primer GAGTCTCCAGCACCAGCAT Reverse Primer GTGCCTACCCTCCCACAACA	60°C	Designed and verified in this study
IRF8 (interferon regulatory factor 8)	Forward Primer ACGCAGGCAAGCAAGACTACA Reverse Primer AATCCGGGCTCTTGTTTCAGA	62°C	Designed and verified in this study
E-cadherin 1 (CDH1)	Forward Primer GGGTTGTCTCAGCCAATGTT Reverse Primer CACCAACACACCCAGCATAG	64°C	Designed and verified in this study
β-actin	Forward Primer CTAGGCACCAGGGCGTAATG Reverse Primer CCACACGGAGCTCGTTGTAG	60-64°C	(Charavaryamat h et al., 2011)

Table 6.2: Role of Differentially expressed (DE) genes from the mesenteric artery (MA) and the effect of high dose IRW (IRW-H) treatment

Gene Name	Type	Effects on cardiovascular function	Fold Change in RNAseq	Fold Change in qPCR
Angiotensin Converting enzyme 2 (ACE-2)	Enzyme	Down regulation <ul style="list-style-type: none"> • Vasoconstriction • Hyperactivity of Renin angiotensin system 	+23.99 fold	+18 fold
ATP-binding cassette, sub-family B (ABCB-1)	Transporter	Down regulation <ul style="list-style-type: none"> • Vasoconstriction • Leukocyte quantity 	+15.26 fold	+7 fold
Interferon regulatory factor 8 (IRF-8)	Transcription regulator	Up regulation <ul style="list-style-type: none"> • Inhibit hyperplasia 	+9.07 fold	+7 fold
E-Cadherin (CDH-1)	Transporter	Up regulation <ul style="list-style-type: none"> • Inhibit hyperplasia 	+19.73 fold	+24 fold
Intercellular adhesion molecule 1 (ICAM-1)	Trans membrane receptor	Down regulation <ul style="list-style-type: none"> • Inhibit vascular inflammation 	-2.90 fold	-8 fold
Vascular cell adhesion molecule -1 (VCAM-1)	Trans membrane receptor	Down regulation <ul style="list-style-type: none"> • Inhibit vascular inflammation 	-2.92 fold	-8 fold

Note: Fold change after RNAseq analysis was performed by calculating log₂fc (log₂fc=log₂treatment/untreated). Upregulation of gene expression is expressed by '+' sign and down regulation by '-' sign.

Fold change for RNA seq = 2^{log₂fc}

Fold change for qPCR = 2^{-ΔΔCT}, - Δ Δ CT= ΔΔCT = [ΔCT (treatment) – ΔCT (untreated)]

Figure 6.1: IRW treatment does not alter body and organ weights of the SHR. Whole body weight (A) over the treatment period and weights of liver (B), kidney, (C) and heart (D) at end of the 18 day experimental period. Data represented as mean \pm SEM from n=5-6 animals per treatment group.

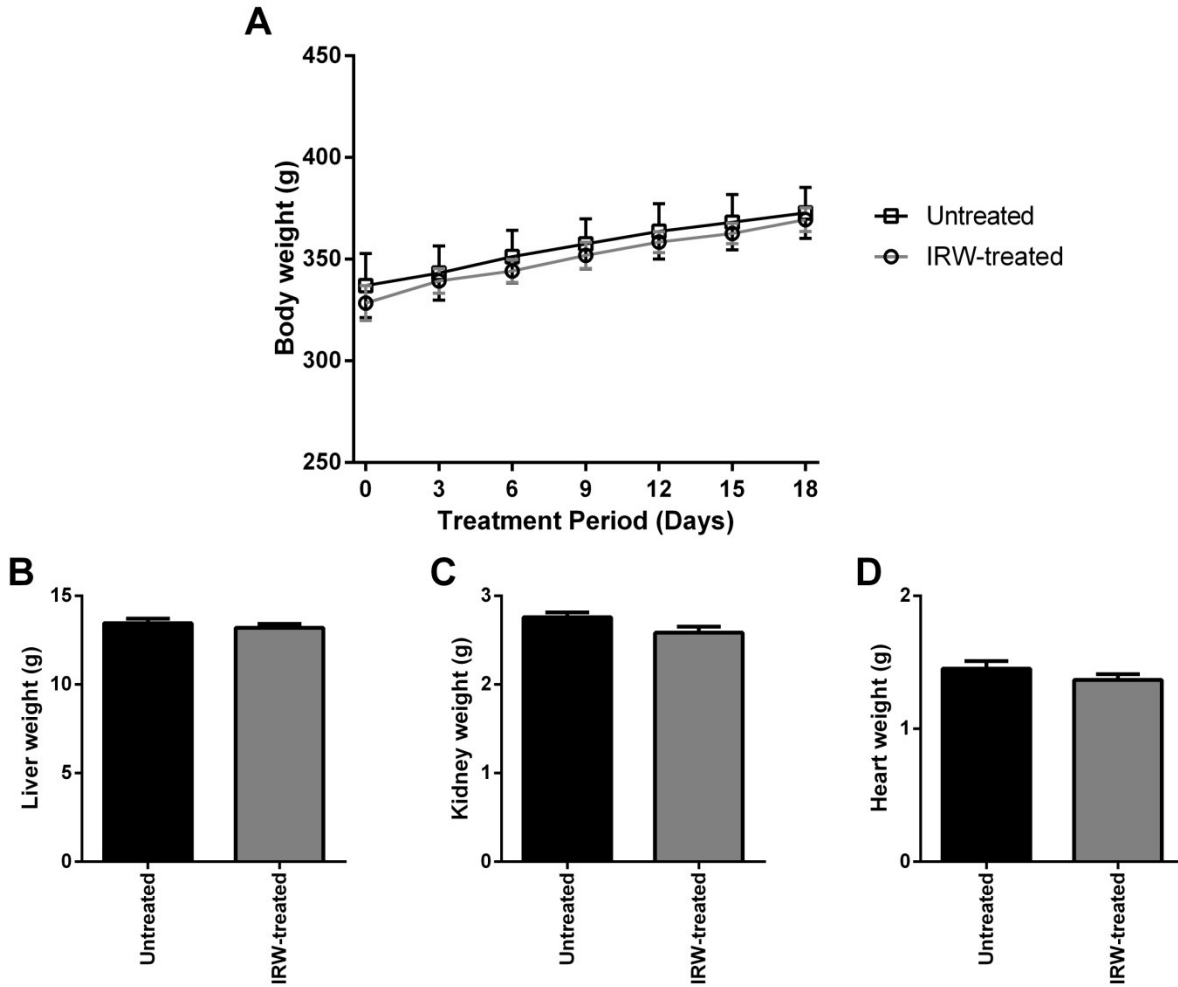


Figure 6.2: Comparison of the number of mRNA detected in kidney (KD) and mesenteric arteries (MA). Total 12764 genes and 13,352 genes were detected in KD and MA, out of that 11,890 genes are common to both the tissues types, 874 genes were exclusive to KD and 1462 genes were exclusive for MA. Data represented as mean \pm SEM from n=6 animals per treatment group for kidney and n=5-6 per treatment for mesenteric arteries.

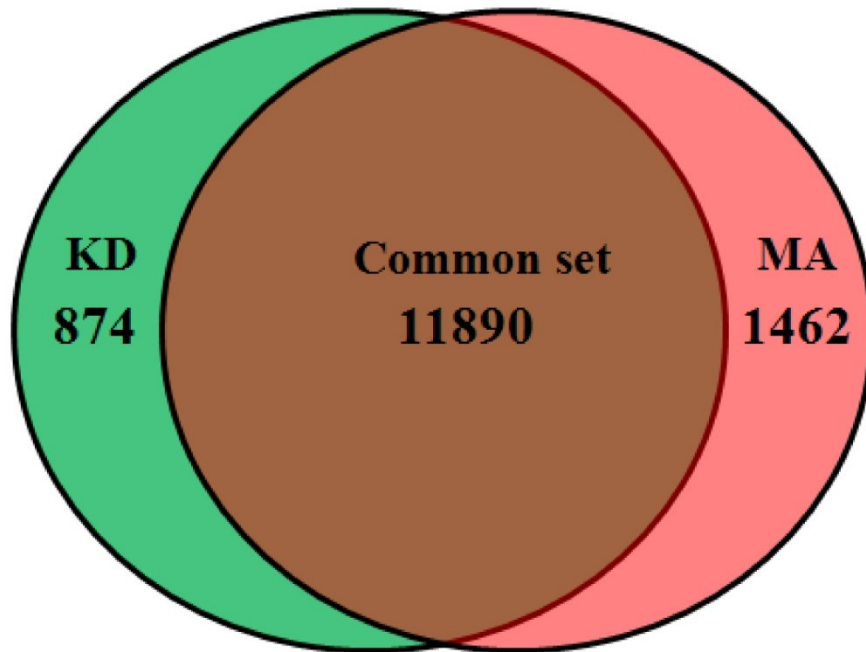


Figure 6.3: Detected differentially expressed (DE) genes in kidney (KD) and mesenteric arteries (MA). The X and Y axes show $\log_2(\text{fold change})$ and $\log_2(\text{normalised reads number})$ of each DE genes, respectively. DE genes detected in the KD shown in green and DE genes detected in MA shown in red. The fold changes of the DE genes from KD are much lesser than gene detected from MA. Data represented as mean \pm SEM from n=6 animals per treatment group for kidney and n=5-6 per treatment for mesenteric arteries. DE genes for kidney ($\log_2\text{fold change}>0$) was used to plot this graph to visualize the expression level difference with DE genes for MA ($\log_2\text{fold change}>1.5$).

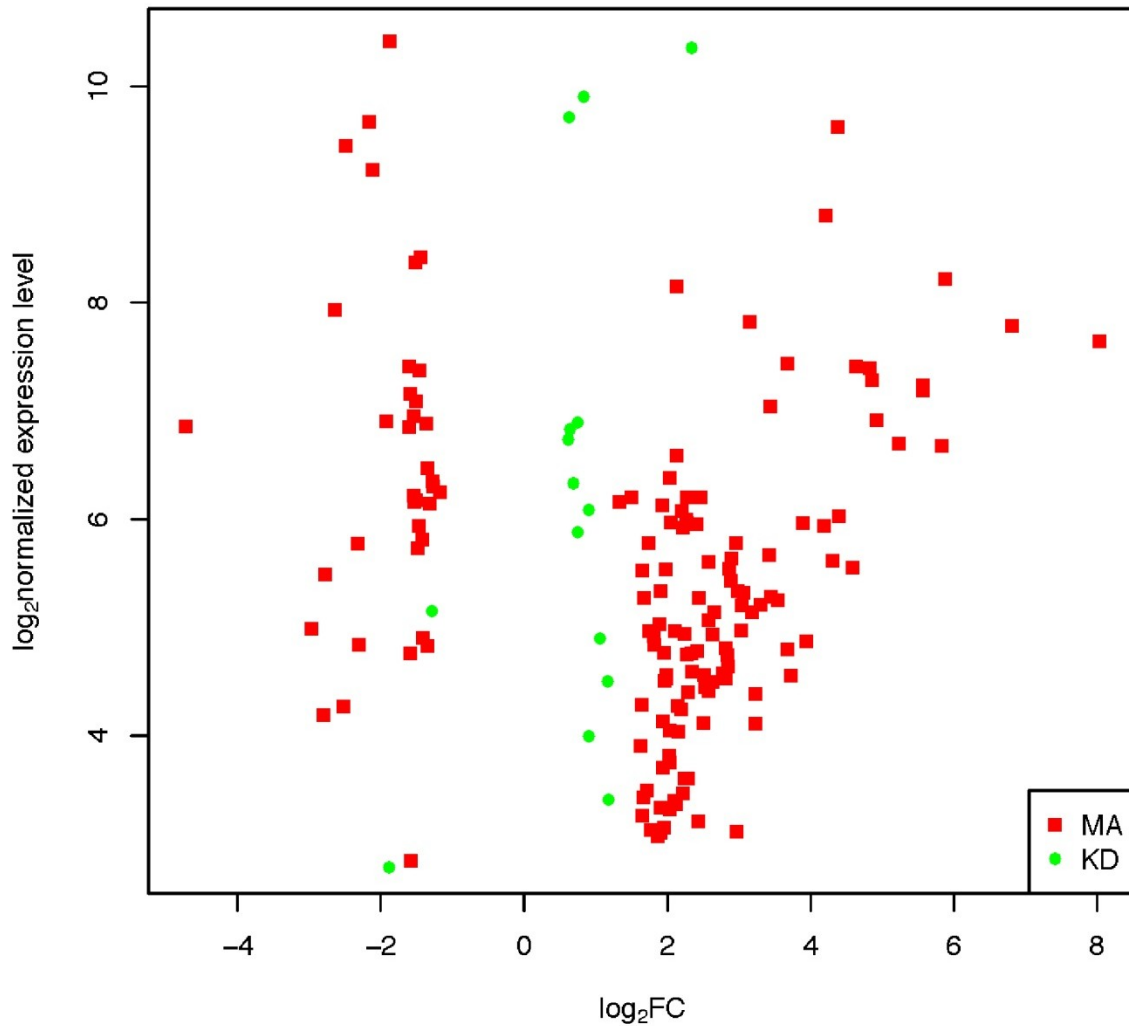


Figure 6.4: Functional role of DE genes from mesenteric arteries (MA). Different functions which can modulate by the DE genes in MA (A), X and Y axes represent $-\log_{10}P$ value and Functions, respectively. The impact of DE genes from MA on cardiovascular disease (B), X and Y axes represent $-\log_{10}P$ value and cardiovascular disease functions, respectively. Results were obtained from IPA analyses from n=5-6 per treatment for mesenteric arteries.

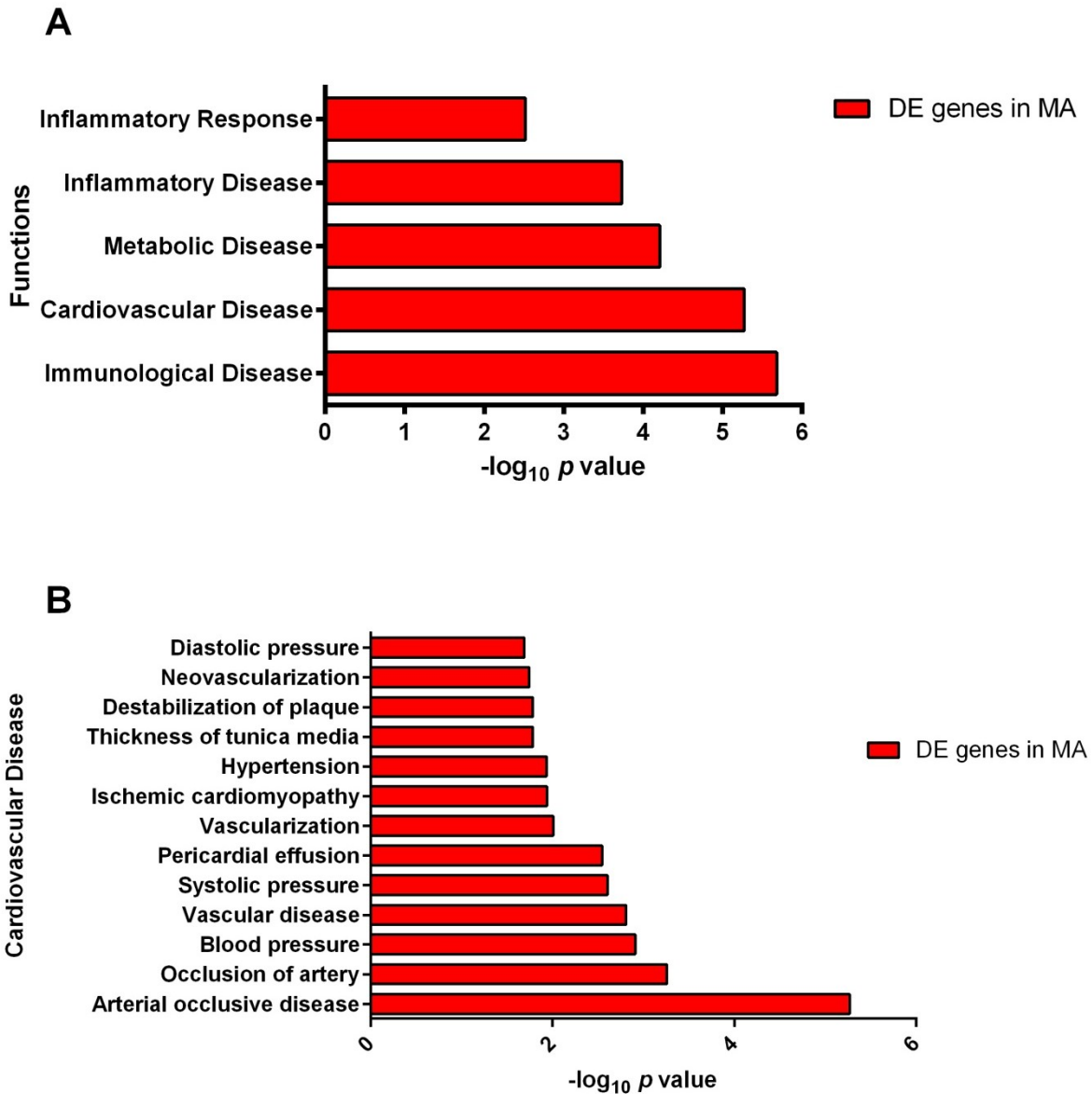


Figure 6.5: Expression of DE genes from mesenteric arteries (MA) detected by RNAseq, validated with qRT-PCR. The expression level of (A) ACE-2, (B) ABCB-1, (C) IRF-8, (D) CDH-1, (E) ICAM-1 and (F) VCAM-1 genes in high dose IRW (IRW-H) treated group were compared to untreated (Untr) group. Y axis represents ΔC_T values for each gene, lower the ΔC_T value indicates higher the gene expression. Data represented as mean \pm SEM from n=5-6 animals per treatment group. * and ** indicate $P < 0.05$, and $P < 0.01$ respectively, as compared to the untreated (Untr) group.

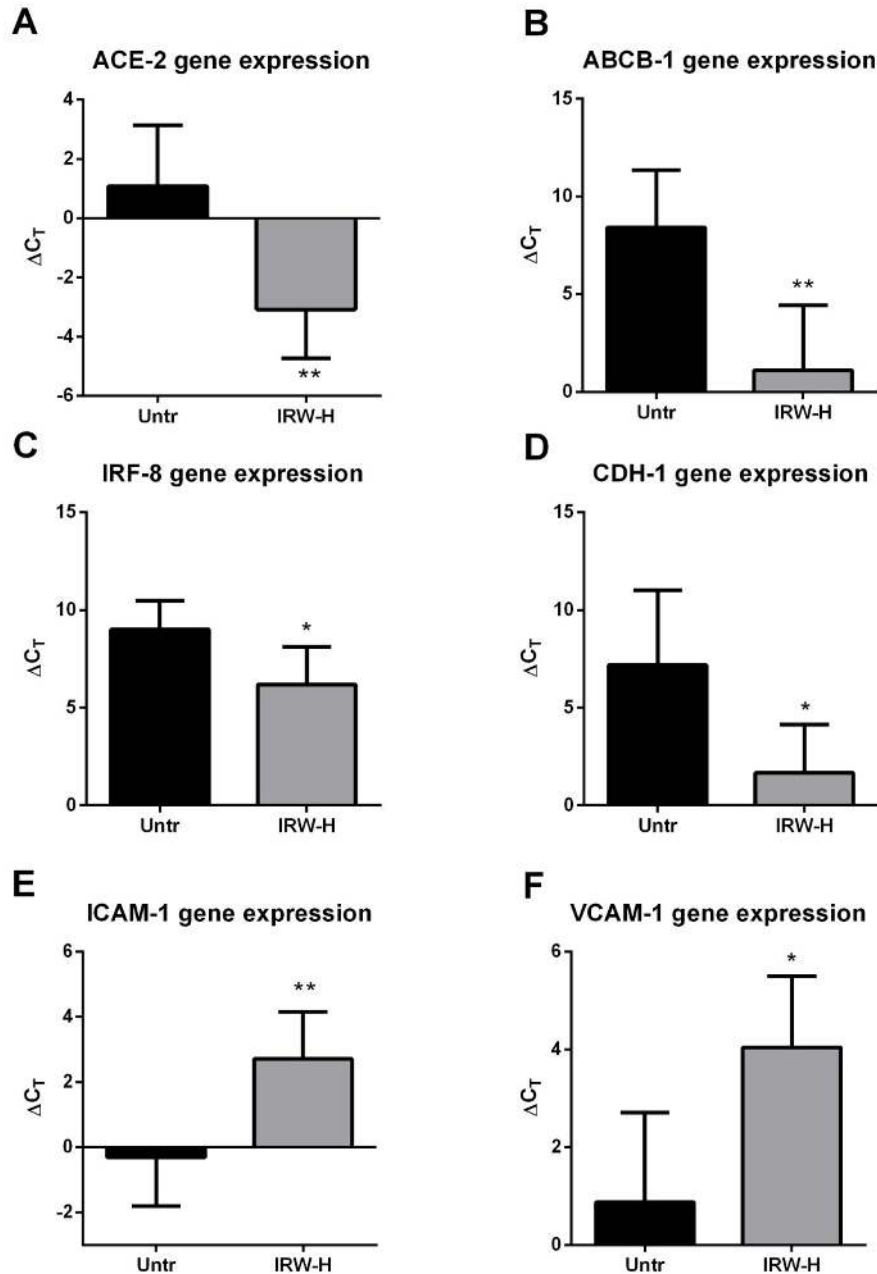
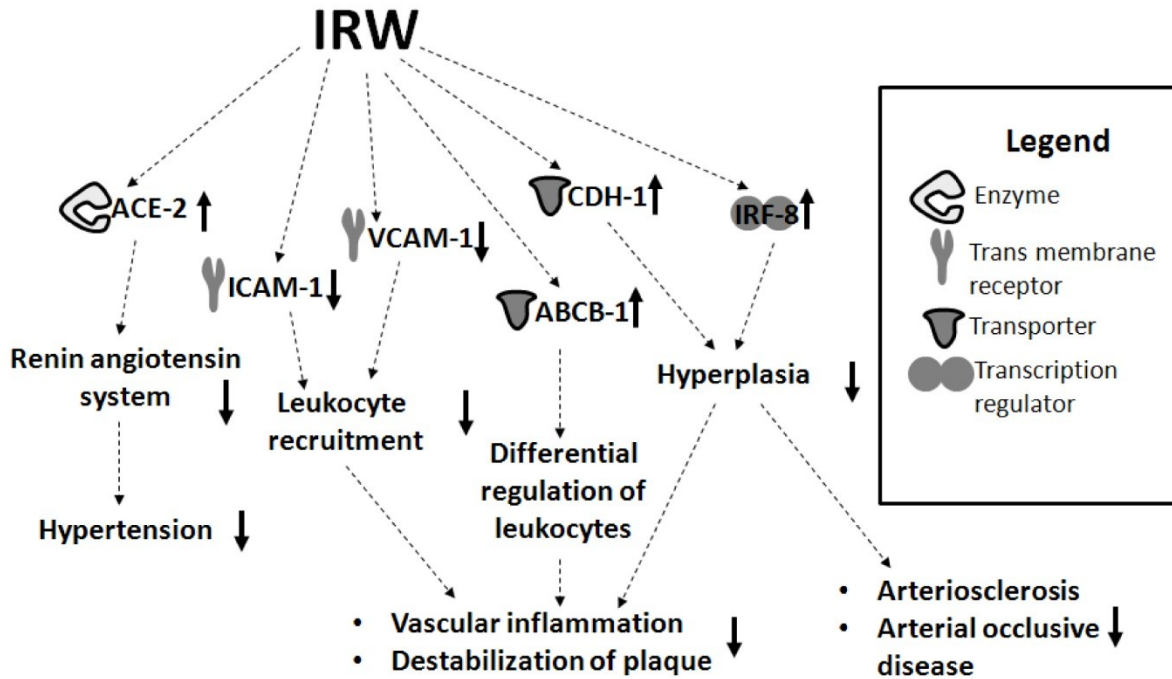


Figure 6.6: A schematic representation of the effect of IRW on various gene expressions and how it can modulate cardiovascular disease function. The DE expressed gene identified from the mesenteric artery of the IRW treated group, further validated through qRT-PCR can modulate various cardiovascular functions. IRW treatment increase the gene expression of angiotensin converting enzyme-2 (ACE-2), which can break down vasoconstrictor angiotensin-II and reduces the activity of renin angiotensin system (RAS), leading to reduced blood pressure. Furthermore, IRW treatment can reduce the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) that decrease the leukocyte recruitment in the vasculature and reduce the risk of vascular inflammation, destabilizing atherosclerotic plaque. Similarly, IRW treatment increased the expression of ATP-binding cassette, sub-family B (ABCB-1) and reduces vascular inflammation through differential regulation of leukocytes. On the other hand, IRW treatment increased the expression of E-Cadherin (CDH-1) and Interferon regulatory factor 8 (IRF-8) that reduces the hyperplasia of vasculature and reduces the risk of arteriosclerosis and associated cardiovascular disorder.



Supplementary Table 6.1

A. Differentially expressed (DE) genes in Kidney after IRW treatment

Gene name	logFC	LogMean	P	FDR
FBJ osteosarcoma oncogene	-1.884748248	2.78261278	4.75E-07	0.001734
Early growth response 1	-1.286228631	5.15001092	0.00011	0.03168
Protein LOC100363408	0.617804868	6.73426499	1.47E-05	0.011636
4-hydroxyphenylpyruvate dioxygenase	0.624236563	9.71159803	0.000164	0.036703
Ribosomal protein L13	0.635150554	6.82969749	2.06E-05	0.015088
Proteasome subunit, beta type 6	0.683308958	6.33278083	6.09E-06	0.006451
Protein Bola2	0.740542874	6.89557646	6.28E-06	0.006451
Glyceraldehyde-3-phosphate dehydrogenase	0.742960786	5.87963012	3.45E-06	0.005056
Glyceraldehyde-3-phosphate dehydrogenase	0.830658247	9.903264	4.94E-06	0.006339
Uncharacterized protein	0.895758435	6.08762135	2.35E-06	0.00403
ArfGAP with dual PH domains 2	0.900480761	3.9933827	1.45E-05	0.011636
Vanin 1	1.055324879	4.89876056	4.37E-05	0.021061
Nitric oxide synthase 3, endothelial cell	1.167685822	4.49935831	6.75E-07	0.001734
Solute carrier family 16, member 6	1.171497997	3.40441626	8.44E-06	0.007885

ENSRNOG00000030478	1.240816372	12.4305464	9.3E-07	0.001911
ENSRNOG00000043866	1.312783667	12.4846917	6.29E-07	0.001734
ENSRNOG00000050614	2.332142771	10.3531364	2.36E-07	0.001734

B. Differentially expressed (DE) genes in Mesenteric artery after IRW treatment

Gene name	logFC	LogMean	P	FDR
Uncharacterized protein	-4.730241059	6.85607846	4.24E-06	0.001896468
Beta globin minor gene	-2.969660414	4.98839547	1.73E-08	6.18166E-05
Beta-globin	-2.80093605	4.18929135	1.50E-06	0.001238174
ENSRNOG00000050614	-2.770843146	5.48580622	1.42E-06	0.001238174
Hemoglobin, alpha 2	-2.641269693	7.9385456	3.53E-07	0.000541426
Kidney androgen regulated protein	-2.525375028	4.27075859	2.26E-05	0.005852268
Hemoglobin alpha, adult chain 2	-2.486128174	9.44672229	1.80E-06	0.001286898
Cytokine inducible SH2-containing protein	-2.321316757	5.77694746	3.89E-05	0.008522965
Cytochrome P450, subfamily 21A, polypeptide 1	-2.303326801	4.8421603	9.47E-08	0.000203218
Neuronatin	-2.163719851	9.66830631	0.000131	0.019652221
Hemoglobin, beta	-2.11795533	9.22255393	5.63E-05	0.010776766
Growth arrest and DNA-damage-inducible, gamma	-1.928714088	6.90716923	3.38E-05	0.008068147

Synuclein, gamma (breast cancer-specific protein 1)	-1.875116937	10.4116357	0.00018	0.023268136
Tubulin polymerization-promoting protein family member 3	-1.607595517	7.4141915	3.94E-05	0.008522965
Spondin 2, extracellular matrix protein	-1.603653932	6.84952838	2.98E-05	0.007266152
NADH-ubiquinone oxidoreductase B9 subunit (Complex I-B9)	-1.596107783	4.75773883	0.000669	0.049680535
Microfibrillar associated protein 5	-1.589438452	7.15913783	5.54E-05	0.010776766
Fatty acid binding protein 3, muscle and heart	-1.57653456	2.84614841	0.000227	0.027360579
Vascular cell adhesion molecule 1	-1.547510031	6.95151531	1.91E-05	0.005257672
Intercellular adhesion molecule 1	-1.538549511	6.21545881	1.16E-05	0.004062404
SRY (sex determining region Y)-box 18	-1.52776583	6.15880993	1.20E-05	0.004062404
Peptidase inhibitor 16	-1.517958896	8.36957402	0.000157	0.022202291
C1q and tumor necrosis factor related protein 9	-1.51080839	6.18149033	3.72E-05	0.008522965
Inhibitor of DNA binding 1	-1.504923486	7.08746204	7.07E-05	0.012620976
Microfibrillar-associated protein 2	-1.485062327	5.73656167	7.30E-05	0.012620976
ENSRNOG00000050514	-1.476739432	5.93777479	0.000614	0.048266859
Indolethylamine N-methyltransferase	-1.45836299	7.37219524	0.000348	0.036575028
Retinol binding protein 7, cellular	-1.452036988	8.41993718	0.000371	0.037514145

Neuron derived neurotrophic factor	-1.417685769	5.81243852	0.00017	0.023268136
Transmembrane protein 88	-1.416876359	4.9066973	0.00034	0.036139788
Glutathione S-transferase mu 2	-1.362095104	6.88587114	0.000447	0.041521791
CD320 molecule	-1.349644602	6.47385196	0.000333	0.035695378
Family with sequence similarity 69, member B	-1.348361952	4.8346431	0.000483	0.043134627
Biogenesis of lysosomal organelles complex-1, subunit 1	-1.318013054	6.1486714	0.000353	0.036737955
Apelin receptor	-1.275596131	6.35378876	0.000362	0.037294486
Cold shock domain containing E1, RNA binding	-1.267133383	6.3030252	0.000573	0.048266859
Receptor (G protein-coupled) activity modifying protein 2	-1.176160512	6.25072598	0.000691	0.049680535
Heat shock protein 1, alpha; heat shock protein 90, alpha (cytosolic), class A member 1	1.332155298	6.16005683	0.000523	0.045982425
ARP2 actin-related protein 2 homolog (yeast)	1.490182407	6.20457775	0.000408	0.039463492
DNA (cytosine-5-)-methyltransferase 3 alpha	1.632962584	3.90455676	0.000642	0.049218868
Chromodomain helicase DNA binding protein 7	1.636359449	4.28719429	0.000492	0.043575434
Ubiquitin carboxyl-terminal hydrolase CYLD; cylindromatosis (turban tumor syndrome)	1.64597993	3.26229707	0.000615	0.048266859
Transferrin receptor	1.657073137	5.52743419	0.000178	0.023268136

GRAM domain containing 1B	1.661300085	3.42734258	0.000695	0.049680535
Pantothenate kinase 3	1.671462449	5.27258918	0.000315	0.034436658
Centaurin-delta 1 (Cnt-d1); ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	1.717285681	3.49177197	0.000469	0.042490155
Cytochrome b-245, beta polypeptide	1.734471363	5.78224406	0.000616	0.048266859
Leucine-rich repeat kinase 2	1.747848455	4.96749926	0.000649	0.049399746
Coxsackie virus and adenovirus receptor	1.775800786	3.13054669	0.000548	0.047429638
Glutamine fructose-6-phosphate transaminase 1	1.808495595	4.91768276	0.000534	0.046528517
StAR-related lipid transfer (START) domain containing 4	1.816733588	4.84002201	5.75E-05	0.010816914
Isopentenyl-diphosphate delta isomerase 1	1.862121878	3.07035287	0.000101	0.016110016
Deoxyribonuclease 1-like 3	1.893417773	5.02731172	0.00069	0.049680535
Senataxin	1.901885811	5.33658054	0.000591	0.048266859
Tumor protein p53 inducible nuclear protein 1	1.905599201	3.10347183	0.000279	0.0318208
NEDD4 binding protein 2	1.908701881	3.33431253	0.000675	0.049680535
RIKEN cDNA D930015E06	1.929975552	6.12655508	0.00043	0.040843333
82-kD FMRP Interacting Protein; similar to mKIAA1321 protein	1.942273589	3.70945334	0.000368	0.037514145

p21 protein (Cdc42/Rac)-activated kinase 1	1.942679158	4.1336389	0.000389	0.03826468
Phosphoinositide-3-kinase adaptor protein 1	1.947791479	4.76602256	0.000618	0.048266859
Piezo-type mechanosensitive ion channel component 2	1.949235298	3.15311879	0.000656	0.049558205
Abhydrolase domain containing 2	1.957384057	4.50361009	0.000174	0.023268136
Dmx-like 1	1.969783973	5.53579861	0.000312	0.034436658
Tnf receptor-associated factor 3	1.98474475	4.56591397	0.000621	0.048266859
SAM domain and HD domain, 1	1.991899772	4.53232224	0.000629	0.048528113
Vacuolar protein sorting 13 homolog A (S. cerevisiae)	2.020348161	3.81663545	0.000601	0.048266859
Pleckstrin homology domain-containing, family A (phosphoinositide binding specific) member 2	2.028682135	3.754657	0.000424	0.040570676
Diacylglycerol kinase, alpha	2.035304398	6.38294882	0.000136	0.020009932
Mucosa associated lymphoid tissue lymphoma translocation gene 1	2.035349609	3.32226203	0.000597	0.048266859
24-dehydrocholesterol reductase	2.035764481	4.0490432	0.000143	0.020519763
Dedicator of cytokinesis 8	2.045065164	5.97428532	0.000101	0.016110016
T-cell activation GTPase activating protein	2.099046875	3.39990665	0.000172	0.023268136
Ras association (RalGDS/AF-6) domain family	2.101810232	4.96981749	0.000139	0.020143552

member 2				
Fyn-related kinase	2.117171118	3.37063634	0.000319	0.034527718
ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	2.128119172	6.5891214	0.00056	0.047654616
Histocompatibility 2, class II antigen E alpha	2.131571534	8.14915061	0.000683	0.049680535
Coiled-coil domain containing 88C	2.147165519	4.27477926	0.000471	0.042490155
laminin, gamma 2	2.150632189	4.03766557	0.000596	0.048266859
Occludin	2.194431611	4.24498182	0.000261	0.030128915
AT-hook transcription factor	2.201501705	6.07796354	0.000461	0.042299981
Family with sequence similarity 151, member A	2.212624917	3.46819778	0.000284	0.031891259
Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1	2.219910475	5.92039956	0.00018	0.023268136
Spleen tyrosine kinase	2.23870834	4.940426	9.86E-05	0.016110016
Phosphoinositide-3-kinase, catalytic, gamma polypeptide	2.241455626	3.60230535	0.000257	0.029914433
Cd53 molecule	2.266834203	5.99227707	0.000374	0.037533425
SMG1 homolog, phosphatidylinositol 3-kinase-related kinase	2.270809058	6.19766964	0.00038	0.037788929
Rho GTPase activating protein 30	2.271514873	5.96702347	0.000285	0.031891259

Protein LOC	2.276231884	4.75390932	9.42E-05	0.015779075
Tenascin C (Tnc)	2.281717482	3.60206646	0.000606	0.048266859
Cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMP-N-acetylneuraminic acid monooxygenase) pseudogene	2.282833362	4.40524187	0.000665	0.049680535
Cathepsin E	2.336403974	4.75920957	0.000591	0.048266859
Sestrin 3	2.345760718	4.5969526	1.33E-05	0.004062404
Serine/threonine kinase 17b	2.410006139	5.95682646	3.48E-06	0.001896468
phospholipase C, gamma 2	2.419266429	4.78201599	6.83E-05	0.012409798
NLR family, pyrin domain containing 1	2.433694237	3.20961354	0.000684	0.049680535
FLJ00354 protein	2.443096981	5.27781052	0.000449	0.041521791
Dedicator of cytokinesis protein 10 (Protein zizimin 3)	2.468980595	6.20523274	6.77E-05	0.012409798
Topoisomerase (DNA) II alpha	2.500834369	4.11834769	8.91E-05	0.015169247
Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	2.51842893	4.5636939	0.000186	0.023317707
Rap guanine nucleotide exchange factor (GEF) 6	2.530366502	4.45474273	4.69E-05	0.009683776
SAM and SH3 domain containing 3	2.56988971	5.61106943	0.000701	0.049822467
Erythrocyte protein band 4.1-like 3	2.572357135	4.40869288	0.000254	0.029914433

Baculoviral IAP repeat-containing 3	2.574595854	5.06639892	0.000102	0.016110016
Dipeptidylpeptidase 4	2.627075492	4.93253808	0.00012	0.018450262
Interleukin 4 induced 1	2.636330568	4.50037143	0.000441	0.041480296
Desmoplakin	2.661077245	5.13970826	7.22E-05	0.012620976
Epithelial stromal interaction 1 (breast)	2.778312163	4.57448288	0.000161	0.022403843
mKIAA0386 protein	2.807237023	4.81183124	2.29E-05	0.005852268
Septin 6	2.813009964	4.53321793	4.91E-05	0.00993813
Nucleoporin 210	2.841756091	4.74501213	0.000217	0.026450594
Macrophage expressed gene 1	2.853375679	4.64564158	0.00019	0.023483082
Cytohesin 1 interacting protein	2.86339943	5.54440458	0.000109	0.016913998
Histocompatibility 2, O region alpha locus	2.88679972	5.43065724	0.000555	0.047618572
Transcription factor 7, T-cell specific	2.890714	5.63809682	0.000187	0.023317707
Cytoplasmic FMR1 interacting protein 2	2.955601913	5.78127791	3.81E-05	0.008522965
Serine/threonine protein kinase MST4	2.96470046	3.11059404	8.18E-06	0.003027131
Lymphocyte protein tyrosine kinase; lymphocyte-specific protein tyrosine kinase	2.975345744	5.33623164	0.000241	0.028721
Pleckstrin and Sec7 domain containing 4	3.032816143	4.97156934	2.02E-05	0.00540412
CD22 molecule	3.037327371	5.20605277	0.000187	0.023317707

Max dimerization protein 1	3.06001954	5.3262907	3.15E-06	0.001896468
Protein tyrosine phosphatase, receptor type, C	3.142270965	7.82409725	3.97E-05	0.008522965
Interferon regulatory factor 8	3.182594567	5.139105	4.16E-06	0.001896468
FYN binding protein (FYB-120/130)	3.226914444	4.38708617	7.97E-06	0.003027131
integrin alpha 4	3.23672061	4.1139067	1.31E-05	0.004062404
Chromosome 10 open reading frame 64	3.297903327	5.21450605	1.24E-05	0.004062404
ArfGAP with coiled-coil, ankyrin repeat and PH domains 1	3.418506772	5.66861934	4.41E-05	0.009283224
Serine (or cysteine) proteinase inhibitor, clade B, member 1a	3.433317373	7.04311962	2.35E-06	0.001575787
Protein tyrosine phosphatase, receptor type, C-associated protein	3.441143647	5.28000462	0.000132	0.019652221
Organic solute transporter alpha	3.543918	5.25244859	0.000399	0.038925968
keratin 8	3.676611213	7.43507826	5.48E-05	0.010776766
Class II transactivator	3.677392846	4.80034197	3.39E-06	0.001896468
Solute carrier family 23, member 2	3.721633709	4.55102166	1.42E-06	0.001238174
Tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	3.893405864	5.96289528	1.22E-06	0.001238174
ATP-binding cassette, sub-family B (MDR/TAP),	3.931917205	4.8704263	3.72E-06	0.001896468

member 1				
ENSRNOG00000030506	4.18720889	5.94031647	7.57E-06	0.003006883
Immunoglobulin kappa constant	4.216432529	8.80883042	1.38E-05	0.004097413
Cadherin 1	4.302928332	5.61395967	1.67E-05	0.004836426
Hypothetical protein LOC678701	4.37562488	9.62532669	3.70E-06	0.001896468
Transmembrane 4 L six family member 4	4.389935003	6.02821542	4.13E-06	0.001896468
Angiotensin I converting enzyme 2	4.584574234	5.55163249	4.81E-07	0.000644771
Fc fragment of IgG binding protein	4.632760091	7.41405043	1.81E-05	0.005102252
Immunoglobulin joining chain	4.826557749	7.39982322	2.53E-07	0.000452458
Apolipoprotein A-IV	4.852540668	7.28405532	1.25E-05	0.004062404
Epithelial cell adhesion molecule	4.923948117	6.9174606	5.53E-06	0.00228125
Zymogen granule protein 16	5.223635082	6.70011537	2.84E-05	0.007077016
Fatty acid binding protein 1, liver	5.561678534	7.24001313	1.73E-06	0.001286898
Mucin 2	5.561831677	7.1905816	4.46E-06	0.001913068
Cadherin 17	5.832826799	6.68015765	5.71E-07	0.000680321
Apolipoprotein A-I	5.878393375	8.22189805	6.88E-08	0.00018455
Fatty acid binding protein 2, intestinal	6.812856213	7.78497909	1.81E-09	9.69936E-06

Chloride channel accessory 4	8.03300465	7.64111585	1.03E-10	1.10657E-06
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Note:

*logFC= \log_2 fold change = \log_2 (treated/untreated)

logMean= (Mean values of treatment + Mean values of untreated)/2

P= Probability of obtaining a test statistic result

FDR= False Discovery Rate (adjusted p value for multiple comparison)

Note: Gene name indicates in 'RED-Colour' represents Up-regulation and 'GREEN Colour' represents down-regulation

Supplementary Table 6.2

Differentially expressed genes identified in the mesenteric artery modulating cardiovascular functions

Gene abbreviation	Name	Location	Type	Functions	logFC
Kap	kidney androgen regulated protein	Extracellular Space	other	diastolic blood pressure	-2.52537503
HBA1/HBA2	hemoglobin, alpha 1	Cytoplasm	transporter	Blood pressure, abnormal morphology of vascular system	-2.48612817
VCAM1	vascular cell adhesion molecule 1	Plasma Membrane	transmembrane receptor	Attachment of endothelial cells	-1.54751003
ICAM-1	intercellular adhesion molecule 1	Plasma Membrane	transmembrane receptor	Attachment of endothelial cells	-1.53854951
SOX18	SRY (sex determining region Y)-box 18	Nucleus	transcription regulator	Attachment of endothelial cells	-1.52776583
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	Nucleus	transcription regulator	Vasculogenesis	-1.50492349
GSTM1	glutathione S-transferase mu 1	Cytoplasm	enzyme	Vasculogenesis	-1.3620951
APLNR	apelin receptor	Plasma Membrane	G-protein coupled receptor	Contractility of cardiac muscle, cardiogenesis	-1.27559613
RAMP2	receptor (G protein-coupled) activity modifying protein 2	Plasma Membrane	transporter	Vasculogenesis, cardiogenesis, blood pressure	-1.17616051
<u>CHD7</u>	chromodomain helicase DNA binding protein 7	Nucleus	enzyme	Vasculogenesis, cardiogenesis	1.63635945
<u>CYLD</u>	cylindromatosis (turban tumor syndrome)	Nucleus	transcription regulator	Migration of endothelial cells	1.64597993
<u>TFRC</u>	transferrin receptor	Plasma Membrane	transporter	Cardiogenesis	1.65707314
<u>CYBB</u>	cytochrome b-245, beta polypeptide	Cytoplasm	enzyme	Blood pressure	1.73447136
<u>LRRK2</u>	leucine-rich repeat kinase 2	Cytoplasm	kinase	Blood pressure	1.74784845
<u>CXADR</u>	coxsackie virus and adenovirus receptor	Plasma Membrane	transmembrane receptor	Cardiogenesis	1.77580079
<u>PAK1</u>	p21 protein (Cdc42/Rac)-activated kinase 1	Cytoplasm	kinase	Angiogenesis	1.94267916

<u>DGKA</u>	diacylglycerol kinase, alpha 80kDa	Cytoplasm	kinase	Vasculogenesis, angiogenesis, movement of endothelial cells	2.0353044
<u>PIK3CG</u>	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma	Cytoplasm	kinase	Attachment of endothelial cells	2.21991047
<u>SYK</u>	spleen tyrosine kinase	Cytoplasm	kinase	Occlusion of artery, attachment of endothelial cells	2.23870834
<u>CD53</u>	CD53 molecule	Plasma Membrane	other	Peripheral occlusion of artery	2.2668342
<u>KCNN4</u>	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	Plasma Membrane	ion channel	Blood pressure	2.51842893
<u>BIRC3</u>	baculoviral IAP repeat containing 3	Cytoplasm	enzyme	Morphology of cardiovascular system	2.57459585
<u>DPP4</u>	dipeptidyl-peptidase 4	Plasma Membrane	peptidase	Movement and migration of endothelial cells	2.62707549
<u>DSP</u>	desmoplakin	Plasma Membrane	other	Cardiogenesis	2.66107724
<u>CYTIP</u>	cytohesin 1 interacting protein	Cytoplasm	other	Occlusion of artery	2.86339943
<u>MXD1</u>	MAX dimerization protein 1	Nucleus	transcription regulator	Migration of endothelial cells	3.06001954
<u>IRF8</u>	interferon regulatory factor 8	Nucleus	transcription regulator	Hyperplasia of vascular cells, occlusion of artery	3.18259457
<u>FYB</u>	FYN binding protein	Nucleus	other	Occlusion of artery	3.22691444
<u>ITGA4</u>	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	Plasma Membrane	transmembrane receptor	Occlusion of artery	3.23672061
<u>ABCB1</u>	ATP-binding cassette, subfamily B	Plasma Membrane	Transporter	Occlusion of artery, blood pressure	3.9319172
<u>CDH-1</u>	E-Cadherin	Plasma Membrane	transporter	Hyperplasia of vascular cells	4.30292833
<u>ACE2</u>	angiotensin I converting enzyme 2	Plasma Membrane	peptidase	Blood pressure, Contractility of cardiac muscle	4.58457423

Note: Gene name indicates in 'RED-Colour' represents Up-regulation and 'GREEN Colour' represents down-regulation

CHAPTER 7 – General Discussion and Future Directions

7.1 Foundation of present research

Cardiovascular diseases (CVD) are the major causes of mortality in the present century (WHO, 2011). Hypertension or high blood pressure is a well-defined risk factor for the development of CVD (WHO, 2013). Currently, application of pharmaceutical drugs is the main therapeutic approach employed for the treatment of hypertension, which is however, considered to be the most expensive component of the total healthcare cost (Public health agency, Canada 2011). In addition, pro-longed use of these antihypertensive drugs is associated with significant adverse side effects (Hall et al., 2012; Zaman, Oparil & Calhoun, 2002). Therefore, functional foods or nutraceuticals have gained tremendous research attention as alternative strategies for the treatment and management of hypertension. Bioactive peptides from food have been previously demonstrated beneficial effect against CVD, such as peptides with anti-hypertensive, cholesterol-lowering, anti-thrombotic, anti-inflammatory, and anti-oxidant activities (Balti, et al., 2012; Turpeinen, et al., 2009). Thus, food derived bioactive peptides exhibiting multiple bioactive functionalities can counteract the disease progression at multiple points. These bioactive peptides were identified from food protein through extensive activity-guided purification method, which is labor-intensive and costly. Under such circumstances, we developed a fast and cheap *in silico* digestion and quantitative structure and activity relationship (QSAR) based method to predict potent bioactive peptides with angiotensin converting enzyme (ACE) inhibitory activity (Majumder & Wu, 2010). ACE is one of the key enzymes of renin angiotensin system (RAS) that can modulate blood pressure in human body (Michel, 2004). Through this newly developed method, our previous study identified three tri-peptides IRW (Ile-Arg-Trp, molecular weight:

473.58 Da), IQW (Ile-Gln-Trp, molecular weight: 445.51 Da) and LKP (Leu-Lys-Pro, molecular weight 356.46 Da) from enzymatic digestion of the ovotransferrin with *in vitro* angiotensin converting enzyme (ACE) inhibitory properties (Majumder & Wu, 2010). IRW, IQW, and LKP were successfully purified from ovotransferrin hydrolysate through multi-step chromatographic purification, comprising of cation exchange chromatography and reverse-phase high performance liquid chromatography (RP-HPLC), and their sequences were analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; (Majumder & Wu, 2011). The main aim of the present research was to evaluate the efficacy of these peptides in more biologically and physiologically (*ex vivo* and *in vivo*) relevant systems and to elucidate the molecular mechanisms of action of their observed activities. The work also contributed to the elucidation of the structural characteristics and additional bioactivity of these peptides. Furthermore, our work has provided a potential platform for the utilization of egg proteins by the functional foods and the nutraceutical industries.

7.2 Key findings of the present research

The key findings of this thesis are outlined below:

1. Cardiovascular diseases are often initiated by the disruption of the vascular homeostasis. Endothelial cells (EC) have some definite physiological functions by which they regulate vascular homeostasis or vascular balance. Increased oxidative stress and vascular inflammation are the key mediators of endothelial dysfunction. Ang II and TNF are the most abundant pro-inflammatory compounds that can trigger the inflammatory response and generation of superoxide ($O_2^{\cdot-}$) in the endothelial cells. ACE inhibitors were shown previously to decrease the markers of inflammation as well as can reduce the increased levels of oxidative stress (Brown & Vaughan, 1998; Mertens, Vanderheyden, Michotte &

Sarre, 2010; Soehnlein et al., 2005). Therefore the main objective of the first experimental study of this thesis was to evaluate the antioxidant or anti-inflammatory properties of ovotransferrin-derived ACE inhibitory peptides (IRW, IQW, and LKP) in endothelial cells. To achieve this specific objective, HUVECs were used as a model system and the anti-inflammatory and anti-oxidant properties of these three peptides (IRW, IQW, and LKP) were evaluated on TNF induced HUVEC monolayers. The results from this study indicated that pre-treatment of IRW (50 $\mu\text{mol/L}$) could significantly inhibit the TNF-induced vascular inflammation via down-regulating the expression of ICAM-1 and VCAM-1. However, IQW (50 $\mu\text{mol/L}$) treatment only significantly down-regulated the expression of ICAM-1, not VCAM-1. Both IRW (50 $\mu\text{mol/L}$) and IQW (50 $\mu\text{mol/L}$) exhibited anti-oxidant effect as shown by reduction of TNF-induced superoxide generation. The anti-inflammatory effects of IRW and IQW appeared to be mediated by the NF- κB pathway. IRW treatment inhibited the translocation of p65 and p50, but interestingly IQW treatment only inhibited the translocation of p50 but not p65. Further study revealed that the structural integrity of these peptides (IRW and IQW) was essential for their anti-inflammatory and anti-oxidant activities, since dipeptides or the combination of constituent amino acids did not exhibit the same effect. Therefore the first experimental study (Chapter 3) of this thesis demonstrated that IRW and IQW possess anti-inflammatory and anti-oxidant activity.

2. Efficacy of IRW as a novel anti-hypertensive agent was evaluated on a well-characterized animal model of hypertension; SHR. SHR develops persistent hypertension at an early age (~12-14 weeks) and remains hypertensive throughout their lives (Trippodo & Frohlich, 1981; Zicha & Kunes, 1999). Moreover, adult SHR (16-17 weeks) show

increased RAS activity, increased levels of oxidative stress in the vasculature and a pronounced pro-inflammatory phenotype (Liu, 2009; Reaves, Beck, Wang, Raizada & Katovich, 2003; Sriramula, Cardinale, Lazartigues & Francis, 2011; Wu & Juurlink, 2002). The *in vivo* effects of IRW were evaluated through oral administration in adult male SHR. The results showed that high dose (at 15 mg/kg BW) treatment of IRW over a period of 18 days significantly reduced mean arterial pressure (MAP) by 40 mmHg compared to the untreated animals. Reduction of blood pressure at high dose IRW was accompanied by the restoration of circadian variations in blood pressure. Impaired circadian variations or nocturnal dipping can contribute to various cardiovascular events, such as myocardial ischemia, acute myocardial infarct, and sudden cardiac death (COCA, 1994; Portaluppi, Tiseo, Smolensky, Hermida, Ayala & Fabbian, 2012; Shimamura, Nakajima, Iwasaki, Hayasaki, Yonetani & Iwaki, 1999). Therefore, restoration of nocturnal dipping by IRW treatment might be helpful to prevent associated cardiovascular complications and end organ damage. The *ex vivo* isolated vessel (mesenteric artery) experiments suggested that IRW treatment did not affect the PE induced vascular constriction but significantly enhanced the MCh induced vascular relaxation. MCh mediated vascular relaxation is multifactorial, earlier research had shown the involvement of NO, prostaglandins and endothelial derived hyperpolarizing factor (EDHF) (Morton, Rueda-Clausen & Davidge, 2010). Among these, NO is greatly affected by increased oxidative stress and is impaired in SHR (Bagnost et al., 2010). NO is a relatively stable gas and one of the major products of endothelium cells through NOS. The role of nitric oxide (NO) in MCh-dependent relaxation on vessels was studied in the presence/absence of the NOS inhibitor L-NAME. The experiment revealed that,

incubation with the NOS inhibitor L-NAME did not alter vasodilation in the untreated and low dose IRW groups, but vasorelaxation in the high dose animals was significantly decreased, suggesting NO dependent vasorelaxation was restored after high dose IRW treatment. Subsequently, it was also found that high dose IRW treatment upregulated eNOS expression in mesenteric arteries, which could be the reason for enhanced NO mediated relaxation indicating the role of NO in vascular relaxation after IRW treatment. Furthermore, high dose IRW treatment also reduced angiotensin II (Ang II) and increased bradykinin levels in blood plasma, indicating that IRW could act as an ACE inhibitor in an *in vivo* system. The previous chapter demonstrated that IRW treatment reduces TNF-mediated upregulation of ICAM-1 and VCAM-1 in an endothelial cell culture system. A similar effect was observed in animals; IRW decreased expression levels of the inflammatory adhesion molecules (ICAM-1 and VCAM-1) in vascular tissues. This phenomenon was accompanied by decreased levels of cytokines/chemokines IL-6 and MCP-1 in blood plasma, suggesting generalized anti-inflammatory effects of IRW *in vivo*. Our study further showed that high dose IRW could reduce nitrotyrosine levels in both aorta and kidneys, suggesting an anti-oxidant effect of IRW on these tissues. Therefore, chapter 4 demonstrated that IRW treatment could reduce the blood pressure in hypertensive animals and the *in vivo* anti-hypertensive effects of orally given IRW appear to be mediated through several different pathways, such as increased NO mediated vasodilation, regulating RAS through ACE inhibition and reducing vascular inflammation. Moreover, IRW could restore the impaired circadian variations of blood pressure in these hypertensive animals.

3. After evaluating the *in vivo* effect of IRW, it was imperative to evaluate the efficacy of two other bioactive peptides (IQW and LKP) identified from egg white protein ovotransferrin. The same animal model, SHRs was used for this experiment at a dosage of 15 mg/kg BW. The results showed that both IQW and LKP treatment decreased mean blood pressure (MAP) by 19 mmHg and 30 mmHg respectively compared to untreated; though both IQW and LKP showed antihypertensive effect but the reduction of blood pressure is much more greater in IRW treatment (40 mmHg) . Plasma Ang II level was reduced after these treatments, indicating the ACE inhibitory activity of these peptides. Vessel function studies revealed that IQW and LKP treatment did not affect the PE vessel constriction but significantly enhanced MCh induced vessel relaxation. While incubation with the NOS inhibitor L-NAME had no effect in the untreated groups but vasorelaxation in both the IQW and LKP treated groups were significantly enhanced compare to the L-NAME treated vessels, indicating the crucial role of NO in this process. But unlike IRW, either IQW or LKP treatment did not altered eNOS expression in mesenteric arteries. Furthermore IQW but not LKP reduced the expression of inflammatory adhesion molecule ICAM-1 expression and nitrotyrosine levels in arteries, suggesting additional protective effects against inflammation and oxidative/nitrosative stress. Therefore, the present study (Chapter 5) demonstrated that orally administered IQW and LKP significantly lowered the blood pressure in adult male SHR animals. However, the anti-hypertensive effect of IQW and LKP appear to be mediated through similar pathways involving increased NO mediated vasodilatation and regulating RAS through ACE inhibition, with some additional beneficial effects mediated by IQW with anti-inflammatory and anti-oxidant effect.

4. Results from Chapter 4 and 5 exhibited that IRW is the most effective anti-hypertensive peptide among these three peptides. Therefore IRW was chosen for further study. In the last part of the thesis (Chapter 6), to investigate the gene expression profile, mRNA was extracted from the tissues (kidney and mesenteric arteries) of both IRW (high dose: 15 mg/kg BW) treated and untreated SHR. Previous studies had shown that bioactive compounds/peptides might exert *in vivo* effect through differential gene expression (Kivimäki, Ehlers, Siltari, Turpeinen, Vapaatalo & Korpela, 2012). The differentially expressed (DE) genes were identified by comparison of gene expression (mRNA fold change) in tissues from both untreated and IRW treated animals through RNA-sequencing. Initial results revealed that genes from mesenteric arteries showed greater changes on IRW treatment (compared to those from the kidney) and many of these affected genes could modulate cardiovascular functions. Further functional studies with Ingenuity pathway (IPA, Ingenuity Systems, www.ingenuity.com) server revealed that DE genes from mesenteric artery could significantly alter various cardiovascular disease functions. Most importantly, the study identified that IRW treatment up-regulates the expression of ACE-2. In addition to cleaving Ang-II to Ang₁₋₇ and exerting vasorelaxation, ACE-2 can also convert Ang-I to Ang₁₋₉ prior to the action of ACE (Castro-Chaves, Cerqueira, Pintalhao & Leite-Moreira, 2010; Kuba, Imai, Ohto-Nakanishi & Penninger, 2010). Therefore the up regulation of ACE-2 mRNA expression suggests that IRW treatment could exhibit vasorelaxation by reducing the amount of vasoconstrictor Ang-II in blood plasma of the SHR. Additionally, IRW treatment also significantly reduced the expression of ICAM-1 and VCAM-1, which is perfectly aligned with the previous findings described in chapters 4 and 5. Furthermore, quantitative

polymerase chain reaction (q-PCR) results further validated the findings of RNA-sequencing data and exhibited that IRW treatment could significantly increase (18 fold) the gene expression of ACE-2 and significantly decrease (7-8 fold) the gene expression of ICAM-1 and VCAM-1. Therefore, the final experimental study (Chapter 6) demonstrated that the possible mechanism of anti-hypertensive action of IRW treatment.

In conclusion, the research from this thesis demonstrated that egg protein ovotransferrin derived peptides (IRW, IQW, and LKP) possess anti-hypertensive properties in an adult male rodent model of human essential hypertension. IRW is the most potent candidate among these three peptides tested; this peptide shows anti-inflammatory, anti-oxidant, and ACE-2 up regulatory properties that might contribute to its blood pressure lowering effect. IRW reduced Ang-II and increased bradykinin levels in blood, indicating possible ACE inhibitory effect of IRW; its decreased Ang-II level could also be due to degradation of Ang-II by increased ACE-2 expression, decreased Ang-II level leads to vasodilation (*Figure 7.1A*). Similarly, IRW also ameliorated endothelial dysfunction by inhibiting vascular inflammation (reduced expression of ICAM-1 and VCAM-1) and reducing oxidative stress (superoxide level), which would also promote vasodilation (*Figure 7.2B*). Our research contributes to our understanding about the underlying mechanisms of action of ACE inhibitory peptides in blood pressure lowering effect. Further studies are, however, required to establish the clinical efficacy and safety of these peptides for long-term use in humans.

7.3 Recommendations for future research

Based on the results of present research, the recommended further studies are listed below:

1. While the present study validates the anti-hypertensive properties of these peptides in a rodent model, further research is required to outline the limitations of these peptides.
 - a. Further studies with a longer treatment period involving younger animals and also in animals of both sexes are required to determine the preventive effects, if any, of these peptides.
 - b. It is also important to thoroughly study the bioavailability or bio-accessibility of these peptides.
 - c. A detailed study involving hypertensive animals from different age groups is required to evaluate the half-life and the pharmacokinetics of these peptides. Results from this study will be helpful to selectively choose different dosage requirements for different purpose.
 - d. A long-term (over a period of months) study involving both normotensive and hypertensive animals are essential to evaluate the chronic toxicity profiles of these peptides.
2. Apart from animal study, it is also essential to evaluate the interaction of these peptides with different food matrices. This result will provide the necessary information about the feasibility for food industries about the large-scale production and practical application of these peptides in the human food system.
3. Finally, human clinical trials with hypertensive as well as normotensive volunteers involving different ethnic group are essential to judge the ultimate efficacy of these peptides for further application for the human society.

Results from these recommended studies will further justify the use of these peptides as an anti-hypertensive agent and will be helpful to commercialize functional foods or nutraceuticals product for the prevention and management of hypertension.

7.4 References

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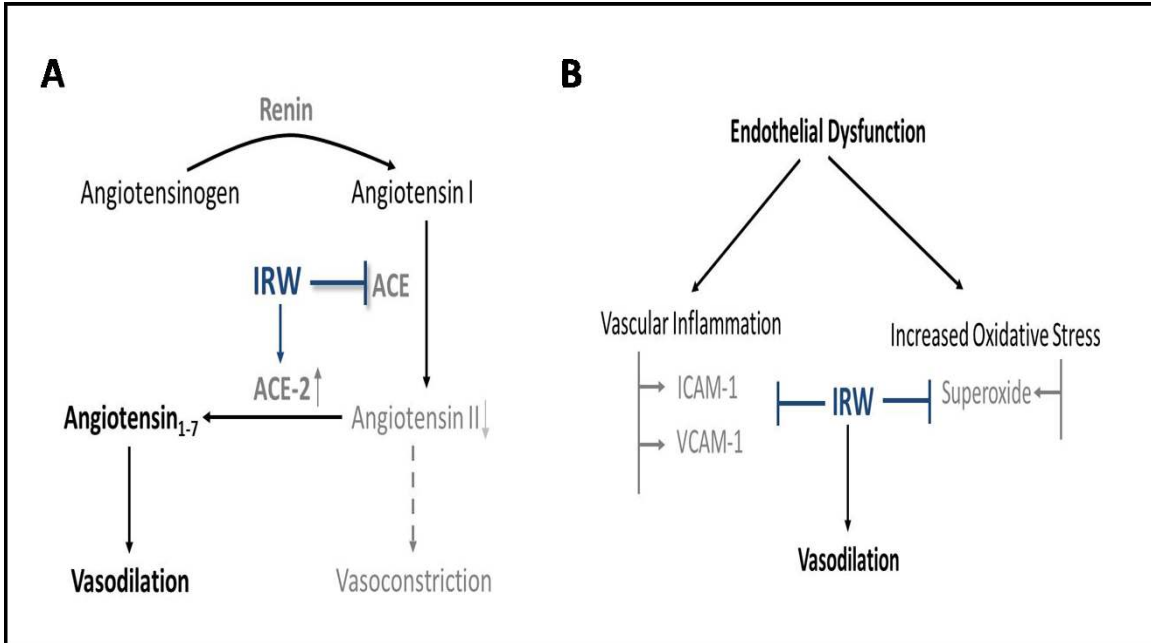
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Figure 7.1: A schematic representation of the vasodilatory effects of IRW. (A) In the renin angiotensin system IRW inhibits the action of ACE and reduces the production of Ang-II, IRW also increases the expression of ACE-2 that further converts ACE-2 to Ang₁₋₇ and exerts vasodilation. (B) IRW inhibits the expression of inflammatory adhesion molecule ICAM-1 and VCAM-1 and also reduces the formation of superoxide, thus exerts vasodilation by modulating endothelial function.



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