The Regulatory Roles of Peptide IRW on angiotensin-converting enzyme 2 (ACE2) in High-Fat Diet-Induced Mice and Spontaneously Hypertensive Rats

by

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Abstract

Metabolic syndrome (MetS) refers to a cluster of various risk factors commonly associated with cardiovascular diseases (CVDs) and diabetes. Up-regulation of angiotensin-converting enzyme 2 (ACE2), a key member of the renin-angiotensin system (RAS), is generally protective against CVDs and diabetes. IRW, a tripeptide derived from ovotransferrin, was previously studied to reduce blood pressure in spontaneously hypertensive rats (SHRs) via a mechanism of up-regulation of ACE2 in artery. This peptide was also able to improve glucose intolerance in a high-fat diet (HFD)-induced insulin-resistant mouse model, but ACE2 was not affected in the skeletal muscle, the main tissue of glucose metabolism.

Study #1 of the thesis was to investigate the regulatory role of the peptide IRW on aortic ACE2 and its associated signaling pathways in HFD-induced insulin-resistant mice.

In the aorta of HFD mice treated with IRW (at a dose of 45 mg/kg body weight), ACE2 level was significantly increased, while the abundance of angiotensin II receptor (AT1R) and angiotensin-converting enzyme (ACE) was significantly reduced, in comparison to the HFD group. IRW supplementation also improved the abundance of glucose transporter 4 (GLUT4) and the expression of AMP-activated protein kinase (AMPK), Sirtuin 1 (SIRT1), and endothelial nitric oxide (eNOS) in IRW-treated mice. Moreover, IRW down-regulated the inflammatory pathways of endothelin-1 (ET-1) and p38 mitogen-activated protein kinases (P38 MAPK). ACE2 knockdown in vascular smooth muscle cells (VSMCs) reduced the levels of AMPK and eNOS. Our results indicate that the up-regulation of aortic ACE2 by the peptide IRW in HFD-induced insulin-resistant mice is responsible for activating pathways associated with vasodilation of blood vessels, potentially contributing to the improvement of insulin resistance and glucose metabolism.

Study #2 was aimed to understand the regulatory role of peptide IRW on epithelial cadherin (E-cadherin), a transmembrane glycoprotein that enables specific cell-cell adhesion, and its interactions with ACE2. Our previous transcriptome analysis showed that gene expression of E-cadherin was significantly elevated in IRW-treated SHRs. Up-regulation of E-cadherin has been linked to reduced hyperplasia, anti-apoptotic, anti-migratory, and anti-epithelial-mesenchymal transition (EMT) effects in the vasculature. In SHRs, IRW treatment also increased the protein level of E-cadherin, while that of Zinc finger protein SNAI1 (Snail1) was decreased. Similar trend was also observed in VSMCs. Treatment with IRW led to a notable reduction in Orai Calcium Release-Activated Calcium Modulator 1 (Orai1) levels in cells, although no significant change was observed in SHRs. In VSMCs, both angiotensin II (Ang II) stimulation and ACE2 knockdown resulted in a significant decrease in E-cadherin levels. The expression of E-cadherin was significantly increased when VSMCs were treated with either Tamoxifen or IRW. Tamoxifen is commonly utilized for the treatment of breast cancer due to its ability to activate the E-cadherin, thereby causing an increase in the expression of E-cadherin mRNA and protein levels.

Taken together, our studies supported that peptide IRW up-regulates ACE2 in artery to exert its beneficial signaling pathways in both animal models.

PREFACE

This thesis is an original work by Fatemeh Ashkar and has been written as per the guidelines given by the Faculty of Graduate Studies and Research, University of Alberta. The concept of the research work in this thesis is originated from my supervisor Dr. Jianping Wu. Research grants of this thesis were funded by Natural Sciences and Engineering Research Council (NSERC) of Canada, Egg Farmers of Canada, and Global Egg Corporation to Dr. Wu. The figure in this thesis is prepared and drawn by Fatemeh Ashkar. The experimental protocol for the animal study was approved by the Animal Care and Use Committee at the University of Alberta (Protocol # 1402) and (Protocol # AUP 00001571) in accordance with the guideline issued by the Canada Council on Animal Care. The thesis consists of five chapters. Chapter 1 provides a general introduction and the objectives of the thesis. Chapter 2 is a literature review on several subjects, including metabolic syndrome and hypertension and their association with RAS in response to IRW. Chapter 3 has been published as "Tripeptide IRW Improves AMPK/eNOS Signaling Pathway via Activating ACE2 in the Aorta of High-fat-Diet-Fed C57BL/6 Mice" in Journal of Biology. Chapter 4 evaluates effect of tripeptide IRW on E-cadherin/snail signaling pathway through ACE2. Chapter 5 provides overall conclusions and discussion with its significance in health management for chronic diseases treatment.

Dr. Jianping Wu greatly contributed to the experimental design, data interpretation, thesis preparation, and edits. Dr. Khushwant Singh Bhullar has assisted me in experimental design and editing (Chapters 3). Dr. Ilekuttige Priyan Shanura Fernando assisted me in the cytoplasmic measurement of calcium (Chapters 4). Mr. Xu Jiang provided technical support in animal studies including animal surgery, animal care and isolation of mesenteric arteries. I was responsible for literature search relevant for the above studies, designing and preforming experiments, data collection and analysis, and drafting the thesis.

DEDICATIONS

This thesis is dedicated to my beloved family and friends.

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LIST OF ABBREVIATIONS

ACE: Angiotensin-converting enzyme

ACE2: Angiotensin-converting enzyme 2

AHA: American Heart Association

AMPK: AMP-activated protein kinase

Ang-(1–7): Angiotensin-(1-7)

Angiotensin II: Ang II

ANOVA: Analysis of variance

AT1R: Angiotensin II Receptor

AT2R: Angiotensin II Receptor

BCA: Bicinchoninic acid

Ca^{2+}: Calcium

CβS: Cystathionine-β-synthase

CVDs: Cardiovascular diseases

DMEM: Dulbecco's modified Eagle medium

E-cadherin: Epithelial cadherin

ECM: Extracellular matrix

EGFR: Epidermal growth factor receptor

EMT: Epithelial-mesenchymal transition

eNOS: Endothelial nitric oxide synthase

ER: Endoplasmic reticulum

ER-α: Estrogen receptor-α

ERK1/2: Extracellular signal-regulated kinase

EPC: Endothelial progenitor cells

ESCC: Esophageal squamous cell carcinoma

ET-1: Endothelin-1

FBS: Fetal bovine serum

FGFR: Fibroblast growth factor receptor

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GLUT4: Glucose transporter 4

HDL: High-density lipoprotein

HFD: High-fat diet

HOMA-IR: Homeostatic Model Assessment for Insulin Resistance

HSMCs: Human aortic smooth muscle cells

IP3R: Inositol-3-phosphate receptor

IRS-1: Insulin receptor substrate 1

LDL: Low-density lipoprotein

MAPK: Mitogen-activated protein kinase

MasR: Mas receptor

MetS: Metabolic syndrome

MMPs: Matrix metalloproteinases

N-cadherin: Neural cadherin

NCEPATP III: National Cholesterol Education Program Adult Treatment Panel III

NF-κB: Nuclear factor kappa B

NO: Nitric oxide

Orai1: Orai Calcium Release-Activated Calcium Modulator 1

PASMCs: Pulmonary artery smooth muscle cells

PI3K: Phosphoinositide-3-kinase

PKC: Protein Kinase C

PTCs: Proximal tubular cells

PPARγ: Peroxisome proliferator-activated receptor gamma

qPCR: Quantitative PCR

RAS: Renin- angiotensin system

ROS: Reactive oxygen species

SIRT: Sirtuin 1

SHRs: Spontaneously hypertensive rats

SHRSP: Spontaneously Hypertensive Rats-Stroke Prone

SHHRs: Spontaneously Hypertensive and Hyperlipidemic rats

Snail1: Zinc finger protein SNAI1

SOCE: Store-operated calcium entry

STIM1: Sensor stromal interaction molecule 1

T-cadherin: Truncated cadherin

T2DM: Type 2 diabetes mellitus

TLR-4: Toll-like receptors

TGF-β: Transforming growth factor beta

TNF-α: Tumor necrosis factor-α

VCAM-1: Vascular cell adhesion molecule

VSMC: Vascular smooth muscle cells

CHAPTER 1- GENERAL INTRODUCTION AND THESIS OBJECTIVES

1.1 General Introduction

Metabolic syndrome (MetS) has become a significant issue in public health and is characterized by combination of factors including abnormal glucose metabolism, increased central fat, raised blood pressure, and abnormal cholesterol levels (Zimmet et al., 2005). It has been revealed that about 25% of people around the globe suffer from MetS (Prasad et al., 2012). In preclinical animal studies, high-fat diets (HFD) are frequently employed to induce obesity and insulin resistance, both of which are closely linked to MetS (Aoqui et al., 2014; Elzinga et al., 2021). HFD has been associated with the initiation of MetS, which might be the result of oxidative stress and long-term inflammation caused by lipids. These alterations can result in cardiovascular problems, including endothelial dysfunction (Panchal et al., 2011).

Hypertension could be linked to proliferation and migration of vascular smooth muscle cells (VSMCs) (Lacolley et al., 2012; Liao et al., 2018b; Mills et al., 2020). Prolonged and significant elevation in blood pressure results in vascular remodeling and impairment of vascular function (Belo et al., 2015). Remodeling process could be accompanied by abnormal growth, inflammation, exposure to oxidative elements, and migration of the cells (Touyz and Schiffrin, 2000).

The Renin-Angiotensin System (RAS) is vital for the development of hypertension. Angiotensin converting enzyme (ACE) is the enzyme responsible for the production of angiotensin II (Ang II), a known vasoconstrictor which causes the constriction of blood vessels, resulting in increased blood pressure (Liao et al., 2019). Angiotensin-converting enzyme 2 (ACE2) works in opposition to ACE, breaking down Ang II into non-hypertensive components such as angiotensin-(1-7) (Ang-(1-7)) (Liao et al., 2018a). Research has demonstrated that ACE2 helps counteract the negative impacts of Ang II, providing anti-hypertensive, anti-oxidant and anti-inflammatory benefits within and beyond the cardiovascular system (Liao et al., 2018a). The presence of MetS, characterized by hyperglycemia, hypertension, hyperinsulinemia, and obesity, leads to an elevation in the expression of RAS components, specifically the Ang II- angiotensin II receptor (AT1R) axis (Jahandideh and Wu, 2020).

Extensive research has been conducted on the possibility of food derived bioactive as an alternative to pharmaceuticals for various diseases (Majumder et al., 2013b). IRW (Ile-Arg-Trp), a tri-peptide with ACE inhibitory activity, was found in the protein from egg white called ovotransferrin (Majumder et al., 2013a; Son et al., 2018). Previously, we observed no significant differences in ACE2 expression in skeletal muscle of HFD mice that were treated with IRW. However, IRW

enhanced ACE2 expression in the aorta of spontaneously hypertensive rats (SHRs). On the other hand, IRW improved glucose hemostasis and insulin sensitivity in the skeletal muscle of insulin resistance model (de Campos Zani et al., 2022). Further, IRW has been shown to have anti-inflammatory and antioxidant properties, which could be beneficial in the prevention of endothelial dysfunction, a major contributor to the occurrence of cardiovascular diseases (CVDs) (Majumder et al., 2013a). The impact of IRW on RAS specifically aorta ACE2 and their contribution with signaling pathways in the aorta of HFD-induced obese mouse model is still unknown.

Previous research has also demonstrated that IRW treatment is able to enhance the expression of the CDH-1 gene in mesenteric artery, thereby lessening the likelihood of hyperplasia of blood vessels and the related CVDs (Majumder et al., 2015). Also, ACE2/Ang-(1-7)/ Mas receptor (MasR) pathway has the ability to impede the function of transcription factors known as Snail1, which have been associated with the progression of epithelial-mesenchymal transition (EMT) (Loh et al., 2019; Yu et al., 2016). It was revealed that activation of the ACE2/Ang-(1-7)/Mas pathway can hinder the metastasis of cancer cells by suppressing Store-Operated Calcium Entry (SOCE) and increasing the expression of epithelial cadherin (E-cadherin) (Yu et al., 2016). Further research is necessary to deeper understanding of the association of E-cadherin with ACE2/Ang-(1–7)/Mas axis influencing proliferation and migration.

1.2 Hypothesis and objectives

Since it has been demonstrated that the peptide IRW increases the expression of aortic ACE2 in SHRs, but not the expression of ACE2 in the skeletal muscle of an HFD-induced insulin-resistant mouse model, we hypothesized that IRW regulates ACE2 and its associated signaling pathways in the aorta of the HFD-induced insulin-resistant mouse model.

Since the ACE2/Ang-(1-7)/MasR pathway has been shown to inhibit the activity of transcription factors associated with the development of EMT, proliferation, and migration, we hypothesized that the ACE2/Ang-(1-7)/Mas pathway is involved in regulating E-cadherin and its related pathways.

The specific objectives of the research are:

1. To investigate the regulatory effect of IRW on RAS, particularly aortic ACE2 and their association with insulin signaling pathway and endothelial function in the HFD-induced obese mice model.

2. To identify the impact of IRW on E-cadherin and investigate their interaction with the ACE2/Ang-(1–7)/Mas axis and related regulatory pathways involved in vascular remodeling, proliferation, and migration *in vivo* and *in vitro.*

1.3 Chapter format

The thesis consists of five chapters, each with a brief description as follows:

Chapter 1 provides an overview of the MetS and proliferation and migration related to hypertension, and their interaction with RAS, as well as the potential influence of IRW in mitigating these conditions. Additionally, the thesis hypothesis and objectives are outlined.

Chapter 2 presented a literature review that is relevant to this thesis. It covers topics including MetS, hypertension, the roles of ACE2/Ang-(1-7)/MasR axis in regulating metabolic complications, endothelial functions, and proliferation. Additionally, the chapter explored the molecular mechanisms of bioactive peptide IRW against MetS, vascular remodeling, and migration.

Chapter 3 investigated the effectiveness of IRW on RAS in the aorta of mice with insulin resistance induced by HFD. The supplementation of IRW (45 mg/Kg body weight) to the HFD mice improved the level of ACE2 in the aorta, implying that the aorta might be a specific target of IRW that could affect ACE2 activity in MetS. Furthermore, when ACE2 was knocked down in VSMCs, a significant reduction in the markers phospho AMP-activated protein kinase (p-AMPK) and phospho endothelial nitric oxide synthase (p-eNOS) was observed, suggesting that ACE2 plays a regulatory role in AMPK and eNOS. Our results indicated that IRW increased ACE2 levels and alleviated AT1R receptor effects via multiple signaling pathways in the mouse aorta, including ACE2/AMPK/ Sirtuin 1(SIRT1)/eNOS, ACE2/AMPK/ glucose transporter 4 (GLUT4), and ATIR/endothelin-1(ET-1)/ p38 mitogen-activated protein kinases (P38 MAPK).

Chapter 4 studied the effect of IRW on E-cadherin, Zinc finger protein SNAI1 (Snail 1), intracellular calcium (Ca^{2+}) concentration, and Orai Calcium Release-Activated Calcium Modulator 1 (Orai1) signaling pathways related to vascular remodeling, proliferation, and migration. In IRW treated group (15 mg/kg body weight for 7 days), Snail1 expression decreased while E-cadherin expression increased compared to untreated group, with no major changes to Orai1 expression proposing the modulatory role of IRW on hypertension complications. Ang II, ACE2 knockdown, and A779 (MasR antagonist) reduced the E-cadherin levels in VSMCs,

showing that IRW treatment could regulate E-cadherin pathways by targeting ACE2/Ang-(1-7)/Mas axis in vitro.

Chapter 5 presents the conclusion and discussions of this thesis, as well as the limitations and potential opportunities for future research.

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CHAPTER 2-LITERATURE REVIEW

2.1. Metabolic syndrome (MetS) definition and epidemiology

MetS is a combination of disorders that can increase an individual's risk of developing cardiovascular disease (CVD), insulin resistance, diabetes mellitus, as well as vascular and neurological complications (Swarup et al., 2021). According to the National Cholesterol Education Program Adult Treatment Panel III (NCEPATP III), MetS is defined as the presence of three or more of the following risk factors: obesity (high waist circumference), high triglyceride concentration (>150 mg/dL or drug treatment), low high-density lipoprotein (HDL) cholesterol (<40 mg/dL in men and <50 mg/dL in women), high blood pressure (>130 mmHg systolic or >85 mmHg diastolic) and fasting blood glucose (>100 mg/dL) (Hernández-Camacho and Hernández-Camacho, 2017). MetS has emerged as a worldwide epidemic and a major public health concern (Wang et al., 2020).

Recent research has shown that a quarter of people throughout the world have suffered from MetS (Prasad et al., 2012). It is generally believed that MetS is highly prevalent in western countries, estimated that over 70% of the adult population has at least one clinical symptom of MetS (Panagiotakos and Polychronopoulos, 2005). Within a decade, the global obesity rate will rise to 16.2 billion people, and the number of people suffering from type 2 diabetes mellitus (T2DM) is expected to double (Genser et al., 2016; Yarizadeh et al., 2022).

2.2. Hypertension definition and epidemiology

Hypertension is a major preventable risk factor for CVD and all-cause mortality around the globe (Mills et al., 2020). Hypertension is diagnosed when a person's systolic/diastolic blood pressure is higher than 130/80 mmHg, rather than the conventional threshold of 140/90 mmHg (Whelton et al., 2018). Pathophysiological aspects have been linked to the cause of essential hypertension. These include over activity in the sympathetic nervous system, production of hormones and substances that cause the body to retain sodium, long-term high sodium intake, increased or inappropriate renin secretion which results in more angiotensin II (Ang II) and aldosterone, deficits in vasodilators, abnormalities of resistance vessels, diabetes mellitus, insulin resistance, obesity, increased activity of vascular growth factors, changes in adrenergic receptors which affect heart rate, inotropic properties of the heart and vascular tone, and modifications in cellular ion transport such as calcium (Ca^{2+}) (Oparil et al., 2003). A sustained and elevated blood pressure can have long-term negative impacts on vascular health, including remodeling of the vascular walls, changes in the extracellular matrix (ECM), and transformation of vascular smooth muscle cells

(VSMCs) (Belo et al., 2015). ECM is significant in vascular remodeling as it assists in detaching VSMCs from the matrix, which in turn allows for migration and proliferation of endothelial cells, and inflammatory cells into the arterial wall (Briones et al., 2010; Chen et al., 2013; Hua and Nair, 2015; Lemarié et al., 2010).

High blood pressure is the most frequent cause of death in the world (Kumar, 2013). Hypertension has become more widespread, particularly in lower to middle-income countries (Heidenreich et al., 2011). It is estimated that over one billion adults suffer from high blood pressure (Yang et al., 2023). The number of people with high blood pressure is expected to reach two billion by 2025 (Feng et al., 2022). According to the American Heart Association (AHA), it is anticipated that approximately 41.4% of adults in the United States will have hypertension by the year 2030 (Kulkarni, 2021). A thorough understanding of the epidemiology of hypertension could be valuable in reducing the incidence of related illnesses and mortality.

2.3. The association of renin-angiotensin system (RAS) with hypertension and MetS

RAS is a key regulator of blood pressure, cardiac function, and fluid homeostasis (Son et al., 2018). Aside from the overall RAS, there are localized RAS such as in the brain, pancreas, heart, fat, and skeletal muscle (Son et al., 2018). Angiotensin converting enzyme (ACE) is responsible for the production of angiotensin II (Ang II), a powerful vasoconstrictor in the body (Liao et al., 2018a). Excess levels of Ang II can contribute to the abnormal proliferation and migration of vascular cells, as well as cause oxidative stress and inflammation in the vascular wall (Liao et al., 2019). The Ang II is involved in vascular remodeling, largely through its angiotensin II receptor (AT1R). This remodeling of the blood vessels can result in an increase of peripheral resistance and can cause high blood pressure (Liao et al., 2018b). Ang II could bind to particular cell surface receptors known as the angiotensin II receptor (AT2R). When these receptors in endothelial cells and the VSMCs are activated, they counteract the effects of AT1Rs by causing vasodilation, releasing nitric oxide (NO), and hindering vascular smooth muscle growth (Wassmann and Nickenig, 2006). Another RAS enzyme, angiotensin-converting enzyme 2 (ACE2) converts Ang II into angiotensin-(1-7)(Ang-(1-7)), which possesses strong anti-inflammatory and anti-hypertensive properties (Liao et al., 2018a). ACE2 acts to oppose the damaging effects of Ang II by engaging with the Mas receptor (MasR) receptor (Liao et al., 2019).

RAS plays a substantial role in the development and advancement of insulin resistance and MetS (Frigolet et al., 2013; Menikdiwela et al., 2020; Putnam et al., 2012). There is evidence indicating

that metabolic disorders, including diabetes and obesity, are linked to the increased expression of RAS elements like Ang II, ACE, and AT1R (Skov et al., 2014). Ang II has the ability to hinder adipocyte differentiation, which can affect insulin sensitivity, reduce adiponectin secretion and increase pro-inflammatory adipokines (Marcus et al., 2013). Additionally, Ang II enhances the production of reactive oxygen species (ROS), which can damage different tissues and pathways in the body, resulting in further inflammation, impaired insulin signaling, and endothelial dysfunction (Jahandideh and Wu, 2020). Oxidative stress can also damage the pancreatic β-cells, which can decrease insulin secretion and increase fasting blood glucose levels. It is clear that RAS overproduction is an important factor in the development of MetS (Jahandideh and Wu, 2020).

High-fat diets (HFD) is commonly used in preclinical animal studies to induce obesity and insulin, which are associated with MetS (Aoqui et al., 2014; Elzinga et al., 2021). Additionally, HFD is a significant factor contributing to endothelial dysfunction *in vivo* (Ashkar et al., 2023). This might be due to the oxidative stress and chronic inflammation resulting from elevated levels of lipids in the body (Panchal et al., 2011). HFD mice showed increased weight and visceral fat along with hypercholesterolemia and elevated blood pressure. Moreover, mice with MetS presented with insulin resistance and systemic inflammation, as indicated by elevated tumor necrosis factor-(TNF-α) and reduced adiponectin levels (Aoqui et al., 2014). In the HFD fed C57BL/6 mice, which have developed obesity and hypertension, demonstrate a decrease in the expression of ACE2 and an elevation in the levels of Ang II in their visceral adipose tissue (Gupte et al., 2008; Rahmouni et al., 2004). These might change cardiovascular function and structure (Panchal et al., 2011).

2.4. Bioactive peptides

A number of bioactive food components, including dietary fiber, omega-3 fatty acids, phytochemicals, and others, have health-promoting properties. In addition to improving human health and preventing chronic diseases, food protein-derived bioactive peptides have major impacts on various biochemical pathways (Li et al., 2018). These peptides can regulate vital functions, including those related to hypertension, infections, antithrombotics, immunomodulatory functions, opioids, antioxidants, and minerals by acting on a variety of mechanisms (Chakrabarti et al., 2018). There is also strong evidence that milk-derived peptides and casein hydrolyzate inhibit ACE in vitro and have antihypertensive effects in rodent models. Additionally, inhibition of renin, sympathetic stimulation, enhanced endothelial function, and ACE2 activation have been proposed as mechanisms of action. In addition to milk, peptides and protein hydrolysates prepared

from pea, egg, and rice were also reported to show antihypertensive effects (Duffuler et al., 2022). Moreover, bioactive peptides from soy, black soybean, and milk protein could reduce weight gain and food intake by increasing satiety, reducing appetite, and increasing energy expenditure (Erdmann et al., 2008; Hall et al., 2003; Jang et al., 2008; Nishi et al., 2003). Also, there is a number of peptides, hydrolysates, and proteins, for instance peptides derived from casein glycomacropeptide and soy bean can improve insulin sensitivity through multiple signaling pathways (Li et al., 2018). Milk-derived tripeptide, Egg-derived tripeptide, and soy-derived peptide showed anti-inflammatory activity via various pathways (Chakrabarti and Wu, 2015; Huang et al., 2010; Kovacs-Nolan et al., 2012; Majumder et al., 2013a; McConnell et al., 2015). Although, bioactive peptides showed beneficial impacts on blood pressure, inflammation, obesity, and T2DM, more studies are requiered to explore the exact machanism, target pathways, and function of these paptides.

2.5. Regulatory role of tripeptide IRW on RAS in hypertension and MetS

Egg-derived peptides have antioxidant, anti-inflammatory, and ACE inhibitory properties, suggesting that they could be used in the prevention and treatment of CVDs (Huang et al., 2010; Majumder et al., 2013a). IRW (Ile-Arg-Trp) was first identified as an ACE inhibitory peptide originating from ovotransferrin, a protein found in egg white (Huang et al., 2010).

In a previous study using a HFD model, the expression of ACE2, AT1R, and MasR in the skeletal muscle of HFD mice did not show any notable difference with or without IRW (45 mg/kg body weight) treatment. Nevertheless, a decrease in the abundance of ACE was observed in the groups treated with IRW in comparison with HFD (de Campos Zani et al., 2022). However, spontaneously hypertensive rats (SHRs) treated with a daily dose of 15 mg/kg body weight of IRW orally showed a notable decrease in their blood pressure and an increase in the expression of ACE2 (Majumder et al., 2015). The ACE2 protein levels in both the aorta and the kidney were significantly higher in the group receiving IRW treatment showing ACE2 activator effect of IRW (Liao et al., 2018a). Further research on SHRs revealed that the antihypertensive effect of IRW was due to activation of ACE2/Ang-(1-7)/MasR axis, as co-administration of A779 (MasR antagonist) at a dose of 48 μg/kg body weight eliminated the blood pressure-lowering effect of IRW. Additionally, oral administration of IRW at a 15 mg/kg body weight dose decreased Ang II and increased ACE2 and Ang-(1-7) levels, but had no effect on ACE (Liao et al., 2019). Recent research also has revealed IRW's ability to act as an antioxidant, anti-inflammatory, and antihypertensive agent in various

pathways (Huang et al., 2010; Liao et al., 2018a; Son et al., 2018) (Figure 2.1). Therefore, in order to enhance our understanding of the regulatory role of IRW on RAS, further studies are required to examine the impact of IRW on the RAS, specifically ACE2, and their correlation with insulin signaling pathway, endothelial function, and hypertension complications in the aorta.

2.6. The modulatory role of endothelia's markers in the initiation and development of MetS

Hypertension, diabetes, and hypercholesterolemia are related to endothelial dysfunction, which can initiate or contribute to the development of MetS (Espinola-Klein et al., 2011; Panchal et al., 2011). Endothelial dysfunction is characterized by impaired vasodilation of the endothelium, reduced arterial flexibility, and accelerated atherosclerosis development (Kraemer-Aguiar et al., 2008). Endothelial dysfunction is also characterized by a decline in the production of mediators made by the endothelium, as well as an increase in the secretion of inflammatory markers (Espinola-Klein et al., 2011). Numerous factors such as oxidative stress, hyperglycemia, advanced glycation products, free fatty acids, inflammatory cytokines, and adipokines can lead to an impairment of the endothelium's ability to perform its normal physiological and protective functions (Kaur, 2014). Thus, biomarkers such as NO, insulin, Sirtuin1 (SIRT1), AMP-activated protein kinase (AMPK), mitogen-activated protein kinases (MAPK), and endothelin-1 (ET-1), which are playing crucial roles in the functioning of the endothelium in MetS are discussed below.

2.6.1 Nitric oxide (NO)

NO, a molecule produced in vascular endothelium by activation of endothelial NO synthase (eNOS), is an important determinant of endothelial function (Muniyappa and Sowers, 2013). Endothelial dysfunction is shown by a decrease in NO resulting in impaired vasodilation (Tziomalos et al., 2010). NO's vasodilatory actions are due to a decrease in the intracellular Ca^{2+} concentrations of VSMCs. This is a result of NO-mediated guanylate cyclase activation and the formation of cyclic guanosine monophosphate (cGMP). Furthermore, phosphorylation of eNOS at Serine 1177 by different serine kinases, such as Akt, AMPK, and protein kinase A (PKA), can also increase the production of NO independent of Ca^{2+} (Dudzinski and Michel, 2007). The lack of other vasodilators like prostacyclin and endothelium-derived hyperpolarizing factors, as well as an increased production or activity of vasoconstrictors such as ET-1 and Ang II, are also thought to be involved in endothelial dysfunction (Deanfield et al., 2007). It is widely accepted that a decrease in the activity of eNOS, a type of vascular enzyme, can result in impaired function of the endothelium, a factor in the development of hypertension and atherosclerosis (Gielis et al., 2011;

Kietadisorn et al., 2012). Our previous research revealed that administering 15 mg/Kg body weight of IRW for 18 days increased eNOS levels and improved vasorelaxation through NO production in SHRs (Majumder et al., 2013b). Moreover, A779 infusion (48 μg/kg body weight per hour) for seven days in SHRs reduced the IRW treatment's effect on the phosphorylation of Akt and eNOS in the aorta. This suggests that IRW activates the ACE2/Ang-(1-7)/MasR axis in SHRs, thus enhancing the phosphorylation of Akt/eNOS and increasing endothelium-dependent vasorelaxation (Liao et al., 2019). While IRW has been shown to enhance eNOS activity in the aorta of SHRs, there is currently no evidence available to indicate the impact of IRW on RAS, specifically ACE2, to regulate eNOS and endothelial function in the HFD mouse model. Further investigation is required to indicate the ability of IRW in RAS and consequently eNOS regulation in the aorta of HFD in mouse insulin resistant model (Figure 2.2).

2.6.2 Insulin

Insulin resistance, a characteristic of MetS, can reduce the vascular response and increase the risk of cardiovascular issues. The relationship between endothelial function and insulin signaling is a key when it comes to diseases like hypertension, obesity, and diabetes. Therefore, it is advisable to take action on metabolic pathways that can help with both improving insulin sensitivity and endothelial function (Del Turco et al., 2013). Insulin also encourages the production and distribution of NO by endothelial cells which causes vasodilation (Kim et al., 2006; Tziomalos et al., 2010). It is possible that insulin resistance can lead to decreased production of NO in the endothelium, which can then cause accelerated atherosclerosis by promoting vasoconstriction, inflammation, and thrombosis (Jansson, 2007). Furthermore, insulin resistance has been known to impair endothelial function by activating MAPK-dependent pathway, stimulating oxidative stress, and releasing ET-1 (Dudzinski and Michel, 2007; Henriksen et al., 2011; Tziomalos et al., 2010). The results of the IRW treatment (45 mg/kg body weight) of the mice with HFD showed a decrease in a fasting insulin, which was in line with a reduction in Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (de Campos Zani et al., 2022). Additionally, it was seen that an egg white hydrolysate positively impacted insulin sensitivity in the muscle and adipocyte tissue of rats that were fed a HFD through enhancing AKT abundance (Jahandideh et al., 2019) (Figure 2.3). This evidence suggests that IRW has the potential to regulate insulin levels. However, it is needed to directly investigate the effects of IRW on endothelial function in mesenteric arteries in the context of insulin signaling pathway.

2.6.3 AMPK

AMPK is a heterotrimeric serine/threonine protein kinase made up of three parts: an alpha (α) component responsible for carrying out reactions, and two other regulatory subunits, beta (β) and gamma (γ). The α-subunit of AMPK has the kinase domain, along with a prominent Thr172 residue that is catalyzed by an upstream kinase. The β-subunit holds a carbohydrate-binding module that allows AMPK to be linked to glycogen. The γ-subunit has four cystathionine-β-synthase (CβS) domains that bind adenine nucleotides, allowing AMPK to respond to the ATP-to-AMP ratio and regulate AMPK (Herzig and Shaw, 2018). AMPK has been associated with expanding the diameter of both large and small arteries without the involvement of the endothelium, as well as controlling the proliferation and inflammation of VSMCs (Rodríguez et al., 2021). AMPK is a widely preserved enzyme that plays a role in controlling the metabolic activity of cells and organs. Activation of endothelial AMPK triggers the phosphorylation of eNOS at Ser1177, resulting in the production of NO which causes the dilation of both large and small blood vessels (Ewart and Kennedy, 2011). Substances like metformin and adiponectin, which had the ability to activate and phosphorylate AMPK, were known to bring about a relaxation of the aorta (Deng et al., 2010; Zou et al., 2004). It appeared that angiotensin II might be able to suppress AMPK, which might explain why it had negative consequences on vascular remodeling (Stuck et al., 2008). Statins might also have positive impacts on endothelial progenitor cells (EPC). Activation of AMPK induced by a statin might be beneficial in encouraging EPC differentiation through the stimulation of eNOS, which is likely to be significant in replenishing parts of arteries affected by stenting or silent plaque rupture (Li et al., 2008b). Also, our recent study showed that the administration of IRW resulted in an elevation in the phosphorylation of AMPK at Thr172 site in skeletal muscle in comparison to the HFD (Figure 2.4) (de Campos Zani et al., 2022). Although there is evidence supporting the potential of IRW to regulate AMPK without affecting ACE2 in skeletal muscle, which might be connected to endothelial function, it is important to note that no studies have been conducted to demonstrate the specific impact of IRW on RAS to modulate AMPK in mesenteric arteries. Further studies are necessary to obtain a comprehensive understanding the impact of RAS on AMPK and endothelial function in mesenteric arteries.

2.6.4 SIRT1

SIRT1 has been found to increase longevity and insulin sensitivity in response to a calorie-restricted lifestyle, as well as having a positive effect on endothelial biology (Schenk et al., 2011).

SIRT1 is an enzyme that is dependent on nicotinamide adenine dinucleotide (NAD^+) and is responsible for deacetylation, which affects numerous genes related to metabolic activities such as fatty acid oxidation, mitochondrial activity, and the gluconeogenesis (Zhou et al., 2022). It is believed that endothelial SIRT1 plays a role in atherosclerosis and may be linked to various beneficial processes, including the inhibition of apoptosis caused by oxidation of low-density lipoprotein (LDL), regulation of eNOS expression, and the enhancement of endothelial relaxation (Potente et al., 2007). Research showed that overexpressing SIRT1 prevented oxidative stress-induced endothelial senescence and improved endothelial-dependent vasodilation by deacetylating eNOS (Mattagajasingh et al., 2007; Ota et al., 2007). Furthermore, SIRT1 and AMPK collaborate to maintain cell stress and energy balance, as well as protect the cardiovascular system (Cheang et al., 2014; Hou et al., 2008). The lack of SIRT1 and p-AMPK in insulin resistance could lead to endothelial dysfunction (Karpe and Tikoo, 2014). Interestingly, oxidative stress had been observed to down-regulate SIRT1, leading to eNOS acetylation and decreased production of NO in endothelial cells (Arunachalam et al., 2010). Treatment with SIRT1 activators has been tested as a potential therapy for coronary artery disease, as SIRT1 has been shown to inhibit ischemia-induced endothelial dysfunction by regulating eNOS expression and acting as a cardioprotective molecule (Figure 2.5) (Askin et al., 2020). In order to comprehensively understand the impact of IRW on endothelial function and insulin signaling, it is crucial to investigate the effect of IRW on SIRT1 in mesenteric arteries.

2.6.5 MAPK

MAPKs are protein Serine/Threonine kinases that are responsible for converting extracellular signals into a vast array of cellular responses. All eukaryotic cells have multiple MAPK pathways, which work together to control gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation (Cargnello and Roux, 2011). Angiotensin has been shown to activate the MAPK pathways in endothelial cells, resulting in increased serine phosphorylation of insulin receptor substrate 1 (IRS-1), impaired phosphoinositide-3-kinase (PI3K) activity, and ultimately endothelial dysfunction and potentially apoptosis (Yusuf et al., 2001). Ang II has been shown to quickly stimulate the phosphorylation of p38 MAPK in cultured VSMCs, as well as in cardiac and vascular tissue (Bao et al., 2007). Hyperglycemia and hyperinsulinemia are associated with activation of MAPK signaling pathways (Favaro et al., 2008; Yang et al., 2008). This can result in decreased NO production, increased secretion of ET-1, activation of cation pumps, increased

expression of vascular cell adhesion molecule (VCAM-1), and impair insulin-stimulated translocation of glucose transporter 4 (GLUT4) in adipocytes, all of which are associated with endothelial dysfunction (Jansson, 2007; van den Oever et al., 2010). Recently, it was shown that 50 μM of IRW inhibited the phosphorylation of p38 in Ang II-stimulated A7r5 cells, suggesting a selective role of IRW in regulating MAPKs (Liao et al., 2018b). However, as a result of IRW treatment (15 mg per kg body weight), the increased Ang-(1-7) did not inhibit extracellular signal-regulated kinase (ERK)1/2 MAPK phosphorylation in SHRs aorta (Liao et al., 2019) (Figure 2.6). These findings suggest that additional investigation is necessary to explore potential regulatory mechanisms involved in the endothelial function in mesenteric arteries.

2.6.6 ET-1

As opposed to NO, endothelins that circulate in the body have the ability to cause vasoconstriction (Barton and Yanagisawa, 2008). Endothelial cells and VSMCs are the main source of ET-1 production (Barton and Yanagisawa, 2008). ET-1, known as the strongest vasoconstrictor, is derived from a preproET-1 precursor that is divided into an inactive peptide consisting of 38 amino acids and further transformed into the active 21 amino acid ET-1. This peptide binds to its two G-protein coupled receptors, ETA and ETB, which are located on endothelial cells (ETB), VSMCs, and fibroblasts (ETA and ETB) (Iglarz and Clozel, 2007). Both ETB and ETA receptors were found to raise ROS production (Iglarz and Clozel, 2007). The interplay between NO and ET-1 is of great importance in a number of pathophysiological conditions (Majumder and Wu, 2015). The decreased access to NO is linked to a rise in ET-1 production. On the other hand, NO hinders the ET-1 pathway through a variety of mechanisms (Bourque et al., 2011). Caveolin-1, the main component of caveolae, appears to have a role in controlling eNOS activity (Minshall et al., 2003). In diseases, ET-1 stimulates the production of caveolin-1, which subsequently reduces eNOS activity (Kamoun et al., 2006; Karaa et al., 2005). Mice with ET-1 overexpression in their endothelium had difficulty with vasorelaxation in their resistance vessels and higher ROS production (Amiri et al., 2004). ET-1 has been found to increase serine phosphorylation of IRS-1 in VSMCs, resulting in a decrease in PI3K activity. Additionally, ET-1 has been found to impair insulin-stimulated GLUT4 translocation in adipocytes (van den Oever et al., 2010). High levels of insulin have been demonstrated to increase the creation of ET-1 *in vitro* and *in vivo*. The same harmful effects were observed with the stimulation of the vascular system's RAS including Ang II (Manrique et al., 2014). Hence, NO's lower availability and an overabundance of Ang II can lead

to impaired endothelial function, which in turn raises blood pressure (figure 2.7) (Majumder and Wu, 2015). However, such investigations will provide valuable insights into the potential influence of bioactive peptides such as IRW on ET-1 and endothelial function and its potential as a therapeutic approach to counteract the negative effects of the HFD.

2.7. The relation of vascular glucose uptake and MetS

Skeletal muscle glucose uptake involves both insulin-dependent and insulin-independent pathways. Insulin stimulates the phosphoinositide-3-kinase (PI3K)/AKT cascade, resulting in GLUT4 being moved to the plasma membrane, consequently increasing glucose uptake (Zhou et al., 2017b). Activation of AMPK, which can be seen in muscle contraction, increases the movement of GLUT4 to the cell membrane, allowing for an independent increase in the amount of glucose that enters the cell, regardless of insulin levels (Bergeron et al., 1999; Kurth-Kraczek et al., 1999). In many tissues that express GLUT4 (like heart muscle, skeletal muscle, and fat), most of the molecules remain inside an intracellular vesicular compartment until prompted by insulin or a different stimulus. This causes them to be transported to and embedded in the cellular membrane, allowing for glucose to be absorbed (Gould and Holman, 1993). GLUT4 is a vital component in controlling glucose metabolism, maintaining glucose homeostasis, and keeping body glucose levels in equilibrium (Manna et al., 2017). Furthermore, a decrease in plasma membrane content of GLUT4 has been found to contribute to acute myocardial glucose metabolic disorder (Wang et al., 2018). A recent study demonstrated that administering IRW 45 mg/kg to mice on a HFD for 8 weeks could lead to improved glucose tolerance, likely due to the involvement of both insulin dependent through AKT and insulin independent through AMPK pathways. IRW could affect certain pathways in the body, such as AKT/GLUT4 and AMPK/GLUT4, which increase the amount of glucose taken up by skeletal muscle (de Campos Zani et al., 2022). AMPK dysregulation has been observed in conditions of metabolic distress, such as obesity and diabetes. This dysregulation can lead to a range of unfavorable health outcomes, including glucose intolerance and insulin resistance (Rodríguez et al., 2021). Research demonstrated that adding 100 μM of IRW to Ang II or TNF-α-treated L6 skeletal muscle cells helped to combat insulin resistance. TNF-α diminished the efficacy of insulin signaling and GLUT4 translocation, which are necessary for glucose uptake, however, pre-treating with IRW for two hours was able to restore these impairments (Son et al., 2018; Son and Wu, 2019).

Recent research has revealed the involvement of GLUT4 in VSMCs in the process of glucose absorption. In diabetic rats, there was a reduction in the amount of GLUT4 of VSMC and the uptake of glucose (Atkins et al., 2001). Reduced GLUT4 expression was linked to a significant decrease in basal glucose uptake in aortas from mouse model of hypertension induced by Ang II (Park et al., 2005). It was observed that the GLUT4 antagonist, indinavir (25 mol/L and 50 mol/L), impeded glucose uptake in the aorta, signifying that around half of the glucose uptake in the VSMCs in the body is regulated by GLUT4.

SIRT1 and AMPK have emerged as key insulin-independent signaling molecules, believed to be capable of stimulating insulin sensitivity and glucose metabolism (Cao et al., 2016; Ruderman et al., 2013). A recent report suggests that insulin resistance is associated with a state of deficiency in vascular SIRT1 and p-AMPK, both of which may contribute to endothelial dysfunction (Karpe and Tikoo, 2014). Protein Kinase C (PKC) activation has been observed in metabolic disorders and has been found to phosphorylate AMPK at the inhibitory site Ser487, thus reducing its activity (Heathcote et al., 2016). Metformin administration to mice has been shown to increase AMPK phosphorylation in the aorta, which in turn increases eNOS activity, resulting in enhanced synthesis and bioavailability of NO in the aorta (Haye et al., 2020). Metformin has been shown to act on $ONOO^-$ level, leading to the activation of AMPK/PI3K signaling in cultured bovine aortic endothelial cells (Zou et al., 2004). Despite the previous study has shown that IRW (45 mg/kg body weight for 8 weeks) improved AMPK and glucose tolerance and GLUT4 in the skeletal muscle, no evidence on the effect of AMPK on GLUT4 in VSMCs has been reported yet (de Campos Zani et al., 2022). The mechanism through which IRW regulate GLUT4 in VSMCs, both in the presence and absence of aortic ACE2 expression has yet to be determined.

2.8. Complications of hypertension

Hypertension is identified as one of the risk factors associated with MetS (Jahandideh and Wu, 2020). A substantial and enduring rise in blood pressure leads to vascular remodeling and dysfunction (Belo et al., 2015). This process requires changes to cellular processes such as cell growth, migration, death and the production/breakdown of the ECM (Majumder and Wu, 2015). Matrix metalloproteinases (MMP) are enzymes that break down proteins found in the ECM and are activated by both zinc and Ca^{2+} (Visse and Nagase, 2003). MMPs are implicated in Ang II-induced hypertension, causing an increase in blood pressure, tissue fibrosis, and migration of VSMCs (Schmid-Schönbein, 2011). IRW showed anti-inflammatory and antioxidant properties

on vascular endothelial cells, likely due to increased ACE2 expression and activity. This could lead to improved vascular tone, and subsequently, a reduction in blood pressure (Liao et al., 2018a). Our previous research has shown that IRW treatment may help to reduce proliferation, oxidative stress, and inflammation in VSMCs when stimulated with Ang II. This is achieved by upregulating eNOS and modulating the expression of MMP9. The effects of IRW (50 μM) on the migration of Ang II-stimulated A7r5 cells were linked to decreased expression of MMP9 and inactivation of the P38 MAPK signaling pathway. Additionally, the ability of IRW to hinder migration was found to be reliant on the AT1R (Liao et al., 2018b). These findings suggest that IRW might be effective in decreasing vascular fibrosis, migration, and remodeling (Liao et al., 2018b; Wu, 2020).

2.8.1 The relation of E-cadherin and hypertension complications

The CDH1 gene, located on chromosome 16q22.1, is a tumor suppressor that produces a 120-kDa protein known as epithelial cadherin (E-cadherin) (Gall and Frampton, 2013). E-cadherin belongs to cadherins family which are an extensive group of glycoproteins located either in the membrane or attached to it, that enable specific cell-cell adhesion, relying on Ca^{2+} ions for the process (Van Roy and Berx, 2008). The inner surface of blood vessels is covered with a single layer of endothelial cells connected by intercellular junctions. If vascular E-cadherin is absent, the junctions between endothelial cells weaken, leading to impaired functioning (Shenoy, 2019). Cadherins not only serve a major purpose in adhesion, but also interact with numerous proteins in the cell and are involved in processes such as migration, proliferation, survival, and differentiation (Frismantiene et al., 2018b). A mutation of the CDH1 gene and the subsequent lack of the E-cadherin protein results in epithelial-mesenchymal transition (EMT) which causes the cell to be unable to adhere to other cells and to lose its polarity at the top (Shenoy, 2019). An immune-histochemical study and western blot analysis revealed that the expression of E-cadherin was lower in the renal medulla from SHR rats compared to control rats. These findings suggest that the renal medulla from SHR rats exhibited a progression of EMT (Negishi et al., 2018). It is widely accepted that the loss of E-cadherin is an essential element of invasion and metastasis, as evidenced by the fact that the transfection of E-cadherin can suppress the invasive phenotype of carcinoma cells *in vitro* (George and Beeching, 2006). During cancer progression, E-cadherin often switches to neural cadherin (N-cadherin), which can lead to more aggressive tumors and greater metastasis

(Wang et al., 2019). As indicated by these findings, further investigation is warranted to explore the potential correlation between E-cadherin and hypertension complications.

2.8.2 Expression and function of E-cadherin in SMC

The functions of E-cadherin in SMCs have yet to be investigated thoroughly, though it has been speculated to have an associative role. Previous research has examined alterations in the cell adhesion that take place during the switching of SMC phenotypes. Specifically, the upregulation of truncated cadherin (T-cadherin) promotes dedifferentiation. Conversely, the depletion of T-cadherin has been found to enforce differentiation, leading to distinct effects on the cell adhesion (Frismantiene et al., 2018a). Analysis of cell adhesion-relevant genes in human aortic SMCs with silenced T-cadherin showed a slight increase in the expression of E-cadherin, which reflects a differentiated non-proliferative and non-migratory effects (Frismantiene et al., 2018a). Thus, this study uncovered the presence of E-cadherin expression in SMCs with anti-proliferative and anti-migration effects. Upon further investigation, it was discovered that E-cadherin was down-regulated in response to a mitogenic concentration of mildly oxidized LDL. This downregulation of E-cadherin was accompanied by an activation of the transcription of proliferative genes of human aortic SMCs (Bedel et al., 2008). Under certain pathological conditions, such as the presence of ROS, VSMCs can transition to a highly proliferative state, where they are no longer expressing markers like E-cadherin. This alteration is a vital step in the proliferation and migration of VSMCs, which is involved in the formation and advancement of CVDs, including atherosclerosis (Liu et al., 2020).

2.8.3 Regulation of migration and proliferation through E-cadherin and related pathways

Multiple markers are implicated in the regulation of E-cadherin and its associated cascades. Zinc finger proteins belonging to the Slug/SNAI1 (Snail) family can inhibit the transcription of the E-cadherin gene, while their activity is stimulated by signals from the fibroblast growth factor receptor (FGFR). Expression of Slug/Snail proteins causes a decrease in the cell-cell adhesion and an increase in the cell migration (Nelson and Nusse, 2004). Research has shown that epidermal growth factor receptor (EGFR) activation can interfere with E-cadherin functioning (Fedor-Chaiken et al., 2003; Hazan and Norton, 1998). E-cadherin was observed to have a suppressing effect on EGFR-induced MAPK signaling in the typical human urothelial cells, while concurrently activating PI3K/AKT signaling (Du et al., 2014). Moreover, EGFR/ ERK signaling pathway is abnormally activated when E-cadherin is reduced, and is also strongly associated with invasion

and migration that is dependent on MMP in cancers (Fedor-Chaiken et al., 2003). Previous research has demonstrated that E-cadherin has an inhibiting effect on nuclear factor kappa B (NF-κB) activation. Loss of E-cadherin and associated cell-cell connections has been known to trigger NF-κB signaling, whereas an increase in E-cadherin has been proven to decrease its activity (Du et al., 2014). A recent study revealed higher levels of phosphorylated p38 and ERK and markers induced EMT in the Ang II treated proximal tubular cells (PTCs). Also, E-cadherin suppression occurred, which caused the epithelial cells to lose their adhesion and transform into fibroblast-like shapes. In this study, losartan (Angiotensin II receptor blocker) treatment has a notable effect on decreasing the levels of p38, ERK, and Zinc finger protein SNAI1 (Snail1), while increasing the expression of E-cadherin (Costantino et al., 2019). Another study also revealed that the mRNA levels of Snail were higher in the aorta of spontaneously hypertensive and hyperlipidemic rats (SHHRs) that consumed the HFD suggesting that the expression of Snail genes might elevate with the progression of fibrosis (KAWAGUCHI et al., 2016).

Ca^{2+} is a vital secondary messenger that plays a vital role in the homeostasis and is essential for the development and maintenance of many physiological functions, such as movement, secretion, and gene transcription (Berridge et al., 2003). It was indicated that Ca^{2+} influx is responsible for regulating the morphological transformation of the cell from a stationary to a motile form, known as EMT (Berridge et al., 2003). EMT in MCF7 breast cancer cells is associated with an increase in Ca^{2+} influx into the cell, which leads to a decrease in E-cadherin protein levels and an increase in cell migration (Schaar et al., 2016). The detection of depletion of ER calcium reserves is accomplished by a membrane sensor named sensor stromal interaction molecule 1 (STIM1), which then initiates the opening of a plasma membrane Ca^{2+} channel known as Orai Calcium Release-Activated Calcium Modulator 1 (Orai1). This pathway is referred to as store-operated calcium entry (SOCE) (Lewis, 2001). Orai1 is an integral membrane protein located in the plasma membrane, characterized by four transmembrane domains (Kang et al., 2021). Studies have found that STIM1 and Orai1 may have an impact on the proliferation, migration, or both of breast cancer, glioblastoma, renal cell carcinoma, and esophageal squamous cell carcinoma (ESCC) (Kim et al., 2014; McAndrew et al., 2011; Motiani et al., 2013; Yang et al., 2009). It was shown that the expression of Orai1 was higher in older human aortas. In addition, STIM1 and Orai1 proteins had a negative association with endothelial function in the mesenteric artery. Additionally, the amount of Orai1 was connected to the inflammatory TNF-α and endothelial dysfunction, indicating its

potential role in age-related microvascular impairment (El Assar et al., 2022). Further, the Orai1 protein was present in greater amounts in the aorta of stroke-prone SHRs compared to the Wistar-Kyoto rats. An increase in Orai1 activity may explain why there is a lack of control over intracellular Ca^{2+} concentrations in cases of hypertension (Giachini et al., 2009). It was shown that Orai1 knockdown up-regulated the expression of E-cadherin in human colorectal cancer cells (Kang et al., 2021). It is also suggested that SOCE might suppress E-cadherin expression through activating Snail1, which can promote metastasis (figure 2.8) (Yu et al., 2016).

2.8.4 Regulatory role of RAS on E-cadherin and related pathways

RAS plays a key role in regulating cardiovascular homeostasis and blood pressure (Brunner et al., 1972). Previous research has demonstrated that overexpression of ACE2 or treatment with Angiotensin-(1–7) can reduce tumor cell migration and invasion (Yu et al., 2016). ACE2 was reported to increase the expression of E-cadherin, a representative marker of EMT, both *in vitro* and *in vivo* (Xu et al., 2017). Further, overexpression of ACE2 or treatment with Ang-(1–7) resulted in an increase in E- cadherin expression in MDA-MB-231 cells. However, when ACE2 was knocked down, the E-cadherin expression was reduced in MCF-7 cells; this effect could be reversed by supplementing with Ang-(1–7) (Liu et al., 2012). Overexpression of ACE2 and treatment with Ang-(1–7) significantly reduced lung fibrosis and EMT and elevated the level of E-cadherin. However, these effects were reversed when MLN-4760 (a specific ACE2 inhibitor) and A779 were administered (Li et al., 2020) suggesting the regulatory role of ACE2/Ang-(1–7)/Mas axis on E-cadherin expression.

Ang II, which opposes the effect of Ang-(1–7), has been found to activate SOCE. This activation of SOCE may lead to the upregulation of NF-κB signal pathways and the subsequent transcription factor Snail1, resulting in the suppression of E-cadherin expression and promoting cancer metastasis (Yu et al., 2016). It was indicated that the expression of Orai1 via the NF-κB pathway was significantly higher in Ang II-treated human coronary smooth muscle cell (HCSMC) suggesting that Ang II might induce proliferation (Liu et al., 2020). In addition, Ang-II increased the intracellular Ca^{2+} concentration by activating the SOCE pathway in VSMC line derived from the medial layer of rat thoracic aorta. This pathway is linked to the inositol-3-phosphate receptor (IP3R) causing the depletion of endoplasmic reticular (ER) Ca^{2+} and subsequently activating the STIM1/Orai1 complex. It had been proposed that Ca^{2+} signaling might play a role in Ang-II expression in VSMCs, and STIM1 or Orai1 might be involved in this response (Simo-Cheyou et

al., 2017). Additionally, knockdown of ACE2 protein has been found to increase SOCE activity in MCF-7 cells. The effects of ACE2 or Ang-(1–7) on SOCE activity could be reversed by A-779. These findings suggest that a decrease in the activity of the ACE2/Ang- (1–7)/Mas axis stimulates SOCE, resulting in increased intracellular Ca^{2+} influx, migration, and metastasis (Figure 2.2) (Yu et al., 2016). Further studies should be conducted to investigate the correlation of RAS with E-cadherin in hypertension and related pathways.

2.8.5 Regulatory role of Tamoxifen on E-cadherin and related pathways

Tamoxifen, *trans*-1-(4-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene and its derivate like 4-hydroxytamoxifen were the first type of antiestrogen approved for treating estrogen-positive breast cancers and has been used for the last thirty years (Ahmad, 2018; Ishii et al., 2008). Tamoxifen is also used for treatment of infertility and retroperitoneal fibrosis (Akram et al., 2007; Steiner et al., 2005). A study observed the preservation of bone mineral density in postmenopausal patients with breast cancer during Tamoxifen treatment. Furthermore, Tamoxifen has the potential to provide benefits in treating postmenopausal osteoporosis, specifically in a specific group of normal women (Turken et al., 1989). In addition, in a randomized 2-year clinical trial, administration of Tamoxifen to postmenopausal women diagnosed with breast cancer demonstrated mostly positive effects on their lipid and lipoprotein profiles. These effects could potentially account for the reduction in adverse events and mortality associated with coronary heart disease observed in patients undergoing adjuvant Tamoxifen treatment (Love et al., 1991). Tamoxifen also has functions, such as antifungal, antioxidant, and antiviral, anti-angiogenesis, and triggering intracellular Ca^{2+} release (Dolan et al., 2009).

It was shown that 4-hydroxytamoxifen activated the E-cadherin promoter, which in turn led to increased expression of E-cadherin mRNA and protein levels (Mauro et al., 2010). Research has demonstrated that Tamoxifen can decrease toll-like receptor 4 (TLR-4), transforming growth factor-beta (TGF-β), and inflammation due to NF-κB, while impeding Snail activation and boosting the expression of E-cadherin in rats with breast cancer (Kusmardi et al., 2021). NF-κB is responsible for suppressing E-cadherin, so preventing this pathway may result in an increase of E-cadherin expression. Moreover, Tamoxifen has the potential to prevent the activation of Slug and Snail, which are proteins that decrease E-Cadherin level by blocking TGF-β pathway, thus boosting the E-cadherin level (Kusmardi et al., 2021).

Tamoxifen is a widely used for the treatment of breast cancer patients whose tumors have estrogen receptor-α (ER-α). However, resistance to this kind of therapy can develop, either at the beginning or during the treatment (Kurebayashi, 2003; Schiff et al., 2003). Tamoxifen resistance has been associated with an increase in the levels of certain EGFR ligands and EGFR receptors. This elevation leads to the activation of a number of downstream kinases, such as ERK1/2 MAP kinase and PKC (Kim et al., 2015). The decreased expression of E-cadherin in Tamoxifen-resistant cells may be due to increased methylation of its promoter. It is well-established that methylation of gene promoters can lead to their suppression (Wang et al., 2019). This evidence verifies that Tamoxifen has the ability to serve as both an agonist and an antagonist of E-cadherin (Figure 2.2) (Mauro et al., 2010).

2.8.6 The role of food components on E-cadherin and related pathways

In the past decade, significant advancements in our comprehension of the molecular and biochemical mechanisms of E-cadherin have emerged. Although standard medications could be utilized for the regulation of E-cadherin and related pathways, drug resistance can occur, causing treatment failure and disease recurrence. Several studies have provided preclinical evidence indicating the influence of various food components, including polyphenols, vitamins, herbs, and minerals, on the E-cadherin pathways and interaction (Table 1). The primary strategy employed focused on modifying molecular events and signaling pathways associated with crucial cellular processes such as survival, proliferation, metastasis, apoptosis, and angiogenesis (Bhosale et al., 2020). Food components hinder the invasion and migration of cells by utilizing a wide range of mechanisms. These mechanisms include reducing the expression of MMP, altering regulators involved in EMT, inhibiting transcription mediated by NF-κB, inducing the expression of E-cadherin, and changing the expression of Snail, Slug, and p53 (Tafrihi and Nakhaei Sistani, 2017; Zhou et al., 2016). Similarly, the results of our most recent study indicated that food-derived peptide IRW caused a significant increase in CDH-1 expression in mesenteric artery. This increase in CDH-1 could prevent the hyperplasia, tissue fibrosis , and vascular remodeling of endothelial and VSMCs (Majumder et al., 2015). Further research is required to investigate the impact of bioactive peptides such as IRW on the E-cadherin/Snail1 and SOCE signaling pathways. Additionally, the interaction between E-cadherin and the ACE2/Ang-(1-7)/Mas axis in response to IRW needs to be explored, as it might have impacts on proliferation and migration in both *in vivo* and *in vitro*.

2.9. Conclusion

The ACE2/Ang-(1-7)/MasR axis has been acknowledged to possess multiple regulatory roles, positioning it as a promising target for the development of novel cardiovascular therapies. Bioactive peptides derived from food proteins have demonstrated the potential to be utilized in functional foods or nutraceuticals to alleviate chronic diseases. The research area of bioactive peptides targeting MetS, particularly endothelial function and insulin signaling in mesenteric arteries, is experiencing significant growth. The bioactive peptide IRW, which originates from ovotransferrin, a protein present in egg white, has demonstrated anti-inflammatory, anti-oxidant, and anti-hypertensive properties. The identification of peptides that target cells through RAS has presented promising opportunities for exploring novel pathways in the management of MetS. However, the current evidence regarding the effectiveness of tripeptide IRW on the RAS associated with MetS, particularly in mesenteric arteries is limited.

MetS represents a cluster of both metabolic and cardiovascular symptoms, with hypertension and vascular disorders serving as core components of this syndrome (Mendizábal et al., 2013). Persistent high blood pressure triggers various biological responses in arteries. These changes are manifested through differential gene expression and arrangement of the ECM, ultimately driving the process of remodeling and migration (Humphrey, 2021). Complications arising from hypertension could have an impact on markers involved in adhesion, such as E-cadherin, as well as the related pathways (Frismantiene et al., 2018b). Despite the demonstrated anti-hypertensive and anti-inflammatory effects of IRW, there is currently insufficient evidence regarding the impact of bioactive peptides like IRW on ACE2/Ang-(1–7)/MasR axis modulating E-cadherin and related pathways. The aim of this study is to bridge the existing knowledge gap regarding the ACE2/Ang-(1-7)/MasR axis and bioactive peptides. The findings from this research have the potential to expand our insights into IRW and implicate novel ACE2/E-cadherin pathways on disease prevention and treatment.

2.10. References

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Table 1. Preclinical evidence of food components on E-cadherin pathway

Substance	Model (animal or cell)	Outcomes	References
Trifolium pratense L. Sproutsis (high contents of isoflavone aglycons—biochanin A and formononetin, which are 4′-O-methylated plant precursors to genistein and daidzein)	MDA-MB-231 cells	Up-regulated the mRNA level of E-cadherin in the cells, indicating their enhancement of cell-cell type adhesion.	(Zakłos-Szyda and Budryn, 2020)
β-Sitosterol (major components of corn silk)	Human lung adenocarcinoma epithelial cells, A549	Blocked the changes of the TGF-β1-induced morphological shape and protein expression of EMT markers, N-cadherin, vimentin, and E-cadherin, Suppressed EMT by inhibiting the TGF-β1/Snail pathway.	(Park et al., 2019)
Methyl donor treatment (L-methionine, choline chloride, folic acid and vitamin B12)	Panc-1 adenocarcinoma cell line	Reduced aggressive and proliferative phenotype of pancreatic adenocarcinoma cell line, which resulted in reduction of phospho-Erk1/2 levels and proliferation rate, Decreased the production of the pro-inflammatory cytokine IL-17a and the transcription factor NF-κB, Elevated E-cadherin expression.	(Kiss et al., 2022)
Quercetin	SW480 cells	Inhibited the EMT process induced by TGF-β1. Rescued the morphological changes and EMT-like phenotypes in TGF-β1-activated cells. Increased expression of E-cadherin and decreased expression of Vimentin and Twist1.	(Feng et al., 2018)
Grape-derived phytochemical resveratrol	Human DU145 and PC-3 CaP cells	Suppressed epithelial prostate cancer cell migration by attenuating the control of EMT and a significant up-regulation on E-cadherin protein.	(Hsieh and Wu, 2020)

Annurca apple polyphenol extract	MDA-MB-231 cells	Increased E-cadherin/N-cadherin protein ratio, Induced the switch from N-cadherin to E-cadherin expression and reduced vimentin levels, Changed the cell morphology highlighting an evident transition from the mesenchymal to epithelial phenotype with decreased migratory features.	(Vuoso et al., 2020)
phloretin	MDA-MB-231 cells	Inhibited cell growth and arrested the cell cycle in a p53 mutant-dependent manner. Decreased the migratory activity of the cells through the activation of E-cadherin	(Wu et al., 2018)
Green tea	Colorectal-cancer SW480, SW620, and LS411N cells	Inhibited cell proliferation by the induction of apoptosis against colorectal-cancer cells by down-regulating the expression of STAT3, Increased in E-cadherin expression.	(Luo et al., 2021)
Olive leaf extract	Immortalized mesothelial cell line MeT-5A	Reduced the TGFβ1-induced activation of Smad2/3 signaling and the mitigation of ERK, c-Jun N-terminal kinase (JNK), and p38 MAPK phosphorylation, Increased the activity of the E-cadherin promoter, Reduced the nuclear content of its co-repressor Snail.	(Lupinacci et al., 2019)
Polyphenol-rich coffee extracts and chlorogenic acid	SW480 and HT-29 CRC cells	Modulated the activity of the Wnt/β-catenin pathway, Increased the CDH1 expression.	(Villota et al., 2022)
Hispolon, isolated from Inonotus hispidus and many tropical mushrooms	MCF-7 cell	Suppressed the migration of breast cancer cells via suppressing the ROS/ERK/Slug/E-cadherin pathway.	(Zhao et al., 2016)
Curcumin	Human endometrial cancer cells	Down-regulated MMP-2, Up-regulated E-cadherin, Inhibited the proliferation and invasion of cancer cells.	(Sun et al., 2018)
unfertilized egg white	SW480 cells	Decreased cell survival, inhibited cell cycle in G0/G1, decreased colony formation and sphere formation and inhibited migration	(Monji et al., 2021)

		ability, Increased the E-cadherin expression.	
Genistein	HT-29 cells	Suppressed the migration of colon cancer cells by reversal the EMT via suppressing the NF-κB/slug/E-cadherin pathway.	(Zhou et al., 2017a)
Piperine extracted from Piper Nigrum	MCF-7 cell	Prevented angiogenesis, migration, and invasion. Suppressed MMP-9 and vascular endothelial growth factor (VEGF), Increased E-cadherin expression.	(Zare et al., 2020)
Caffeic acid p-nitro-phenethyl ester	MDA-MB-231 cells and BALB/c athymic nude mice	Inhibited the growth and metastasis, Down-regulated the proteins expression of p-EGFR, p-STAT3, p-Akt, MMP-2, and MMP-9, increased the expression of E-cadherin.	(Huang et al., 2019)
plant-based medicinal food composed of six medicinal and edible plants (Coix seed, Lentinula edodes, Asparagus officinalis L., Houttuynia cordata, Dandelion, and Grifola frondosa	SGC-7901, a human GC cell line and nude mice (BALB/c nu/nu, SPF level	Up-regulated the mRNA expression levels of E-cadherin, Down-regulated those of N-cadherin, MMP-2, MMP-9, and Snail in tumor tissues.	(Chen et al., 2021)
Grape seed-derived polyphenols extract	Rat model with prostatic deficits	Increased prostatic level of E-cadherin, Decreased levels of fibronectin.	(Lei et al., 2017)
Epigallocatechin-3-gallate	Endometriosis mouse	Inhibited the growth of endometrial lesion, Affected the expression of E-cadherin on the cell membrane. Reduce the status of DNA methylation of E-cadherin promoter region.	(Guan et al., 2020)

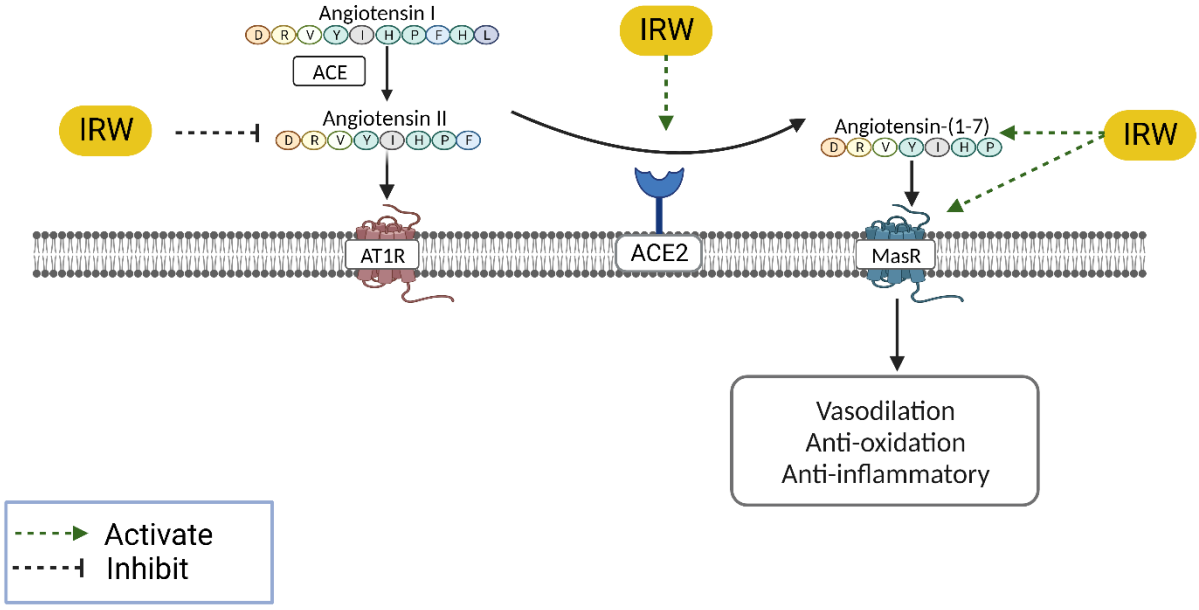

Figure 2. 1 The impact of IRW on the RAS.

ACE plays a crucial role as the primary enzyme responsible for producing the vasoconstrictor Ang II. The negative effects of Ang II are mediated by AT1R. ACE2 operates in the counter-regulatory branch of the system to counteract the detrimental effects of Ang II. This is accomplished by producing Ang-(1-7), which binds to MasR. IRW increases in the expression of ACE2, Ang-(1-7), and MasR and decreases Ang II, revealing IRW's ability to act as an antioxidant and anti-inflammatory agent.

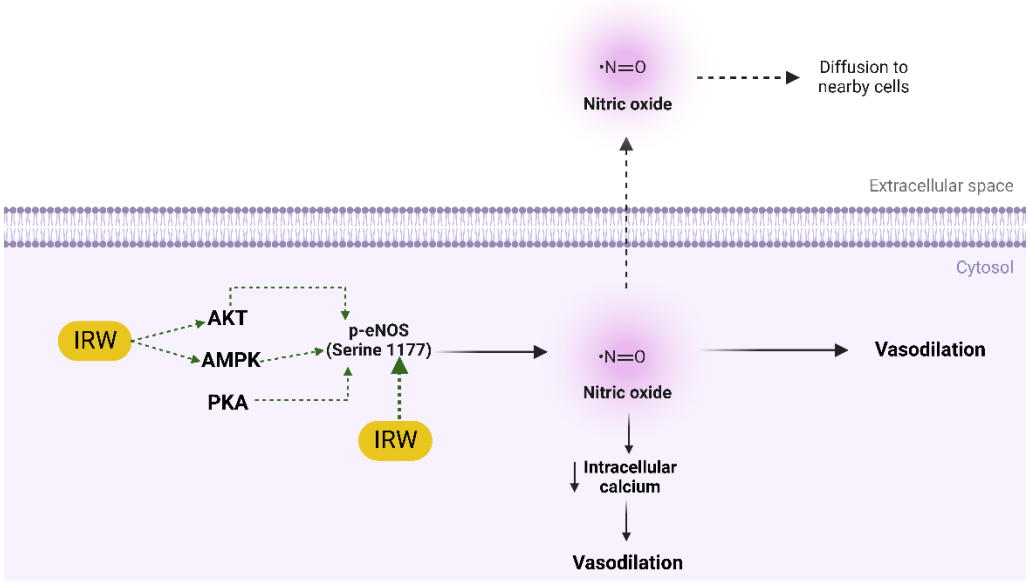

Figure 2 2. The effect of NO on endothelial function.

The vasodilatory effects of NO are attributed to the decrease in intracellular Ca^{2+} concentrations in VSMCs. Additionally, phosphorylation of eNOS at Serine1177 by various serine kinases, such as Akt, AMPK, and PKA, can independently enhance NO production even in the absence of Ca^{2+}. On the other hand, a decrease in eNOS activity contributes to endothelial dysfunction, which is a key factor in the development of hypertension and atherosclerosis. IRW has been shown to increase eNOS levels and promote vasorelaxation by stimulating NO production. Furthermore, IRW enhances the phosphorylation of Akt/eNOS and AMPK improving endothelium-dependent vasorelaxation.

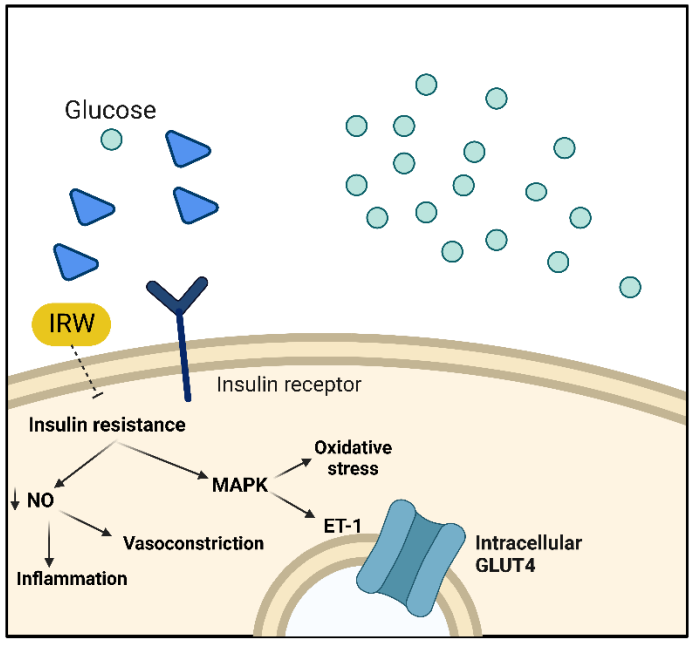

Figure 2. 3 The effect of insulin on endothelial function.

Insulin resistance can result in reduced nitric oxide (NO) production in the endothelium, which in turn promotes vasoconstriction and inflammation. Moreover, insulin resistance is known to impair endothelial function through the activation of the MAPK-dependent pathway, induction of oxidative stress, and release of ET-1. However, treatment with IRW demonstrates a decrease in insulin resistance, indicating potential benefits in improving endothelial function.

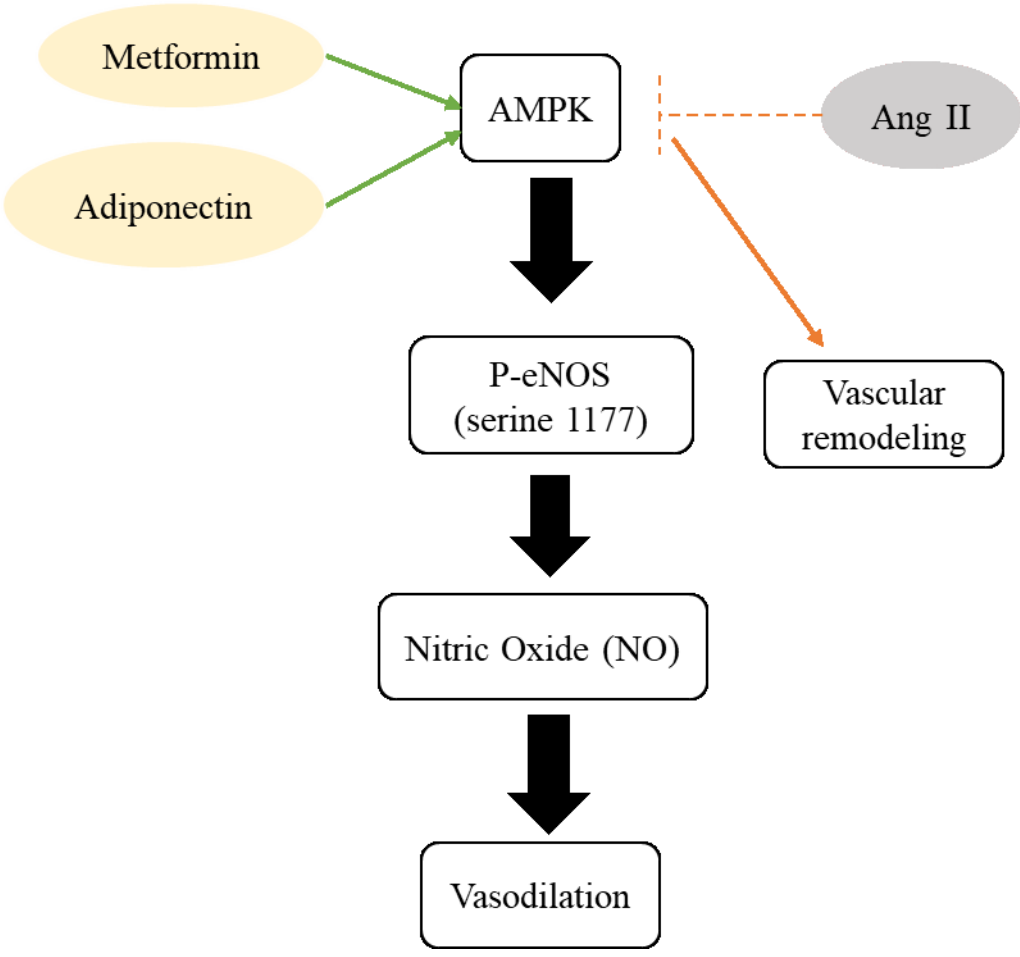

Figure 2. 4 The effect of AMPK on endothelial function.

Endothelial AMPK activation leads to the phosphorylation of eNOS at Ser1177, leading to the production of NO and subsequent vasodilation. Substances such as metformin and adiponectin, known for their ability to activate and phosphorylate AMPK, have been shown to induce relaxation of the aorta. It seems that angiotensin II has the potential to inhibit AMPK, which can have detrimental effects on vascular remodeling.

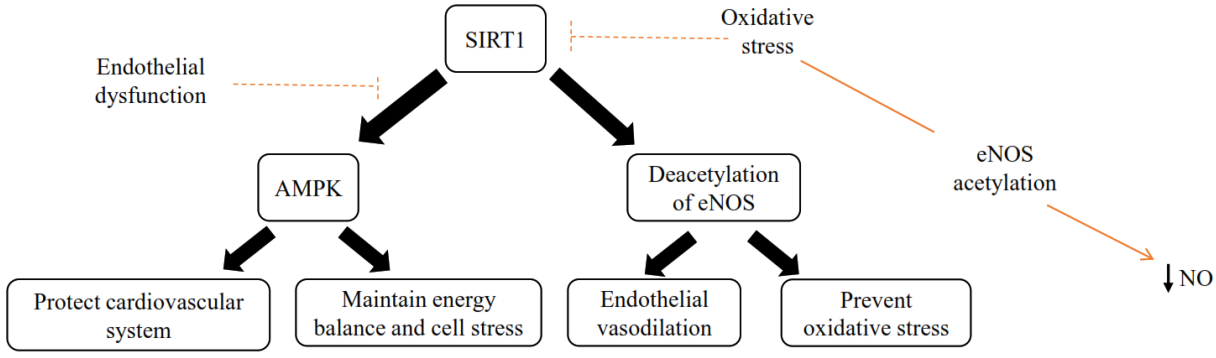

Figure 2. 5 The effect of SIRT1 on endothelial function.

Elevated levels of SIRT1 prevents endothelial senescence induced by oxidative stress and enhanced endothelial-dependent vasodilation by deacetylating eNOS. Additionally, SIRT1 and AMPK work together to maintain cellular stress and energy balance while providing protection for the cardiovascular system. The absence of SIRT1 and p-AMPK in insulin resistance can contribute to endothelial dysfunction. Interestingly, oxidative stress has been observed to down-regulate SIRT1, resulting in eNOS acetylation and reduced production of NO in endothelial cells.

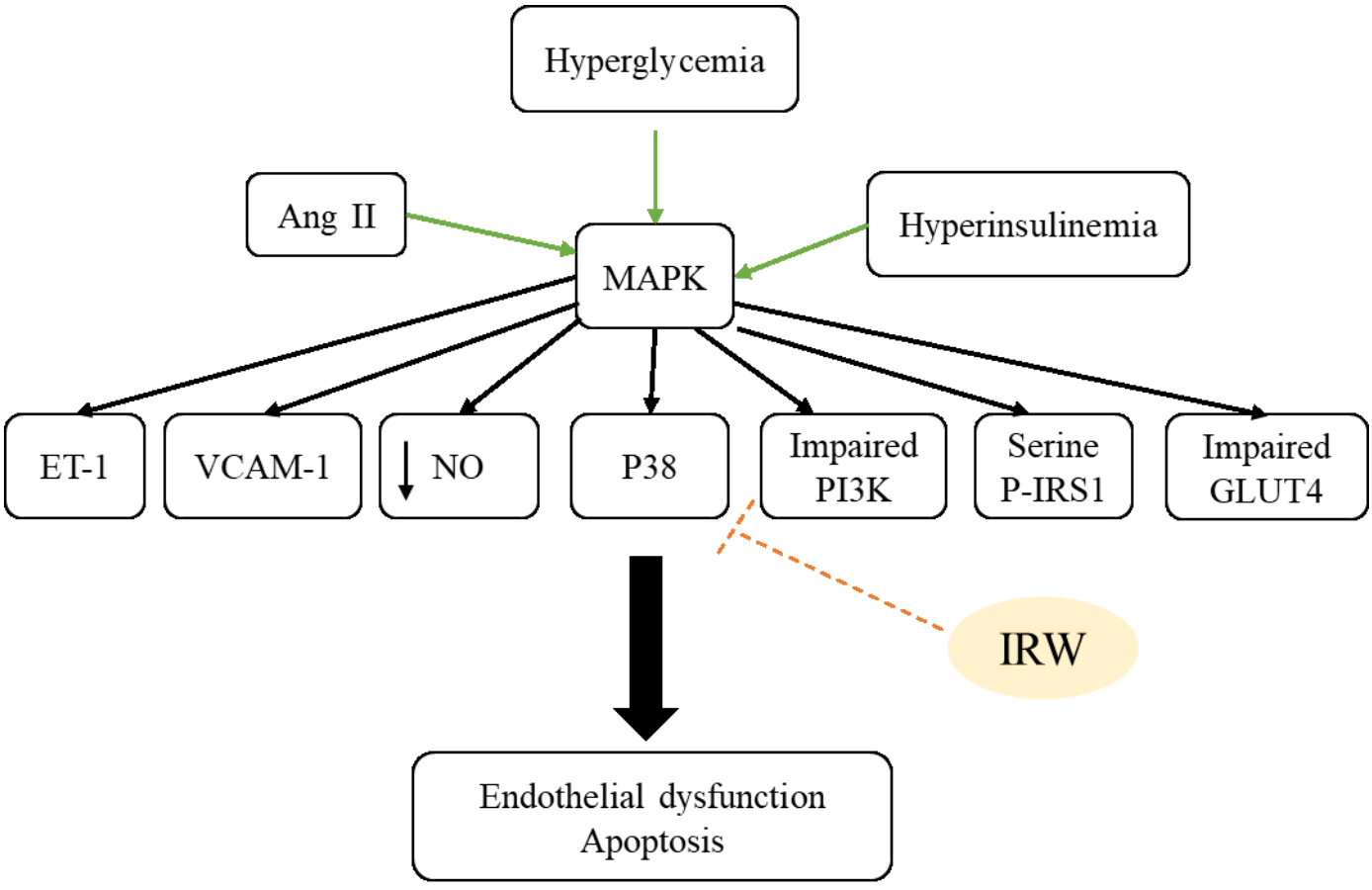

Figure 2. 6 The effect of MAPK on endothelial function.

Activation of Ang II is able to activate the MAPK pathways in endothelial cells, leading to increased serine phosphorylation of IRS-1, impaired activity of PI3k, and ultimately causing endothelial dysfunction and potentially apoptosis. Hyperglycemia and hyperinsulinemia are associated with the activation of MAPK signaling pathways, which can lead to reduce NO production, increased secretion of endothelin-1 (ET-1), elevated expression of VCAM-1, and impaired insulin-stimulated translocation of GLUT4 in adipocytes, all of which contribute to endothelial dysfunction. IRW inhibits the phosphorylation of p38 in cells stimulated by Ang II.

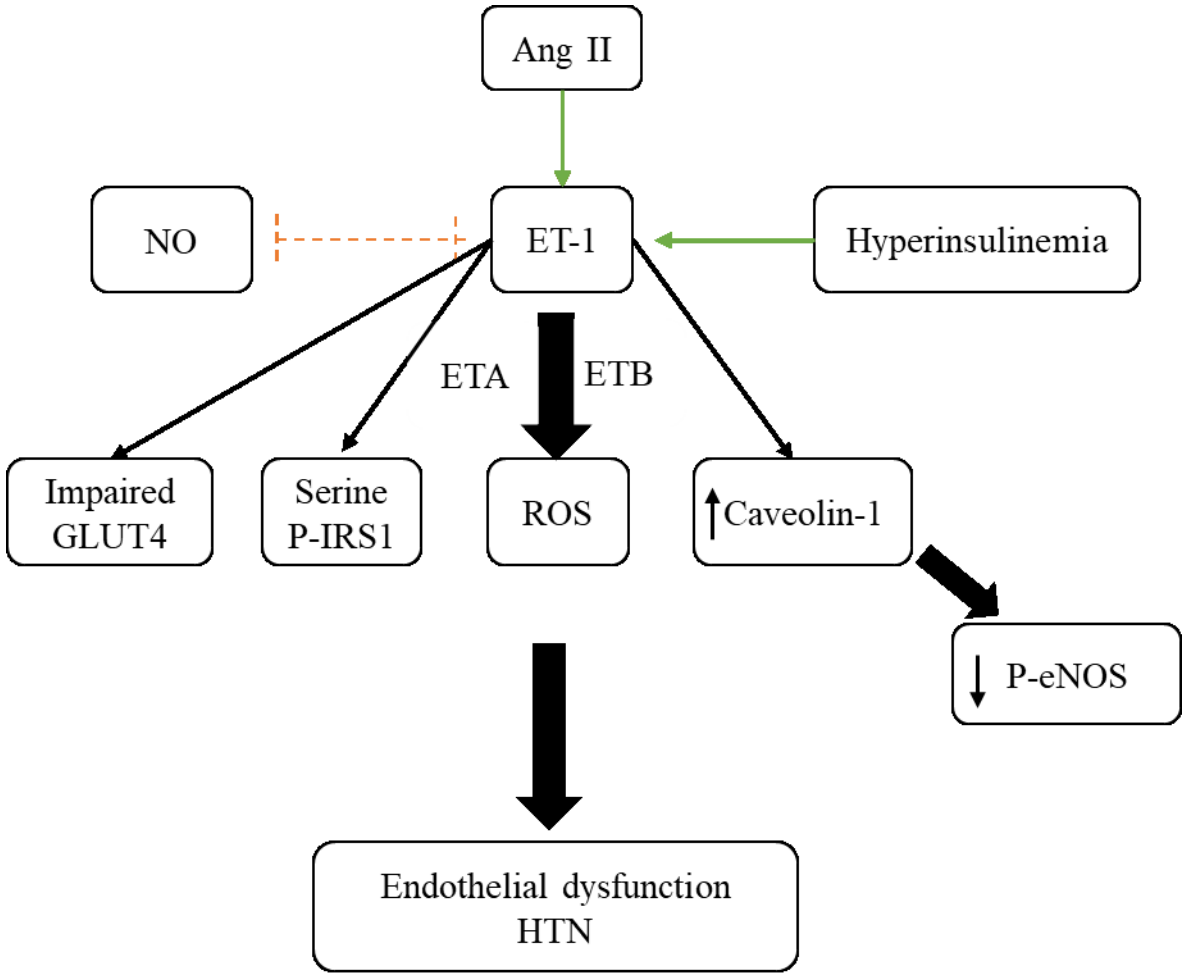

Figure 2. 7 The effect of ET-1 on endothelial function.

ET-1 binds to ETA and ETB receptors, which can increase the production of ROS. The reduced availability of NO is associated with an elevation in ET-1 production. Conversely, NO impedes the ET-1 pathway through various mechanisms. In diseases, ET-1 promotes the synthesis of caveolin-1, leading to a decrease in eNOS activity. ET-1 has been observed to enhance serine phosphorylation of IRS-1 in VSMCs, resulting in decreased activity of PI3k. Moreover, ET-1 impairs the translocation of GLUT4 in response to insulin stimulation in adipocytes. High levels of insulin and Ang II can augment the production of ET-1. Therefore, a decrease in NO availability and an excess of Ang II can contribute to impaired endothelial function, subsequently raising blood pressure.

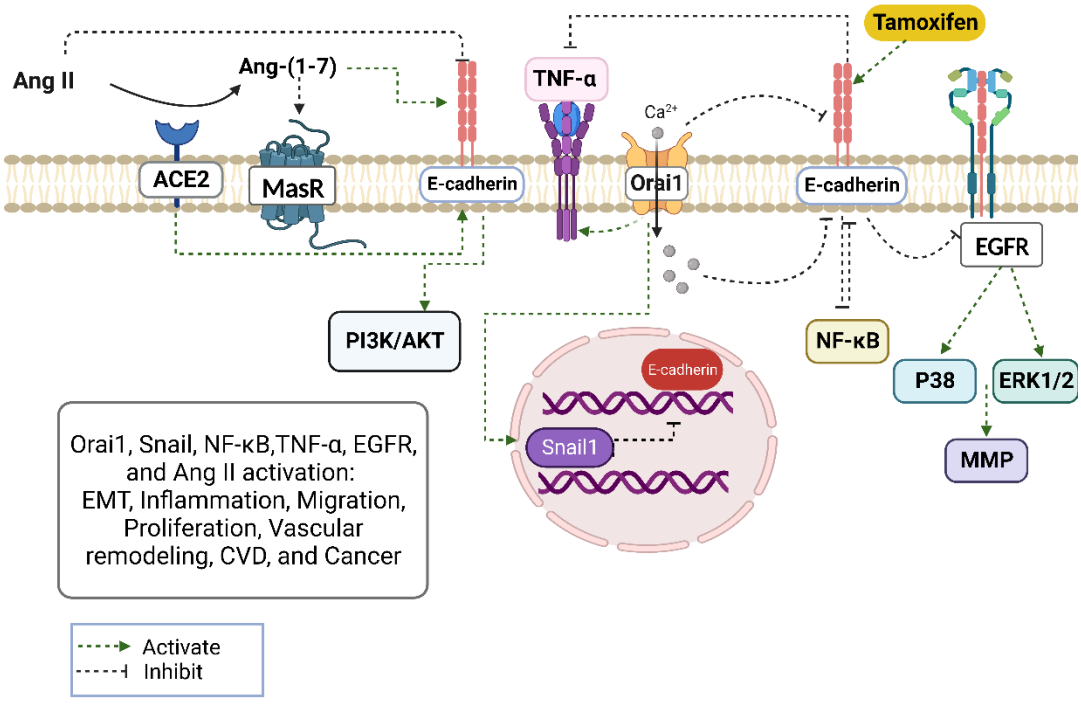

Figure 2. 8 The effect of different factor on E-cadherin expression.

ACE2 or treatment with Ang-(1–7) lead to an increase in the expression of E-cadherin. Additionally, the activation of the E-cadherin promoter by Tamoxifen results in elevated levels of E-cadherin mRNA and protein levels. Conversely, Tamoxifen resistance has been linked to the activation of various downstream kinases, which in turn suppress the expression of E-cadherin. The suppression of E-cadherin is attributed to AngII, NF-κB, Slug/Snail family, and an increase in intracellular Ca^{2+} influx. Notably, E-cadherin acts as an inhibitor of NF-κB activation. The functioning of E-cadherin can be interfered with by the activation of EGFR. E-cadherin was observed to suppress EGFR-induced MAPK signaling while concurrently activating PI3K/AKT signaling. Moreover, a reduction in E-cadherin triggers the activation of the EGFR/ERK signaling pathway, which is dependent on MMP. Orai1 plays a role in migration and inflammation by activating the Slug/Snail family and TNFα and increasing intracellular Ca^{2+}. The decrease in E-cadherin is associated with EMT, invasion, migration, inflammation, vascular remodeling, CVD, and cancer.

CHAPTER 3- TRIPEPTIDE IRW IMPROVES AMPK/ENOS SIGNALING PATHWAY VIA ACTIVATING ACE2 IN THE AORTA OF HIGH-FAT-DIET-FED C57BL/6 MICE

3.1. Introduction

Metabolic syndrome (MetS) is the combination of risk factors associated with cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM), including hypertension, dyslipidemia, impaired glucose tolerance, and obesity (Jahandideh and Wu, 2022). Visceral obesity and insulin resistance, the main pathophysiologic features of MetS, are strongly associated with high blood pressure (Grundy, 2016). A key additional component of insulin resistance is the renin–angiotensin system (RAS), largely known for its role in blood pressure regulation (Marcus et al., 2013). The major biologically active stress component generated by this system is angiotensin II (Ang II) (Son et al., 2018). While angiotensin-converting enzyme (ACE) is responsible for the generation of Ang II, angiotensin-converting enzyme 2 (ACE2) is involved in the conversion of Ang II into angiotensin-(1-7) (Ang-(1-7)). Ang-(1-7) displays anti-inflammatory and anti-hypertensive properties, which antagonize Ang II's harmful vascular impacts (Liao et al., 2019; Serfozo et al., 2020). The expression of local RAS components, especially the Ang II–angiotensin II receptor (AT1R) axis, is enhanced by hyperglycemia, hypertension, hyperinsulinemia, and obesity associated with the MetS (Jahandideh and Wu, 2020). ACE2 was observed to reduce insulin resistance by decreasing the production of Ang II, potentially providing a therapeutic approach for the management of MetS (Wu, 2020).

Bioactive food components, such as bioactive peptides, have attracted the interest of global food scientists for their potential in the prevention and treatment of MetS (Li et al., 2018). IRW (Ile-Arg-Trp), a bioactive tripeptide, was identified and characterized from egg white protein ovotransferrin as an activator of ACE2 in hypertensive animals (Liao et al., 2018a; Wu, 2020). Thus, IRW could shift the balance from vasoconstrictor and inflammatory actions of Ang II towards cytoprotective and vasorelaxant effects via ACE2 activation (Liao et al., 2018a; Liao et al., 2018b; Wu, 2020). Recently, studies showed that the expressions of glucose transporter 4 (GLUT4) and glucose uptake were reduced in hypertension and diabetes (Atkins et al., 2001; Marcus et al., 1994). Moreover, Ang II-mediated hypertension mouse models showed lower GLUT4 expression and glucose absorption in the mice aorta (Park et al., 2005), further supporting an underlying interplay between hypertension and T2D.

In our previous high-fat diet (HFD) model study, we noticed no significant difference in ACE2 expression in skeletal muscle of HFD mice treated with or without IRW (de Campos Zani et al., 2022), refuting a role of local ACE2 activation. However, it should be noted that ACE2 is

significantly up-regulated in mesenteric and aorta arteries of spontaneously hypertensive rats (SHRs) (Atkins et al., 2001), which prompted us to further conduct a study to investigate the role of aorta ACE2 in response to IRW treatment. Although skeletal muscle is the major site of glucose metabolism, vascular function, especially endothelial dysfunction, plays a key role in the pathogenesis of hypertension (van den Oever et al., 2010) as well as insulin resistance and T2D (van Sloten et al., 2014). HFD is widely used to induce obesity and insulin resistance in preclinical animal studies (Elzinga et al., 2021) and is also a key contributor to endothelial dysfunction *in vivo* (Majumder et al., 2013b). HFD can induce chronic inflammation via oxidative stress that disrupts vascular structure and function, resulting in endothelial and physiological dysfunction, and initiate the onset and development of MetS (Panchal et al., 2011). Therefore, in order to gain further insight into the regulatory role of aorta ACE2 in the insulin signaling pathway and endothelial function, this study aims to investigate the effect of IRW on RAS, particularly ACE2, and their association with signaling pathways in the aorta of an HFD-induced obese mouse model.

3.2. Materials and Methods

3.2.1 Chemicals and reagents

Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle medium (DMEM), Opti-MEM I Reduced Serum Medium, fetal bovine serum (FBS), and antibiotics penicillin and streptomycin were purchased from Gibco/Invitrogen (Carlsbad, CA, USA). Lipofectamine 2000 Transfection Reagent and interference RNA (siRNA; 10 μm/L) were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Triton-X-100 was procured from VWR International (West Chester, PA, USA). The synthesized tripeptide IRW (>99.8% purity) was purchased from Genscript (Piscataway, NJ, USA). All other reagents and chemicals of analytical grade were provided by Sigma-Aldrich (St Louis, MO, USA).

3.2.2 Animal model study

Animal Care and Use Committee of the University of Alberta approved our study (protocol# 1402) in line with the Canadian Council on Animal Cares standard. Eighteen male C57BL/6 mice, aged 4 weeks, were obtained from Charles River Canada and given ad libitum access to food and water for one week. The environment was regulated with a 12:12 h light–dark cycle, with 60% humidity and 23 °C temperature. Six of the mice were fed a low-fat diet (10% kcal from fat) and the rest were provided with a HFD (45% kcal from fat) over 6 weeks. Following this period, the mice were randomly separated into three groups: low-fat diet, HFD, and HFD plus IRW (45 mg/kg body

weight/day). All three groups had *ad libitum* access to food and water for 8 weeks, with the HFD plus IRW group additionally receiving IRW (45 mg/kg body weight/day) for the duration.

3.2.3 Tissue collection

Before euthanasia, all animals were fasted for 16 h before being injected intraperitoneally with insulin (2 IU/kg body weight) to stimulate insulin signaling. Blood was collected via cardiac puncture after animals were euthanized with CO_2. In order to obtain plasma, blood was centrifuged at 3000× *g* for 15 min and stored at −80 °C. Aortic vascular smooth muscle tissues were collected, washed with ice-cold saline, weighed, immediately frozen in liquid nitrogen, and stored at −80 °C for further analysis.

3.2.4 Protein extraction and western blotting

Tissue proteins were extracted by protein extraction buffer (20 mm Tris, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM sodium fluoride, and 1% NP-40) containing a 1% (*v*/*v*) protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Then, the homogenates were centrifuged at 15,000× *g* for 15 min at 4 °C. Protein concentrations in the supernatants were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Samples (calibrated to the same protein mass) were loaded on a 9% separating gel and transferred to a nitrocellulose membrane (diameter 0.45 μm; Bio-Rad, Montreal, QC, Canada) for incubation with antibodies of ACE (Abcam, Toronto, ON, Canada), ACE2 (Abcam, Toronto, ON, Canada), AT1R (Novus biologicals, Oakville, ON, Canada), endothelial nitric oxide synthase (eNOS; BD Biosciences, San Jose, CA, USA), phospho-eNOS (p-eNOS Ser1177, Abcam), phospho AMP-activated protein kinase (p-AMPKα Thr172, cs2535), phospho p38 mitogen-activated protein kinases (p38 MAPK, NOVUS Biologicals), phospho extracellular signal-regulated kinase (p-ERK1/2, Cell Signaling Technology, Whitby, ON, Canada), endothelin-1 (ET-1, Abcam), Sirtuin 1 (SIRT1; 9475, Cell signaling Technology, Danvers, MA, USA), peroxisome proliferator-activated receptor gamma (PPARγ, cs2430), and GLUT4 (Abcam, Toronto, ON, Canada). All bands were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam). Goat-anti-rabbit IRDye 680 RD or Donkey-anti-mouse 800 CW secondary antibodies were used to visualize the bands in a Licor Odyssey Bio Imager, with the fluorescence signal quantified using Image Studio Lite 5.2 (Licor Biosciences, Lincoln, NE, USA).

3.2.5 Cell culture

In this study, the A7r5 cell line (ATCC CRL1444, Manassas, VA, USA) was used between passages 8 and 11. The cell culture was performed in 12-well plate(s) in a humidified atmosphere with 95% air/5% CO_2 at 37 °C. The cells were grown in DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin and streptomycin) at 5% CO_2 level. Once reaching ~80% confluence, the cell culture media were replaced with DMEM supplemented with 1% FBS and antibiotics and then treated with 50 μM IRW for 24 h to measure protein expressions of eNOS and AMPK in the cells treated with or without IRW.

3.2.6 SiRNA transfection

To silence ACE2, siRNA and lipofectamine 2000 transfection reagent were used in A7r5 cells. The cells were placed overnight in non-antibiotic DMEM with 10% FBS after reaching 50% confluence. For ACE2 knockdown, the non-antibiotic DMEM for cell culture media was changed to serum-reduced Opti-MEM media. Each targeting well was treated by ACE2 siRNA (80 pmol) containing 0.8 μL of transfection reagent. After 6 h of incubation, non-antibiotic DMEM with 10% FBS was replaced with serum-reduced Opti-MEM media for 24 h. The efficiency of knockdown was at least 70%. After 24 h, cells were placed in the non-antibiotic medium (DMEM + 1% FBS) and then treated with 50 μM of IRW to measure protein expressions of eNOS and AMPK in the cells treated with ACE2 siRNA.

3.2.7 RT-PCR

Total RNA was isolated from the aorta of mice with TRIzol solution and 1 μg of total RNA was then used to synthesize cDNA via the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The expression of targeted genes was measured with quantitative PCR (qPCR), using GAPDH as an internal control, and the MIQE guidelines for qPCR were followed for all experiments and analyses.

3.2.8 Statistics

The results are presented as mean ± SEM of a minimum of three independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) coupled with Dunnett's test by Prism 6 statistical software (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

3.3. Results

3.3.1 IRW treatment up-regulated ACE2 and diminished ACE and AT1R expression in the aorta

ACE2-Ang-(1-7)-Mas receptor (MasR) plays an imperative role in inhibiting oxidation, proliferation, and inflammation in vascular smooth muscle cells (VSMCs). To explore whether IRW could improve ACE2 in the aorta of HFD mice, both protein and RNA levels of ACE2 were examined following treatment of IRW (45 mg/kg body weight) for 8 weeks. Protein and RNA levels of ACE2 were significantly increased in the IRW group compared to the HFD ($p < 0.01$) (Figure 1). Ang II exerts detrimental effects mainly through AT1R (Liao et al., 2018b); the levels of ACE and AT1R were also examined. Our results showed that IRW significantly decreased ACE and AT1R levels in IRW-treated mice compared to the HFD ($p < 0.05$) (Figure 3.1).

3.3.2 IRW enhanced AMPK/SIRT1/eNOS cascade in aorta of HFD mice via aortic ACE2 activation

We previously showed that IRW treatment significantly improved endothelial function in SHR via ACE2 activation (Liao et al., 2016; Majumder et al., 2013a; Majumder et al., 2013b). There is strong evidence that endothelial dysfunction plays a causal role in the development of insulin resistance and the progression of diabetes mellitus (Li et al., 2018; Xu and Zou, 2009). Thus, biomarkers associated with endothelial function such as AMPK, PPARγ, SIRT1, and eNOS were detected. In the next experiments, we examined whether IRW treatment induces AMPK/SIRT1/eNOS cascade in the aorta of HFD mice. AMPK and SIRT1 both regulate each other and share many common target molecules in MetS (Ruderman et al., 2010). HFD feeding reduced PPARγ expression ($p < 0.01$). Treatment with IRW significantly elevated the p-AMPK (The172), SIRT1, and p-eNOS (Ser1177) protein expression ($p < 0.05$); however, no significant changes were observed in the total PPARγ abundance (Figure 3.2). The correlation of ACE2 with AMPK and eNOS was further evaluated by ACE2 knockdown experiment in A7r5 cells. Interestingly, ACE2 knockdown significantly decreased the expression of p-AMPK and p-eNOS in the VSMCs ($p < 0.01$) (Figure 3.3). Overall, these results show that p-AMPK and p-eNOS are positively regulated by ACE2 but negatively associated with insulin resistance.

3.3.3 IRW improved GLUT4 in aorta of HFD mice

GLUT4 is an important glucose transporter to uptake glucose in skeletal muscle (Park et al., 2005). Moreover, the effectiveness of GLUT4 is reliant on the activity of AMPK in skeletal muscle (Haye

et al., 2020). As shown in Figure 3.4, the protein level of GLUT4 was reduced significantly in the aorta of HFD, whereas GLUT 4 translocation to the plasma membrane was higher in the IRW group ($p < 0.01$) compared to the HFD group.

3.3.4 IRW down-regulated ET1/MAPK pathway

MAPK signaling pathways are activated in VSMCs by ET-1, which is a mediator of Ang II-mediated signaling (Bouallegue et al., 2007; Zhang et al., 2016). Next, signal transduction of ET-1/MAPK in the aorta of HFD was investigated. IRW treatment in HFD significantly decreased the levels of ET-1 ($p < 0.01$) and p38 MAPK ($p < 0.05$), which are key members of migration and proliferation remodeling of the vascular system. However, the IRW group showed a significant increase in p-ERK expression compared to the HFD group ($p < 0.05$) (Figure 3.5). This result suggested that IRW at least partially was involved in the modulatory effects of the Ang II–AT1R axis on ET-1/MAPK (P38) in the aorta of mice.

3.4. Discussion

MetS has attracted great attention because the prevalence of obesity and related chronic diseases have increased globally (Nguyen et al., 2022; Yarizadeh et al., 2022). Recent findings have revealed that approximately 25% of the global population suffers from MetS (Prasad et al., 2012). It is possible to treat these diseases with synthetic drugs; however, this can lead to some unfavorable consequences. The tripeptide IRW has been shown to improve glucose tolerance and lower fasting blood glucose and insulin concentrations in the skeletal muscle of mice fed HFD via insulin-dependent signaling and independent pathways (de Campos Zani et al., 2022). Moreover, the anti-inflammatory and antioxidant activities of IRW, along with its ability to up-regulate eNOS and nitric oxide (NO), support its ability to mediate vasorelaxation of blood vessels (Majumder et al., 2013b). In the current study, we provided evidence that IRW could enhance ACE2 level in the aorta of HFD mice, proposing that the aorta might be a specific target of IRW to affect ACE2 activity in MetS. Additionally, ACE2 knockdown caused a marked attenuation of p-AMPK and p-eNOS markers in A7r5 cells, indicating the regulatory role of ACE2 on AMPK and eNOS. These findings suggested that IRW with an increase in ACE2 level and mitigation of the AT1R receptor via multiple signaling pathways including ACE2/AMPK/SIRT1/eNOS, ACE2/AMPK/GLUT4, and ATIR/ET-1/P38 MAPK in the mouse aorta might be a therapeutic agent in MetS. This study was the first to demonstrate that IRW could be linked to MetS through a novel pathway characterized by aortic ACE2 activation.

The RAS has a significant impact on the initiation and progression of insulin resistance (Frigolet et al., 2013). There is evidence that metabolic complications, such as diabetes and obesity, are associated with the upregulation of RAS components such as angiotensinogen, ACE, and AT1R (Skov et al., 2014). ACE2 has been found to counteract the effects of Ang II, resulting in lower blood pressure and a decreased risk of developing CVD (Gheblawi et al., 2020). Despite no effect of IRW on ACE2 and AT1R levels of skeletal muscle being observed on the insulin resistance model (de Campos Zani et al., 2022), we found a decrease in ACE and AT1R protein expression in the aorta of HFD mice treated with IRW, suggesting the modulatory role of IRW on aortic RAS, which is related to MetS. On the other hand, our previous study showed that oral intake of IRW improved the amount and activity of ACE2 in the blood, as well as the amount of ACE2 protein in the aorta of SHR rats (Liao et al., 2018a; Liao et al., 2019). Similarly, IRW increased both ACE2 RNA level and protein expression in the aorta of the insulin-resistant mouse model. This could be attributed to the fact that the genes of the RAS elements were mainly expressed in the organs, such as the heart, brain, kidneys, and aorta (Li et al., 2008a). Therefore, ACE2 might provide novel insight into the molecular mechanism associated with MetS.

AMPK activation was recently proposed as a potential therapeutic target for the prevention and amelioration of insulin resistance and T2D (Mackenzie and Elliott, 2014; Ruderman et al., 2013). Our prior study demonstrated an increase in p-AMPK and PPARγ in the skeletal muscle of HFD mice treated with IRW, but ACE2 was unaffected, suggesting that IRW could potentially improve glucose metabolism independently of ACE2 in the skeletal muscle of HFD mice (de Campos Zani et al., 2022). Moreover, it was observed that the endothelial-dependent vasodilatory response is impaired in insulin resistance (Abbasnezhad et al., 2019). The endothelial AMPK/eNOS pathway accounted for adequate endothelial function in the whole aorta (García-Prieto et al., 2015). Activation of AMPK in endothelial cells by metformin led to the phosphorylation of eNOS at Ser1177, resulting in the production of NO and subsequent dilation of the mice's aorta (Ewart and Kennedy, 2011; Haye et al., 2020). NO is a critical player in vascular homeostasis and maintenance of blood pressure (Machha and Schechter, 2012), and the development and progression of diabetes mellitus are related to the alteration in eNOS expression and activity (Takahashi and Harris, 2014; Triggle and Ding, 2010; Xu and Zou, 2009). Furthermore, upon treatment with rosiglitazone, an insulin sensitizer, the diabetic mouse indicated an increase in the release of adiponectin, which activated AMPK/eNOS signaling pathways in the aorta, subsequently reducing oxidative stress and

amplifying NO bioavailability (Balakumar and Kathuria, 2012). This allowed for an improvement in the mouse's endothelial function due to PPARγ activation (Balakumar and Kathuria, 2012). Our study also indicated the ability of IRW in increasing p-AMPK and p-eNOS in the aorta of HFD mice. These results are in accordance with our previous study that IRW led to a rise in p-eNOS and NO-mediated dilation of the mesenteric arteries of SHRs (Majumder et al., 2013b). Although the protein expression of PPARγ decreased in HFD mice, IRW treatment did not exhibit any effect on PPARγ protein abundance, suggesting that IRW might only be able to regulate PPARγ in the skeletal muscle of the mice with HFD-induced MetS. On the other hand, our study showed reduced levels of p-AMPK and p-eNOS in ACE2 knockout VSMCs, which is in accordance with a recent study indicating that ACE2 deficiency caused a decrease in cardiac p-AMPK levels in knockout rats (Zhang et al., 2017). Thus, our results suggested that IRW, particularly affecting aortic ACE2, could play a crucial role in the MetS by modulating the ACE2/AMPK/eNOS pathway.

SIRT1 plays an important role in endothelial biology as well. Deacetylating eNOS by SIRT1 prevents oxidative stress-induced endothelial senescence and increases endothelial-dependent vasodilation (Mattagajasingh et al., 2007; Ota et al., 2007). Additionally, SIRT1 and AMPK work together to regulate cell stress and energy balance and protect the cardiovascular system (Karpe and Tikoo, 2014). According to previous research, a deficiency in SIRT1 and p-AMPK has been linked to endothelial dysfunction (Karpe and Tikoo, 2014). Our findings suggested that IRW was able to increase AMPK levels, which was regulated by aortic ACE2, and subsequently led to an increase in SIRT1 expression in HFD mice. This could potentially influence MetS and associated pathways.

Our recent study revealed that activation of AMPK without affecting ACE2 promoted GLUT4 translocation to the cell membrane, which ultimately stimulated glucose uptake in skeletal muscle (de Campos Zani et al., 2022). According to the inhibition of basal glucose uptake in the mice aortas by GLUT4 antagonist (indinavir), it was demonstrated that ~50% of basal glucose uptake in VSMCs was mediated by GLUT4 *in vivo* (Park et al., 2005). In addition, it was shown that VSMC GLUT4 levels and glucose uptake were decreased in rats with diabetes mellitus (Atkins et al., 2001). Similarly, we observed the improvement of membrane localization of GLUT4 in the aorta of HFD mice treated with IRW. Additionally, we observed the regulatory role of ACE2 on AMPK, proposing that the ACE2/AMPK/GLUT4 signaling pathway in the aorta might play a role in regulating MetS. Although evidence demonstrated that AMPK had a positive influence on

GLUT4, no proof of modulating GLUT4 by AMPK was reported in VSMCs. The exact mechanism by which IRW enhances GLUT4 in VSMCs with and without aortic ACE2 expression remains unknown; thus, further research is required to explore other possible pathways.

ET is a potent vasoconstrictor and stimulates the renin–angiotensin–aldosterone system. The ET-1 receptor being triggered causes the MAPK cascade to occur, which is a key signaling event in endothelial function. MAPKs are serine/threonine protein kinases playing a significant role in mediating Ang II-induced signaling in VSMCs (Bouallegue et al., 2007; Liao et al., 2016). Hyperinsulinemia could trigger MAPK pathways, leading to reduced NO production and higher ET-1 release, which are linked to impaired endothelial function (Jansson, 2007; van den Oever et al., 2010). It was shown that expressions of p-ERK and several upstream signaling p38 proteins in the MAPK pathway in the HFD renal tissues were elevated significantly compared to the normal group (Ye et al., 2019). Previously, IRW reduced the phosphorylation of p38 when VSMCs were stimulated with Ang II, implying that IRW could have an important function in regulating MAPKs through the AngII/AT1R pathway (Liao et al., 2018b). However, IRW treatment did not prevent ERK1/2 MAPK phosphorylation even with an increase in Ang-(1-7) in the aorta of SHRs (Liao et al., 2019). Consistent with these results, the current study demonstrated that HFD only induced an increase in ET-1 and P38 MAPK in the aorta, which could be countered by IRW treatment *in vivo*. No significant decrease in ERK1/2 expression was observed after IRW treatment in the aorta of HFD mice, suggesting that further investigation into the endothelial function related to MetS is required. Further functional studies in isolated mice aortic rings, histopathology of the VSMCs in the aorta, and immunohistochemistry to identify protein nuclear localization should be considered to provide strong evidence for the potential use of IRW as a treatment.

3.5. Conclusions

This research indicated that ACE2 in the aorta of HFD obese mice might play an important role in maintaining the ACE2/AMPK/eNOS signaling pathways. Therefore, aortic ACE2 might be a potential focus of IRW to improve glucose uptake and vasodilation of blood vessels in MetS. Moreover, IRW might play a crucial role in the suppression of AT1R/ET-1/ p38 MAPK signaling pathways related to MetS *in vitro* and in *vivo* models. Overall, our study is the first to link the effect of bioactive peptides on MetS with the local RAS, in particular, ACE2 in the aorta of HFD mice. Furthermore, our data suggest that IRW holds strong potential for modulating metabolic

complications by aortic ACE2 regulation *in vivo*. Thus, this makes IRW worthy of further investigation as a therapeutic agent for MetS in the aorta.

3.6. References

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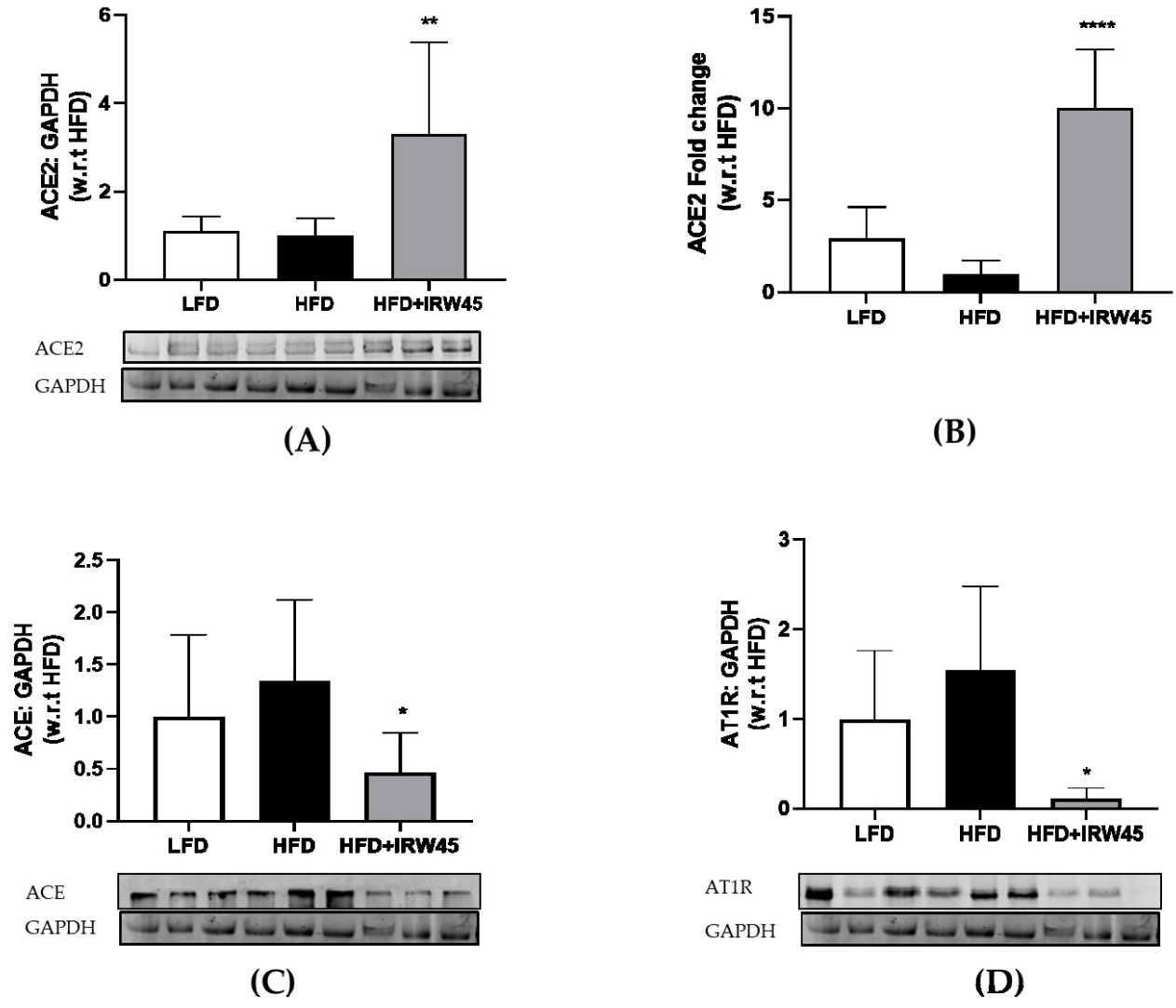

Figure 3. 1 Effect of IRW on protein and RNA expression of ACE2 and protein expression of ACE and AT1R in aorta of HFD mice.

(A) Quantification and Western blots of ACE2. (B) q-PCR quantification of ACE2. Quantification and Western blots of (C) ACE and (D) AT1R. ACE2, ACE, and AT1R were normalized to GAPDH. Data were analyzed by ANOVA coupled with Dunnett's test. Data expressed as mean ± SEM of n = 6 mice. *, $p < 0.05$, **, $p < 0.01$, and ****, $p < 0.0001$ versus HFD. HFD: high-fat diet; LFD: low-fat diet; w.r.t HFD: with regard to HFD.

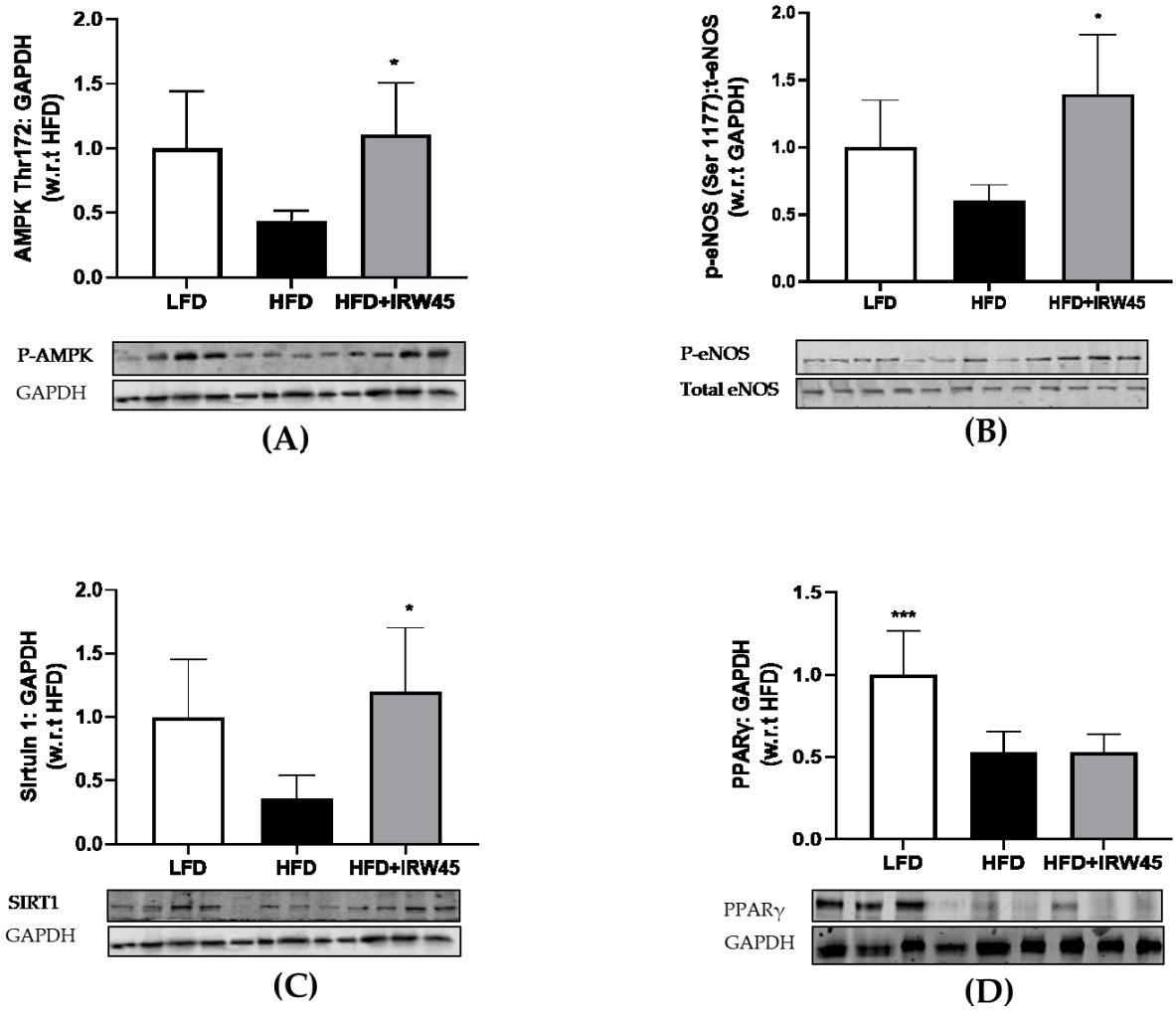

Figure 3.2 Effect of IRW on expression of p-AMPK, p-eNOS, Sirtuin1, and PPARγ in aorta of HFD mice.

Quantification and Western blots of (A) p-AMPK, (B) p-eNOS, (C) Sirtuin 1, and (D) PPARγ. P-AMPK, Sirtuin1, and PPARγ were normalized to GAPDH. p-eNOS was normalized to Total eNOS. Data were analyzed by ANOVA coupled with Dunnett's test. Data expressed as mean ± SEM of $n = 4$ for p-eNOS, Sirtin 1, and p-AMPK, and $n = 6$ for PPARγ. *, $p < 0.05$, ***, and $p < 0.001$ versus HFD. HFD: high-fat diet; LFD: low-fat diet; w.r.t HFD: with regard to HFD.

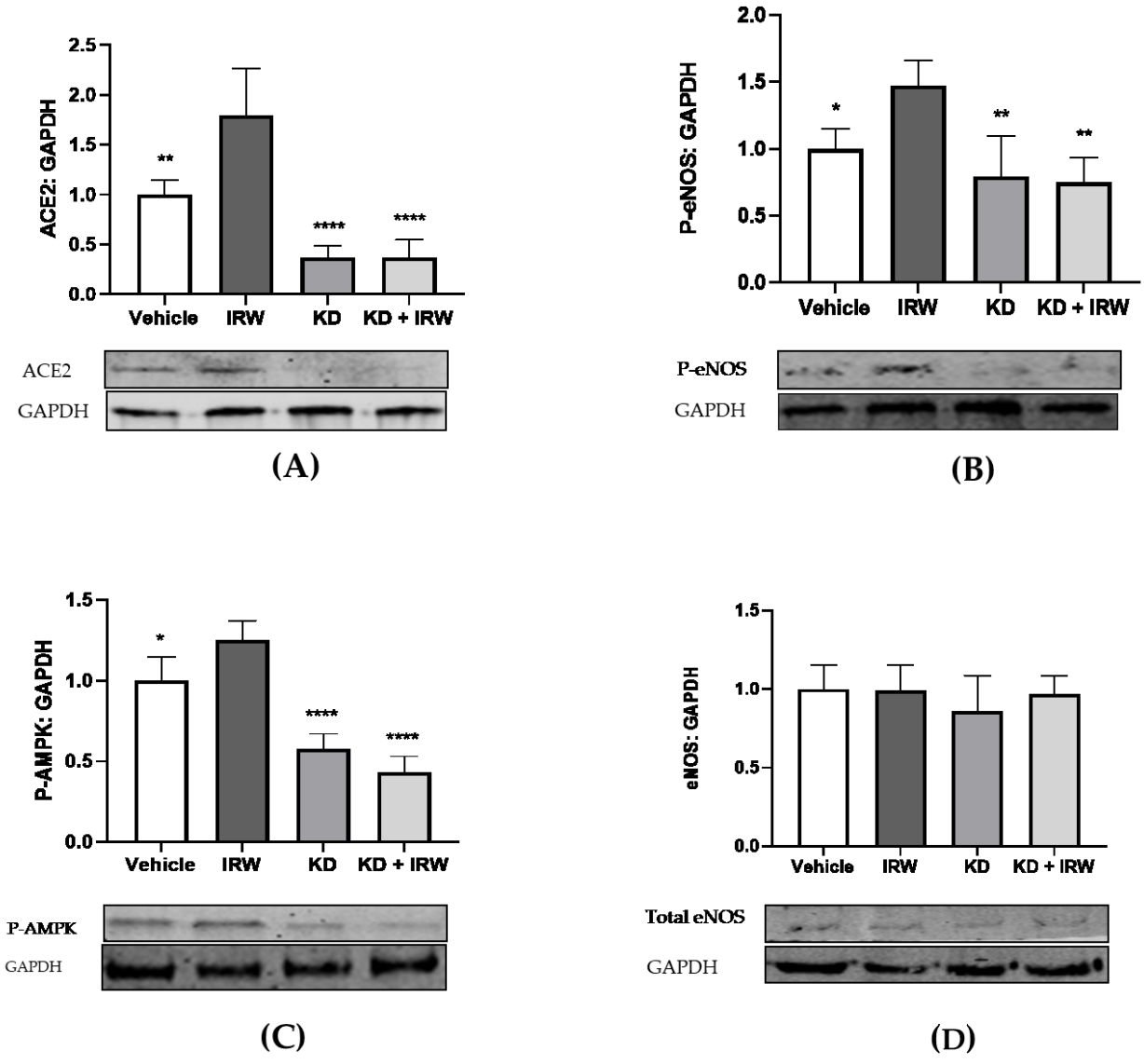

Figure 3. 3 Effect of IRW on expression of ACE2, p-eNOS, p- AMPK, and Total eNOS in vehicle, IRW-treated, and ACE2 knockdown (KD) VSMCs.

ACE2 knockdown in A7r5 cells were conducted using ACE2 siRNA. Afterwards, cells were treated with 50 µM of IRW for 24 h. The cells were then lysed and western blotting of the lysates was performed. Quantification and Western blots of (A) ACE2, (B) p-eNOS, (C) p-AMPK, and (D) Total eNOS. ACE2, p-AMPK, p-AMPK, and Total eNOS were normalized to GAPDH. Data were analyzed by ANOVA coupled with Dunnett's test. Data expressed as mean ± SEM of four independent experiments. *, $p < 0.05$, **, $p < 0.01$, and ****, $p < 0.0001$ versus IRW-treated group.

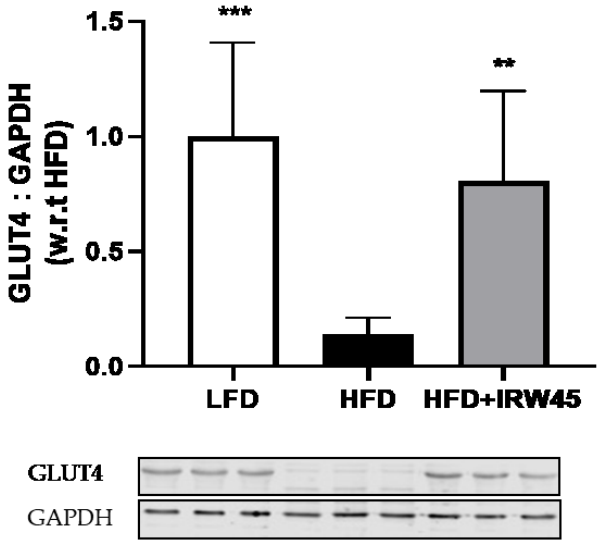

Figure 3. 4 Effect of IRW on expression of GLUT4 in aorta of HFD mice.

Quantification and Western blots of GLUT4. GLUT4 was normalized to GAPDH. Data were analyzed by ANOVA coupled with Dunnett's test. Data expressed as mean ± SEM of n = 6 mice. **, $p < 0.01$ and ***, $p < 0.001$ versus HFD. HFD: high-fat diet; LFD: low-fat diet; w.r.t HFD: with regard to HFD.

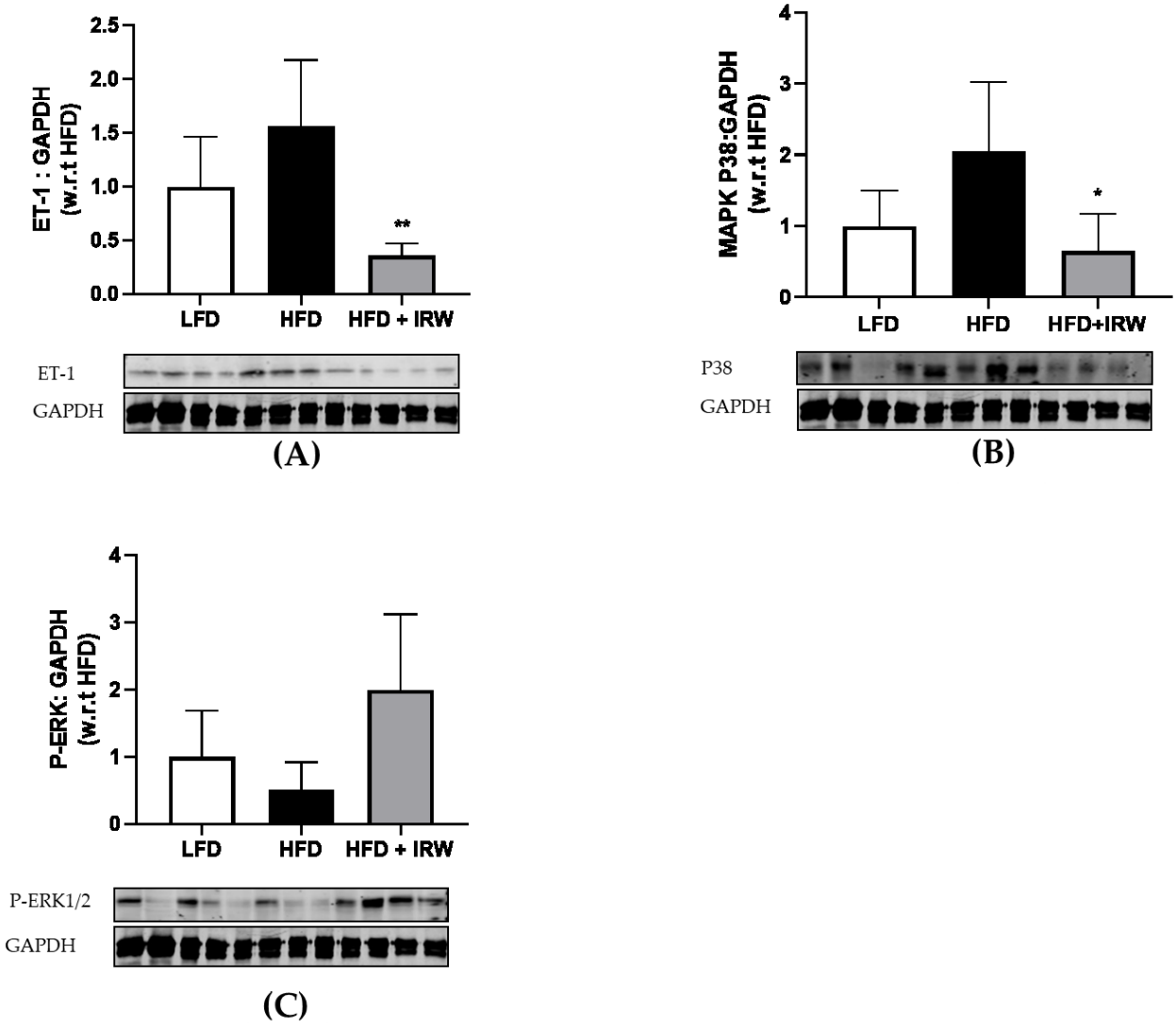

Figure 3. 5 Effect of IRW on expression of ET-1, MAPK P38, and P-ERK1/2 in aorta of HFD mice.

Quantification and Western blots of (**A**) ET-1, (**B**) MAPK P38, and (**C**) p-ERK. ET-1, MAPK P38, and p-ERK were normalized to GAPDH. Data were analyzed by ANOVA coupled with Dunnett's test. Data expressed as mean ± SEM of n = 4 mice. *, $p < 0.05$, ** and $p < 0.01$ versus HFD. HFD: high-fat diet; LFD: low-fat diet; w.r.t HFD: with regard to HFD.

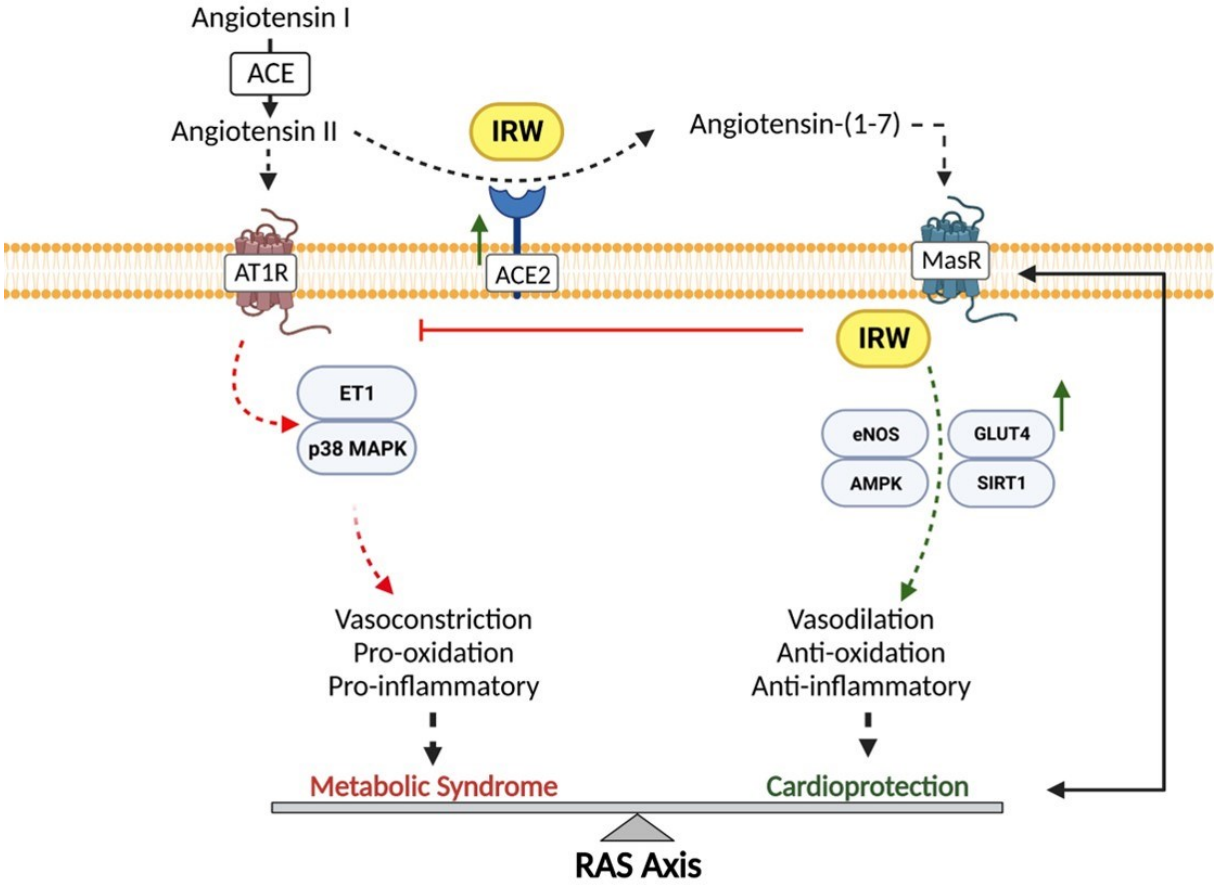

Figure 3. 6 The effect of IRW on regulation of RAS by IRW and their associated pathways. Upregulation of aortic ACE2 by the peptide IRW is responsible for the activation of pathways associated with vasodilation of blood vessels, anti-oxidation, and anti-inflammatory responses. These pathways may play a role in improving insulin resistance and glucose metabolism in HFD-induced insulin-resistant mice. On the other hand, IRW has an inhibitory effect on vasoconstriction, pro-oxidation, and pro-inflammatory pathways.

CAPTER 4- MODULATION OF E-CADHERIN/SNAIL SIGNALING PATHWAY BY IRW THROUGH ACE2

4.1. Introduction

Vascular smooth muscle cells (VSMCs) are specialized cells found in the vasculature that can adjust the internal diameter of the vessels and regulate blood pressure through contraction and relaxation (Liao et al., 2018b). VSMCs are known to undergo remodeling in the presence of high blood pressure, which is characterized by excessive proliferation, inflammation, oxidative stress, and migration (Belo et al., 2015; Touyz and Schiffrin, 2000). As a significant factor in cardiovascular diseases (CVDs), hypertension is associated with abnormal VSMC migration and proliferation (Lacolley et al., 2012; Liao et al., 2018b; Mills et al., 2020).

Epithelial cadherin (E-cadherin), a transmembrane glycoprotein encoded by the CDH1 gene, is responsible for calcium (Ca^{2+})-dependent cell–cell adhesion (Van Roy and Berx, 2008). E-cadherin is an essential component of adherens junctions, providing a tight connection between adjacent epithelial cells and maintain intercellular integrity and connections (Van den Bossche et al., 2012). Besides its vital role in adhesion, cadherins are able to interact with a variety of proteins in the cell and affect processes like migration, proliferation, survival, and differentiation (Frismantiene et al., 2018b). A recent study has shown that oxidation of low-density lipoprotein (LDL) decreased E-cadherin, resulting in an increase in the proliferation of human aortic smooth muscle cells (HSMCs) (Bedel et al., 2008). Matrix metalloproteinases (MMPs) are responsible for breaking down the extracellular matrix (ECM), including E-cadherin (9), which would cause the migration and proliferation of SMCs (Galis and Khatri, 2002). Moreover, the transcription of the E-cadherin gene can be suppressed by the transcription factors of the Zinc finger protein SNAI1 (Snail) family; consequently, it can promote Epithelial-mesenchymal transition (EMT) and cell migration (Loh et al., 2019).

The coordination of Ca^{2+} at the interface between successive modules of the cadherin ectodomain is believed to regulate the adhesive interactions of cadherins (Courjean et al., 2008). Ca^{2+} plays a crucial part in the fluids inside and outside of a living cell, helping to maintain the cell's homeostasis and other physiological functions (Berridge et al., 2000). It can be released from the endoplasmic reticulum (ER) to the cytosol and organelles. When the ER calcium reserves are depleted, this is detected by a membrane sensor, which then triggers the opening of Orai Calcium Release-Activated Calcium Modulator 1 (Orai1) in a process known as store-operated calcium entry (SOCE) (Sharma and Elble, 2020). It has been demonstrated that activation of Orai1 in the aorta of Spontaneously Hypertensive Rats-Stroke Prone (SHRSP) disrupted the regulation of

intracellular Ca^{2+} levels (Lu et al., 2022). Also, Orai1 knockdown significantly up-regulated E-cadherin expression in human colorectal cancer cells (SW480 cells) (Kang et al., 2021). It is suggested that SOCE might activate Snail1 to suppress E-cadherin expression, which can promote metastasis (Yu et al., 2016).

Interest in food protein-derived bioactive peptides is on the rise as they appear to have the potential to manage and prevent chronic diseases (Jiang and Wu, 2022; Majumder et al., 2013a). The egg peptide Ile-Arg-Trp (IRW) was found to enhance angiotensin-converting enzyme 2 (ACE2) expression in the VSMCs and reduce blood pressure in spontaneously hypertensive rats (SHRs) primarily through the ACE2/angiotensin-(1-7) (Ang-(1–7))/ Mas receptor (MasR) axis (Fan and Wu, 2021). IRW has also been shown to possess anti-inflammatory and antioxidant effects on vascular endothelial cells, likely having a positive effect on vascular tone (Liao et al., 2018a). In our previous study, IRW treatment has been shown to increase the expression of CDH-1 gene in mesenteric arteries, which may reduce the risk of hyperplasia of vasculature and associated cardiovascular disorders (Majumder et al., 2015). In another study, it was observed that IRW could limit the migration of VSMCs in response to angiotensin II (Ang II) stimulation, as demonstrated by the lowering of MMP levels and mitogen-activated protein kinase (MAPK) in A7r5 cells (Liao et al., 2018b). Moreover, our lab discovered that IRW was capable of suppressing Ang II-induced inflammation in A7r5 cells (Liao et al., 2016). The ACE2/Ang-(1–7)/MasR pathway also appears to inhibit the activity of transcription factors Snail1, which have been linked to the development of EMT and migration (Loh et al., 2019; Yu et al., 2016). Additionally, ACE2/Ang-(1–7)/Mas axis activation was found to inhibit breast cancer cell metastasis by deactivating SOCE and increasing E-cadherin expression (Yu et al., 2016). These findings suggest that the ACE2/Ang-(1–7)/Mas pathway is likely to be involved in regulating the E-cadherin and SOCE pathways. The aim of this study is to explore the effect of IRW on E-cadherin /Snail 1 and SOCE signaling pathways and the interaction of E-cadherin with ACE2/Ang-(1–7)/Mas axis, which might influence proliferation and migration in both *in vivo* and *in vitro*.

4.2. Experimental section

4.2.1 Chemicals and Reagents

All calcium measurements and confocal microscopy were conducted with HEPES-PSS buffer containing 140 mM NaCl, 10 mM glucose, 5 mM KCl, 5 mM HEPES, 1.5 mM $CaCl_2$, and 1 mM $MgCl_2$, with a pH of 7.4. Fluo-4AM, Pluronic F-127, Dulbecco's modified Eagle medium

(DMEM), fetal bovine serum (FBS), and penicillin–streptomycin and gentamicin antibiotics were acquired from Invitrogen (Carlsbad, CA, USA). A stock solution of Fluo-4AM was made by dissolving it in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA). Dulbecco's phosphate-buffered saline (PBS) and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triton-X-100 was acquired from VWR International (West Chester, PA, USA) supplied. IRW with a minimum purity of 99% was synthesized by Genscript (Piscataway, NJ, USA).

4.2.2 Animal study

Male SHRs between 12 and 14 weeks old, weighing 290.0±10 g, were obtained from Charles River in Senneville, Canada. These animals were kept in the University of Alberta animal core facility for a week to acclimate, with a 12:12 hour light: dark cycle, controlled humidity and temperature, and access to rat chow and water ad libitum. This procedure was approved by the University of Alberta Animal Welfare Committee (Protocol # AUP 00001571) and complied with Canada Council on Animal Care guidelines and the Guide for the Care and Use of Laboratory Animals by the United States National Institutes of Health. The animals were separated into two groups: Untreated (n=4) and IRW (15 mg/kg body weight, n=4). IRW was given orally once per day for 7 days, dissolved in 20 mL of Ensure (Abbott Nutrition, QC, Canada) as previously described (Majumder et al., 2013b).

4.2.3 Cell culture

A7r5 rat aorta VSMC cells, obtained from ATCC (cat# CRL-1444, Manassas, VA, USA) between passages 3 and 10, were grown in DMEM supplemented with 10% FBS and 1% antibiotics until 70% confluence. Then, the cells were placed in a quiescent DMEM medium supplemented with 1% FBS and 1% antibiotics. IRW (25 μM, 50 μM, and 100 μM) or Tamoxifen (0.5 μM, 1 μM, and 2 μM) was incubated for 24 h. Next, to investigate the effect of Ang II and A779 (MasR antagonist), the cells were treated with 50 μM of IRW 1 hour before adding 1 μM Ang II or 1 μM of A779 (Cambridge, MA, USA).

4.2.4 ACE2 knockdown

Invitrogen's Lipofectamine 2000 transfection reagent and small interfering RNA (siRNA) were used to silence ACE2 in A7r5 cells. Cells were seeded in non-antibiotic DMEM with 10% FBS and incubated overnight. The cell culture media was then replaced with serum-reduced Opti-MEM and transfected with 80 pmol of either ACE2 siRNA, which contains 0.8 μL of transfection.

Following incubation for 6 h, the serum-reduced Opti-MEM was replaced with non-antibiotic DMEM with 10% FBS for 24 h. The knockdown efficiency of ACE2 was at least 70%. Cells were then placed in a non-antibiotic quiescing medium (DMEM + 1% FBS) and treated with 50 μM of IRW.

4.2.5 Protein extraction and western blotting

At the end of the experiment, the A7r5 cells were lysed in boiling Laemmle's buffer with 50 mM of DTT (a reducing agent) and 0.2% Triton-X-100 to extract samples for Western blotting. Proteins from the aortas were extracted using a protein extraction buffer (10 mM $Na_4P_2O_7$, 100 mM sodium fluoride, 5 mM EDTA, 1% NP-40, and 20 mM Tris) which included 1% (v/v) of a protease inhibitor cocktail (from Sigma, St. Louise, MO). After homogenization, the mixture was centrifuged at 10,000 Xg for 10 min at 4 ℃. Protein concentration was measured with the bicinchoninic acid (BCA) assay (ThermoFisher, Waltham, MA, USA). Samples of cell lysates and total aortas were separated using 9% SDS-PAGE and then moved to nitrocellulose membranes. The membrane was then immunoblotted with antibodies. Protein bands for E-cadherin (Novus Biologicals, Littleton, CO, USA), 4 hydroxy Tamoxifen (Sigma-Aldrich, St. Louis, MO, USA), Snail 1 (Novus Biologicals, Littleton, CO, USA), and Orai1 (Novus Biologicals, Littleton, CO, USA) were normalized to the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam, Waltham, Boston, USA). Goat anti-rabbit IRDye 680RD and Donkey anti-mouse 800CW from Licor Biosciences (Lincoln, NE, USA) were employed as the secondary antibody. Protein bands were identified with the Licor Odyssey BioImager (Licor Biosciences, Lincoln, NE, USA), and the amount was calculated by densitometry with the Image Studio Lite 5.2 (Licor Biosciences, Lincoln, NE, U.S.A).

4.2.6 Cytoplasmic measurement of calcium

To determine cytoplasmic Ca^{2+} levels in the aorta, 2-mm sections of aortic rings were flipped over and treated with 5 M Fluo-4AM with 5 M Pluronic F-127 (ThermoFisher, Waltham, MA, USA) for two h at 37°C. Later, the aortic rings were washed out in HEPES-PSS solution for 15 minutes (Esfandiarei et al., 2011). For in vitro measurement of calcium signals, subconfluent VSMCs were grown on 24 well culture dishes 48 h before each experiment. Cells were then treated with 50 μM IRW for 24 h. Next, treated and untreated cells were loaded with Fluo-4AM at 37°C for 20 min and subsequently with DAPI at 37°C for 10 min, followed by a 15- to 20-min wash in HEPES-

PSS buffered physiological saline solution and fluorescence was visualized in a fluorescence microscope (Olympus IX81, Olympus Canada Inc., Ontario, CA).

Images from tissue were acquired on Olympus FV3000 scanning confocal fluorescent and laser microscope (Tokyo, Japan). All parameters (laser intensity, gain, etc.) were maintained constant during the experiment. The tissue was illuminated using a high-gain photomultiplier tube to collect the emission (505–550 nm). The measured changes in Fluo-4 fluorescence level are proportional to the relative changes in Ca^{2+}cytoplasm.

4.2.7 Statistics

Statistical analysis was done using GraphPad Prism (from La Jolla, CA, USA). The data was displayed as the average ± SEM (standard error of the mean) from 4 animals. The results of the western blotting analysis were examined by unpaired Student's t-test (figure 4.4) or one-way analysis of variance (ANOVA) along with Dunnett's multiple comparisons test (figures 4.1, 4.2, and 4.3). The confocal and fluorescent images were analyzed offline with ImageJ 4.0 Software (figures 4.5 and 4.6). A p-value of less than 0.05 was seen as statistically significant.

4.3. Results

4.3.1 IRW promoted the expression of E-cadherin while down-regulated the expression of Snail1 and Orai1 in vitro

Up-regulation of E-cadherin has been associated with anti-apoptotic, anti-migratory, and anti-EMT effects in the vasculature (Loh et al., 2019). To explore whether IRW was involved in the regulation of E-cadherin, A7r5 cells were treated with 25, 50, and 100 μM of IRW. In our study, protein levels of E-cadherin in A7r5 cells were significantly up-regulated by 50 μM and 100 μM of IRW treatment ($p<0.01$; Figure 4.1 A**)**. Moreover, evidence suggested that transcription factors such as Snail1, which directly bind to the E-cadherin gene, suppress E-cadherin transcription and facilitate cell migration and proliferation (Loh et al., 2019). Also, Orai1-mediated SOCE sensitized cells to apoptosis and proliferation (Sharma and Elble, 2020). Our data showed that treatment of the A7r5 cell with 100 μM of IRW countered Snail1 and Orai1 significantly by decreasing their protein expression compared to vehicle ($p<0.05$) **(**Figure 4.1 B&C**)**.

4.3.2 ACE2 knockdown abolished E-cadherin expression in vitro

We further evaluated the dependence between ACE2 and E-cadherin by knocking down ACE2 expression with siRNA, as there appears to be a link between the increased expression of ACE2 and E-cadherin by IRW. It was found that ACE2 knockdown significantly diminished the E-

cadherin protein expression in A7r5 cells ($p<0.01$). Meanwhile, IRW treatment could not restore the expression of E-cadherin in ACE2-knockdown cells **(**Figure 4.2 A**)**. These results supported that E-cadherin is ACE2-dependent in VSMCs.

4.3.3 E-cadherin expression was down-regulated in A7r5 cells treated with A779

Our previous study showed that administration of a combination of A779 with IRW eliminated the IRW's ability to reduce blood pressure in SHRs, reinforcing the essential role of ACE2/Ang-(1-7)/MasR axis in IRW's role (Wu, 2020). As illustrated by Figure 4.1 A, IRW treatment significantly enhanced E-cadherin expression ($p<0.05$). However, the protein expression of E-cadherin in A7r5 cells via IRW treatment was mitigated by A779 treatment ($p<0.05$; Figure 4.2 B). The above results indicated the regulatory role of IRW on E-cadherin expression through the ACE2/Ang-(1-7)/MasR axis in VSMCs.

4.3.4 E-cadherin's expression was diminished in Ang II stimulated A7r5 cells

In VSMCs, Ang II activates a series of signaling pathways which leads to the expression of downstream proteins, resulting in migration and vascular remodeling (Liao et al., 2018b). As expected, treatment with 1 μM of Ang II for 24 h significantly decreased the protein level of E-cadherin in A7r5 cells without IRW treatment **(**Figure 4.2 C**)**. However, IRW was not able to increase the E-cadherin level in A7r5 cells stimulated by Ang II ($p<0.01$). This suggests that Ang II could be responsible for reducing E-cadherin levels in VSMCs.

4.3.5 IRW has the same effectiveness as Tamoxifen in terms of E-cadherin expression in vitro

Studies confirm that Tamoxifen significantly reduces hyperplasia and apoptosis *in vitro* and *in vivo* (Hiscox et al., 2011; Nazarali and Narod, 2014). Also, the direct involvement of E-cadherin in the enhanced cell-cell adhesion upon 4 hydroxy Tamoxifen treatment in MCF7 cells was reported (Mauro et al., 2010). Therefore, we evaluated the E-cadherin expression using different concentrations of Tamoxifen to determine the most effective dosage in VSMCs. As shown in Figure 4.3 A, the expression of E-cadherin significantly increased when treated with 0.5 μM ($p<0.01$) and 1 μM ($p<0.05$) Tamoxifen, as compared to 2 μM in A7r5 cells. Moreover, E-cadherin expression rose comparably at 0.5 μM Tamoxifen ($p<0.01$) and 100 μM IRW ($p<0.01$).

4.3.6 IRW-treated rats increased E-cadherin while reduced Snail1 expression, yet there was no change in Orai1

It was reported that cell migration could be blocked by reducing the activity of certain molecules and pathways that are involved in the migration signaling pathway, such as Snail1, and/or by

increasing the level of the protein E-cadherin (Loh et al., 2019). In our study, IRW treatment significantly increased E–cadherin expression but decreased the protein levels of Snail1, but no effect on Orail in the aorta of SHRs ($p<0.05$) **(**Figure 4.4**)**. An influx of Ca^{2+} is believed to be caused by an opening of Orai1 channels that allows for SOCE (Kang et al., 2021). In our study, the intracellular Ca^{2+} concentration reduced significantly in both SHRs and VSMCs treated with IRW compared to the untreated group ($p<0.01$) **(**Figures 4.5 & 4.6**)**.

4.4. Discussion

Hypertension is a key factor for CVDs, contributing to 30% of all deaths worldwide (Majumder et al., 2015). Hypertension is linked to changes in the structure of blood vessels, which is called remodeling. This remodeling is composed of alterations at the cellular level, such as changes in the proliferation, migration, and inflammation of VSMCs (Touyz et al., 2003). Cell–cell contacts are an essential survival signal for many cell types, including VSMCs (George and Beeching, 2006). E-cadherin is a Ca^{2+}-dependent protein and a well-known growth and invasion suppressor that functions as an epithelial cell-cell adhesion molecule (Van Roy, 2014; Van Roy and Berx, 2008). Also, CDH-1 (E-cadherin is encoded with CDH1 gene) upregulation has been shown to be effective in inhibiting the hyperplasia of both endothelial and VSMCs (Armstrong and Bischoff, 2004; Lee et al., 2006). It was shown that the decrease in E-cadherin expression was related to the progression of EMT in the renal medulla of SHRs. In addition, it has been reported that ACE2/Ang-(1-7)/MasR axis might be associated with E-cadherin expression. It was suggested that ACE2 might increase E-cadherin expression and reduce biomarkers associated with EMT and migration *in vivo* and *in vitro* studies (Xu et al., 2017). IRW (Ile-Arg-Trp) was previously shown to modulate renin- angiotensin system (RAS), inhibit excessive proliferation, eliminate superoxide production, and ameliorate vascular inflammation, oxidative stress, and remodeling in VSMCs (Wu, 2020). The present study discovered that IRW regulated E-cadherin pathways by targeting ACE2/Ang-(1–7)/Mas axis in VSMCs. Additionally, IRW was found to regulate intracellular Ca^{2+} concentration involved in vascular remodeling, proliferation, migration, and hypertension.

RAS plays a vital role in maintaining cardiovascular homeostasis and regulating blood pressure (Liao and Wu, 2021). Upon stimulation by Ang II, VSMCs undergo aberrant proliferation, oxidative stress, inflammation, migration, and vascular remodeling, ultimately resulting in increased peripheral resistance (Liao et al., 2016). On the other hand, ACE2 is responsible for counteracting the negative impacts of Ang II, as well as providing beneficial properties such as

reducing blood pressure, eliminating oxidative stress, and minimizing inflammation in the cardiovascular system (Liao et al., 2018a). A recent study revealed that in SHR proximal tube cells (PTCs), Ang II treatment led to the activation of the MAPK signaling pathway, indicating that Ang II-induced migration by the MAPK signaling pathway on SHR PTCs (Costantino et al., 2019). Furthermore, Ang II treatment of SHR PTCs led to a suppression of E-cadherin expression and an upregulation of markers associated with EMT (Costantino et al., 2019). Previously, it has been shown that IRW can reverse the activation of MMP and MAPK in Ang II-treated cells, suggesting that it might inhibit the migration of VSMCs when they are stimulated by Ang II (Liao et al., 2018b). Consistent with other studies, our study's results suggested that E-cadherin expression is reduced upon Ang II stimulation. A recent study has revealed that IRW up-regulated ACE2/Ang-(1-7)/MasR axis was linked to antioxidant, anti-inflammatory, and anti-migratory effects (Wu, 2020). In our study, A-779, an antagonist of the MasR accompanied by IRW, reduced the expression of E-cadherin in A7r5 cells. In a previous study, overexpression of ACE2 or treatment with Ang-(1–7) in MDA-MB-231 cells resulted in an increase in E-cadherin expression. However, when ACE2 was knocked down, E-cadherin expression was decreased in MCF-7 cells (Yu et al., 2016). Likewise, in our study, the expression level of E-cadherin was diminished when ACE2 was knocked down in VSMCs. Our study confirmed that IRW played an important role in regulating E-cadherin protein levels by targeting the ACE2/Ang-(1–7)/Mas axis *in vitro*.

Tamoxifen and 4-hydroxytamoxifen are commonly employed as a therapy for the treatment of a variety of cancers, particularly breast cancer (Karagol et al., 2007; Kuruppu et al., 1998; Wegman et al., 2005). Tamoxifen has been shown to reduce toll-like receptors (TLR)-4, transforming growth factor beta (TGF-β), and inflammation caused by nuclear factor kappa B (NF-κB) while preventing the activation of Snail and increasing the expression of E-cadherin in the rats with breast cancer (Kusmardi et al., 2021). In addition, it has beneficial effects on osteoporosis and reduces the chance of heart disease (Akram et al., 2007; Steiner et al., 2005; Turken et al., 1989; van Bommel et al., 2006). 4-hydroxytamoxifen has been shown to boost the levels of E-cadherin mRNA and protein in MCF7 breast cancer cells, which in turn affects the adhesion of the cells (Mauro et al., 2010). On the other hand, many patients developed signs of Tamoxifen resistance, making the effective management of breast cancer more difficult since the cancer was more likely to invade and spread to other parts of the body (Cai et al., 2021; Wang et al., 2019). In a word, the resistance to Tamoxifen treatment might be associated with a decline in E-cadherin levels and an

increase in the markers related to migration (Kim et al., 2015). These results suggested that Tamoxifen could be effective in regulating E-cadherin.

Recently, interest in discovering bioactive food components has been heightened because of the undesirable consequences of synthetic drugs (Jiao et al., 2019). IRW, a bioactive peptide isolated from egg ovotransferrin, has been shown to regulate the expression of various adhesion molecules and inflammatory cytokines in cells without causing any adverse side effects (Majumder et al., 2015). Our findings indicate that IRW raised E-cadherin levels *in vitro*.

E-cadherin, encoded with the CDH1 gene, is a key element in controlling the adhesion among cells, such as tight junction and adherens junction (Campbell et al., 2017). Research has revealed that E-cadherin is down-regulated in response to mildly oxidized LDL, which is accompanied by the activation of pro-proliferative gene transcription (Bedel et al., 2008). Pathological conditions, such as the presence of reactive oxygen species (ROS), can cause VSMCs to transition to a highly proliferative state and cease to express contractile markers like E-cadherin. This alteration is a critical step in the proliferation and migration of VSMCs, which is linked to the formation and progression of CVDs (Liu et al., 2020). Also, it was shown that CDH1 expression was reduced in the proliferation of pulmonary artery smooth muscle cells (PASMCs) and HSMCs (Bedel et al., 2008; Zhai et al., 2020). Our latest research showed that IRW treatment caused a noteworthy rise in the gene expression of CDH1 found in the mesenteric artery (Majumder et al., 2015). Similarly, we observed that the aorta of SHRs treated with IRW exhibited reduced E-cadherin levels suggesting the regulatory role of IRW on E-cadherin, which might connect to the hyperplasia and proliferation reduction. Although some studies proposed that E-cadherin might be linked to SMCs in some way, the role of E-cadherin has yet to be studied in depth.

Snail family, zinc finger proteins, can block the E-cadherin gene's transcription in response to signals from the fibroblast growth factor receptor (FGFR) (Nelson and Nusse, 2004). Decreasing expression of E-cadherin is a common occurrence during EMT and migration, and the repressor Snail is a key contributor to this process (Yu et al., 2015). It was found that Snail mRNA levels were greater in the aorta of Spontaneously Hypertensive and Hyperlipidemic rats (SHHRs) that were fed high-fat diet, indicating that Snail gene expression could be increased in fibrosis progresses (KAWAGUCHI et al., 2016). In accordance with a previous study, our results showed the reduction of Snail1 expression in the aorta of SHRs and VSMCs after IRW treatment,

proposing that IRW might play an essential role in the modulation of Snail 1 and subsequent complications of hypertension.

Calcium is essential for a wide range of physiological processes, including maintaining balanced levels inside cells, movement, secretion, and activating gene expression (Berridge et al., 2003). Higher levels of Ca^{2+} entering the cells is a key factor in stimulating the proliferation of VSMC (Guo et al., 2012). The elevated Ca^{2+} concentrations found in the SMCs of SHRs could be due to a rise in the Ca^{2+} stored in the sarcoplasmic reticulum, which may lead to increased vascular tone caused by Ca^{2+}-releasing mechanisms (Lompré, 1999). Our study revealed a decrease in intracellular Ca^{2+} concentration in the aorta of both IRW-treated SHRs and VSMCs, suggesting that IRW is able to regulate hypertension and its associated complications by modulating Ca^{2+} levels. Activation of SOCE through Orai1 in SMCs is essential for generating prolonged cytosolic Ca^{2+} signals and restoring intracellular Ca^{2+} reserves (Giachini et al., 2009). Evidence was provided that Orai1 protein was more abundant in the aorta of stroke-prone SHRs compared to the Wistar-Kyoto rats. This increase in Orai1 activity could explain why intracellular Ca^{2+} concentrations are not properly regulated in hypertension (Giachini et al., 2009). Ang-II has been proposed to cause an increase in intracellular Ca^{2+} concentration in the VSMC line derived from the rat thoracic aorta by activating the SOCE pathway, which leads to the depletion of endoplasmic reticular Ca^{2+} and the subsequent activation of the Orai1. Ca^{2+} signaling has been suggested to play a role in Ang-II expression in VSMCs, in which Orai1 is potentially involved in this response (Simo-Cheyou et al., 2017). Also, SOCE could activate Snail1 to suppress E-cadherin expression, causing metastasis (Yu et al., 2016). Although our study has shown the Ca^{2+} reduction in both aorta and VSMCs and a decrease in the Orai1 expression in VSMCs, no significant changes in the protein expression of Orai1 were observed after IRW treatment *in vivo*. Further investigation into Orai1 activity should be conducted, as it has the potential to affect intracellular Ca^{2+} levels. Moreover, our study suggested that IRW could affect the expression and activity of other Ca^{2+} channels playing important roles in regulation of intracellular Ca^{2+}.

4.5. Conclusion

In conclusion, these results suggest a key role of IRW in the regulation of E-cadherin, Snail 1, and Orai1 signaling pathways related to vascular remodeling, proliferation, and migration. Also, IRW is able to regulate E-cadherin by targeting the ACE2/Ang-(1-7)/MasR axis. Moreover, Orai1/ Ca^{2+} pathway might be regulated by IRW both *in vitro* and *in vivo*. These discoveries might highlight

potential opportunities to utilize IRW as a novel therapeutic agent to diminish the adverse effects of synthetic medicine for future studies.

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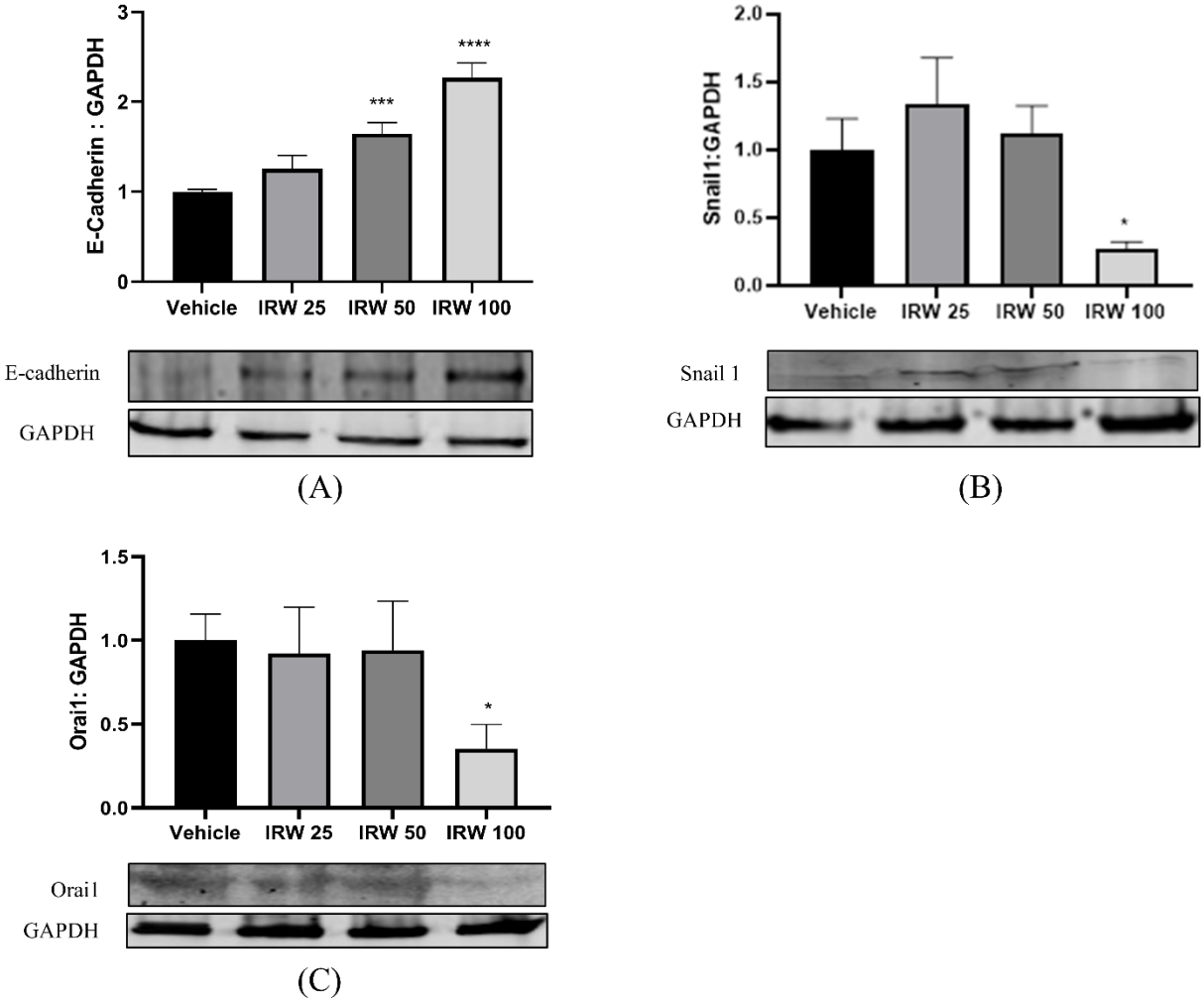

Figure 4. 1 The effect of different concentrations (25 µM, 50 µM, and 100 µM) of IRW on E-cadherin (A), Snail1 (B), and Orai1 (C) levels.

Confluent monolayers of A7r5 cells were treated with different doses of IRW for 24 hrs. Cells were lysed at the end of the experimental period and lysates were used in western blotting for E-cadherin, Orai1, Snail1, and GAPDH. Data was analyzed by one-way ANOVA along with Dunnett's multiple comparisons test. Data expressed as mean ± SEM of four independent experiments. *, $p < 0.05$, ***, $p < 0.001$, and ****, $p < 0.0001$ versus vehicle.

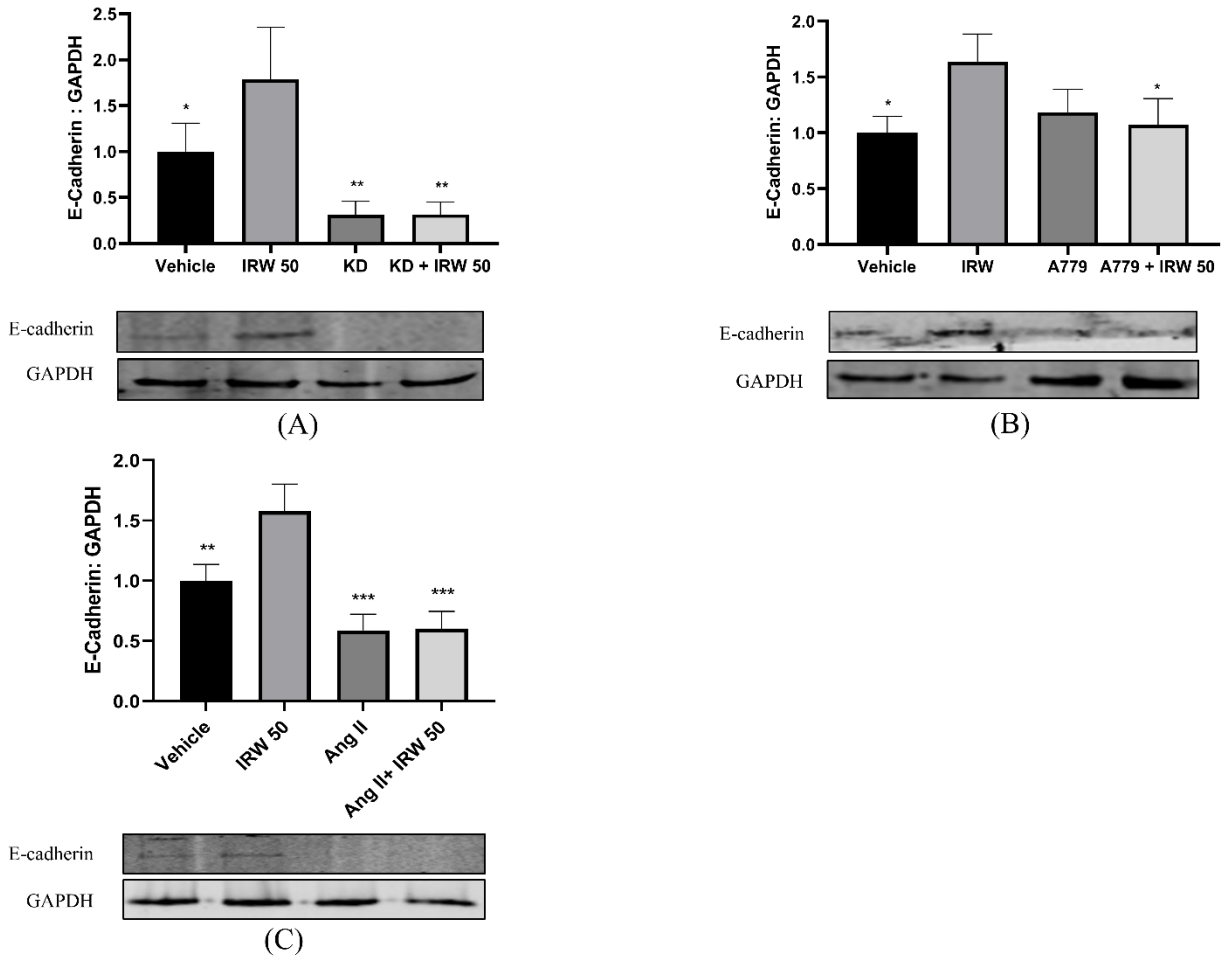

Figure 4. 2 E-cadherin expression in the A7r5 cells treated by siRNA; Knock down (KD) (A), A779 (MasR antagonist, B), and Ang II (C) in A7r5 cells.

ACE2 knockdown in A7r5 cells were conducted using ACE2 siRNA. Next, cells were treated with 50 µM of IRW for 24 hrs. Also, cells were pre-treated with 50 µM of IRW for 1 h prior to 23 hrs stimulation with 1 µM of Ang II or A779. Cells were lysed and immunoblotted for E-cadherin and GAPDH. Data was analyzed by one-way ANOVA along with Dunnett's multiple comparisons test. Data expressed as mean ± SEM of four independent experiments. *, $p < 0.05$, **, $p < 0.01$, ***, and $p < 0.001$ versus IRW treated group.

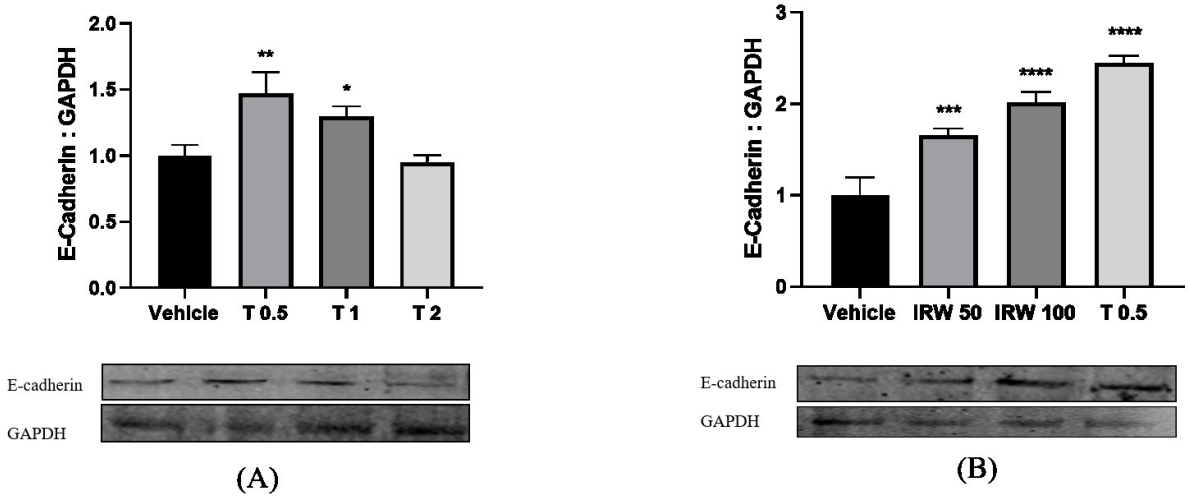

Figure 4. 3 The effect of different concentrations of Tamoxifen alone (A) and comparison concentrations of IRW and Tamoxifen (B) on E-cadherin expression in A7r5 cells.

Confluent monolayers of A7r5 cells were treated with different doses of Tamoxifen and IRW for 24 hrs. Cells were lysed and immunoblotted for E-cadherin and GAPDH. Data was analyzed by one-way ANOVA along with Dunnett's multiple comparisons test. Data expressed as mean ± SEM of four independent experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, and ****, $p < 0.0001$ versus vehicle. T 0.5: Tamoxifen 0.5 µM; T 1: Tamoxifen 1 µM; T 2: Tamoxifen 2 µM.

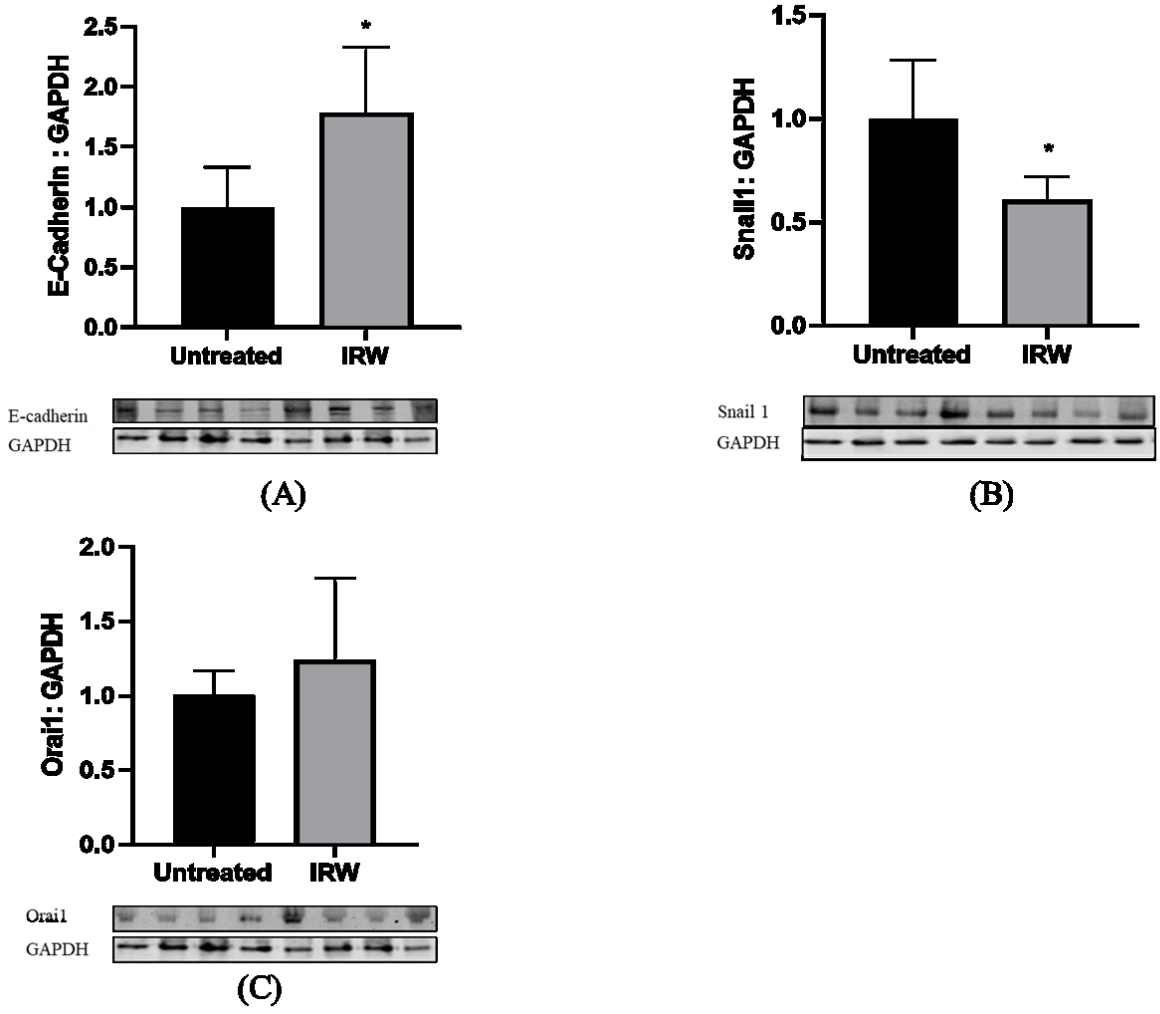

Figure 4. 4 The effect of IRW on the E-cadherin (A), Snail1 (B), and Orai1 (C) levels in the aorta of SHRs.

Data was analyzed by one-way ANOVA along with Dunnett's multiple comparisons test. Data expressed as mean ± SEM of n = 4. *, $p < 0.05$ versus untreated.

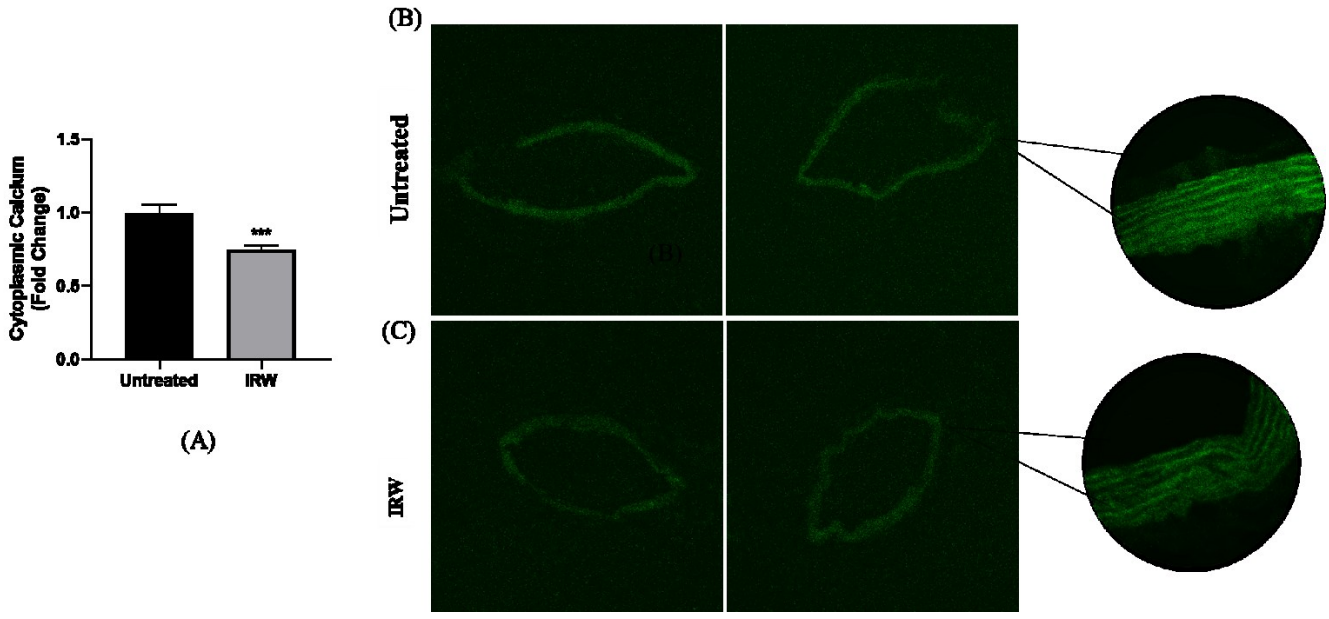

Figure 4. 5 Effect of IRW on the fluorescence intensity of intracellular calcium in the aorta of SHRs in four independent groups.

SHRs were orally fed with IRW (15 mg/kg body weight) for 7 days. Following sacrifice, aorta were collected, frozen, sectioned and immunostained for Ca^{2+}. (A) Represented the confocal imaging of the fluorescence intensity of the intracellular calcium in the aorta of SHRs in untreated groups. (B) Represented the confocal imaging of the fluorescence intensity of the intracellular calcium in the aorta of SHRs in IRW-treated groups (C). Confocal images were analyzed offline with ImageJ 4.0 Software. ***, $p < 0.001$ versus untreated.

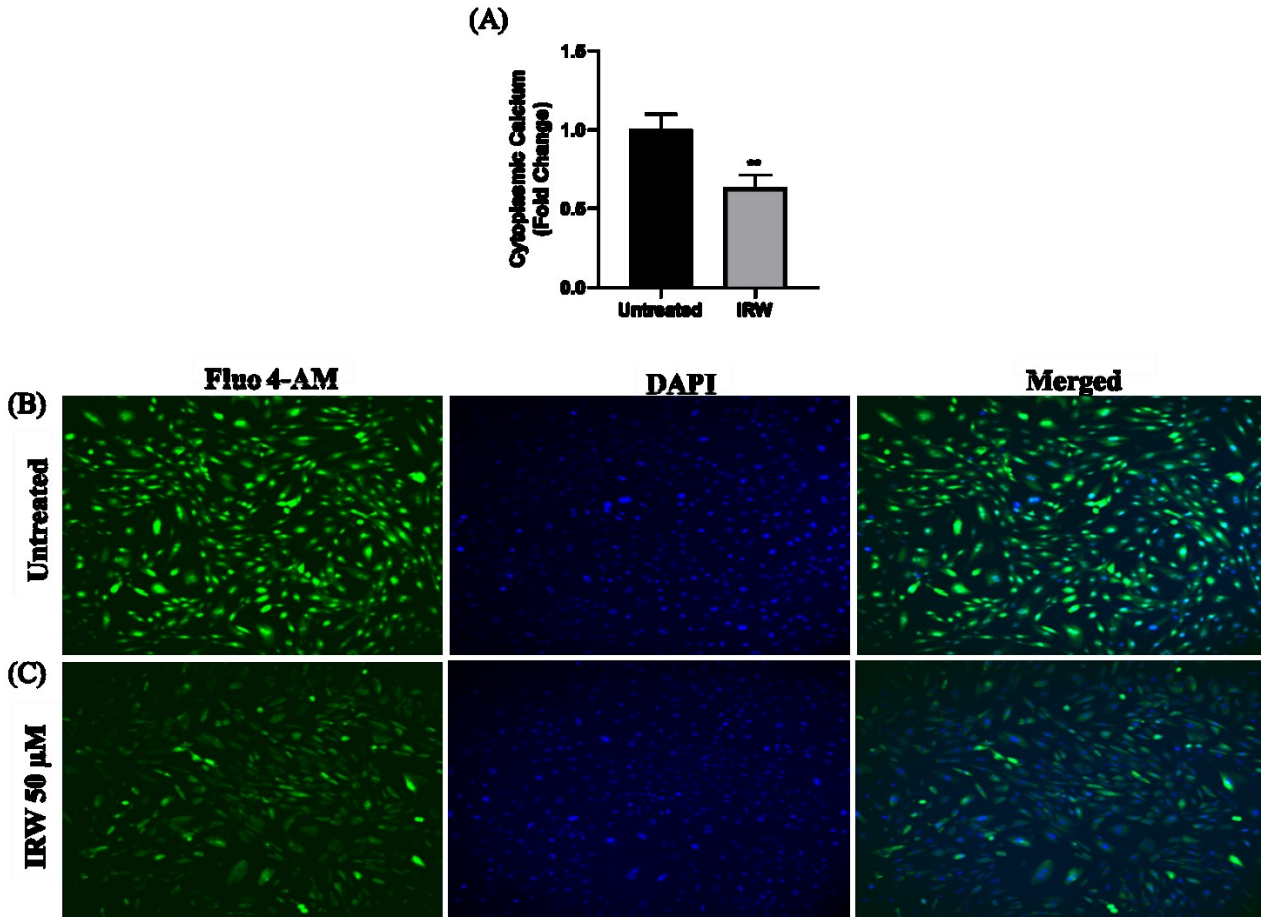

Figure 4. 6 Effect of IRW on the fluorescence intensity of intracellular calcium in cultured rat aortic A7r5 smooth muscle cells in four independent groups.

Cells were treated with 50 µM of IRW for 24 hrs. Treated and untreated cells were loaded with Fluo-4AM for 20 min and subsequently with DAPI for 10 min, then visualized by fluorescent microscopy. (A) The changes in intracellular calcium were assayed by Fluo 4-AM with an inverted microscope equipped with a 10 × objective. (B) Represented images of the fluorescent intensity of intracellular calcium in the untreated group (C) Represented images of the fluorescent intensity of the intracellular calcium in the IRW-treated group. Confocal images were analyzed with ImageJ 4.0 Software. **, $p < 0.01$ versus untreated.

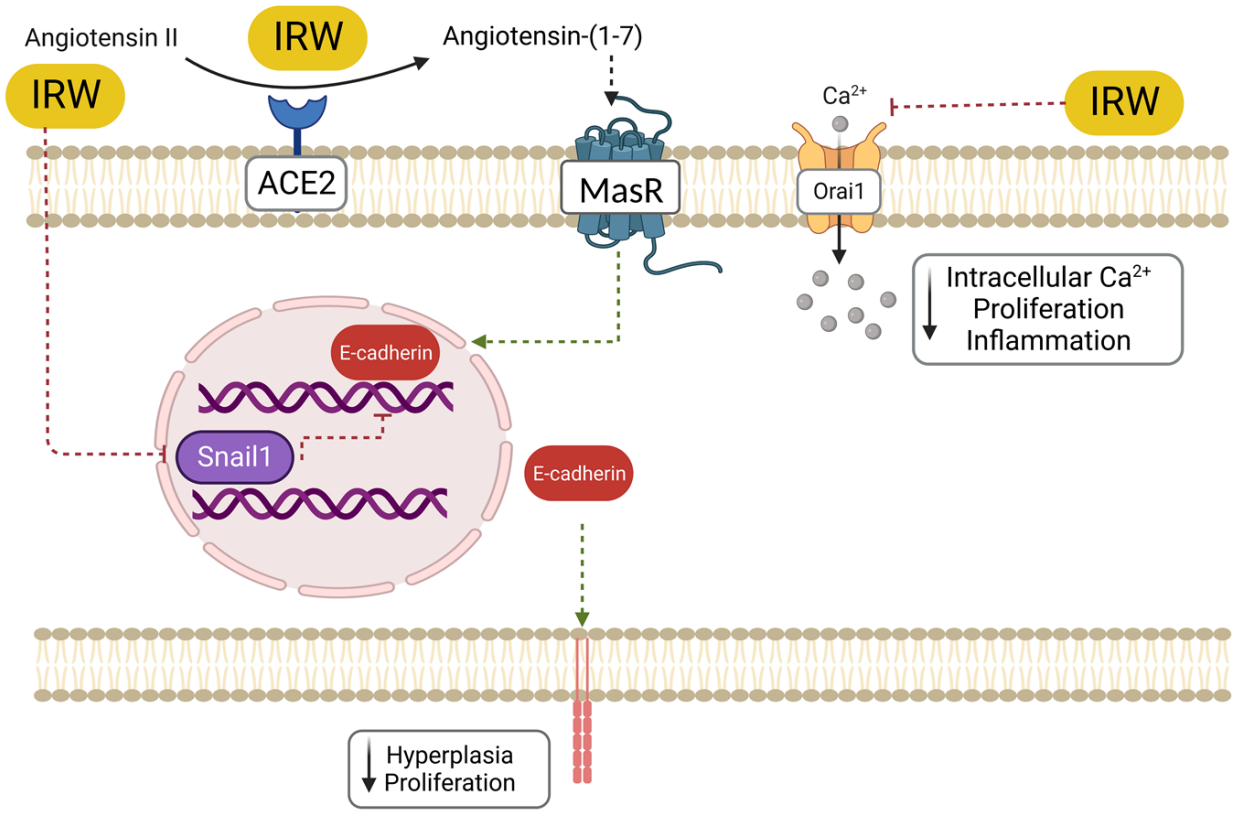

Figure 4. 7 The effect of IRW on RAS and related pathways.

IRW regulated E-cadherin pathways by targeting ACE2/Ang-(1–7)/Mas axis in VSMCs. IRW played an essential role in the modulation of Snail1 and subsequent complications of hypertension such as hyperplasia and proliferation. Additionally, IRW was found to regulate Orai1 and intracellular Ca^{2+} concentration involved in vascular remodeling, proliferation, inflammation, migration, and hypertension.

CHAPTER 5- THESIS SUMMARY AND DISCUSSION

5.1. Key findings of the present research

The overall objectives of the research were to investigate the regulatory role of IRW on the angiotensin-converting enzyme 2 (ACE2)/angiotensin-(1-7) (Ang-(1–7))/Mas axis and their associated signaling pathways in metabolic syndrome (MetS) and hypertension.

The key findings of each study are listed below:

5.1.1 IRW regulated aortic ACE2 against MetS in high-fat diet (HFD)-induced insulin-resistant model

MetS is a term used to describe a collection of risk factors that are often linked to cardiovascular diseases (CVDs) and diabetes (Jahandideh and Wu, 2022). Upregulation of ACE2, an important component of the renin-angiotensin system (RAS), is generally considered to have a protective effect against CVDs and diabetes (Gheblawi et al., 2020). Previous studies have shown that the peptide IRW can increase the expression of ACE2 in spontaneously hypertensive rats (SHRs) (Liao et al., 2018a; Liao et al., 2019). However, it does not have the same effect on skeletal muscle ACE2 in a mouse model with insulin resistance induced by a HFD (de Campos Zani et al., 2022). This study revealed a decrease in angiotensin-converting enzyme (ACE) and angiotensin II receptor (AT1R) protein expression, while an increase in the ACE2 RNA and protein levels in aorta of HFD mice treated with IRW. Although the protein expression of peroxisome proliferator-activated receptor gamma (PPARγ) decreased in HFD mice, IRW treatment did not exhibit any effect on PPARγ protein abundance. IRW was able to increase phospho AMP-activated protein kinase (p-AMPK, Thr172) levels and phospho endothelial nitric oxide synthase (p-eNOS, serine 1177) and subsequently led to an increase in Sirtuin 1(SIRT1) expression in HFD mice. ACE2 knockdown significantly decreased the expression of p-AMPK and p-eNOS in the vasculae smooth muscle cells (VSMCs). The improvement of membrane localization of GLUT4 in the aorta of HFD was observed in the mice treated with IRW. HFD only induced an increase in endothelin-1 (ET-1) and phospho p38 mitogen-activated protein kinases (P38 MAPK) in the aorta, which could be countered by IRW treatment in HFD-induced insulin-resistant model. This study showed the modulatory effect of IRW on aortic RAS, which is associated with MetS. Further, these findings indicated that IRW, with a specific impact on aortic ACE2, might have a significant role in MetS by influencing multiple signaling pathways such as ACE2/AMPK/SIRT1/eNOS, ACE2/AMPK/GLUT4, and ATIR/ET-1/P38 MAPK.

5.1.2 IRW through targeting ACE2/Ang-(1–7)/Mas axis and related pathways might be essential for regulating vascular remodeling

VSMCs are recognized to undergo remodeling in response to sustained elevation in blood pressure , a process marked by heightened proliferation, inflammation, oxidative stress, and migration (Touyz and Schiffrin, 2000). It was demonstrated that treatment with IRW led to an elevation in the expression of the CDH-1 gene in mesenteric arteries. This increase in CDH-1 expression has the potential to lower the risk of hyperplasia and related cardiovascular disorders (Majumder et al., 2015). In this study, IRW treatment resulted in the upregulation of Epithelial cadherin (E-cadherin) expression while downregulating the expression of Zinc finger protein SNAI1 (Snail1) and Orai Calcium (Ca^{2+}) Release-Activated Calcium Modulator 1 (Orai1) in VSMCs. When ACE2 was knocked down or when A779 (Mas receptor (MasR) antagonist) and angiotensin II (Ang II) were applied to the VSMCs, the protein expression of E-cadherin was decreased. Treatment with 4-hydroxy Tamoxifen increased the expression of E-cadherin, but it did not have any impact on ACE2 expression, either alone or in combination with IRW. In SHRs treated with IRW, there was an increase in E-cadherin expression and a decrease in Snail1 expression in the aorta of SHRs, while Orai1 expression remained unchanged. The intracellular concentration of Ca^{2+} significantly decreased in both SHRs and IRW-treated VSMCs compared to the untreated group. These findings suggested that IRW was capable of regulating E-cadherin by targeting the ACE2/Ang-(1-7)/MasR axis. IRW played a crucial role in regulating the signaling pathways of E-cadherin, Snail1, and Orai1, which were associated with vascular remodeling, proliferation, and migration.

5.2. Significance of this research

Our understanding of the regulatory role of peptide IRW and its underlying mechanisms is still in its early stage. This current study is the first to demonstrate a potential link between IRW and MetS through a novel pathway involving the activation of aortic ACE2. Our findings suggest that the upregulation of aortic ACE2 by IRW in HFD-induced insulin-resistant mice is responsible for the activation of pathways associated with vasodilation in blood vessels. This activation may contribute to the improvement of insulin resistance and glucose metabolism. Therefore, the results of this study support the further investigation of IRW as a potential therapeutic agent for treating MetS. Moreover, IRW exerted its regulatory effects on E-cadherin and related pathways by targeting the ACE2/Ang-(1–7)/Mas axis in VSMCs. Both *in vitro* and *in vivo* results showed that

IRW modulated the intracellular concentration of Ca^{2+} playing a significant role in vascular remodeling, proliferation, migration, and hypertension.

5.3. Future research prospect

It is important to acknowledge that the thesis have certain limitations in its research approach.

1. This study suggested that ACE2/AMPK/GLUT4 signaling pathway in the aorta might have a role in regulating MetS. There is currently no reported evidence of AMPK directly modulating GLUT4 in VSMCs. The exact mechanism by how IRW enhances GLUT4 in VSMCs, both with and without aortic ACE2 expression, remains unknown. Therefore, further research is necessary to investigate other potential pathways involved in this process.
2. ERK1/2 serves as a critical factor in promoting the proliferation of endothelial cells (Kliche and Waltenberger, 2001). No significant decrease in expression of ERK1/2 was observed in the aorta of HFD mice following IRW treatment. These findings indicate the need for further investigation of IRW treatment into the endothelial function associated with MetS.
3. Administration of IRW did not result in significant alterations in the protein expression of Orai1 *in vivo*. However, there was a significant decrease in intracellular Ca^{2+} levels. Therefore, further investigation is necessary to explore the activity of Orai1 and other Ca^{2+} channels, as they have the potential to affect intracellular Ca^{2+} levels.
4. To provide robust evidence for the potential therapeutic application of IRW, it is recommended to conduct additional functional studies using isolated mouse aortic rings, histopathology assessments of VSMCs in the aorta, and immunohistochemistry to determine the nuclear localization of specific proteins. These additional investigations will contribute to a better understanding of the efficacy of IRW as a treatment option.
5. Additional research is necessary to comprehend the bioavailability and metabolism of tripeptide IRW *in vivo*. It is possible that the physiological effects observed *in vivo* are due to the metabolites of the peptide, but this remains to be determined.
6. In order to establish strong evidence for the potential therapeutic application of IRW, conducting research on cancer cells to measure markers involved in vascular remodeling and migration is recommended.
7. To enhance the statistical power of the study, it is advisable to increase the sample size to gain a more precise estimate of the true effects. This helps in improving the study's

reliability, strengthens the confidence in the results obtained, and increasing the accuracy and robustness of the findings.

8. The outcomes derived from both *in vitro* and *in vivo* investigations of IRW are promising, indicating their potential efficacy in addressing complications associated with MetS. However, there is a lack of research conducted on human subjects. As a result, randomized clinical trials are essential to validate the effectiveness and safety of IRW.
9. The study was restricted to adult male SHRs and adult HFD mice, which narrowed its scope. Therefore, exploring the the regulatory role of IRW on the ACE2/ (Ang-(1–7))/Mas axis and their associated signaling pathways in female SHRs and female HFD-induced obese mice models would be valuable.

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