APELIN-APJ SIGNALING IN HYPERTENSIVE CORONARY ARTERIES

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ABSTRACT

Apelin is an endogenous ligand for APJ receptors, which are highly expressed throughout the cardiovascular system, including coronary arteries. Apelin causes endothelium-derived nitric oxide (NO) –dependent relaxation of coronary arteries under physiological conditions, but little is known about regulation of coronary vasomotor tone by apelin under pathological conditions. This research addresses the critical gap in understanding of the apelin signaling in coronary circulation under normal and pathological conditions.

Evidence from this study suggests that apelin could not provide beneficial vasodilatory effects in hypertensive coronary arteries. Moreover, apelin impairs endothelium-dependent relaxations to vasodilator, acetylcholine. Impaired apelin-APJ signaling in hypertensive coronary arteries is possibly through defective production or release of NO from coronary endothelial cells rather than inhibiting effects of NO in coronary arterial smooth muscle cells.

My next aim was to understand the mechanisms involved in the loss of apelin response in hypertensive arteries. The results suggested that the APJ receptor signaling via GRK2 pathway is possibly responsible for the impaired apelin response in hypertensive coronary arteries. Interestingly, APJ receptor biased agonist, CMF-019, -induced relaxation in hypertensive coronary arteries and showed no effects on vasodilatory response to acetylcholine. My results also suggest the possible impairment of PI3K/AKT/eNOS pathway mediated by GRK2 activation in hypertensive coronary arteries.

My final aim was to check whether apelin signaling is impaired in coronary arteries only under hypertensive conditions or if it occurs in other disease conditions. My data suggests that apelin signaling is impaired in coronary arteries exposed to cigarette smoke extract (CSE), a model for secondhand smoke exposure. Interestingly, similar to hypertensive coronary arteries, apelin

lost its beneficial vasodilatory effects possibly through the GRK2 activation in CSE treated coronary arteries.

Overall, this research provides evidence that apelin behaves differently under physiological and pathological conditions. As a point of fact, apelin not only lost its beneficial effects but also might have negative effects under pathological conditions such as hypertension and secondhand smoke exposure. I anticipate that the results from this approach will be useful in improving the therapeutic strategies with apelin and other APJ receptor agonists that are aimed to alleviate different cardiovascular disorders.

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DEDICATION

To my family and friends

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LIST OF ABBREVIATIONS

ACE-2 ....angiotensin converting enzyme-2

ACh ....acetylcholine

Akt..protein kinase B

Ang II ....angiotensin 2

APJ ....apelin receptor

BK_{Ca} ....large conductance calcium activated potassium channel

BSA...bovine serum albumin

CAD ....coronary artery disease

cAMP ....cyclic adenosine mono phosphate

CSE ....cigarette smoke extract

CVD ....cardiovascular disease

DEA ....diethylamine

DMSO ....dimethyl sulfoxide

EC_{50}..concentration required to produce 50% of maximal response

ELA...Elabela

eNOS..endothelial nitric oxide synthase

ERK..extracellular signal regulated kinases

F13A ....H-Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Ala-OH

GPCR ....G-protein coupled receptor

GRK2 ....G-protein coupled receptor kinase 2

GRK3 ....G-protein coupled receptor kinase 3

5-HT 5-hydroxytryptamine

L-NAME .... N^G-nitro-l-arginine methyl ester

MAP .... mean arterial pressure

MLC .... myosin light chain

NEP .... neprilysin

NLA .... ω-nitro-L-arginine

NO .... nitric oxide

PAH .... pulmonary arterial hypertension

PBS .... phosphate buffer saline

PECAM 1 .... platelet endothelial cell adhesion molecule 1

PI3K phosphoinositide-3-kinase

PKC .... protein kinase C

PLC .... phospholipase C

PSS .... physiological salt solution

PVDF .... polyvinylidene difluoride

SEM .... standard error of mean

SHR .... spontaneously hypertensive rat

SHS .... secondhand smoking

SMA .... smooth muscle actin

TBS .... tris buffer saline

WKY .... Wistar Kyoto rat

CHAPTER 1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death worldwide. Approximately 18 million people died from cardiovascular diseases in 2016, which represent 31% of all global deaths (1). Nearly, 44% of the US adult population is projected to have some form of CVD by 2030 (2). The most common type of cardiovascular disease is coronary artery disease (CAD). Although, CAD is generally considered as a disease mediated by plaque formation in coronary arteries, it is a very complex process (3). The initial stage of CAD involves the dysfunction of endothelial cells that line the coronary arteries and thereby affecting the vasomotor regulation (4). The risk factors for CAD include hypertension, smoking, diabetes mellitus, psychosocial stress to name the least (5). Even the secondhand smoking (SHS) is associated with 31% increased chance of CAD (6). Nearly half of US adults are suffering from hypertension and are at risk of developing CAD (7). The pathophysiological mechanisms underlying CAD is not clearly understood. The development of better therapeutic strategies is thus an urgent need to better tackle the morbidity and mortality associated with cardiovascular diseases. In the last decade, the apelinergic system has garnered considerable attention in the field of cardiovascular research.

Apelin and Elabela (ELA) (also known as apela or toddler) are two endogenous vasoactive peptides that bind to APJ receptors and together they form the apelinergic system (8). APJ receptors are expressed in a wide variety of organs including the brain, heart, lung, spleen, thymus, kidney, and small intestine (9,10). Apelin, a peptide discovered in 1998, was initially identified as the endogenous ligand for APJ receptors. The human apelin gene is located on chromosome Xq25-26.1, which encodes a 77-amino-acid apelin precursor, preproapelin that is cleaved into different isoforms of apelin (11). The second novel endogenous APJ receptor ligand, ELA, was discovered in 2013. ELA gene encodes a 54 amino acid preproprotein that is cleaved into different mature

isoforms of ELA (12). Apelin expression is more widely distributed (brain, heart, lung, adipose tissue, and mammary glands) compared to ELA (embryonic stem cells, kidney, prostate, and heart). Both apelin and ELA are expressed in the cardiovascular system especially in vascular endothelial cells (13,14). Although apelin and ELA show little sequence homology, they exert similar biological responses such as energy metabolism, fluid, and cardiovascular homeostasis (15–17).

Apelinergic System

Apelinergic system is composed of endogenous peptide ligands apelin, Elabela/Toddler, and a single receptor called APJ which belongs to class A of the G-protein-coupled receptor (GPCR) superfamily. APJ receptors were first identified as an orphan receptor in 1993 and deorphanized by the discovery of its cognate ligand, apelin, followed by the recent discovery of a second cognate ligand, ELA (11,12,18). APJ receptors contain 380 amino acid residues and are located on chromosome 11q12. APJ receptor subtypes are not present in mammals but two APJ receptor subtypes (APLNRa and APLNRb) are identified in zebrafish (19,20). APJ receptors have a typical GPCR structure with the seven transmembrane domains and consensus post-translation modification sites for glycosylation, palmitoylation, and phosphorylation by cAMP-dependent protein kinase (18). APJ receptors contain two glycosylation motifs expected at extracellular loop 2 (Asn175) and N-terminal tail (Asn15) residues which are involved in agonist binding. APJ receptors have multiple potential palmitoylation sites in the intracellular C-terminal region which are important in receptor function and expression. APJ receptors undergo phosphorylation at Ser348 residue in C-terminus which is key for interactions with GRK2/5 (G-protein-coupled receptor kinase), ß-arrestin and receptor internalization (21). APJ receptors show approximately 40-50% sequence homology with angiotensin (AT1) receptors, but it does not interact with angiotensin II (Ang II). Ang II treatment of rat APJ receptors does not affect forskolin-stimulated

cyclic adenosine monophosphate production (cAMP) production whereas apelin showed inhibitory effects at sub-nanomolar concentrations (22). However, APJ receptors can undergo heterodimerization with AT1 receptors to allosterically modify and antagonize the AT1 receptor-mediated responses which suggest that apelin could induce beneficial cardiovascular effects by negative regulation of Ang II/AT1R system (23). APJ receptors are broadly expressed in both human and rodent tissues with the highest expression in the pulmonary system and moderate expression in cardiovascular as well as central nervous systems (24). In the cardiovascular system, APJ receptors are expressed in cardiomyocytes as well as in intima, media, and adventitia layers of coronary arteries (25–27).

APJ signaling profile is complex and continues to be under active investigation. Multiple G-protein subunits are known to be involved in APJ signaling. APJ signaling via pertussis toxin-sensitive Gi/o proteins can activate PI3K/ Akt pathway resulting in the endothelial nitric oxide synthase (eNOS) activation and extracellular signal-regulated kinases (ERK) or the p70 S6 kinase phosphorylation and inhibit the forskolin-stimulated cAMP production (28–30). APJ receptor signaling can activate G-protein-independent pathway which involves the activation of GRK2 mediated signaling. GRK2 is a multimeric protein involved in the desensitization of APJ receptors (31). Moreover, GRK2 is known to regulate many signaling pathways including Akt/eNOS/NO (**Figure 1)** (32,33)**.** APJ receptor activation can induce cardiac muscle contractility via Gαq/11 subunits involving the activation of phospholipase C (PLC) and protein kinase C (PKC) (34). Furthermore, apelin-APJ signaling via Gα13 induces myocyte enhancer factor 2 (MEF2) transcription factors by cytoplasmic translocation of histone deacetylase 4 (HDAC 4) and HDAC 5 in cardiovascular development (35). The wide expression and multiple signaling mechanisms associated with the apelinergic system make them involved in the regulation of a variety of

biological functions such as body fluid homeostasis, energy metabolism, angiogenesis, and cardiovascular homeostasis including myocardial contractility as well as blood pressure regulation (15–17,36–41).

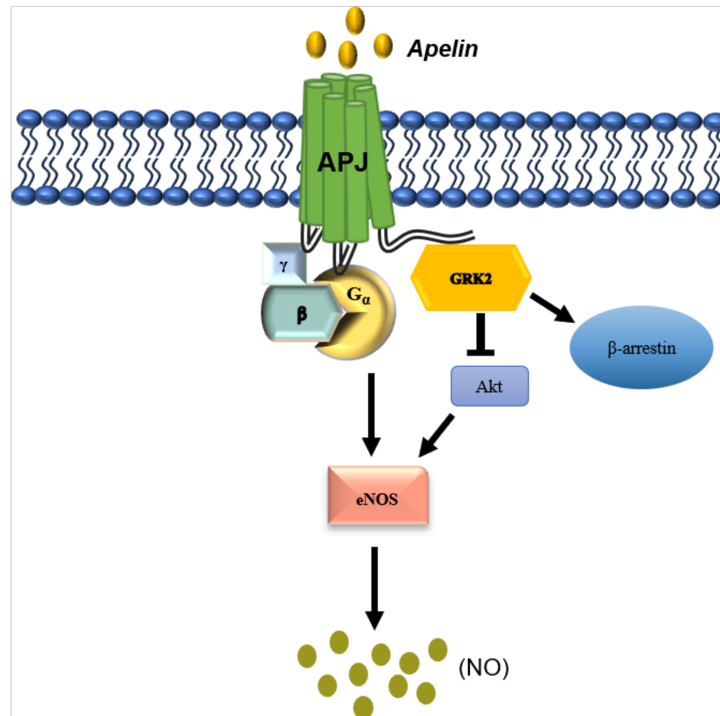

Figure 1. APJ receptor signaling via G-protein-dependent and -independent pathways.

Apelin is the first endogenous ligand for APJ receptors which was isolated from bovine stomach tissue extracts. The genes encoding human, mouse, and rat apelin are located on chromosome Xq25–26.1, XA3.2 and Xq3 respectively (11,18). The active isoforms of apelin are generated from a 77 amino acid precursor protein, preproapelin, proposed to contain the secretory signal sequence in hydrophobic rich N-terminal region (42–44). Preproapelin undergoes post-translational modification at N-terminal residues by endopeptidases to form proapelin (55 aa) which is further sequentially cleaved to four mature active isoforms such as apelin-36, - 17, -13, and -12. Apelin-13 can further undergo N-terminus glutamine residue cyclization to form [Pyr1-apelin-13]. A recent study demonstrated that proapelin (apelin-55) can activate APJ receptors and

induce similar maximum ERK phosphorylation response and potency to the shorter isoforms such as apelin-17 and apelin-36 which makes apelin-55 the longer bioactive isoform of apelin (45). Apelin isoforms have a conserved C-terminal region which is responsible for APJ receptor binding, internalization, and biological activity (46–48). A recent study showed that proprotein convertase subtilisin kexin subtype 3 (PCSK3) (also known as furin), can preferentially and specifically hydrolyze proapelin directly to apelin-13 without apelin-17 and apelin-36 production in adipocytes (49). The most abundant apelin isoforms in human plasma are apelin-13, Pyr1-apelin-13 and apelin-17, whereas the apelin-36 level is much lower (50,51). In human cardiac tissue, apelin-13 and Pyr1-apelin-13 are the more potent and predominant isoforms (52,53). Apelin gene is mainly localized to vascular endothelial cells of both resistant and conduit arteries.

Apelin has a short plasma half-life (4-8 min) as it undergoes proteolytic degradation which decreases the efficiency of peptides (54,55). The first enzyme implicated in apelin degradation is angiotensin-converting enzyme type-2 (ACE2). ACE2 removes C-terminal phenylalanine (Phe) residue of apelin isoforms with high catalytic efficiency. Cleavage of apelin-13, Pyr1-apelin-13, and apelin-17 by ACE2 leads to the generation of apelin -12, Pyr1-apelin-12, and apelin-16 respectively with reduced functional activity (56–58). Interestingly, evidence suggests that ACE2-apelin forms a feedback loop by which apelin maintains and upregulates the expression of ACE2 in vivo (59). Moreover, it is hypothesized that upregulation of ACE2 by apelin might provide beneficial effects in Coronavirus disease 2019 (COVID19) disease by down-regulation of the renin-angiotensin system (60). Neprilysin (NEP), a zinc-dependent metalloprotease, is another enzyme known for apelin degradation. RPRL (Arg2-Leu5) motif is the degradation site for neprilysin to generate fully inactive apelin fragments (61,62). Interestingly, NEP has been identified as a potential therapeutic target for heart failure, and the fact that NEP inactivates apelin

peptides might provide a better understanding of the beneficial effects of NEP inhibition (63). Recent evidence suggests that human protease plasma kallikrein (KLKB1) can cleave the first three N-terminal amino acids (KFR) of apelin-17 similar to NEP at RPRL motif thereby inactivating apelin (64).

ELA is the second endogenous ligand for APJ receptors which was identified as a factor required for early cardiac development of zebrafish embryos in 2013. Human ELA is located on chromosome 4 and consists of 3 exons (12,65). ELA has little sequence homology with apelin but shows a nanomolar affinity for APJ receptors similar to that of apelin (66,67). In silico molecular modeling and docking analysis indicated that ELA binds to APJ receptor via C-terminal hydrophobic moiety in a hydrophobic pocket of the receptor (68). The human ELA gene encodes a 54 amino acid preproprotein with a 22 amino acid signal peptide. The signal peptide is truncated from ELA-54 to form a mature ELA-32 peptide (32-amino acid) which is further cleaved by furin-like endopeptidases to generate ELA-21, ELA-14, and ELA-11 isoforms (12,67). ELA is highly expressed in the early stages of embryogenesis and promotes cardiac development. In adult tissues, ELA expression is limited to the prostate, kidney, and blood vessels (14,65). In the heart, the ELA expression is significantly localized in the coronary endothelium with little presence in cardiomyocytes or vascular smooth muscle cells (68).

Vascular Effects

The regulation of vasomotor tone by the apelinergic system is very complex as it can induce vasodilation or vasoconstriction depending on the vascular bed and experimental conditions. APJ receptors are expressed on both endothelial and smooth muscle layers of blood vessels. The binding of apelin to APJ receptors on endothelial cells promotes vasodilation while vascular smooth muscle cell APJ receptor activation induces vasoconstriction (69). The vasodilatory effects of apelin have been well studied in humans and different animal models. The potency of apelin’s

vasodilatory effects varies depending on the vascular bed, experimental model, and route of administration (70).

In humans, acute apelin infusion causes peripheral and coronary vasodilatation, increases cardiac output, and reduces mean arterial pressure, cardiac preload, and afterload (71). Apelin infusion into forearm brachial artery induced vasodilation in a NO-dependent and prostanoid-independent pathway in vivo in humans. The study demonstrated that apelin response was attenuated by eNOS inhibition but not by prostaglandins inhibition (55,72). Similarly, apelin-induced NO-dependent relaxation in human mesenteric arteries ex vivo (73). These studies suggest that apelin causes vasodilation mainly by the activation of the NO-dependent pathway. Interestingly, Maguire et al. demonstrated that the ex vivo apelin administration in human mammary arteries caused vasodilation which is not affected by NO-synthase inhibitor, N^{G}-nitro-l-arginine methyl ester (L-NAME) whereas cyclooxygenase inhibitor, indomethacin abolished the vasodilator response. These findings suggest that apelin induces relaxation also in a prostanoid-dependent mechanism (52,69).

Several studies demonstrated the vasodilatory properties of apelin in different animal models. Systemic administration of apelin reduces both systolic and diastolic pressure in anesthetized and conscious rats (74–77). Apelin causes vasodilatation by the activation of the PI3K/Akt/eNOS pathway and thereby increasing eNOS activity and NO production in endothelial cells of rat aorta (78). In isolated rat coronary arteries, apelin induces endothelium-dependent relaxation by the activation of the BK_{Ca} channel-mediated through the stimulation of NO (25). Most studies suggest that apelin promotes vasodilation in an endothelium-dependent pathway. However, Gurzu et al demonstrated that apelin can attenuate AT-II-induced contractions even in

endothelium-denuded rat portal vein suggesting that apelin may induce relaxation in an endothelium-independent mechanism by acting directly on vascular smooth muscle cells (79).

Studies showed that ELA also has blood pressure-lowering effects. Similar to apelin, ELA has been demonstrated to cause mean arterial pressure (MAP) reduction in rats in vivo (54). In addition, ELA induces coronary vasodilation at the nanomolar level (66). In isolated mouse aorta, ELA causes relaxation in both endothelium intact and -denuded aortic rings and the endothelium-dependent relaxation was not affected by L-NAME suggesting the NO-independent relaxation response to ELA (14). However, the exact vasomotor regulation mechanism of ELA remained to be elucidated. A recent clinical study demonstrated that ELA is negatively correlated with systolic and diastolic blood pressure (80,81).

In the case of damaged endothelium or denuded endothelium, apelin cause vasoconstrictor response mainly because of the activation of APJ receptors on vascular smooth muscle cells. Apelin isoforms causes vasoconstriction in endothelium-denuded human saphenous veins and mammary arteries in a dose-dependent manner (52,82). In human coronary arteries that lacked functional endothelium, apelin induced vasoconstriction with potency and maximum response comparable to Ang II (83). Nagano et al demonstrated that apelin causes a transient blood pressure increase in ICR mice with L-NAME induced endothelial dysfunction, suggesting that apelin may act as a vasopressor agent under disease conditions (84,85). In asymmetric dimethyl arginine (ADMA, an endogenous competitive inhibitor of eNOS) treated rats, apelin permeates through damaged endothelial cells and promotes vasoconstriction by increasing myosin light chain (MLC) phosphorylation (86). In mouse aortic smooth muscle cells, apelin promotes vasoconstriction via MLC phosphorylation that may involve $G\alpha_{i/o}$ proteins and activation of NHE, NCX, PKC, and myosin phosphate target subunit 1 (MYPT1) (87). Furthermore, apelin-APJ signaling induced

intense vascular contraction via cooperative action with the α1-adrenergic receptor in mice aortas overexpressing APJ receptors (88,89). In rat cerebral arteries, apelin impairs both NO-dependent and -independent relaxation by inhibiting BK_{Ca} channel activity on cerebral arterial smooth muscle cells through activation of APJ receptors on the same (90,91). Apelin may directly act on cerebral smooth muscle cells to induce vasoconstriction via PI3K/Akt-dependent pathway (92).

Studies suggest that apelin can induce distinct peripheral and central actions. As described previously, most of the studies showed the depressor effects of the apelinergic system under normal conditions in peripheral blood vessels. In contrast, direct microinjection of exogenous apelin into the rostral ventrolateral medulla (RVLM) in the brain stem of rats caused chronic blood pressure elevation via stimulation of NAD(P)H oxidase-derived superoxide (93,94). Furthermore, paraventricular nucleus (PVN) ELA microinjection elevated blood pressure mediated by both sympathetic nerve activity and vasopressin release via PI3K/Akt -dependent pathway in spontaneously hypertensive rats (95).

In diabetic mice, apelin treatment improves the vascular tone in response to Ang II and acetylcholine by increasing phosphorylation of Akt and eNOS (96). The antagonistic interaction between apelin/ELA and the RAAS system promotes vasomotor tone under hypertensive conditions (97–99). In animal models and humans with pulmonary arterial hypertension (PAH), apelin infusion reduces pulmonary vascular resistance and increases stroke volume and CO (100–102). Likewise, apelin administration reduced mean pulmonary arterial pressure in canine model of acute pulmonary thromboembolism (103).

The apelinergic system is also involved in other important vascular functions such as cell proliferation, angiogenesis, and cell permeability. Apelin has both proliferative and antiproliferative properties. In rat aortic smooth muscle cells, apelin promoted cell proliferation

through PI3K/Akt-ERK1/2-cyclin-D1 signaling transduction pathway similar to Ang-II (104). Conversely, exogenous apelin treatment showed antiproliferative effects in pulmonary vascular smooth muscle cells under hypoxic conditions via activation of the P13K/Akt/mTOR signaling pathway (105). Angiogenesis has beneficial effects in ischemic cardiovascular diseases. Apelin treatment promotes angiogenesis thereby ameliorating diabetic cardiomyopathy via SirT3-dependent pathways in mice (106). However, apelin-induced retinal and glomerular endothelial cell angiogenesis aggravates retinopathy and nephropathy in type 2 diabetes mellitus patients (77,107,108).

Knowledge Gap

Extensive expression of the apelinergic system in the cardiovascular system indicates the potential role of APJ signaling in cardiovascular function including cardiotonic, diuretic, cardiorenal, and blood pressure regulation effects. The apelinergic system is beneficial in cardiovascular diseases because of positive inotropic and vasodilatory effects. In fact, clinical trials are going on with apelin that are based on its myocardial and vasodilatory protective properties. Activation of the apelinergic system can result in complex vasomotor effects. Depending on the vascular bed and experimental conditions, it can induce vasodilation or vasoconstriction. Some studies suggested the role of the apelin/APJ system in hypercholesterolemia-associated atherosclerosis (109). Activation of APJ receptors causes endothelium-dependent relaxation in many blood vessels including coronary arteries under physiological conditions (25). However, pathological conditions might lead to a shift in the protective role of the apelinergic system. This represents a critical gap in understanding the apelin signaling under normal and pathological conditions and thereby challenging the conventional view of its strictly protective role in the cardiovascular system.

Taking these together, the **central hypothesis of this research is that pathological condition such as hypertension leads to the loss of protective function of apelin in the coronary circulation. Further, apelin/APJ signaling impairment is mediated through the activation of the GRK2 pathway thereby impairing the eNOS activity in hypertensive coronary arteries**. To prove the central hypothesis, I set the following specific aims:

Specific aim 1: To determine the vasomotor response of apelin in spontaneously hypertensive rat (SHR) coronary arteries. The previously published work from our lab showed the NO-mediated endothelium-dependent vasodilatory effects of apelin by the activation of large conductance calcium-activated potassium channels (BK_{Ca}) in vascular smooth muscle cells of coronary arteries under physiological conditions. The vascular effects of apelin are very complex and hence it is not ideal to assume that apelin can have the similar beneficial vasomotor response in coronary arteries even under pathological conditions. Hence, the aim is to study the vasomotor effects of apelin in hypertensive coronary arteries.

Specific aim 2: To determine the molecular mechanism of altered apelin/APJ receptor signaling in coronary arteries of spontaneously hypertensive rats. APJ receptors signal via G-protein independent pathway includes the activation of G-protein coupled receptor kinase 2 (GRK2) (21). Increased GRK2 activity is known to inhibit eNOS activity and NO production in endothelial cells (110,111). The aim here is to establish the involvement of GRK2 as well as understand the molecular mechanisms involved in the altered apelin response in hypertensive coronary arteries.

Specific aim 3: Compare findings from cigarette smoke extract (CSE) (Secondhand smoke model) exposed coronary arteries with those obtained from the hypertension model. Effects of cigarette smoke on apelin signaling in coronary arteries is not studied. Secondhand

smoke exposure can lead to endothelial dysfunction in coronary arteries. Recent study showed an increased GRK2 expression that has been observed in the cultured trophoblasts after cigarette smoke exposure (112). Hence, the aim here is to study whether apelin/APJ signaling is altered by cigarette smoke exposure in rat coronary arteries similar to SHR coronary arteries.

To my knowledge, there are no published reports exploring the vasomotor actions of apelin on coronary arteries under disease conditions including hypertension. Moreover, this research compared results from the hypertensive to the second-hand smoke model which will tell us whether the altered apelin signaling is specific to hypertensive conditions or it is applicable to other disease conditions. My contribution here is expected to be a detailed understanding of the regulation of vasomotor tone by apelin in coronary arteries under hypertensive conditions and its associated functional outcomes, which will help us to better understand the safety profile of apelin-APJ system-based therapies as well as develop new therapeutic strategies in cardiovascular diseases. Considering the fact that apelin and its analogs are currently under clinical trials, this research might provide more relevant insight into the potential limitations of ongoing clinical trials.

CHAPTER 2. APELIN-INDUCED RELAXATION IS IMPAIRED IN CORONARY ARTERIES FROM SPONTANEOUSLY HYPERTENSIVE RATS

Introduction

Apelin is a novel vasoactive adipokine that binds to a G-protein-coupled receptor called APJ. Apelin and APJ receptors are widely expressed in peripheral tissues and the central nervous system making them involved in diverse physiological and pathological processes, especially in the cardiovascular system including coronary arteries (25,42,113). The apelinergic system plays a vital role in maintaining cardiovascular homeostasis because of its positive inotropic and blood pressure regulation effects (114). The preclinical and clinical studies showed various beneficial cardiovascular functions of the apelin such as decreased blood pressure, ventricular preload, and afterload, increased cardiac contractility and protection against ischemia-reperfusion injury, and inhibition of atherosclerosis, aneurysm formation, and cardiomyocyte apoptosis (34,55,71,83,115–117).

Increasing evidence suggests the role of the apelinergic system as a critical factor in vasomotor tone regulation and in the development as well as the progression of vascular pathologies (77,118). In both clinical and preclinical settings, apelin has shown vasopressor effects in the case of lack of functional endothelium. The previously published work from our lab showed the endothelium-dependent NO-mediated vasodilatory effects of apelin by the activation of large conductance calcium-activated potassium channels (BK_{Ca}) in vascular smooth muscle cells of coronary arteries under physiological conditions (25). However, the effects of activating APJ receptor signaling pathways in coronary arteries under disease conditions are largely unknown. Pathological conditions might lead to a shift in the protective role of the apelinergic system in coronary arteries. This represents a critical gap in understanding of the apelin signaling in coronary circulation under normal and pathological conditions. This research is focused on understanding

the vasomotor regulation of apelin in hypertensive conditions. Hypertension is a major risk factor for coronary artery disease, but the pathophysiological mechanisms linking these diseases are not well understood. Since endothelium-dependent relaxations are often blunted in arteries from hypertensive animals, we tested the hypothesis that apelin-induced relaxation is impaired in coronary arteries from spontaneously hypertensive rats (SHR), a well-established animal model for essential hypertension.

The data from this study indicate that apelin failed to cause relaxation in hypertensive coronary arteries, likely due to impaired production or release of NO from endothelial cells rather than interfering with the action of NO on coronary smooth muscle cells. In SHR coronary arteries, apelin instead actively inhibited relaxation to another endothelium-dependent vasodilator, i.e., ACh. APJ receptor signaling may contribute to both the loss of relaxation to apelin itself, as well as the ability of apelin to inhibit endothelium-dependent relaxation to ACh in coronary arteries from SHR.

Materials and Methods

Animals and Tissue Preparation

Experiments were performed on tissues obtained from age matched Wistar Kyoto (WKY) and Spontaneously Hypertensive rats (SHR) (Charles River Laboratories, Wilmington, MA). Rats were housed on a 12-hr/12-hr light/dark cycle at 22 ± 2°C and were provided with food and water ad libitum. All animal protocols used in this study were approved by the North Dakota State University Institutional Animal Care and Use Committee. The animals were anesthetized with isoflurane and hearts were isolated and placed into ice-cold physiologic salt solution (PSS) of the following composition: 118.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 2.5 mM $CaCl_2$, 1.2

mM KH_2PO_4, 0.03 mM EDTA, 5.5 mM glucose, and 25.0 mM $NaHCO_3$. Epicardial coronary arteries were dissected free and cleaned of surrounding tissues.

Western Immunoblotting

Rat coronary arteries were collected and frozen immediately using liquid nitrogen. Tissues were crushed using a mortar and pestle and the powdered tissue was homogenized at 4°C using an IKA Ultra-Turrax T8 homogenizer (IKA Works Inc., Wilmington, NC) in lysis buffer containing protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA). Standard procedures were followed for coronary endothelial cell lysis. Protein estimation was performed using a Pierce BCA protein estimation kit (ThermoFisher Scientific). Equal amounts of protein (30 µg) were separated by SDS–polyacrylamide gel electrophoresis using Mini-PROTEAN precast gels (4-15% gradient; Bio-Rad, Hercules, CA) and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% bovine serum albumin in Tris buffered saline (TBS, pH 7.4), blots were incubated overnight at 4°C with appropriate primary antibodies specific for APJ receptors (sc-517300, Santa Cruz Biotechnology Inc.) and eNOS (32027S, Cell Signaling Technology) using a dilution of 1:100 and 1:1000 respectively. Membranes were washed with TBS-Tween20 four times for 10 minutes followed by incubation with a horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology Inc.). To ensure equal loading, the blots were analyzed for β-actin protein expression using an anti-actin antibody with a dilution of 1:500 (sc-47778, Santa Cruz Biotechnology Inc.). Protein bands and relative densities were measured using an enhanced chemiluminescence light detection kit (Advansta, San Jose, CA).

RT-qPCR

Freshly isolated coronary arteries were frozen immediately in liquid nitrogen and total RNA was isolated using a RNeasy Mini kit, according to the manufacturer's protocol (Qiagen,

Germantown, MD). Standard procedures were followed for coronary endothelial cell lysis and RNA isolation. DNase I treatment was used to avoid genomic DNA contamination. The concentration and purity of RNA was determined using Synergy HTX multi-mode reader (Agilent Technologies, Santa Clara, CA). cDNA was synthesized using 200 ng of RNA and OneScript cDNA Synthesis Kit (Applied Biological Materials). BrightGreen 2XqPCR master mix (Applied Biological Materials) was used to determine expression of APJ and eNOS on the QuantStudio 3 RT-PCR system following manufacturer's instructions. S16 was used as a housekeeping gene. The following primers were used for qRT-PCR analysis ("r" before a primer refers to rat). rAPJ (forward5'-GACACACACCCAGAAGAGTATC-3'; reverse 5'-GAAGAGAGAGGGATGGG-AAATAAG); reNOS (forward 5'-GCTTGGGATCCCTGGTATTT-3'; reverse 5'-GAAG-ATTGCCTCGGTTTGTTG-3') and rS16 (forward 5'-TGCTATCCGGCAGTCTATCT-3'; reverse 5'-CTGATCCTTGAGACTGGCTTATC-3'). All primers were synthesized by IDT (Newark, NJ). Target mRNA expression was normalized against 16S mRNA expression. Data are presented as fold change in mRNA expression.

Isolation of Coronary Endothelial Cells

Coronary artery endothelial cells were isolated using previously established procedures (119), with minor modifications. Briefly, rat coronary arteries were digested in dissociation solution (55 mM NaCl, 6 mM KCl, 80 mM Na-glutamate, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.3) containing elastase (0.5 mg/ml) (Worthington, Lakewood, NJ) and neutral protease (0.5 mg/ml) (Worthington) for 60 minutes at 37°C, followed by collagenase type II (0.5 mg/ml) (Worthington) in the same solution for 2 minutes. The enzyme solution was removed, and arteries were treated with dissociation solution without enzyme for 10 min followed by trituration with a polished Pasteur pipette to produce a suspension of single

endothelial cells. Cells were re-suspended in endothelial cell growth medium MV (EGM MV) (Promo cell, Heidelberg, Germany) with 1% penicillin-streptomycin solution and maintained at 37°C (5% CO_2, 95% air) until 80% confluent. Cells from 2nd to 5th passage were used for experiments. Phenotype stability was confirmed periodically using the endothelial cell marker, platelet endothelial cell adhesion molecule (PECAM 1), and smooth muscle cell marker, smooth muscle α-actin (α-SMA).

Immunofluorescence Microscopy

Rat coronary endothelial cells were grown on 8-well Lab Tek chambers (ThermoFisher Scientific) in EGM MV until 40-50% confluent. Cells were then fixed using 4% paraformaldehyde for 15 min at room temperature and washed thrice with PBS. The cells were permeabilized using 0.1% TritonX-100 in PBS for 15 min and nonspecific antibody binding was blocked with 10% donkey serum (Sigma Aldrich, USA) in PBS for 60 min at room temperature. Cells were incubated overnight at 4°C with PECAM-1(AF3628, R&D Systems) and APJ receptor (ABD43, EMD Millipore) or α-smooth muscle actin (19245S, Cell Signaling Technology) antibodies. For co-localization studies, cells were simultaneously incubated with APJ receptor and PECAM-1 or α-SMA antibodies. Following day cells were incubated with Alexa Fluor 488 (APJ receptor) and Alexa Fluor 555 (PECAM-1 or α-SMA) tagged secondary antibodies against rabbit and goat respectively for 60 min at room temperature. Negative controls were subjected to same process with the exception of addition of primary antibody. Cells were washed with PBS and a drop of mounting medium containing DAPI was added and then a coverslip was placed over the slide. Images were obtained at 40X magnification using LSM 900 confocal microscope (Zeiss, CA).

Vascular Function Studies

Rat coronary arterial rings (120-150 µm; 1.2 mm in length) were mounted in wire myographs (DMT, Aarhus, Denmark) for isometric tension (muscle is under tension, but neither shortens nor lengthens) recording. The myograph chambers were filled with PSS (5 ml), which was maintained at 37°C and continuously aerated with 95% O_2/5% CO_2 throughout the experiment. The arterial rings were stretched up to a resting tension of 6 mN by sequential stretching and then allowed to stabilize for 30 minutes with intermittent washings. Vascular reactivity was established by evoking a contractile response to KCl (60 mM). In some rings, the endothelium was removed by gently rubbing the intimal surface with a human hair. The absence or presence of endothelium was verified with the endothelium-dependent vasodilator, acetylcholine (ACh; 10^{-6} M). Responses to vasodilators used in this study were obtained in arterial rings contracted with 5-hydroxytryptamine (5-HT; 10^{-7} M). When apelin (10^{-7} M) was used as an inhibitor, it was added to the tissues for 5 minutes, after the contraction to 5-HT had stabilized, in order to minimize desensitization of APJ receptors. All inhibitors remained in the myograph solution for the remainder of the experiment. Experiments with untreated control rings were conducted in parallel with rings treated with inhibitors from the same animal.

Drugs

The following drugs were used: acetylcholine, diethyl amine (DEA) NONOate, diltiazem, and 5-HT (Sigma Chemical, St. Louis, MO); apelin-13 (Bachem, Torrance, CA). Drug solutions were freshly prepared in double-distilled water. Drugs were added to the myograph chambers in volumes not greater than 0.02 ml. Drug concentrations are reported as final molar concentrations in the myograph chamber.

Data Analysis

Relaxation responses are expressed as percent of the initial tension induced by 5-HT (10^{-7} M). The EC_{50} values were determined and then converted to their negative logarithms and expressed as the -log molar EC_{50} (pD_2) value. RT-qPCR data were analyzed to quantify relative gene expression by comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$). Immunoblot was analyzed to determine the density of the individual protein band and normalized with respect to intensity of the corresponding β-actin protein band. Results are expressed as means ± SEM, and n refers to the number of animals from which blood vessels were taken, unless otherwise stated. Values were compared by Student's t-test for paired or unpaired observations to determine significance between groups, as appropriate. Values were considered significantly different when $p<0.05$.

Results

Apelin-induced Relaxation in Isolated WKY and SHR Coronary Arteries

Previous work from our lab showed that apelin induces NO-mediated endothelium-dependent relaxation by the activation of BK_{Ca} channels on smooth muscle cells in coronary arteries from SD rats (25). Here, I determined the vasomotor response of apelin in SHR (an animal model for essential hypertension) and WKY (normotensive control) coronary arteries. Consistent with previous reports, apelin (10^{-8} – 3×10^{-6} M) caused concentration-dependent relaxation of WKY coronary arteries contracted with 5-HT (10^{-7} M) (Figure 1). The pD_2 value for apelin was 7.00 ± 0.11 and the maximal relaxation (E_{max}) was 54 ± 4%. By contrast, these concentrations of apelin failed to cause relaxation in isolated SHR coronary arterial rings contracted with 5-HT (10^{-7} M) (n=6, $p<0.05$ vs. WKY) **(Figure 2)**.

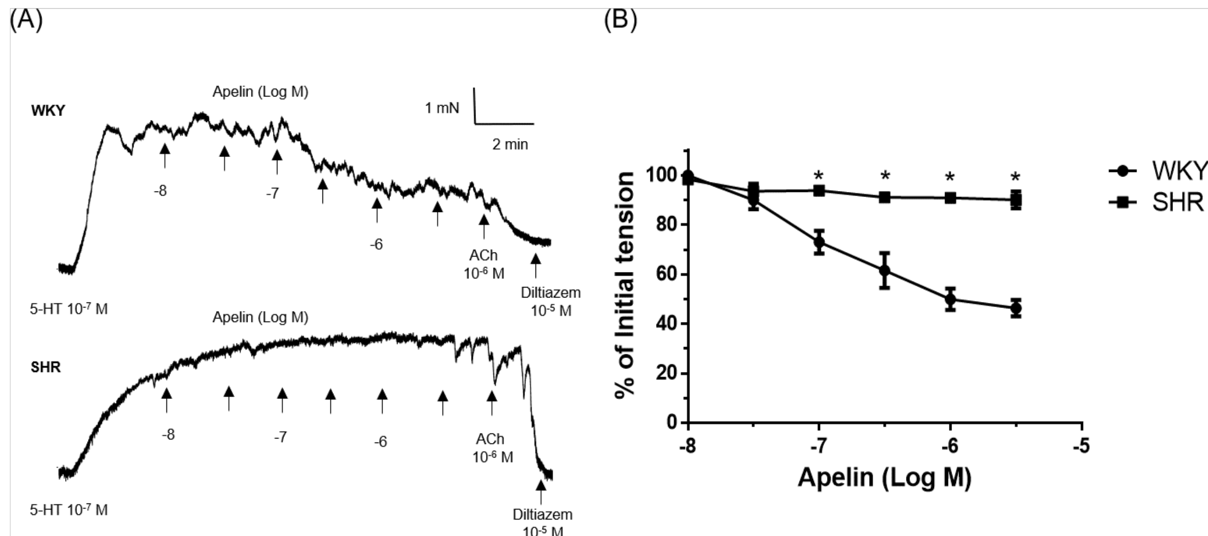

Figure 2. Apelin-induced relaxation is inhibited in isolated coronary arteries under hypertensive conditions.

(A) Representative original tracings of isometric tension recordings from isolated Wistar Kyoto (WKY) and spontaneously hypertensive rat (SHR) coronary arteries. (B) Log concentration-response curves for apelin in producing relaxation of endothelium-intact isolated WKY and SHR coronary arteries. Each point represents the mean ± SEM (n=6). *p<0.05 vs. WKY.

Apelin Inhibits ACh-induced Endothelium-dependent Relaxation but Not DEA NONOate-Induced Endothelium-independent Relaxation in SHR Coronary Arteries

Apelin alone had no effect on ACh-induced relaxation in normotensive WKY coronary arteries (pD_2 = 7.26 ± 0.17 vs 7.04 ± 0.15 without and with apelin, respectively; p>0.05, n=6) **(Figure 3A)**.

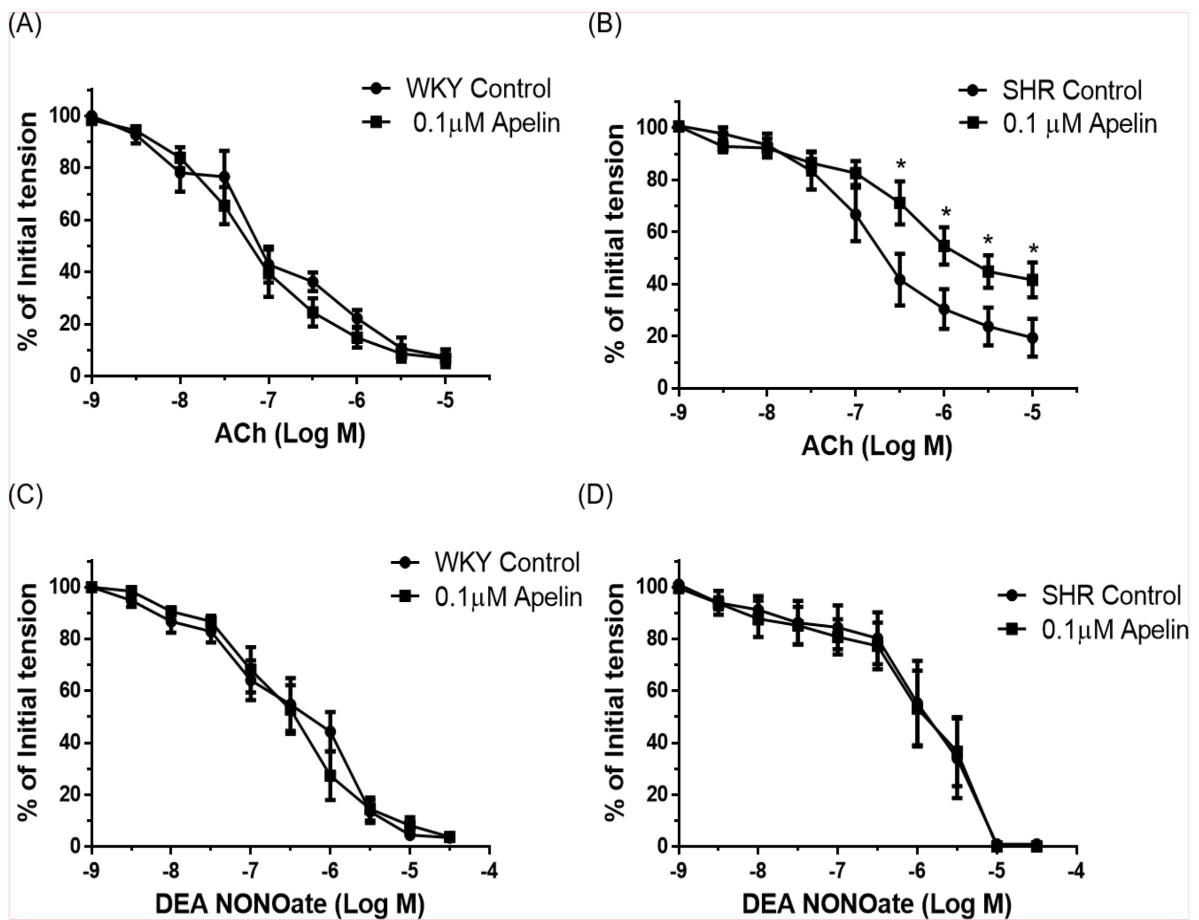

Figure 3. Effect of apelin on Ach-induced endothelium-dependent and DEA-induced endothelium-independent relaxation in normotensive and hypertensive arteries.
(A-B) Log concentration-response curves for ACh in (A) control and (B) SHR coronary arteries in the absence and presence of apelin (10^{-7} M). Log concentration-response curves for DEA in (C) control and (D) SHR coronary arteries in the absence and presence of apelin (10^{-7} M). Each point represents the mean ± SEM (n=6). *$p<0.05$ indicates a significant difference from the corresponding control value.

However, in hypertensive coronary arteries, apelin (10^{-7} M) caused a rightward shift in the concentration-response curve to ACh (pD_2 = 6.83 ± 0.09 vs 6.56 ± 0.16, and E_{max} = 80 ± 7 vs 62 ± 9% relaxation without and with apelin, respectively; $p< 0.05$, n=6) **(Figure 3B)**. Moreover, the concentration-response curve to DEA NONOate (10^{-9} – 10^{-5} M) was unaffected by the presence of apelin (10^{-7} M) in WKY (pD_2 = 6.36 ± 0.21 vs 6.48 ± 0.19) **(Figure 3C)** and SHR coronary arteries

(pD_2 = 6.03 ± 0.98 vs 6.01 ± 0.18) without and with apelin, respectively; p> 0.05, n=6) **(Figure 3D)**.

APJ Receptor and eNOS Expression Is Similar in WKY and SHR Coronary Arteries

APJ receptor expression was established in both SHR and WKY coronary arteries using RT-qPCR and western blot techniques. mRNA and protein expression level of APJ was similar in WKY and SHR coronary arteries **(Figure 4)**.

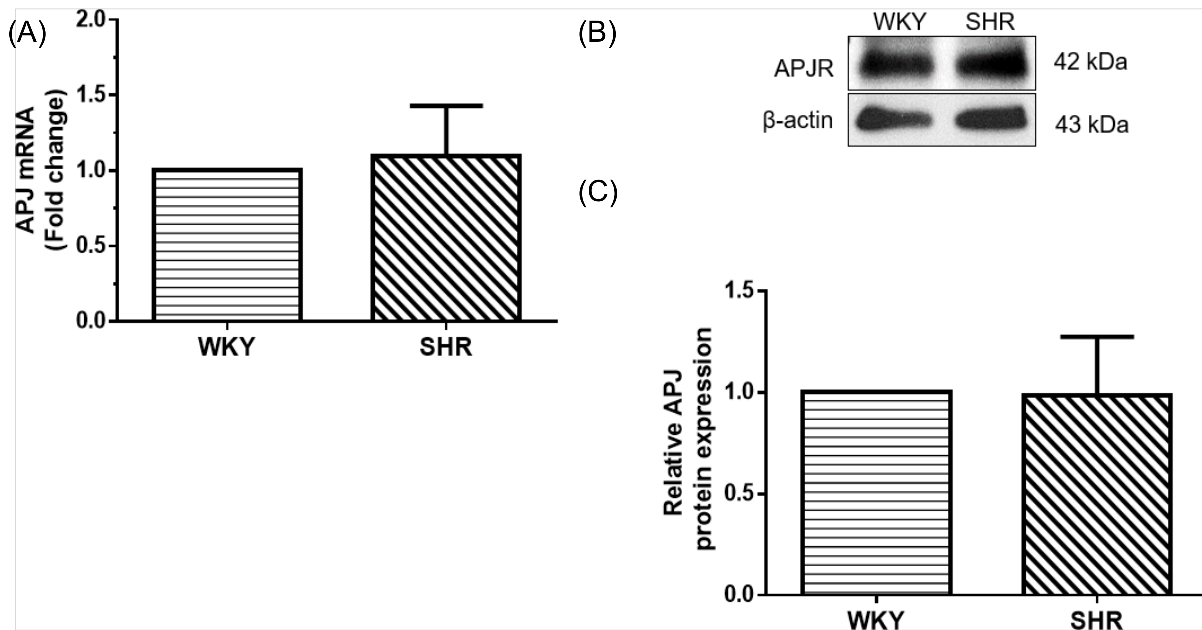

Figure 4. APJ receptor mRNA and protein expression in coronary arteries.
(A) mRNA expression of APJ receptors presented as fold change in WKY and SHR coronary arteries. Data are presented as means ± SEM (n=4). (B) Representative western blot showing expression of APJ receptor in WKY (Lane 1) and SHR (Lane 2) coronary arteries where β-actin was used as loading control and (C) bar graph showing relative expression of APJ receptors in WKY and SHR coronary arteries. Each bar represents means ± SEM (n=3). p>0.05 vs. WKY.

Endothelial cell characterization was performed periodically in WKY and SHR cultured coronary endothelial cells using the endothelial cell marker, platelet endothelial cell adhesion molecule (PECAM 1) **(Figure 5)**.

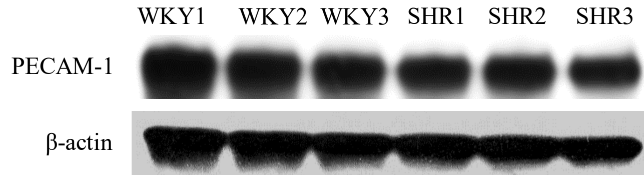

Figure 5. Characterization of endothelial cells.
Representative western blot showing expression of endothelial cell marker, PECAM-1 in WKY (Lane 1-3) and SHR (Lane 4-6) in coronary endothelial cells where β-actin was used as loading control.

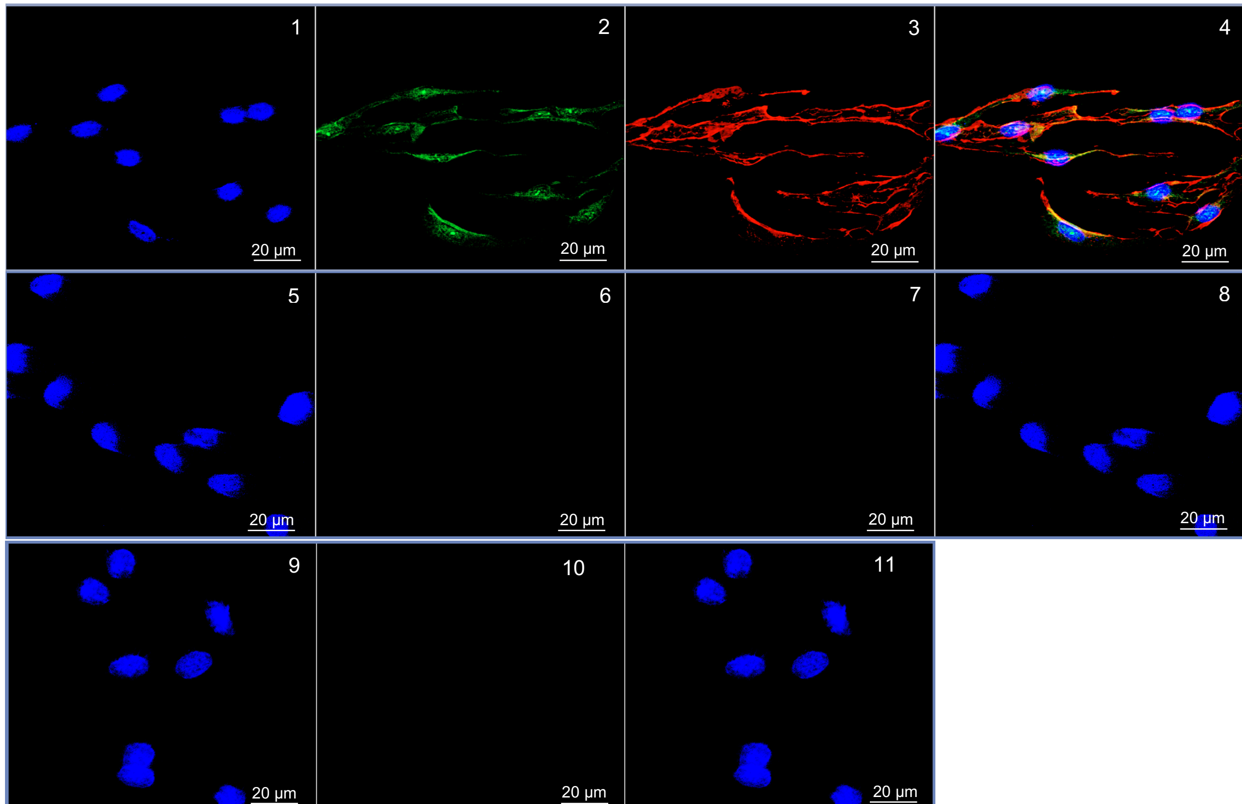

Figure 6. Localization of APJ receptors in cultured WKY coronary endothelial cells.
Representative immunofluorescence images demonstrate APJ receptor expression in endothelial cells immunostained with (1,5,9) DAPI (blue), (2) APJ receptors (green), (3) PECAM-1 (red), (4) merged imaged showing localization of APJ receptors in endothelial cells (yellow), secondary antibody control showing negative staining for (6) APJ receptors, (7) PECAM-1, (8) merged image for secondary control with DAPI, cells stained negative for (10) α-SMA and (11) merged image for α-SMA and DAPI. The images are representative of those obtained from three different animals. Scale bars = 20 µm.

Further, localization of APJ receptors in coronary endothelial cells of WKY and SHR were demonstrated using immunofluorescence confocal microscopy. APJ receptor protein was localized in cultured WKY **(Figure 6.1-6.4)** and SHR **(Figure. 7.1-7.4)** coronary endothelial cells.

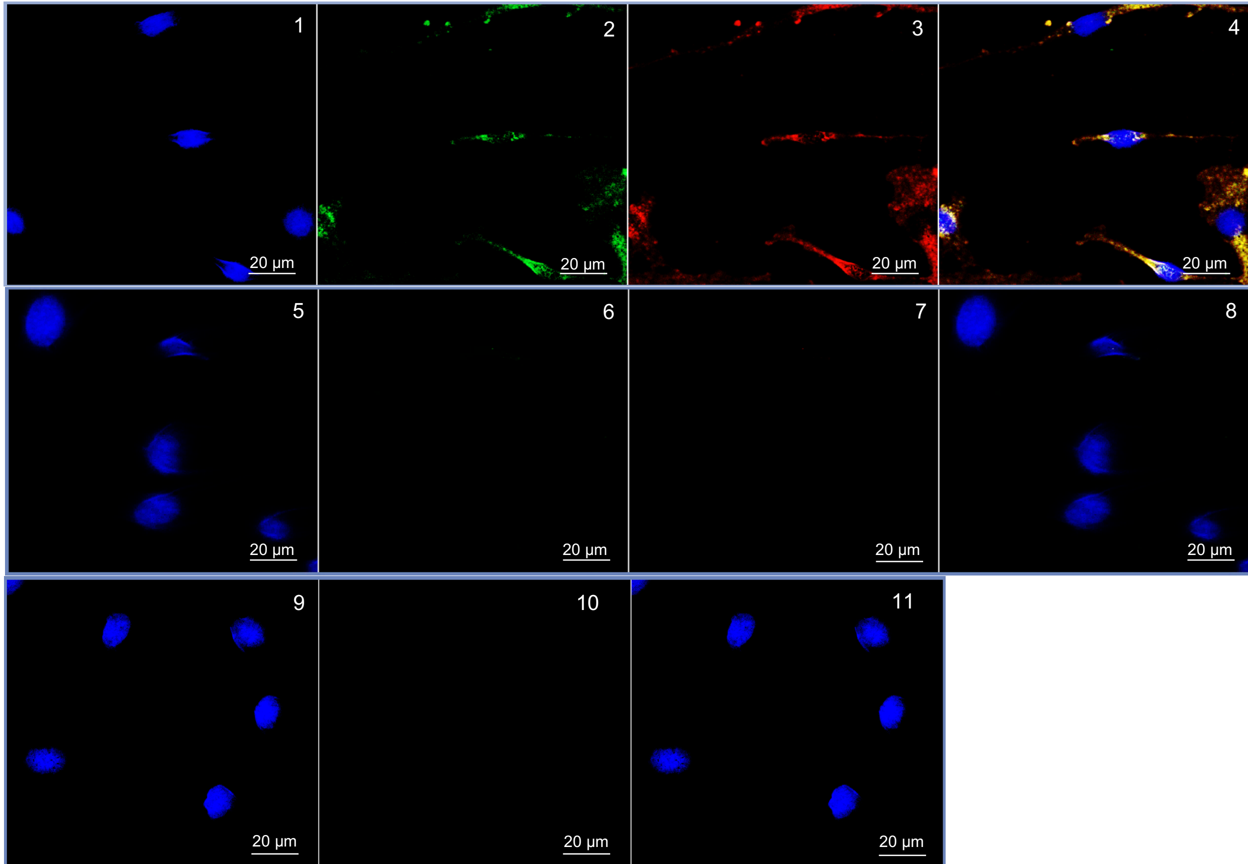

Figure 7. Localization of APJ receptors in cultured SHR coronary endothelial cells. Representative immunofluorescence images demonstrate APJ receptor expression in endothelial cells immunostained with (1,5,9) DAPI (blue), (2) APJ receptors (green), (3) PECAM-1 (red), (4) merged imaged showing localization of APJ receptors in endothelial cells (yellow), secondary antibody control showing negative staining for (6) APJ receptors, (7) PECAM-1, (8) merged image for secondary control with DAPI, cells stained negative for (10) α-SMA and (11) merged image for α-SMA and DAPI. The images are representative of those obtained from three different animals. Scale bars = 20 µm.

The non-specific binding of secondary antibody in the absence of primary antibody (secondary control) for APJ or PECAM-1 was shown in WKY **(Figure. 6.5-6.8)** and SHR **(Figure. 7.5-7.8)** in coronary endothelial cells. WKY **(Figure. 6.9-6.11)** and SHR **(Figure. 7.9-7.11)**

cultured coronary endothelial cells that were stained for smooth muscle cell marker, α-SMA, was used as negative control.

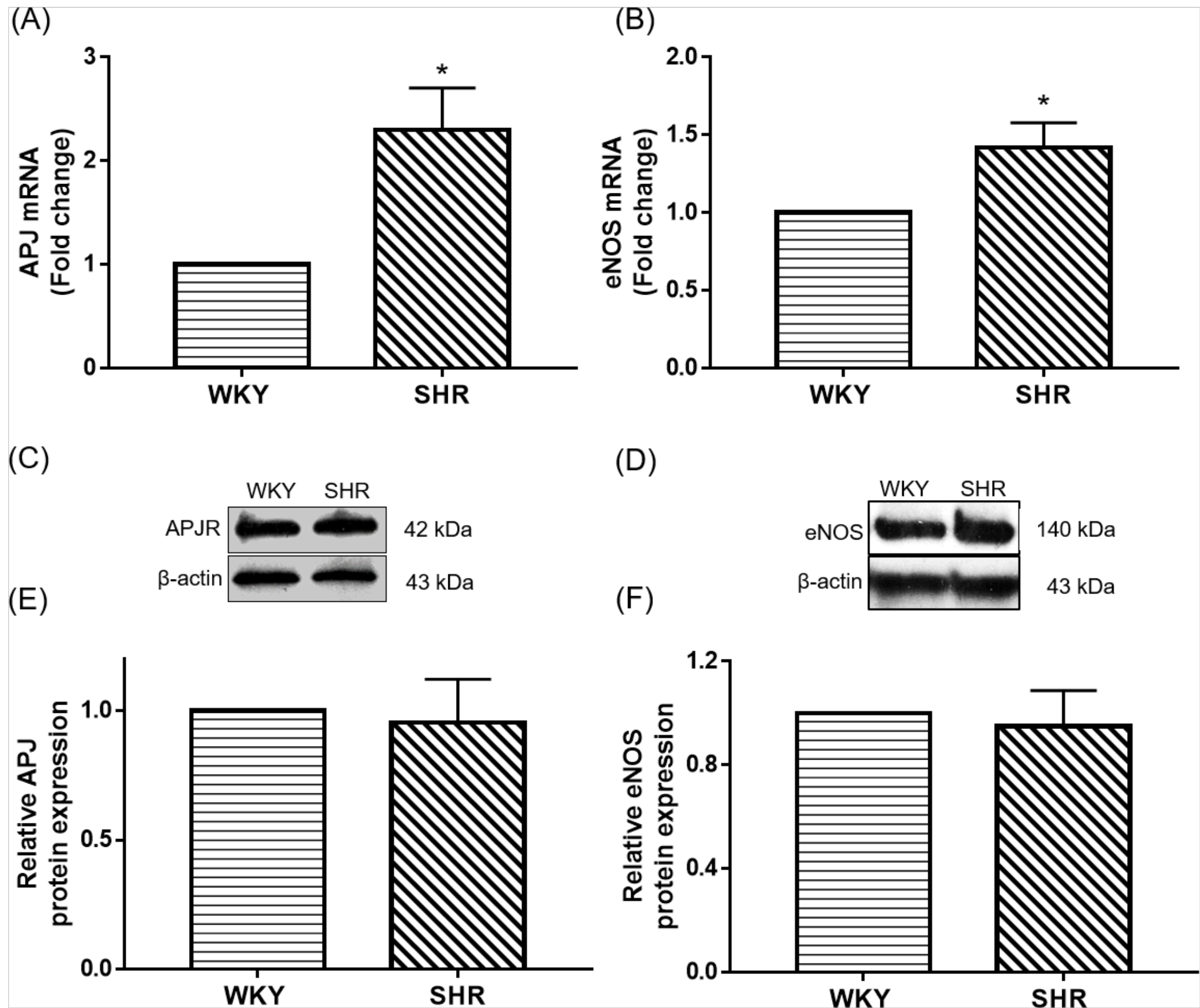

Figure 8. APJ receptor and eNOS expression in coronary endothelial cells.
mRNA expression of (A) APJ receptor and (B) eNOS presented as fold change in WKY and SHR coronary endothelial cells. Data are presented as means ± SEM (n=6). *$p<0.05$ vs. WKY. Representative western blot showing expression of (C) APJ receptor and (D) eNOS protein expression in WKY (Lane 1) and SHR (Lane 2) coronary endothelial cells where β-actin was used as loading control. Bar graph showing relative expression of (E) APJ receptors and (F) eNOS protein in WKY and SHR coronary endothelial cells. Each bar represents means ± SEM (n=4-6). $p>0.05$ vs. WKY.

To evaluate whether change in APJ receptor or eNOS expression level plays a role in altered apelin response in hypertensive arteries, both mRNA and protein expression were

compared in cultured coronary endothelial cells using RT-qPCR and immunoblot techniques. Data showed that APJ receptor and eNOS mRNA increased in cultured SHR coronary endothelial cells in comparison to WKY (**Figure 8A-B**). However, protein level of APJ receptor and eNOS were similar under normotensive and hypertensive conditions in cultured coronary endothelial cells **(Figure. 8C-F)**.

GRK2 Expression is Increased in SHR Coronary Arteries

Previous reports suggest that G-protein coupled receptor kinase (GRK2) is upregulated in hypertensive conditions and is associated with downregulation of eNOS activity and thus NO production in endothelial cells. The expression of GRK2 protein expression was determined using RT-qPCR and western blot analysis. GRK2 mRNA level was similar between WKY and SHR coronary arteries (**Figure 9A)**. However, GRK2 mRNA level was increased in cultured coronary endothelial cells from SHR compared to WKY (**Figure 9B)**. Furthermore, GRK2 protein expression was significantly increased in cultured SHR coronary endothelial cells as compared to normotensive WKY **(Figure 9C, E)**. G-protein-coupled receptor kinase-3 (GRK3) and GRK2 are highly homologous proteins and are involved in GPCR desensitization. Here, I determined the expression level of GRK3 protein in WKY and SHR coronary endothelial cells. GRK3 protein expression level was very low and similar in SHR and WKY cultured coronary endothelial cells **(Figure 9D, F)**.

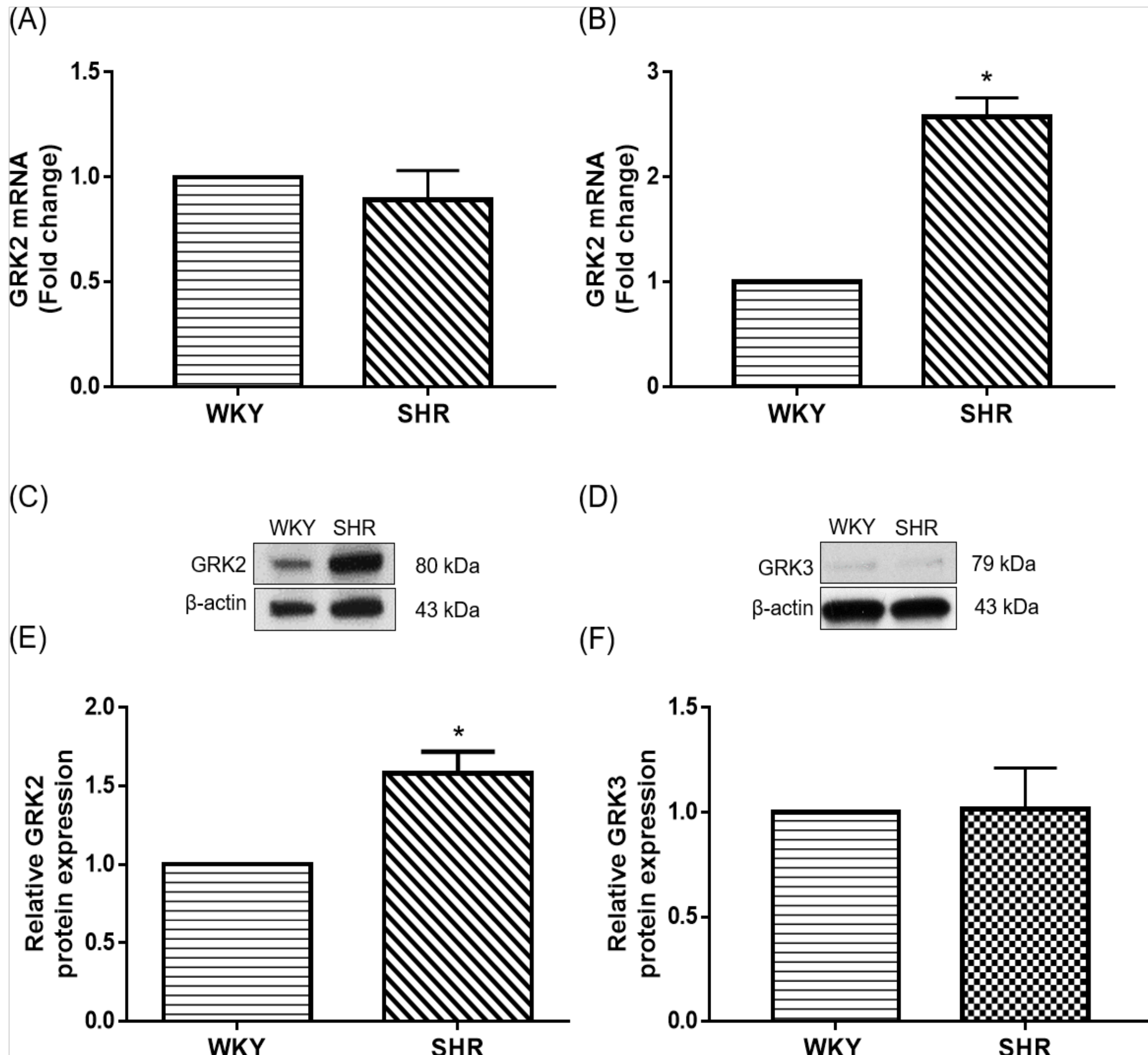

Figure 9. GRK2 expression in cultured coronary endothelial cells.
mRNA expression of GRK2 in (A) coronary arteries and (B) coronary endothelial cells presented as fold change in WKY and SHR. Data are presented as means ± SEM (n=3-5). *p<0.05 vs. WKY. Representative western blot showing expression of (C) GRK2 and (D) GRK3 protein expression in WKY (Lane 1) and SHR (Lane 2) coronary endothelial cells where β-actin was used as loading control. Bar graph showing relative expression of (E) GRK2 and (F) GRK3 protein in WKY and SHR coronary endothelial cells. Each bar represents means ± SEM (n=4-6). *p<0.05 vs. WKY.

Discussion

Data from this study demonstrate that apelin-induced, endothelium-dependent relaxation of isolated coronary arteries is markedly impaired under hypertensive conditions. Moreover, the function of apelin changed in hypertensive arteries, i.e., rather than causing relaxation of coronary

arteries, the peptide instead inhibited relaxation evoked by ACh, an endothelium-dependent vasodilator that acts via the release of NO from endothelial cells in rat coronary arteries (25). Results from APJ receptor, eNOS, and GRK2 expression experiments suggest that GRK2 may be playing a vital role in the altered coronary artery response to apelin that occurs in hypertensive rats. Overall, the findings in this research suggest that apelin could not provide beneficial vasodilatory effects in coronary arteries under pathological conditions (e.g., hypertension). Furthermore, APJ receptor signaling could result in increased vasomotor tone in coronary arteries possibly leading to a reduced coronary blood flow under hypertensive conditions.

Our understanding about apelin-APJ signaling under pathological conditions is not well established. The regulation of vasomotor tone by the apelinergic system is very complex as it can induce vasodilation or vasoconstriction depending on the vascular bed and experimental conditions. The consensus is that apelin has vasodilatory effects in the presence of healthy endothelium and vasoconstrictor effects in the case of damaged endothelium. APJ receptors expression has been reported in endothelial cells and vascular smooth muscle cells in blood vessels (25,91). Apelin caused vasoconstriction in human saphenous veins, mammary arteries and coronary arteries that lacked functional endothelium mainly because of the activation of APJ receptors on vascular smooth muscle cells (52,82,83). Under physiological conditions apelin caused vasodilatation mainly via NO-dependent pathway in human forearm brachial, mesenteric, and coronary arteries (55,72,73). In normotensive rat coronary arteries, apelin induced NO-mediated endothelium-dependent relaxation by the activation of BK_{Ca} channels on coronary smooth muscle cells (25). There are no previous studies on the vasomotor effects of apelin under pathological conditions in coronary arteries. This research first provide evidence that apelin behave differently under pathological conditions (e.g., hypertension) in rat coronary circulation.

As previously reported, under normotensive conditions, apelin causes relaxation of coronary arteries by releasing NO from vascular endothelial cells (25). In contrast, apelin failed to cause relaxation in hypertensive SHR coronary arteries. Another key finding from this present study is that apelin inhibited relaxation response to acetylcholine (ACh), the prototypical endothelium-dependent vasodilator that releases NO from endothelial cells. It is noteworthy that the apelin/APJ receptor signaling system takes on a completely different function in coronary circulation under hypertensive condition. Apelin not only lost its beneficial vasodilatory effects but also apelin itself inhibited another vasodilator (ACh) response in hypertensive arteries. From previous reports, we know that apelin causes relaxation by the activation of BK_{Ca} channels in coronary smooth muscle cells in a NO-dependent manner (25). Here, apelin had no effect on relaxation of SHR coronary arteries in response to NO donor, DEA NONOate. These findings with the NO-donor indicate that apelin does not interfere with the actions of NO on coronary smooth muscle cells. Taken together, the data suggest that coupling between APJ receptor activation by apelin and NO synthesis by eNOS is selectively impaired in endothelial cells from hypertensive coronary arteries.

One possible explanation for impaired apelin signaling would be the changes in APJ receptor expression levels in coronary arteries, especially coronary endothelial cells. There are previous reports which correlated the decreased expression level of APJ receptors in cardiovascular diseases and functional loss of apelinergic system (83,116). This study showed that the mRNA level of APJ receptor was increased in SHR coronary endothelial cells. However, protein level of APJ receptors were similar between normotensive and hypertensive coronary arteries as well as endothelial cells. The difference in gene expression and protein expression data might be due to change in the regulation of transcriptional rate and mRNA degradation between

WKY and SHR coronary endothelial cells. Since apelin induced relaxation is NO-dependent, decreased expression levels of eNOS might be a possible reason for altered apelin signaling in hypertensive coronary arteries. However, the results suggest that the mRNA level was increased in SHR coronary endothelial cells whereas protein levels of eNOS were unaffected in control and SHR coronary endothelial cells. Ample data suggest that eNOS must be phosphorylated to show full functional capacity. eNOS activity can be modulated by its binding to HSP90, serine-threonine kinase and the caveolin (111,120–122). Hence, it is possible that eNOS activity is impaired in hypertensive coronary arteries with regard to apelin signaling.

APJ receptor signaling activates G-protein-dependent and -independent pathway. One such G-protein-independent pathway involves the activation of GRK2 protein (21,72,123). GRK2 is a multidomain protein which is a key component of the G-protein independent intracellular signaling cascade that interacts with a complex array of cellular effectors (124). It is well known that GRK2 activation can impair the eNOS activity under cardiovascular disease conditions (e.g., hypertension) (125). GRK2 can interact with multiple effectors such as Akt, caveolin thereby impair the eNOS activity and NO production in endothelial cells (110,111). Interestingly, GRK2 expression was significantly increased in SHR coronary endothelial cells as compared to normotensive WKY. Evidence suggests that GRK2 is abundantly expressed in vascular smooth muscle cells (111,126). However, GRK2 expression were similar between WKY and SHR coronary arterial tissue indicating that GRK2 upregulation might be specific to coronary endothelial cells. Since GRK2 and GRK3 proteins are highly homologous (127), I determined the expression level of GRK3 in coronary endothelial cells. The results showed that the expression level of GRK3 was less and similar between control and hypertensive coronary endothelial cells whereas GRK2 expression was upregulated in SHR endothelial cells. These data suggest the

increased chance of a potential role for GRK2 protein in the altered apelin response in hypertensive coronary arteries.

In summary, this present research suggests that apelin behaves differently under disease condition (e.g., hypertension). Apelin caused relaxation in normotensive coronary arteries but not under hypertensive conditions i.e., apelin could not provide its beneficial vasodilatory effects in coronary circulation during hypertension. Moreover, apelin inhibits relaxation response to another endothelium-dependent vasodilator, ACh. However, DEA NONOate induced relaxation response was unaffected. These indicate that apelinergic system activation under hypertensive conditions results in defective production or release of NO from coronary endothelial cells. Considering the fact that APJ receptor and eNOS expression was unaltered and GRK2 expression was upregulated in SHR coronary endothelial cells, coupling between APJ receptor and GRK2 activation might be a potential mechanism involved in the impaired apelin response in SHR coronary arteries.

CHAPTER 3. APELIN/APJ RECEPTOR SIGNALING IS IMPAIRED IN SHR CORONARY ARTERIES VIA GRK2 PATHWAY ACTIVATION

Introduction

Hypertension is a major modifiable risk factor for coronary artery disease (128). The pathophysiological mechanisms underlying hypertension as a risk factor for coronary artery disease are not fully understood. Apelin is a vasoactive peptide that binds to APJ receptors, which are highly expressed throughout the cardiovascular system, including coronary arteries. In coronary arteries, APJ receptors are expressed in endothelial cells as well as vascular smooth muscle cells. The reported evidence suggests that apelin causes endothelium-dependent, NO-mediated relaxation of coronary arteries from normotensive animals (25). However, in previous chapter, I have shown that apelin-APJ signaling with respect to vasodilatory response was impaired in hypertensive coronary arteries.

APJ receptors signal via G-protein-dependent and -independent pathways, including activation of G-protein-coupled-receptor kinase 2 (GRK2) (31). GRK2-arrestin system plays a vital role in the desensitization of G-protein coupled receptors including APJ. Clinical and preclinical studies in cardiovascular and metabolic disorders showed increased GRK2 expression and activity thereby contributing to disease progression by different mechanisms (124,129,130). In the vasculature, GRK2 is known to be associated with endothelial dysfunction since it is a negative modulator of eNOS activity and NO bioavailability (33). GRK2 is a multimeric protein that can regulate multiple proteins such as caveolin, ß-arrestin, and Akt thereby impairing the eNOS activity (111,131). It is well established that GRK2 can impair the phosphorylation of Akt resulting in decreased eNOS activity in disease conditions (32,132,133).

Previous reports showed that APJ receptors signal via the PI3-kinase/Akt pathway to activate eNOS and stimulate NO production (78,134). In the portal hypertension model, GRK2

decreased eNOS activity by impairing the Akt/eNOS pathway in sinusoidal endothelial cells (111). In the last chapter, I have shown that GRK2 expression is increased in SHR coronary endothelial cells Considering these facts, I postulated that the mechanism underlying the defective eNOS activity in hypertensive coronary arteries is via the GRK2 pathway activation thereby resulting in impaired Akt/eNOS signaling and NO production.

The results from this study showed that APJ receptor biased agonist, CMF-019, can cause relaxation in normotensive and hypertensive coronary arteries. Apelin and CMF-019 increased eNOS and Akt activity in WKY coronary endothelial cells. CMF-019 induced Akt activity was significantly increased compared to apelin in SHR coronary endothelial cells. Moreover, GRK2 inhibition resulted in the reversal of apelin-induced relaxation response in SHR coronary arteries. Furthermore, GRK2 inhibition markedly increased eNOS and Akt activity in hypertensive coronary arteries.

Materials and Methods

Animals and Tissue Preparation

Experiments were performed on tissues obtained from age matched Wistar Kyoto (WKY) and Spontaneously Hypertensive rats (SHR) (Charles River Laboratories, Wilmington, MA). Rats were housed on a 12-hr/12-hr light/dark cycle at 22 ± 2°C and were provided with food and water ad libitum. All animal protocols used in this study were approved by the North Dakota State University Institutional Animal Care and Use Committee. The animals were anesthetized with isoflurane and hearts were isolated and placed into ice-cold physiologic salt solution (PSS) of the following composition: 118.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.03 mM EDTA, 5.5 mM glucose, and 25.0 mM $NaHCO_3$. Epicardial coronary arteries were dissected free and cleaned of surrounding tissues.

Vascular Function Studies

Rat coronary arterial rings (120-150 μm; 1.2 mm in length) were mounted in wire myographs (DMT, Aarhus, Denmark) for isometric tension recording. The myograph chambers were filled with PSS (5 ml), which was maintained at 37°C and continuously aerated with 95% O_2/5% CO_2 throughout the experiment. The arterial rings were stretched up to a resting tension of 6 mN by sequential stretching and then allowed to stabilize for 30 minutes with intermittent washings. Vascular reactivity was established by evoking a contractile response to KCl (60 mM). In some rings, the endothelium was removed by gently rubbing the intimal surface with a human hair. The absence or presence of endothelium was verified with the endothelium-dependent vasodilator, acetylcholine (ACh; 10^{-6} M). Responses to vasodilators used in this study were obtained in arterial rings contracted with 5-hydroxytryptamine (5-HT; 10^{-7} M). Inhibitors were added to the myograph chamber 20 min prior to contraction with 5-HT. When apelin (10^{-7} M) was used as an inhibitor, it was added to the tissues for 5 minutes, after the contraction to 5-HT had stabilized, in order to minimize desensitization of APJ receptors. All inhibitors remained in the myograph solution for the remainder of the experiment. Experiments with untreated control rings were conducted in parallel with rings treated with inhibitors from the same animal.

Isolation of Coronary Endothelial Cells

Coronary artery endothelial cells were isolated using previously established procedures (119), with minor modifications. Briefly, rat coronary arteries were digested in dissociation solution (55 mM NaCl, 6 mM KCl, 80 mM Na-glutamate, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.3) containing elastase (0.5 mg/ml) (Worthington, Lakewood, NJ) and neutral protease (0.5 mg/ml) (Worthington) for 60 minutes at 37°C, followed by collagenase type II (0.5 mg/ml) (Worthington) in the same solution for 2 minutes. The enzyme

solution was removed, and arteries were treated with dissociation solution without enzyme for 10 min followed by trituration with a polished Pasteur pipette to produce a suspension of single endothelial cells. Cells were re-suspended in endothelial cell growth medium MV (EGM MV) (Promo cell, Heidelberg, Germany) with 1% penicillin-streptomycin solution and maintained at 37°C (5% CO_2, 95% air) until 80% confluent. Cells from 2nd to 5th passage were used for experiments. Phenotype stability was confirmed periodically using the endothelial cell marker, platelet endothelial cell adhesion molecule (PECAM 1), and smooth muscle cell marker, smooth muscle α-actin (α-SMA).

Western Immunoblotting

Cultured coronary endothelial cells were washed in ice cold PBS and collected in lysis buffer (Cell Signaling Technology) supplemented with protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA). Protein estimation was performed using a Pierce BCA protein estimation kit (ThermoFisher Scientific). Equal amounts of protein (30 μg) were separated by SDS–polyacrylamide gel electrophoresis using Mini-PROTEAN precast gels (4-15% gradient; Bio-Rad, Hercules, CA) for 4 hours at 50V and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% bovine serum albumin in Tris buffered saline (TBS, pH 7.4), blots were incubated overnight at 4°C with appropriate primary antibodies specific for phospho-eNOS (9570S), phospho-Akt (9271S), eNOS (32027S), Akt (9272S) (Cell Signaling Technology), phospho-GRK2 (PA5-67507, Invitrogen) and GRK2 (SC-13143, Santa Cruz Biotechnology Inc.) using a dilution of 1:1000. Membranes were washed with TBS-Tween20 four times for 10 minutes followed by incubation with a horseradish peroxidase-linked secondary antibody (NA934V, Cytiva, Marlborough, MA) using a dilution of 1:10000. To ensure equal loading, the blots were analyzed for β-actin protein expression using an anti-actin antibody with a dilution of 1:500 (sc-

47778, Santa Cruz Biotechnology Inc.). Protein bands and relative densities were measured using an enhanced chemiluminescence light detection kit (Advansta, San Jose, CA).

Drugs

The following drugs were used: acetylcholine, diltiazem, and 5-HT (Sigma Chemical, St. Louis, MO); apelin-13 and F13A (H-Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Ala-OH trifluoroacetate salt) (Bachem, Torrance, CA); CMPD 101 (Tocris, Ellisville, MO); and CMF 019 (Aobious, Gloucester, MA). Drug solutions were freshly prepared in double-distilled water with the exception of CMF-019 and CMPD 101, which were dissolved initially in DMSO and followed by further dilutions in double-distilled water. Drugs were added to the myograph chambers in volumes not greater than 0.02 ml. Drug concentrations are reported as final molar concentrations in the myograph chamber.

Data Analysis

Relaxation responses are expressed as percent of the initial tension induced by 5-HT (10^{-7} M). The EC_{50} values were determined and then converted to their negative logarithms and expressed as the -log molar EC_{50} (pD_2) value. Immunoblot was analyzed to determine the density of the individual protein band and normalized with respect to intensity of the corresponding β-actin protein band. Results are expressed as means ± SEM, and n refers to the number of animals from which blood vessels were taken, unless otherwise stated. Values were compared by Student's t-test for paired or unpaired observations to determine significance between groups, as appropriate. Values were considered significantly different when $p<0.05$.

Results

CMF-019 -induced Relaxation in SHR Coronary Arteries

To check whether GRK2 play any role in altered response to apelin in SHR coronary arteries, the vasomotor response of APJ receptor biased agonist, CMF-019 (135,136), which

preferentially activates G-protein-dependent pathway with little effect on GRK2 were determined. Interestingly, unlike apelin, CMF-019-induced relaxation was similar between WKY (pD_2 = 6.93 ± 0.43; n=6) and SHR (pD_2 = 6.89 ± 0.36; n=8) isolated coronary arteries **(Figure. 10)**.

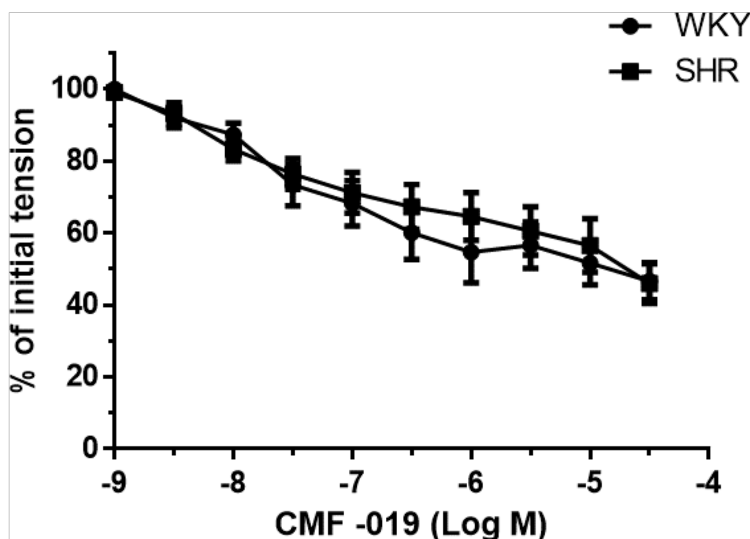

Figure 10. CMF-019-induced relaxation in coronary arteries.
Log concentration-response curves for CMF-019 in producing relaxation of endothelium-intact isolated coronary arteries. Each point represents the mean ± SEM (n=6-8). p>0.05 vs. WKY.

CMF-019 Induced Endothelium-dependent Relaxation in Coronary Arteries

Apelin causes NO-mediated endothelium-dependent relaxation through activation of APJ receptors in SD rat coronary arteries (25). Here we determined the vasodilatory mechanism of CMF-019 in WKY and SHR coronary arteries. CMF-019 caused concentration-dependent relaxation of isolated WKY coronary arteries with intact endothelium but the relaxation response was abolished in endothelium-denuded WKY coronary arteries (% Emax: 53% ± 3%. Vs 7% ± 2% with and without endothelium respectively) **(Figure.11A)**. Likewise, CMF-019-induced endothelium-dependent relaxation in SHR coronary arteries (% Emax: 42% ± 8%. Vs 8% ± 3% with and without endothelium respectively) **(Figure.11B)**. Moreover, in the presence of APJ receptor antagonist, F13A (10^{-7} M), relaxation response to CMF-019 was inhibited in WKY **(Figure. 11C)** and SHR **(Figure. 11D)** coronary arteries.

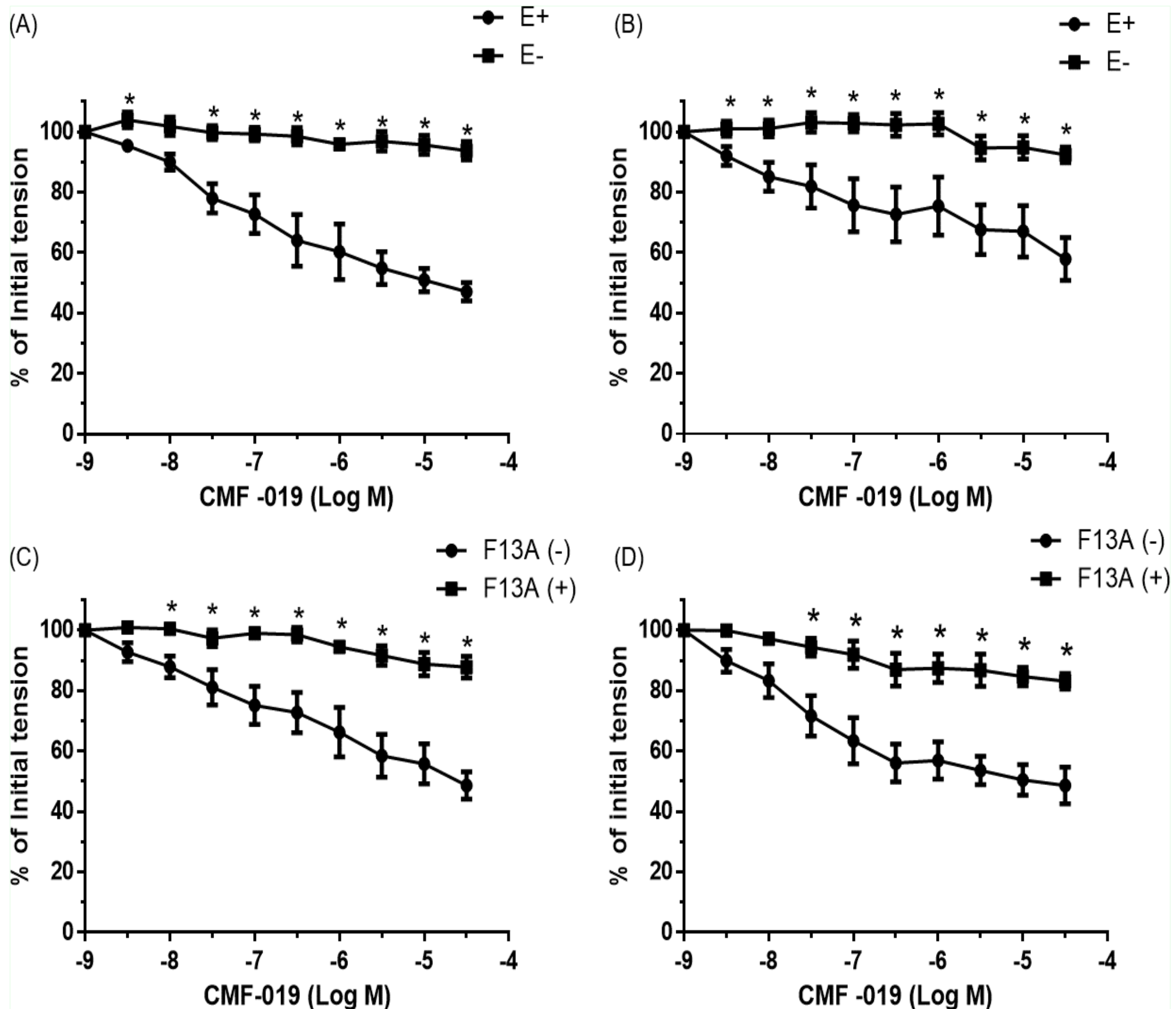

Figure 11. CMF-019 induces endothelium-dependent relaxation through activation of APJ receptors in isolated WKY and SHR coronary arteries.
Coronary arterial rings were suspended in myographs for isometric tension recording. Rings were contracted with 5-HT (0.1 µM) and exposed to increasing concentrations of CMF-019 (A and B) in the presence or absence of endothelium, and (C and D) in the presence and absence of F13A (10^{-7} M) in WKY and SHR coronary arteries, respectively. Data are expressed as mean ± SEM (n=5-6). *p<0.05 vs. CMF-019 alone.

Furthermore, experiments were performed to check whether relaxation response to CMF-019 was NO-dependent. CMF-019-induced relaxations were markedly inhibited in the presence of NLA (3 x 10^{-5} M) in WKY **(Figure. 12A)** and SHR **(Figure. 12B)** coronary arteries.

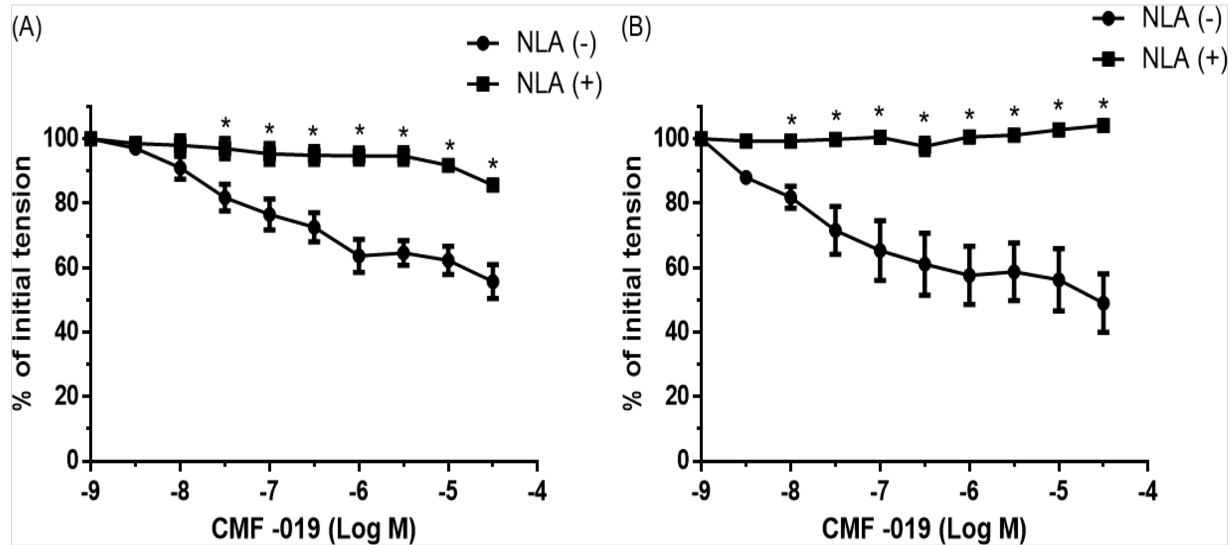

Figure 12. CMF-019-induced nitric oxide-mediated relaxation in isolated WKY and SHR coronary arteries.
Coronary arterial rings were suspended in myographs for isometric tension recording. Rings were contracted with 5-HT (0.1 μM) and exposed to increasing concentrations of CMF-019 (A and B) in the presence and absence of NLA (30 μM) in WKY and SHR coronary arteries, respectively. Data are expressed as mean ± SEM (n=4-6). *$p<0.05$ vs. CMF-019 alone.

Apelin and CMF-019-induced eNOS Phosphorylation in Coronary Endothelial Cells

eNOS activity in cultured coronary endothelial cells was determined by the ratio of phosphorylated eNOS and total eNOS using western blot technique. Serine phosphorylation (Ser^{1177}) of eNOS protein was greatly increased in apelin (10^{-7} M) and CMF-019 (10^{-7} M) treated WKY coronary endothelial cells compared with controls **(Figure 13A-B)**. In contrast, apelin treatment had no significant effect on eNOS phosphorylation in SHR coronary endothelial cells. However, eNOS activity was markedly increased in CMF-019 (10^{-7} M) treated SHR coronary endothelial cells **(Figure 13C-D)**. This data suggests that Ser^{1177} -eNOS activity is significantly increased by apelin and CMF-019 in normotensive coronary endothelial cells. Furthermore, CMF-019 increased Ser^{1177} -eNOS activity whereas apelin had no effect in SHR coronary endothelial cells.

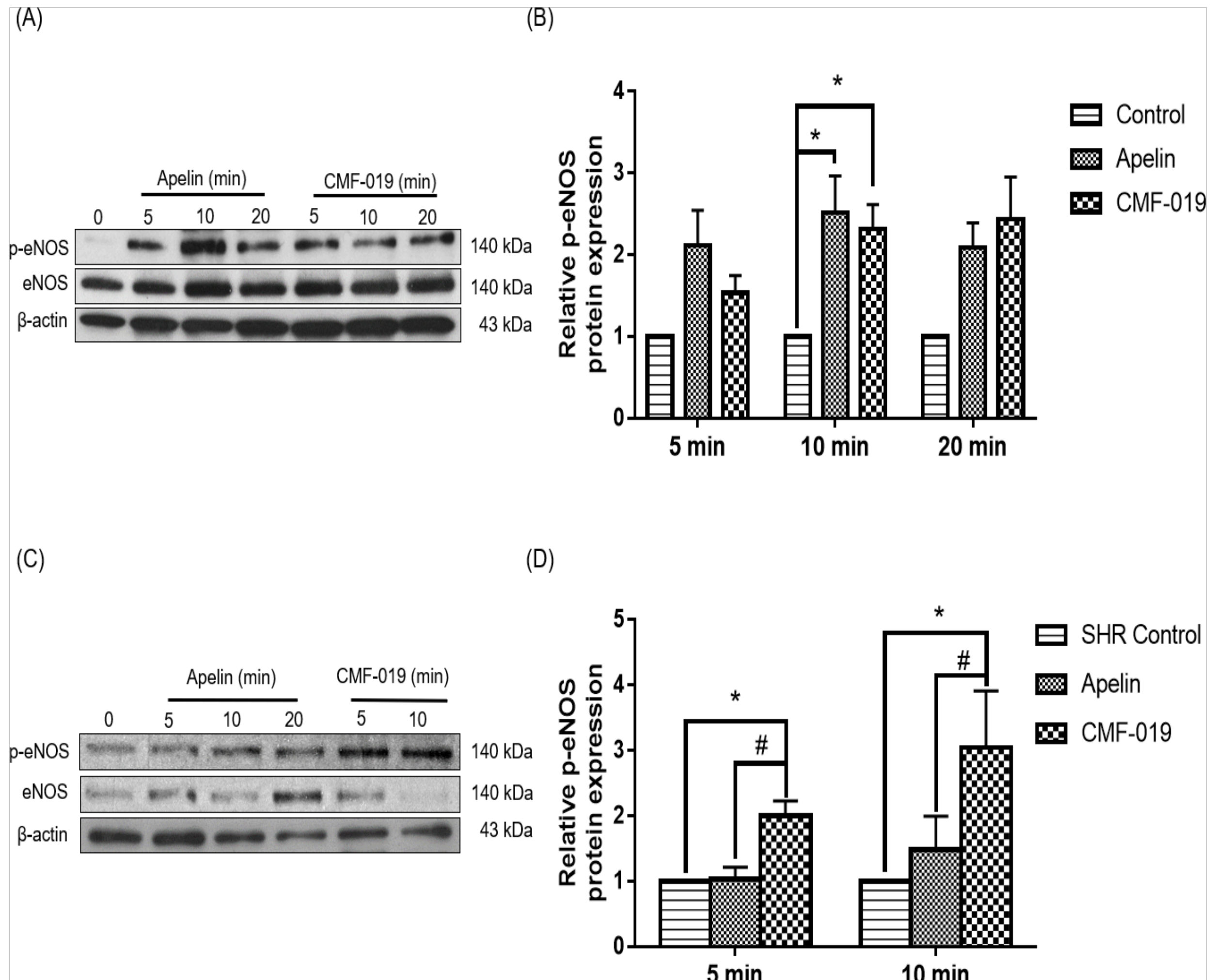

Figure 13. Effects of apelin and CMF-019 on Ser[1177] -eNOS activity in coronary endothelial cells. Representative western blot showing Ser[1177] -eNOS phosphorylation in (A) WKY and (C) SHR coronary endothelial cells treated with apelin (10^{-7} M) or CMF-109 (10^{-7} M) at different time points. β-actin was used as loading control. Bar graph summarizing the effect of apelin or CMF-019 on eNOS activity expressed as p-eNOS/eNOS protein levels in (B) WKY and (D) SHR coronary endothelial cells. Each bar represents means ± SEM (n=4-5). *p<0.05 as compared with non-treated control, #p<0.05 as compared with apelin.

Apelin and CMF-019-increased PI3K/Akt Activity in Coronary Endothelial Cells

PI3K/Akt activity in cultured coronary endothelial cells was determined by the ratio of phosphorylated Akt and total Akt using western blot technique. Ser[473] -Akt phosphorylation was significantly increased in apelin (10^{-7} M) and CMF-019 (10^{-7} M) treated WKY coronary endothelial cells compared with controls **(Figure 14)**.

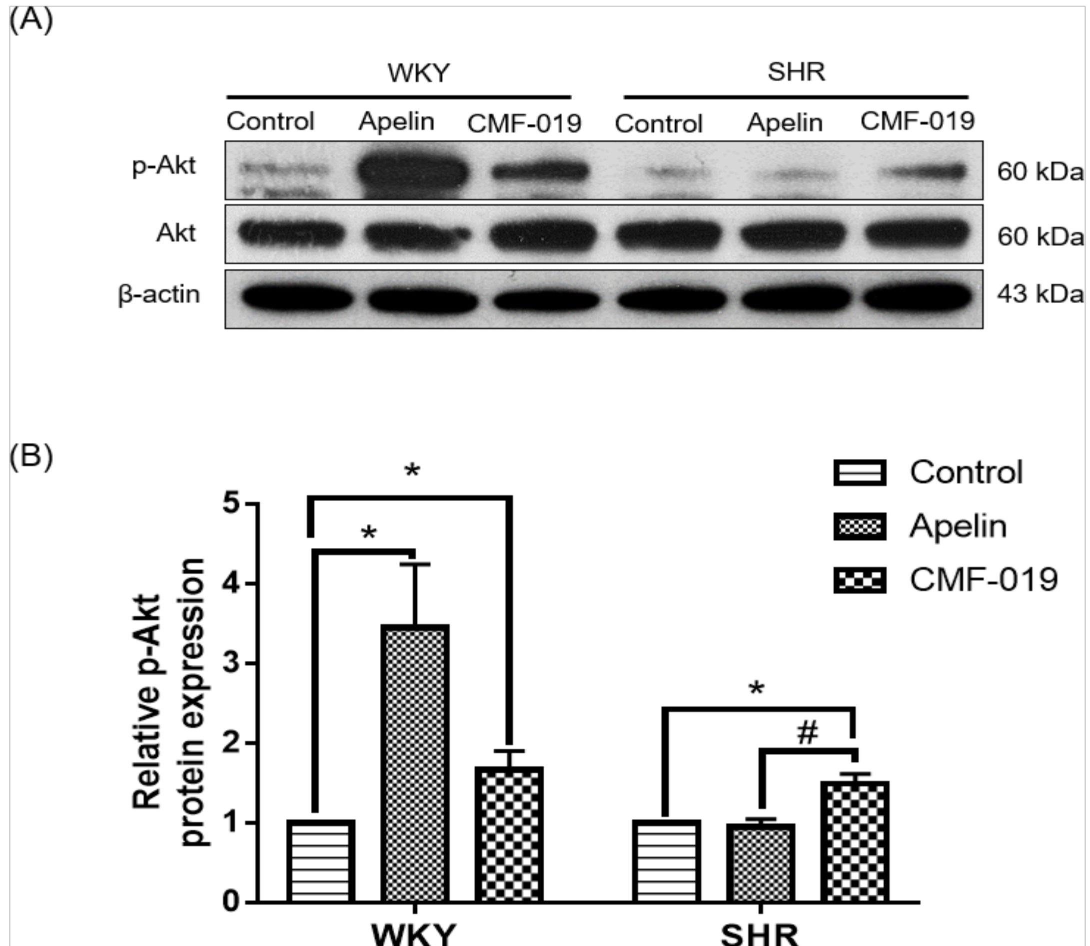

Figure 14. Effects of apelin and CMF-019 on phosphorylation of Ser[473] -Akt in WKY and SHR cultured coronary endothelial cells.
Representative western blot showing Ser[473] -Akt phosphorylation in (A) WKY and SHR coronary endothelial cells treated with apelin (10^{-7} M) or CMF-109 (10^{-7} M) for 10 minutes. β-actin was used as loading control. (B) Bar graph summarizing the effect of apelin or CMF-019 on Akt activity expressed as p-Akt/Akt protein levels in (B) WKY and SHR coronary endothelial cells. Each bar represents means ± SEM (n=5). *$p<0.05$ as compared with non-treated control, #$p<0.05$ as compared with apelin.

In contrast, apelin treatment had no significant effect on Akt phosphorylation in SHR coronary endothelial cells. Interestingly, Ser[473] -Akt activity was markedly increased in CMF-019 (10^{-7} M) treated SHR coronary endothelial cells **(Figure 14)**. This data suggests that PI3K/Akt activity is significantly increased by apelin treatment in WKY coronary endothelial cells but had no effect in SHR. Moreover, APJ receptor biased agonist, CMF-019, treatment significantly increased Akt activity in WKY and SHR coronary endothelial cells.

ACh-induced Relaxation Response Was Unaffected by CMF-019 in SHR Coronary Arteries

In previous chapter we showed that apelin inhibited ACh-induced relaxation in SHR coronary arteries **(Figure 2B)**. Here, effects of APJ receptor biased agonist, CMF-019 (10^{-7} M), on vasodilatory response of ACh was determined. Unlike apelin, CMF-019 had no inhibitory effects on ACh-induced relaxation in SHR coronary arteries **(Figure 15)**.

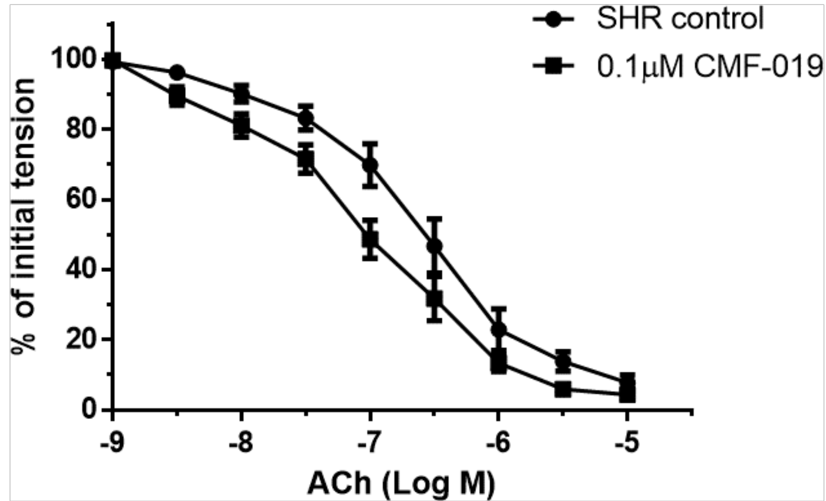

Figure 15. Effect of CMF-019 on ACh-induced relaxation.
Coronary arterial rings were suspended in myographs for isometric tension recording. Rings were contracted with 5-HT (10^{-7} M) and exposed to increasing concentration of ACh in the absence and presence of CMF-019 (10^{-7} M). Data are expressed as mean ± SEM (n=6).

GRK2 Inhibition Restored the Apelin-induced Relaxation in SHR Coronary Arteries

GRK2 is known to impair the eNOS activity in endothelial cells**.** Based on the observation that GRK2 expression was increased in coronary endothelial cells of hypertensive rats (Figure 8), we tested whether the GRK2 inhibitor, CMPD101 (137,138), could rescue apelin-induced relaxation in SHR coronary arteries. Interestingly, treatment of coronary arteries with the GRK2 inhibitor CMPD101 (30 μM) partially restored apelin-induced relaxation in SHR coronary arteries ($pD_2 = 6.56 \pm 0.22$; $E_{max} = 46 \pm 6\%$ relaxation; n=6, in the presence of CMPD101) **(Figure 16)**.

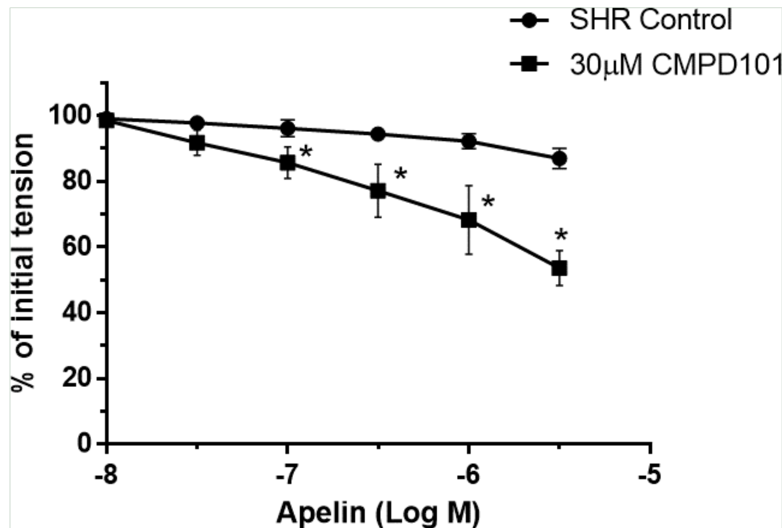

Figure 16. Apelin-induced relaxation is restored in isolated SHR coronary arteries.
Coronary arterial rings were suspended in myographs for isometric tension recording. Rings were contracted with 5-HT (10^{-7} M) and exposed to increasing concentrations of apelin in the presence or absence of CMPD 101 (30 μM). Data are expressed as mean ± SEM (n=6). *p<0.05 vs. apelin alone.

CMPD101 Treatment Increased Apelin-induced eNOS and PI3K Activity in SHR Coronary Endothelial Cells

Here we determined the effect of apelin treatment (10^{-7} M) on eNOS and Akt activity in SHR coronary endothelial cells pretreated with GRK2 inhibitor, CMPD101 (30 μM), for 20 minutes. Apelin significantly increased serine phosphorylation (Ser^{1177}) of eNOS protein in SHR coronary endothelial cells in the presence of CMPD101 but not in the absence of CMPD101 **(Figure 17A)**. Similarly, pretreatment of CMPD101 markedly increased apelin-induced Akt phosphorylation (Ser^{473}) in SHR coronary endothelial cells compared to apelin alone treated cells **(Figure 17B)**. These data suggest that apelin failed to cause eNOS or Akt activity in SHR coronary endothelial cells. However, apelin induced eNOS and PI3K activity was strikingly restored in SHR coronary endothelial cells in the presence of CMPD101 which indicate the role of GRK2 pathway activation in impaired apelin signaling under hypertensive conditions.

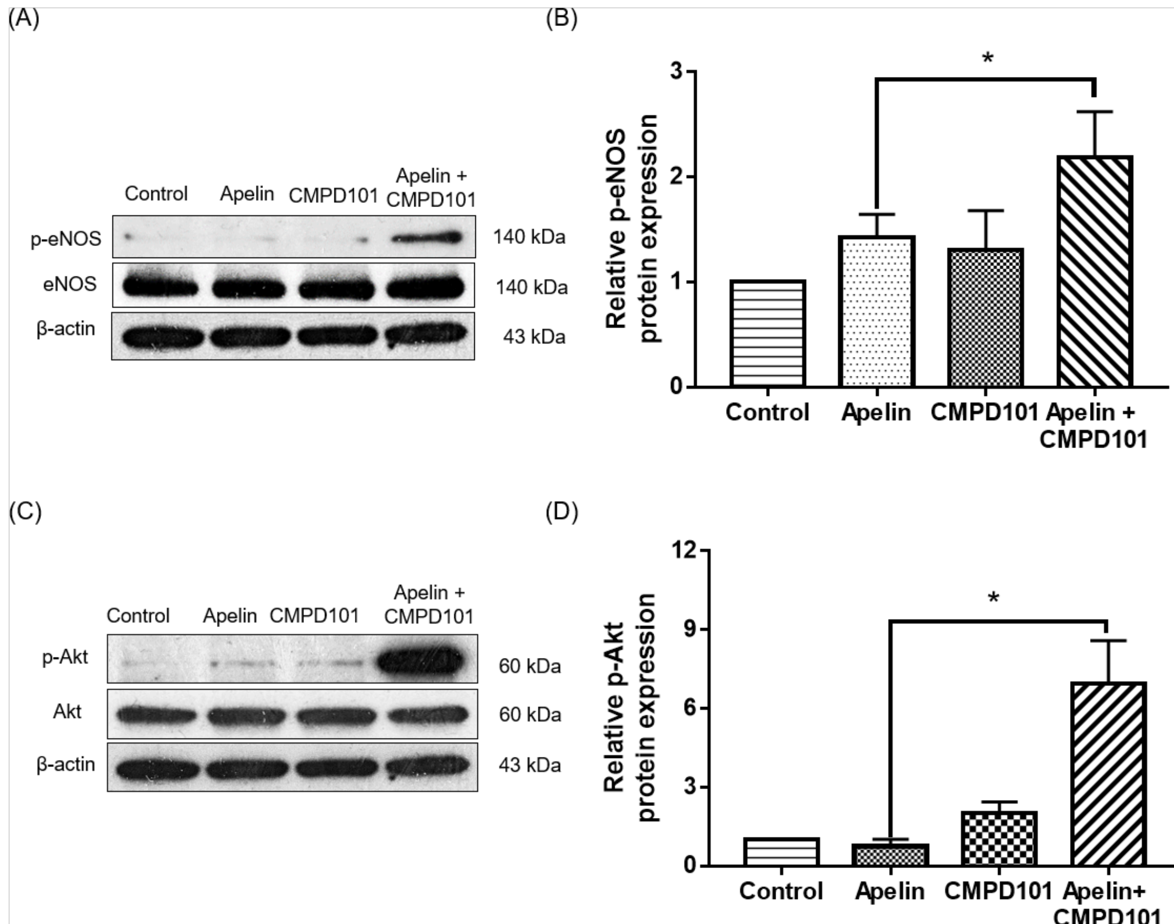

Figure 17. Effects of apelin on Ser^{1177} -eNOS and Ser^{473} -Akt activity in SHR cultured coronary endothelial cells treated with CMPD101.
Representative western blot showing apelin-induced (A) Ser^{1177} -eNOS and (C) Ser^{473} -Akt phosphorylation in SHR coronary endothelial cells treated for CMPD101 (30 μM) for 20 minutes. β-actin was used as loading control. Bar graph summarizing the effect of apelin on (B) eNOS and (D) Akt activity expressed as p-eNOS/eNOS and p-Akt/Akt protein levels respectively in SHR coronary endothelial cells. Each bar represents means ± SEM (n=5-6). *$p<0.05$ as compared with apelin alone.

CMPD101 Treatment Blocked the Inhibitory Effects of Apelin on SHR Coronary Arteries

In previous chapter we showed that apelin inhibited ACh-induced relaxation in SHR coronary arteries **(Figure 3B)**. To determine whether GRK2 play any role in the inhibitory effects of apelin on ACh-induced relaxation, experiments were performed in the presence of GRK2 inhibitor, CMPD101. Interestingly, ACh-induced relaxation was unaffected by apelin (10^{-7} M) in

the presence of CMPD101(30 μM) in SHR coronary arteries (pD_2 = 6.71 ± 0.15 vs 7.17 ± 0.16; without and with CMP101, respectively; $p<0.05$, n=5) **(Figure. 18)**.

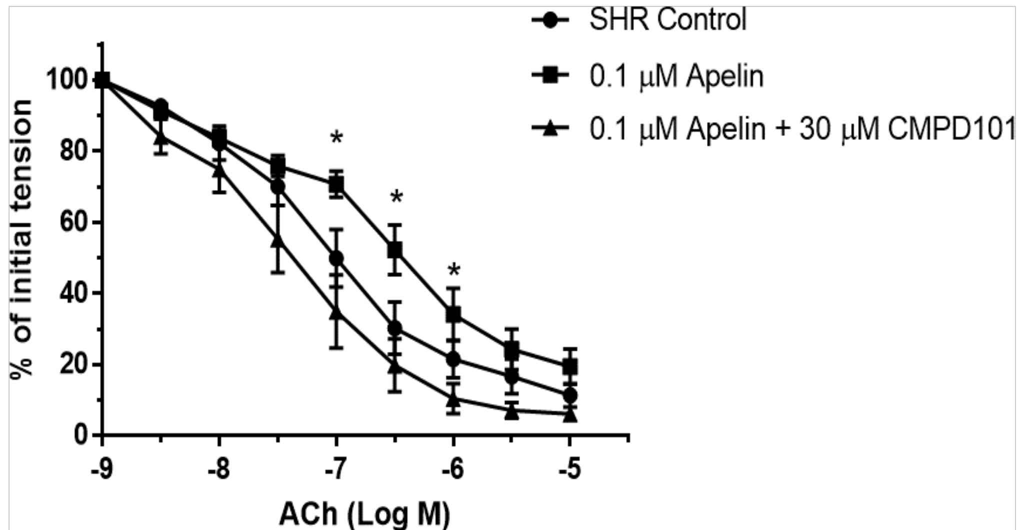

Figure 18. Effect of apelin on ACh relaxation in CMPD101 treated arteries.
Log concentration-response curves for ACh in SHR coronary arteries treated with apelin (10^{-7} M) in the absence and presence of CMPD101 (30 μM). Each point represents the mean ± SEM (n=5). *$p < 0.05$ indicates a significant difference from the corresponding apelin alone value.

Discussion

To our knowledge, this is the first report of a novel mechanism for the regulation of eNOS activity and NO production by apelin-APJ axis in coronary arteries under disease condition (hypertension). Major findings of the present study are 1) Unlike apelin, APJ receptor biased agonist, CMF-019, caused relaxation of coronary arteries under hypertensive conditions 2) Apelin and CMF-109 possibly increases NO production via PI3K/Akt/eNOS pathway activation in normotensive coronary arteries 3) CMF-019 increased eNOS and Akt activity in SHR coronary endothelial cells whereas apelin had no effect 4) Unlike apelin, CMF-019 had no inhibitory effects on ACh-induced relaxation in SHR coronary arteries 5) GRK2 inhibition restored apelin response in SHR coronary arteries 6) GRK2 inhibition increased apelin-induced eNOS and Akt activity in SHR coronary endothelial cells and 7) ACh-induced relaxation was unaffected by apelin in the

presence of GRK2 inhibitor, CMPD101. These findings provide evidence for the GRK2 mediated impairment of apelin response in hypertensive coronary arteries by defective eNOS activity and NO production.

Apelin is a novel vasoactive peptide that has gained tremendous interest in the field of cardiovascular research because of its positive inotropic (increased cardiac contraction) and vasodilatory effects. Apelin is reported to have vasodilatory effects in coronary arteries under normotensive conditions. In the previous chapter, we provided evidence that apelin behaves differently under disease condition (hypertension). We showed that APJ receptor signaling may contribute to both the loss of relaxation to apelin itself, as well as the ability of apelin to inhibit endothelium-dependent relaxation to ACh in coronary arteries from SHR. It has been previously shown that GRK2 expression is increased in hypertensive conditions and thereby plays a vital role in impairing eNOS activity and NO production in endothelial cells (111). Consistent with previous reports, our results **(Figure. 9)** showed that GRK2 expression was upregulated in hypertensive coronary arteries indicating the possible role of GRK2 in impairing the eNOS activity in SHR coronary endothelial cells.

To check the role of GRK2 in altered apelin response in SHR coronary arteries, I used the APJ receptor biased agonist, CMF-019, which preferentially activates G-protein-dependent signaling with little effect on the GRK2 pathway. Data in this study showed that, unlike apelin, CMF-019, -induced relaxation in SHR coronary arteries. Previous work from our lab showed that apelin-induce NO-mediated endothelium-dependent relaxation through activation of APJ receptors in rat coronary arteries. This present study showed that CMF-019-induced relaxation in endothelium intact WKY and SHR coronary arteries. Moreover, CMF-019-induced relaxation was blocked in the presence of eNOS inhibitor (NLA), and APJ receptor antagonist (F13A), indicating

CMF-019 selectively activates APJ receptor thereby leading to NO-mediated relaxation in WKY and SHR coronary arteries. These data confirm that CMF-019 causes endothelium-dependent relaxation similar to apelin.

Endothelial cell-derived NO play a vital role in vascular homeostasis (139). The results we discussed in the previous chapter indicate that eNOS activity and NO production is impaired in SHR coronary endothelial cells. eNOS must be phosphorylated to show full functional capability (140,141). The present study shows that apelin increases eNOS activity in WKY coronary arteries but failed in SHR. In contrast, CMF-019, increased eNOS phosphorylation in both WKY and SHR coronary arteries.

Apelin has been shown to augment Akt activity thereby improving eNOS activity and vascular function in rat aorta (96). To my knowledge, our present study provides the first evidence that apelin activates P13K/Akt/eNOS pathway in normotensive coronary endothelial cells thereby possibly increasing the eNOS activity. Interestingly, CMF-019 strikingly increased Akt activity in WKY and SHR whereas apelin had no significant effect on Akt activity in SHR coronary endothelial cells. Our results in hypertensive coronary endothelial cells are consistent with other data linking Akt to eNOS in reduced eNOS activity. Overall, our present study suggests that apelin and CMF-019 activate P13K/Akt/eNOS pathway in normotensive coronary endothelial cells resulting in vasodilation. Considering the fact that CMF-019 was able to activate PI3K/Akt/eNOS pathway in hypertensive coronary endothelial cells suggests GRK2 activation as a potential mechanism in impaired response to apelin in SHR coronary arteries.

To further evaluate the role of GRK2 in altered apelin response in SHR coronary arteries, I evaluated the vasomotor effects of apelin in coronary arteries pretreated with GRK2 inhibitor, CMPD101. Interestingly, apelin response was significantly restored in the presence of CMPD101

in SHR coronary arteries. To further confirm whether GRK2 inhibition can increase the apelin induced eNOS and Akt activity in SHR coronary endothelial cells, I measured the eNOS and Akt phosphorylation by apelin in CMPD101 treated cells. GRK2 inhibition markedly increased the eNOS and Akt activity in SHR coronary endothelial cells in response to apelin. These results strongly support the role for GRK2 in response to binding of apelin to APJ receptors in hypertensive coronary arteries.

The present research showed that apelin lost its endothelium-dependent relaxation in SHR coronary arteries. Moreover, apelin inhibited endothelium-dependent vasodilatory effect of ACh in SHR coronary arteries. To evaluate the role of GRK2 in ability of apelin to inhibit ACh relaxation in SHR coronary arteries, I checked the ACh response in the presence of APJ receptor G-protein biased agonist, CMF-019. That CMF-019 had no effects on ACh-induced relaxation strongly suggests the role of APJ receptor signaling via GRK2 in SHR coronary arteries. Another striking finding in this present study was that apelin had no inhibitory effects on ACh response in SHR coronary arteries pretreated with GRK2 inhibitor, CMPD101 which further confirmed the role of GRK2 in altered apelin signaling under hypertensive conditions. Further studies are required to understand the mechanism by which APJ receptor signaling impairs the ACh response in SHR coronary arteries. For example, by comparing the eNOS and Akt activity caused by ACh in the presence of apelin or CMF-019 in WKY and SHR coronary endothelial cells will provide us in-depth knowledge about the mechanisms involved in the inhibitory effects of apelin on relaxation response of ACh in hypertension.

In summary, the present study shows that apelin treatment strikingly modulate the vascular tone in coronary arteries. Under physiological conditions apelin improved vascular function by augmenting eNOS activity via PI3K/Akt/eNOS pathway. However, apelin/APJ receptor signaling

takes on an entirely different function under hypertensive conditions where apelin-induced endothelium-dependent relaxation is impaired. Moreover, apelin showed inhibitory effects on ACh-induced relaxation response in SHR coronary arteries. The study provides evidence of a novel mechanism for apelin signaling impairment and associated endothelial dysfunction in coronary arteries under hypertensive conditions. APJ receptor biased agonist (CMF-109) increased eNOS activity in SHR coronary arteries via P13K/Akt/eNOS pathway activation. GRK2 inhibition could rescue apelin response via increasing eNOS activity in hypertension. Overall, apelin signaling is impaired in SHR coronary arteries via GRK2 activation and increase vasomotor tone in coronary circulation. APJ receptor biased agonist might provide better beneficial effects in coronary arteries under pathological conditions (e.g., hypertension).

CHAPTER 4. SECONDHAND SMOKE EXPOSURE IMPAIRS THE VASODIALTORY RESPONSE TO APELIN IN RAT CORONARY ARTERIES

Introduction

The peptide hormone, apelin, dilates coronary arteries and thereby increases coronary blood flow (71). Apelin-induced coronary vasodilation results from the activation of APJ receptors located on endothelial cells in the coronary arterial wall (25). The binding of apelin to APJ receptors stimulates the release of endothelium-derived nitric oxide (NO) (75), which diffuses to the underlying smooth muscle to cause relaxation.

APJ receptors are G-protein-coupled receptors that signal via the PI3-kinase/Akt pathway to activate eNOS and stimulate NO production (78). A substantial body of evidence indicates that APJ receptors can also transduce extracellular signals in a G-protein-independent manner via activation of the GRK/ß-arrestin pathway (21,72,123). Several GRK isoforms have been identified (142,143), with native vascular endothelial cells primarily expressing the GRK2 isoform (111,126). Activation of this G-protein-independent pathway may be particularly relevant in disease states. Indeed, activation of GRK2 inhibits eNOS activity and NO production in endothelial cells (111,132), and increased GRK2 expression and activity in the vasculature is associated with the pathogenesis of the cardiovascular disease (125,144).

Based on the putative beneficial effects of apelin on the heart and coronary circulation, apelin and apelin-like analogs are being investigated for cardiovascular disorders such as heart failure and pulmonary hypertension (117). Surprisingly, the effects of apelin on vasomotor tone in diseased coronary arteries are largely unknown. Cigarette smoking, both active and passive (i.e., second-hand smoke), is a known risk factor for the development of coronary artery disease (145,146). Secondhand smoking (SHS) is associated with 31% increased chance of CAD and the mechanism underlying the adverse effects of smoking on coronary arteries are poorly understood

(6). Even acute exposure to SHS resulted in impaired endothelium-dependent vasodilation in coronary arteries of healthy adults (146). Second-hand smoke exposure has been reported to increase GRK2 expression in isolated trophoblasts (112); however, the effects, if any, of cigarette smoke on GRK2 expression in blood vessels have not been determined. The aim of the study is to check whether secondhand smoke exposure can impair apelin-APJ signaling in coronary arteries by a mechanism similar to SHR coronary arteries. Therefore, we tested the hypothesis that the vasorelaxant effect of apelin is impaired in coronary arteries exposed to cigarette smoke extract, a well-established model of second-hand smoke (147,148).

Materials and Methods

Animals and Tissue Preparation

Experiments were performed on tissues obtained from 12-week-old male Sprague-Dawley rats purchased from Envigo RMS (Indianapolis, IN). Rats were housed on a 12-hr/12-hr light/dark cycle at 22 ± 2°C and were provided with food and water ad libitum. All animal protocols used in this study were approved by the North Dakota State University Institutional Animal Care and Use Committee. The animals were anesthetized with isoflurane and hearts were isolated and placed into ice-cold physiologic salt solution (PSS) of the following composition: 118.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 0.03 mM EDTA, 5.5 mM glucose, and 25.0 mM $NaHCO_3$. Epicardial coronary arteries were dissected free and cleaned of surrounding tissues.

Cigarette Smoke Extract Preparation

Cigarette smoke extract (CSE) was freshly prepared from research grade cigarettes (3R4F) purchased from the Tobacco Research Institute (University of Kentucky, Lexington, KY), using a modified method of Blue and Janoff (149). Briefly, CSE was prepared freshly for each experiment

using a 60 ml syringe controlled by a three-way stopcock, which mimics the gas fluid pathway in the human lung during smoking (150). Cigarette smoke was drawn into the syringe and then slowly bubbled through 10 ml of phosphate buffered saline. The obtained CSE solution was pH corrected (7.4), and then filtered (0.2 μm) to remove bacteria and large particles. The concentration of the CSE solution obtained (stock) was considered as 100% (150). The CSE concentrations used (i.e., 1 & 2%), as well as the incubation period (4 h), were selected based on previous reports of altered protein expression within that time frame in pulmonary endothelial cells (151).

Vascular Function Studies

Rat coronary arterial rings (120-150 μm; 1.2 mm in length) were mounted in wire myographs (DMT, Aarhus, Denmark) for isometric tension (muscle is under tension, but neither shortens nor lengthens) recording. The myograph chambers were filled with PSS (5 ml), which was maintained at 37°C and continuously aerated with 95% O_2/5% CO_2 throughout the experiment. The arterial rings were stretched up to a resting tension of 6 mN by sequential stretching and then allowed to stabilize for 30 minutes with intermittent washings. Vascular reactivity was established by evoking a contractile response to KCl (60 mM). In some rings, the endothelium was removed by gently rubbing the intimal surface with a human hair. The absence or presence of endothelium was verified with the endothelium-dependent vasodilator, acetylcholine (ACh; 10^{-6} M). Responses to vasodilators used in this study were obtained in arterial rings contracted with 5-hydroxytryptamine (5-HT; 10^{-7} M). Inhibitors were added to the myograph chamber 20 min prior to contraction with 5-HT. When apelin (10^{-7} M) or CMF-019 (10^{-7} M) was used as an inhibitor, it was added to the tissues for 5 minutes, after the contraction to 5-HT had stabilized, in order to minimize desensitization of APJ receptors. All inhibitors remained in the

myograph solution for the remainder of the experiment. Experiments with untreated control rings were conducted in parallel with rings treated with inhibitors from the same animal.

Isolation of Coronary Endothelial Cells

Coronary artery endothelial cells were isolated using previously established procedures (119), with minor modifications. Briefly, rat coronary arteries were digested in dissociation solution (55 mM NaCl, 6 mM KCl, 80 mM Na-glutamate, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.3) containing elastase (0.5 mg/ml) (Worthington, Lakewood, NJ) and neutral protease (0.5 mg/ml) (Worthington) for 60 minutes at 37°C, followed by collagenase type II (0.5 mg/ml) (Worthington) in the same solution for 2 minutes. The enzyme solution was removed, and arteries were treated with dissociation solution without enzyme for 10 min followed by trituration with a polished Pasteur pipette to produce a suspension of single endothelial cells. Cells were re-suspended in endothelial cell growth medium MV (EGM MV) (Promo cell, Heidelberg, Germany) with 1% penicillin-streptomycin solution and maintained at 37°C (5% CO_2, 95% air) until 80% confluent. Cells from 2nd to 4th passage were used for experiments. Phenotype stability was confirmed periodically using the endothelial cell marker, platelet endothelial cell adhesion molecule (PECAM 1), and smooth muscle cell marker, smooth muscle α-actin.

Western Immunoblotting

Rat coronary arteries were collected and frozen immediately using liquid nitrogen. Tissues were crushed using a mortar and pestle and the powdered tissue was homogenized at 4°C using an IKA Ultra-Turrax T8 homogenizer (IKA Works Inc., Wilmington, NC) in lysis buffer containing protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA). Standard procedures were followed for coronary endothelial cell lysis. Protein estimation was performed

using a Pierce BCA protein estimation kit (ThermoFisher Scientific). Proteins were separated by SDS–polyacrylamide gel electrophoresis using Mini-PROTEAN precast gels (4-15% gradient; Bio-Rad, Hercules, CA) and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% bovine serum albumin in Tris buffered saline (TBS, pH 7.4), blots were incubated overnight at 4°C with appropriate primary antibodies specific for APJ receptors (sc-517300, Santa Cruz Biotechnology Inc.), or GRK2 (sc-13143, Santa Cruz Biotechnology Inc.), or eNOS (sc-376751, Santa Cruz Biotechnology Inc.) using a dilution of 1:100, followed by incubation with a horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology Inc.). To ensure equal loading, the blots were analyzed for β-actin protein expression using an anti-actin antibody with a dilution of 1:500 (sc-47778, Santa Cruz Biotechnology Inc.) Protein bands and relative densities were measured using an enhanced chemiluminescence light detection kit (Thermo Fisher Scientific, Waltham, MA).

Immunofluorescence Microscopy

Rat coronary endothelial cells were grown on 8-well Lab Tek chambers (ThermoFisher Scientific) in EGM MV until 40-50% confluent. Cells were then fixed using 4% paraformaldehyde for 15 min at room temperature and washed thrice with PBS. The cells were permeabilized using 0.1% TritonX-100 in PBS for 15 min and nonspecific antibody binding was blocked with 10% donkey serum (Sigma Aldrich, USA) in PBS for 60 min at room temperature. Cells were incubated overnight at 4°C with PECAM-1(Santa Cruz Biotechnology Inc.) and APJ receptor (Santa Cruz Biotechnology Inc.) or α-smooth muscle actin (Santa Cruz Biotechnology Inc.) antibodies. For co-localization studies, cells were simultaneously incubated with APJ receptor and PECAM-1 antibodies. Following day cells were incubated with Alexa Fluor 488 (APJ receptor and α-SMA) and Alexa Fluor 555 (PECAM-1) tagged secondary antibodies against mouse and goat respectively

for 60 min at room temperature. Negative controls were subjected to same process with the exception of addition of primary antibody. Cells were washed with PBS and a drop of mounting medium containing DAPI was added and then a coverslip was placed over the slide. Images were obtained at 10X magnification using Lionheart Fx imaging station (Biotek, USA).

Drugs

The following drugs were used: acetylcholine, diethyl amine (DEA) NONOate, diltiazem, and 5-HT (Sigma Chemical, St. Louis, MO); apelin-13 and F13A (H-Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Ala-OH trifluoroacetate salt) (Bachem, Torrance, CA); CMPD 101 (Tocris, Ellisville, MO); and CMF 019 (Aobious, Gloucester, MA). Drug solutions were freshly prepared in double-distilled water with the exception of CMF-019 and CMPD 101, which were dissolved initially in DMSO and followed by further dilutions in double-distilled water. Drugs were added to the myograph chambers in volumes not greater than 0.02 ml. Drug concentrations are reported as final molar concentrations in the myograph chamber.

Data Analysis

Relaxation responses are shown as a percent of initial tension induced by 5-HT (10^{-7} M). The EC_{50} values (concentration of drug that gives half-maximal response) were determined, converted to their negative logarithms, and expressed as -log molar EC_{50} (pD_2). The data from all sets of experiments are presented as mean ± standard error of mean and *n* indicates the number of animals from which blood vessels were taken. The statistical significance was evaluated by paired t-test or two-way ANOVA followed by Bonferroni post hoc analysis, as appropriate. Differences were considered significant when $P < 0.05$.

Results

CSE Exposure Inhibits Apelin-induced Relaxation of Isolated Coronary Arteries

We had previously reported that apelin induces relaxation in rat coronary arteries (25). Here we determined the effect of 4h exposure to 1% or 2% CSE on apelin-induced relaxation in isolated rat coronary arteries. In coronary arteries contracted with 5HT (10^{-7} M), apelin (10^{-8} - 3×10^{-6} M) caused concentration-dependent relaxation in the absence of CSE, but in the presence of 1% and 2% CSE, apelin-induced relaxation was markedly inhibited **(Figure 19)**. The pD2 values for apelin were 7.25 ± 0.16 vs 6.83 ± 0.35 vs 6.36 ± 0.16 and E_{max} values were 43% ± 5% vs 14% ± 4% vs 12% ± 3%; in control, 1%, 2% CSE exposed arteries, respectively ($P < 0.05$, n = 6).

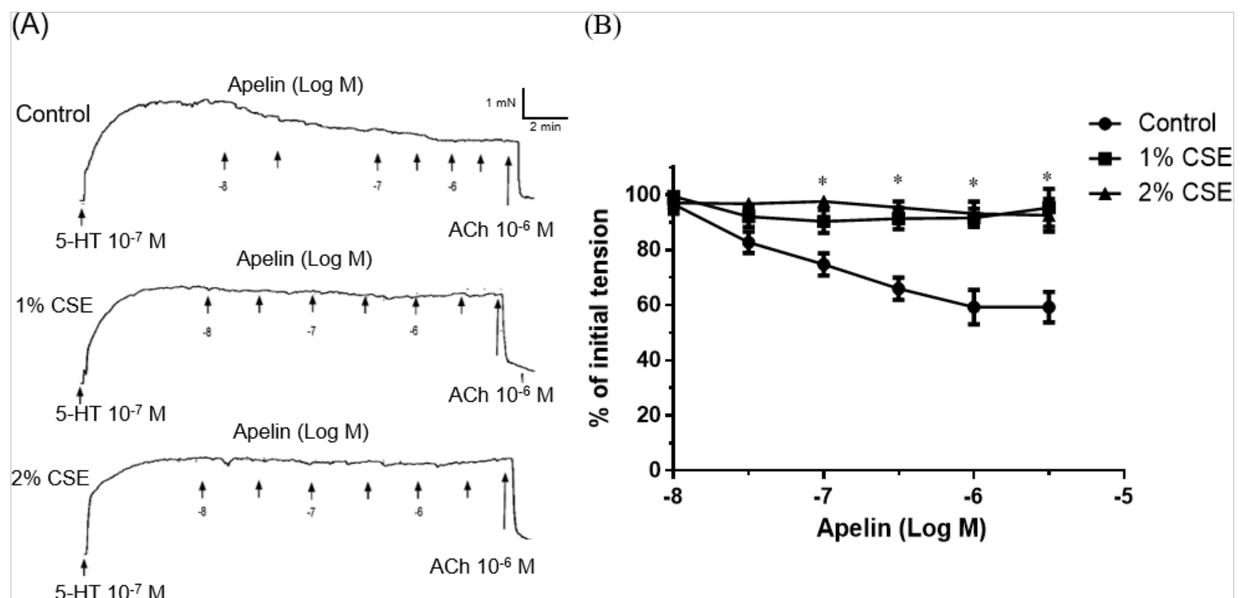

Figure 19. Effect of CSE exposure on apelin-induced relaxation of isolated rat coronary arteries. (A) Representative original tracings of isometric tension recordings from rat isolated coronary arteries (in control and CSE-treated arteries) in response to cumulative addition of increasing concentrations of apelin, followed by acetylcholine (10^{-6} M). (B) Mean data demonstrating apelin-induced relaxation in control, which was abolished in the CSE-treated arteries. Data are expressed as a percentage of the initial increase in tension induced by 5-HT (10^{-7} M). Each point represents the mean ± SEM (n=6). *$p < 0.05$ indicates a significant difference from the corresponding control value. ACh, acetylcholine.

Apelin Inhibits ACh-endothelium-dependent Relaxation but Not DEA NONOate-induced endothelium-independent Relaxation in Rat Coronary Arteries

The relaxation response to a classical endothelium-dependent vasodilator, ACh (10^{-9} - 10^{-5} M), was similar in control and CSE treated arteries (pD2 = 6.97 ± 0.11 vs 6.93 ± 0.37 vs 6.83 ± 0.21 in control, 1%, 2% CSE exposed arteries, respectively; $p > 0.05$, n = 6) (**Figure 20A**). ACh-induced relaxations did not show any significant difference in the presence of apelin (10^{-7} M) in 1% CSE treated arteries (pD2 = 7.20 ± 0.32 vs 7.16 ± 0.25 without and with apelin, respectively; $p > 0.05$, n = 6) **(Figure 20B)**.

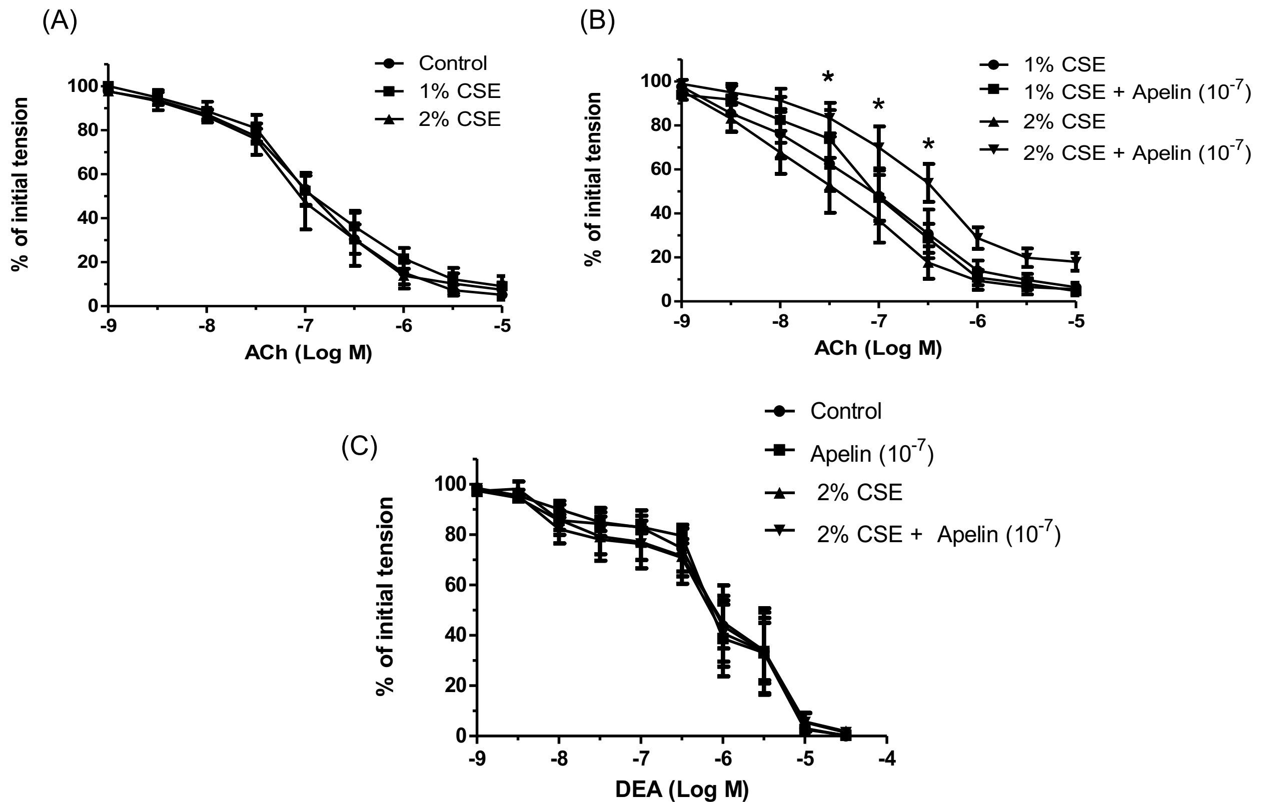

Figure 20. Effect of CSE on Ach-induced endothelium-dependent relaxation and DEA-induced endothelium-independent relaxation in the presence and absence of apelin.
(A-B) Log concentration-response curves for ACh in control and CSE-treated arteries (A) as well as in the absence and presence of apelin (10^{-7} M) in CSE-treated arteries (B). (C) Log concentration-response curves for DEA in control and CSE-treated arteries in the absence and presence of apelin ((10^{-7} M). Each point represents the mean ± SEM (n=6). *$p < 0.05$ indicates a significant difference from the corresponding control value.

By contrast, ACh-induced relaxations were markedly impaired in presence of apelin (10^{-7} M) in arteries treated with higher concentration of CSE (2%) (pD2 = 7.43 ± 0.24 vs 6.78 ± 0.20 without and with apelin, respectively; $P < 0.05$, n = 6 **(Figure.20B)**. However, the relaxation response to NO donor, DEA NONOate (10^{-9} – 10^{-5} M) did not show any difference in the presence of apelin (10^{-7} M) in endothelium-denuded coronary arteries treated with 2% CSE (pD2 = 6.21 ± 0.35 vs 6.22 ± 0.25 without and with apelin, respectively; $P > 0.05$, n = 6) **(Figure.20C)**.

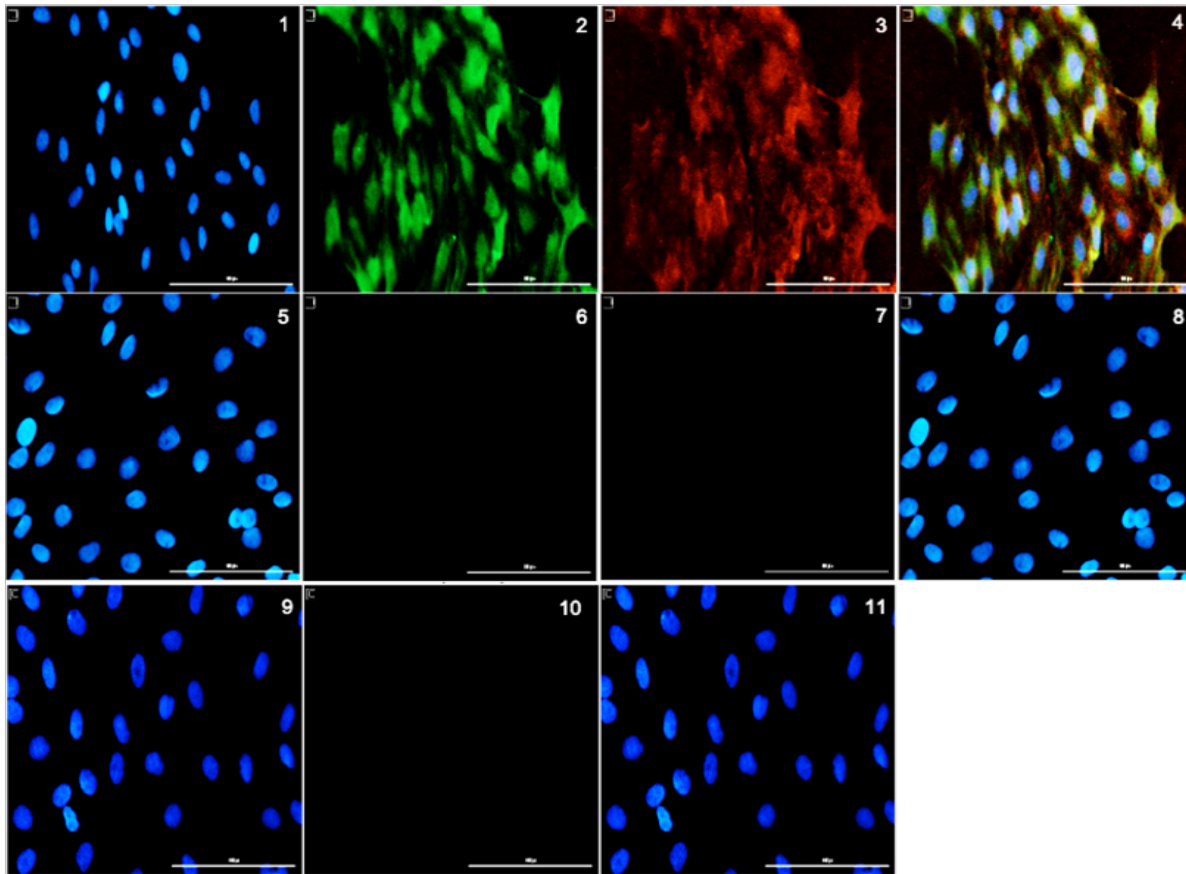

Figure 21. Localization of APJ receptors in coronary endothelial cells.
A) Representative immunofluorescence images demonstrate APJ receptor expression in endothelial cells immunostained with (1,5,9) DAPI (blue) stained nucleus, (2) APJ receptors (green), (3) PECAM-1 (red), (4) merged imaged showing localization of APJ receptors in endothelial cells (yellow), (6) secondary antibody control showing negative staining for APJ receptors, (7) PECAM-1, (8) merged image for secondary control with DAPI, (10) cells stained negative for α-SMA and merged image for α-SMA and DAPI. The images are representative of those obtained from three different animals. Scale bars = 100μm.

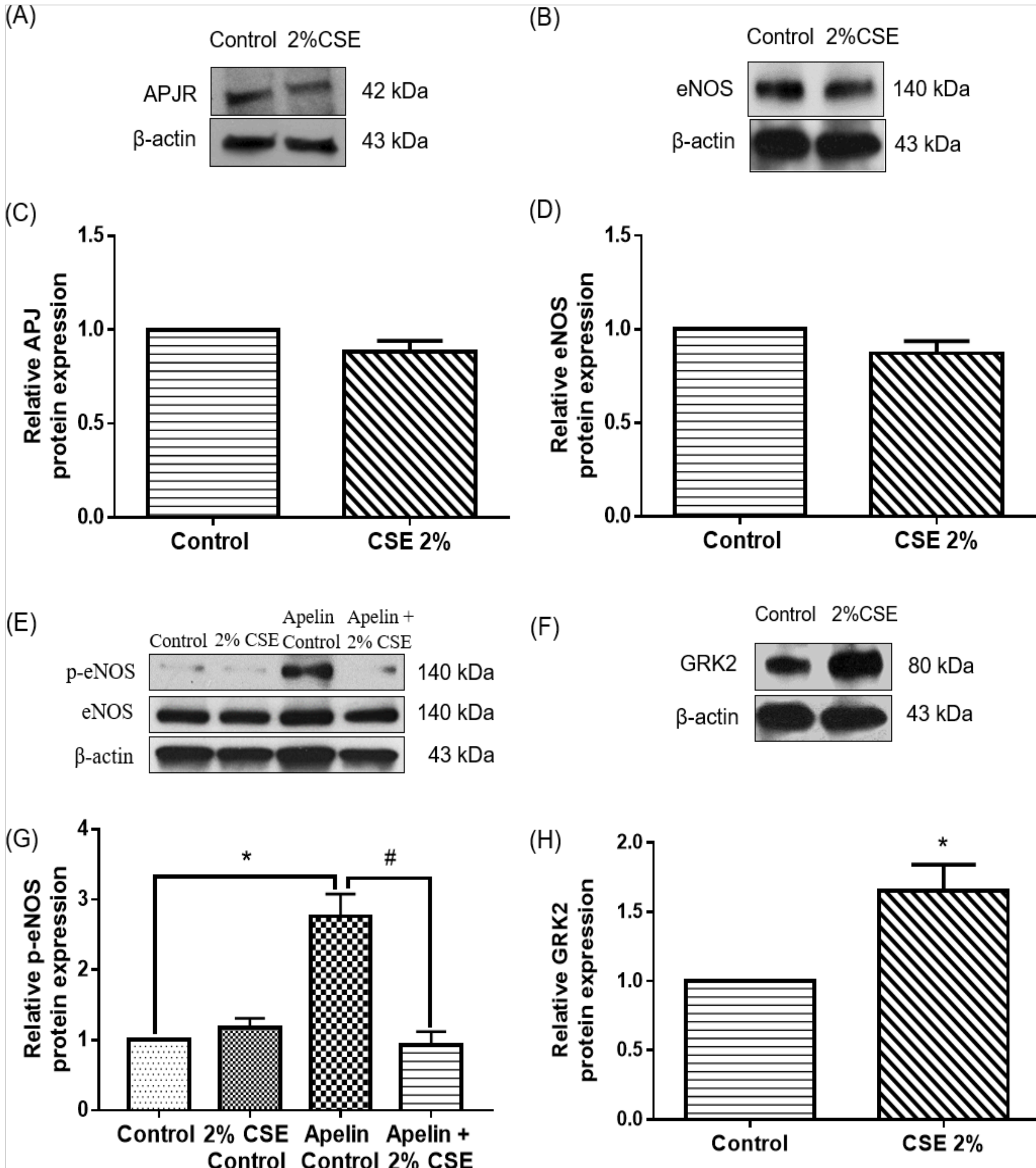

Figure 22. Effect of CSE on eNOS activity and GRK2 expression in coronary endothelial cells. Representative immunoblot showing expression of (A) APJR, (B) eNOS, (E) p-eNOS and (F) GRK2 protein, in control and CSE-treated cultured coronary endothelial cells; β-actin was used as a loading control. Bar graph showing relative expression of C) APJR, (D) eNOS, (G) p-eNOS (expressed as p-eNOS/eNOS protein levels) and (H) GRK2 protein, in control and CSE-treated coronary endothelial cells. Data are represented as means ± SEM (n=3-5). *$p < 0.05$ indicates a significant difference from the corresponding control value. #$p<0.05$ as compared with apelin alone.

GRK2 Expression is Increased in Coronary Endothelial Cells Exposed to CSE

Immunofluorescence studies were performed to characterize coronary artery endothelial cells as well as to check the expression of APJ receptors in endothelial cells. APJ receptor protein localization was detected in cultured primary endothelial cells which showed positive staining for PECAM-1**(Figure 21.1-4)**. The secondary antibody controls showed an absence of non-specific binding of secondary antibody in the absence of primary APJ receptor and PECAM-1antibody **(Fig 21.5-8)**. The cultured cells did not show any fluorescence for α-SMA **(Figure 21.9-11)**. Expression of APJ receptor, eNOS and GRK2 proteins was identified by western immunoblot analysis in cultured rat coronary endothelial cells. APJ receptor and eNOS protein expression was unaffected by CSE exposure **(Figure 22.A-B**). However, apelin, markedly increased the eNOS activity in control coronary endothelial cells but failed in CSE exposed cells (**Figure 22E**) ($p< 0.05$, $n = 3$). GRK2 protein expression was markedly increased in coronary endothelial cells exposed to 2% CSE for four hours **(Figure 22F)** ($p< 0.05$, $n = 4$).

GRK2 Mediates the Inhibitory Effect of CSE on Apelin-induced Relaxation

Based on the observation that GRK2 expression was increased in coronary endothelial cells exposed to 2% CSE **(Figure 22D)**, we tested whether the GRK2 inhibitor, CMPD101 (137,138), could rescue apelin-induced relaxation in coronary arteries exposed to CSE. CMPD101 (3×10^{-5} M) had no effect on the concentration-response curve to apelin in control arteries not exposed to CSE ($pD_2 = 6.89 \pm 0.26$ vs 6.88 ± 0.07 in absence and presence of CMPD101, respectively; $p > 0.05$, $n = 6$) **(Figure 23A)**; however, treatment of coronary arteries with the GRK2 inhibitor restored apelin-induced relaxation in arteries previously exposed to CSE ($pD_2 = 7.01 \pm 0.17$ and $E_{max} = 32\% \pm 4\%$ in the presence of CMPD101) **(Figure 23B)**.

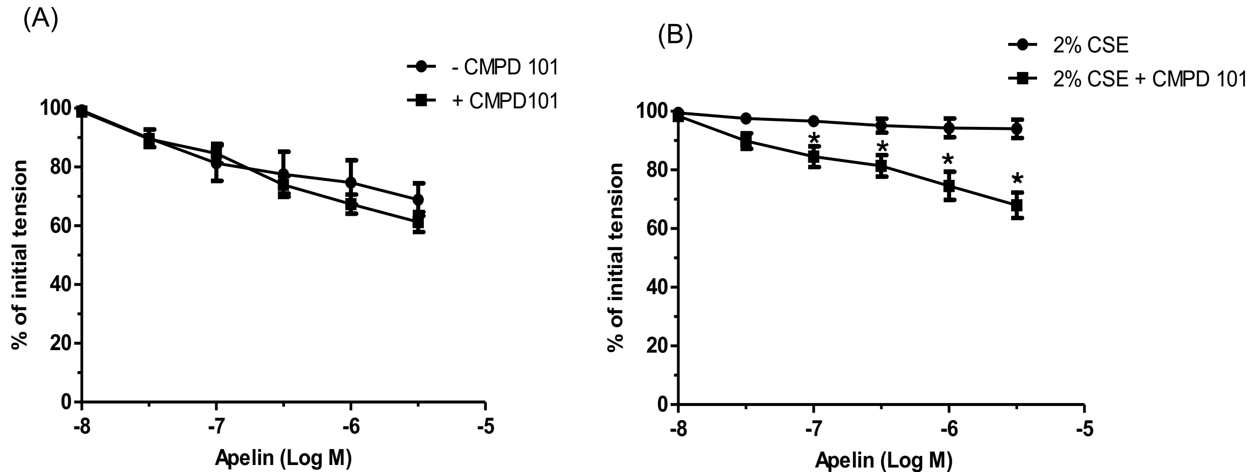

Figure 23. Effect of CSE on apelin-induced relaxation in the presence of GRK2 inhibitor.
Log concentration-response curves for apelin-induced relaxation in the absence or presence of CMPD101 ($3*10^{-5}$ M) in (A) control and (B) CSE-treated coronary arteries. Each point represents the mean ± SEM (n=6). *$p < 0.05$ indicates a significant difference from the corresponding control value.

CMF-019 Induced Endothelium-dependent Relaxation Was Unaffected by CSE Exposure in Rat Coronary Arteries

We also tested the ability of CMF-019, an APJ receptor biased agonist that acts selectively through the G-protein-dependent pathway with little effect on GRK2 (135), to cause relaxation of coronary arteries. CMF-019 (10^{-9} - 10^{-5} M) caused concentration-dependent relaxation of untreated (control) coronary arteries with intact endothelium (**Figure 24A**). The pD_2 value for CMF-019 was 7.27 ± 0.36 and the E_{max} was 40% ± 3%. CMF-019 induced relaxation was abolished by removal of the endothelium and by the APJ receptor antagonist, F13A (10^{-7} M) **(Figure 24B)**, consistent with activation of endothelial APJ receptors (2).

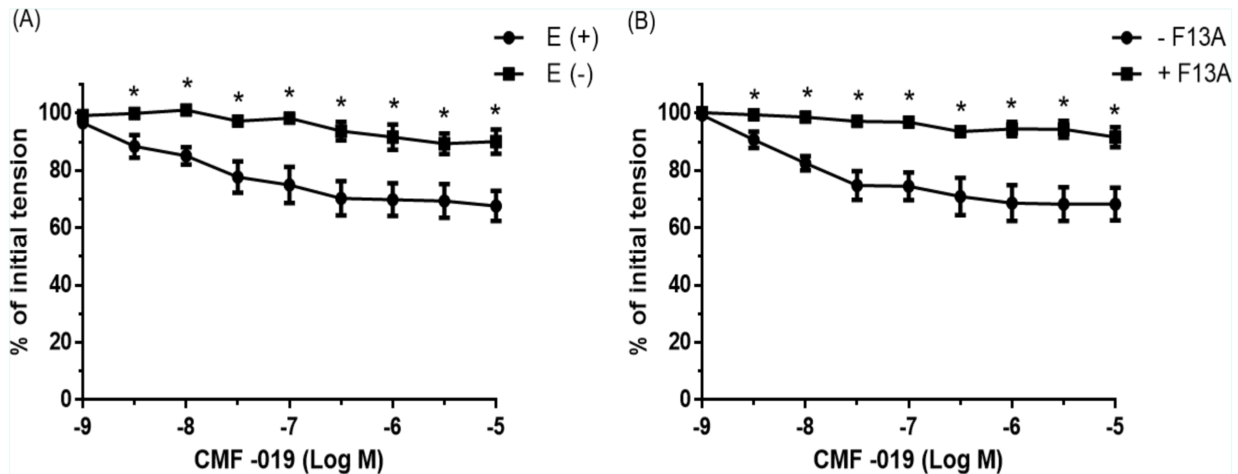

Figure 24. CMF-019-induced endothelium-dependent relaxation mediated through APJ receptor. (A-B) Log concentration-response curves for CMF-019-induced relaxation in coronary arteries with endothelium (E+), which was abolished in endothelium denuded (E-) segments or (B) in the presence of F13A (10^{-7} M). Each point represents the mean ± SEM (n=6). *$P < 0.05$ indicates a significant difference from the corresponding control value.

In contrast to our findings above with apelin **(Figure 19)**, exposure of coronary arteries to 2% CSE had no effect on CMF-019-induced relaxation ($pD_2 = 7.17 \pm 0.24$ vs 6.90 ± 0.22 and E_{max} = 40% ± 6% vs 41% ± 7% in control and CSE-treated arteries, respectively; $p > 0.05$, n = 6) **(Figure 25A)**. The earlier data suggested that apelin could inhibit coronary arterial relaxations mediated by ACh in CSE-treated coronary arteries. Since GRK2 activation can cause inhibition of eNOS and decreased NO production in endothelial cells(111), the ACh response was checked in presence of CMF-019. Interestingly, ACh-induced relaxation was unaffected by CMF-019 ((10^{-7} M) in either control or CSE-treated arteries ($pD_2 = 7.04 \pm 0.21$ vs 6.94 ± 0.18 in control and CSE, respectively; $p > 0.05$, n = 6) **(Figure 25B)**.

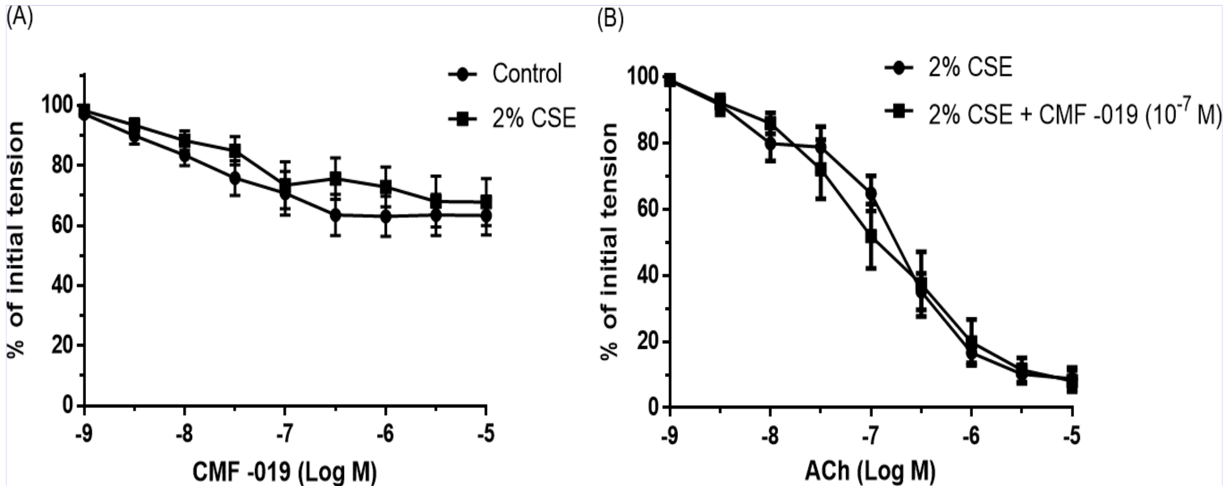

Figure 25. Effect of CSE on CMF-019 induced relaxation and Ach-induced relaxation in the presence or absence of CMF-019.
(A) Log concentration-response curves for CMF-019-induced relaxation in control and CSE-treated coronary arteries. (B) Log concentration-response curves for ACh-induced relaxation in the absence and presence of CMF-019 (10^{-7} M) in CSE-treated arteries. Each point represents the mean ± SEM (n=6).

Discussion

The present study was undertaken to demonstrate the effects of secondhand cigarette smoke on apelin signaling in rat coronary arteries. The results indicate that CSE exposure may contribute to both the loss of relaxation to apelin itself as well as the ability of apelin to inhibit endothelium-dependent relaxation to ACh. Moreover, CSE-induced adverse effects are mediated by G-protein-independent signaling via the GRK2 pathway which might result in impaired release or production of NO from endothelial cells in coronary arteries. The conclusion is based on the findings that (1) CSE-treatment abolished the apelin-induced relaxation in coronary arteries, but the APJ receptor biased agonist, CMF-019-induced endothelium-dependent relaxation was unaffected; (2) CSE exposure impaired the ACh-induced relaxation in the presence of apelin, but not in the presence of CMF-019; (3) CSE-treatment up-regulated the GRK2 expression in rat coronary endothelial cells; (4) In the presence of GRK2 inhibitor, CMPD101, the apelin-induced relaxation was not affected in rat coronary arteries. Results from mechanistic studies provide

evidence that GRK2 may be a pivotal link in the phenotypic switch in the coronary artery response to apelin that occurs in CSE-treated arteries. The findings are significant inasmuch as they suggest that changes in apelin/APJ receptor signaling under pathologic conditions (e.g. exposure to second-hand smoke) could create an environment that favors increased vasomotor tone in coronary arteries, which would predictably lead to a reduction in coronary blood flow and myocardial ischemia. Taken together, this study provides a novel mechanism by which apelin signaling is impaired in coronary arteries of secondhand smoke exposure model similar to hypertensive coronary arteries.

Apelin is generally thought to cause vasodilation in the presence of endothelium (55,152) and vasoconstriction in the absence of endothelium (52,82). In fact, apelin and analogs are in clinical development for treating cardiovascular disease conditions such as pulmonary arterial hypertension and heart failure (117). Moreover, apelin caused NO-mediated endothelium-dependent relaxation in rat coronary arteries by activation of large conductance calcium-activated potassium channel (25). The question arises as to whether such beneficial effects of apelin are maintained in disease conditions. Mechanisms underlying the adverse effects of SHS on coronary arteries are poorly understood. Here, our study focused on the apelin-APJ axis regulated vasomotor tone in the presence of CSE exposure in rat coronary arteries.

Under normal physiologic conditions, apelin causes relaxation of coronary arteries by releasing NO from vascular endothelial cells (25). In coronary arteries exposed to CSE, apelin strikingly failed to cause relaxation of the tissues, whereas the response to acetylcholine (ACh), the prototypical endothelium-dependent vasodilator that releases NO from endothelial cells, was retained. CSE exposure also had no effect on relaxation to the NO donor, DEA NONOate. These findings with ACh and DEA indicate that the impaired response to apelin is unlikely due to a defect

in the intrinsic ability of the endothelial cells to produce bioavailable NO, or in the responsiveness of the underlying smooth muscle cells to NO and are consistent with a phenotypic switch in apelin/APJ receptor pharmacology occurring most likely at the level of the endothelial cells, resulting in impaired signaling between APJ receptor activation and NO production. Moreover, CSE treatment significantly impaired the apelin-induced eNOS activity in coronary endothelial cells. Taken together, the data suggest that coupling between APJ receptor activation by apelin and NO synthesis by eNOS is selectively impaired in endothelial cells from coronary arteries exposed to CSE.

Although the endothelium-dependent relaxation response to apelin is lost in coronary arteries exposed to CSE, it is noteworthy that the apelin/APJ receptor signaling system takes on a completely different function in these blood vessels. Rather than cause relaxation, apelin inhibited endothelium-dependent relaxation in response to ACh. In addition, apelin had no effect on relaxation of CSE-treated coronary arteries in response to DEA NONOate. These findings with the NO-donor indicate that apelin does not interfere with the smooth muscle response to NO. These data suggest that APJ receptors retain their affinity for apelin in CSE-treated coronary arteries but process the apelin signal in a manner that differs from that in control arteries.

Impaired coupling between apelin/APJ receptor activation and NO synthesis by eNOS could be explained, at least in part, by changes in expression of proteins involved in intracellular signaling. Relevant to this possibility, exposure to CSE has been reported to cause increases as well as decreases in protein expression (153). Nonetheless, it not likely that the impaired response to apelin is due a reduction in either APJ receptors or eNOS since expression of these proteins were not altered by CSE exposure. In contrast, GRK2 expression was significantly elevated in CSE-treated coronary endothelial cells. GRK2 is a multidomain protein that interacts with a

complex array of cellular effectors and is a key component of the G-protein independent intracellular signaling cascade (124). However, in disease conditions where GRK2 expression is up-regulated, GRK2 protein interacts with Akt which inhibits the phosphorylation of Akt followed by defective eNOS function in endothelial cells. Furthermore, GRK2 knockdown resulted in increased Akt-mediated NO production in sinusoidal endothelial cell (111). Since increased GRK2 is known to inhibit eNOS activity and NO production in endothelial cells (110), our finding that GRK2 expression is increased in coronary endothelial cells after CSE exposure suggests a potential mechanism for the impaired response to apelin. Indeed, the observation that CMPD101, a potent and selective GRK2 inhibitor (137,138), restored the response to apelin in CSE-treated coronary arteries strongly supports such a role for GRK2 in response to binding of apelin to APJ receptors in coronary arteries exposed to CSE.

In order to further explore the role of the G-protein-independent signaling pathway in the response to apelin in CSE-treated coronary arteries we compared the effects of apelin with CMF-019, a G-protein biased agonist at APJ receptors (136). Our data show that, like apelin, CMF-019 causes endothelium-dependent relaxation of isolated rat coronary arteries and that this response is inhibited by the APJ receptor antagonist, F13A. In contrast to apelin, however, CMF-019-induced coronary artery relaxation was unaffected by exposure to CSE. That the effects of the G-protein biased APJ receptor agonist did not differ in control and CSE-treated coronary arteries is consistent with a role for G-protein independent signaling via GRK2 in the impaired response to apelin in CSE-treated arteries. That CMF-019 had no effect on ACh-induced relaxation further implicates a role for G-protein-independent signaling via APJ receptors under these conditions. However, the underlying mechanism of GRK2 up-regulation by CSE exposure in coronary endothelial cells remains unclear. More studies need to be conducted may be with more focus on

GRK2/PI3K/Akt/eNOS pathway to understand the mechanism by which GRK2 activation affects the NO production in coronary endothelial cells exposed to CSE.

In conclusion, the present study demonstrates that apelin-induced relaxation of coronary arteries is impaired in a model of second-hand smoke exposure. Apelin/APJ receptor signaling via the GRK2 pathway may contribute to both the loss of relaxation to apelin itself as well as the ability of apelin to inhibit endothelium-dependent relaxation to ACh in CSE-treated coronary arteries. The results with CMF-019 suggest that, at least under certain pathologic conditions, APJ receptor biased agonists have the potential to be more effective than apelin itself as therapeutic agents for treating cardiovascular disorders.

CHAPTER 5. SUMMARY AND CONCLUSIONS

The apelinergic system is composed of peptide ligands apelin and, its receptor called APJ receptors. The apelinergic system is widely expressed in peripheral tissues and the central nervous system making them involved in diverse physiological and pathological processes especially in the cardiovascular system (113). The apelinergic system plays a vital role in maintaining cardiovascular homeostasis because of its positive inotropic and blood pressure regulation effects. In the past decade, the apelinergic system has been getting immense attention for its therapeutic potential in cardiovascular and metabolic disorders. Both in preclinical and clinical settings apelinergic system has shown promising therapeutic potential under physiological and disease conditions including heart failure and pulmonary hypertension. Apelinergic system activation has complex effects on blood vessels depending on the vascular bed and pathological conditions. While apelin has pressor effects in the central system, and in the periphery apelin has opposite effects.

Since apelin and its analogues are in clinical trials it is important to study the vascular effects of apelin under physiological and conditions to better understand the safety profile as well as improving apelin based therapies. Current literature provides clinical and preclinical evidence that support the vasodilatory effect of apelin in different arteries under physiological conditions. Binding of apelin to APJ receptors causes endothelium-dependent relaxation in coronary arteries under physiological conditions. However, the regulation of vasomotor tone by apelin under disease conditions are not well studied. Pathological conditions might lead to a shift in the protective role of the apelinergic system in coronary arteries. Considering these facts, the present research was focused to understand the vasomotor regulation of apelinergic system under pathological conditions.

The result of the present study demonstrated that apelin causes relaxation in normotensive coronary arteries but had no effect in hypertensive arteries. Moreover, instead of causing relaxation, apelin inhibited ACh-induced endothelium-dependent relaxation in SHR coronary arteries. However, endothelium-independent relaxation by NO-donor, DEA NONOate, was unaffected by apelin treatment. Apelin is known to cause NO release from endothelial cells thereby activating BKCa channels on coronary smooth muscle cells resulting in vasodilation. My study here provide evidence that apelin/APJ receptor signaling is impaired in hypertensive coronary arteries possibly through the defective production or release of NO from SHR coronary endothelial cells. These findings raise the concern of endothelial dysfunction by altered apelin signaling rather than protective role in hypertension.

The binding of apelin to the APJ receptor has been shown to cause NO-mediated relaxation in coronary arteries. Hence, I compared the expression levels of APJ receptor and eNOS between normotensive and hypertensive coronary endothelial cells. Since the APJ receptor and eNOS protein expression were similar in coronary endothelial cells, I concluded that the altered apelin signaling in hypertensive coronary arteries is not because of any differences in APJ receptor and eNOS expression level in endothelial cells. eNOS is a major weapon of endothelial cells that produce vasoprotective NO thereby fighting vascular disease (139). eNOS protein must be activated by phosphorylation to show its full functional capability. Several studies showed that impaired eNOS activity results in endothelial dysfunction and vascular disease. GRK2 is a multidomain protein involved in the desensitization of different GPCRs including APJ receptors. Clinical and preclinical studies showed that GRK2 levels and activity is increased in different tissues contribute to the progression of cardiovascular diseases including hypertension (124). Evidence suggests that GRK2 inhibition improved endothelial dysfunction by restoring eNOS and

NO availability in animal models of hypertension (154). Interestingly, GRK2 expression was upregulated in SHR coronary endothelial cells indicating the role of GRK2 in altered apelin response in SHR coronary arteries.

The role of GRK2 in impaired apelin signaling was evaluated using the APJ receptor biased agonist, CMF-019. CMF-109 selectively activates G-protein-dependent pathway with the least effects on GRK2. My data suggests that CMF-019 induces NO-mediated endothelium-dependent relaxation in WKY coronary arteries. Unlike apelin, CMF019 caused relaxation in SHR coronary arteries. Moreover, apelin and CMF-019 increased eNOS activity in WKY coronary endothelial cells. Consistent with the functional study, CMF-019 increased eNOS activity in SHR coronary endothelial cells whereas apelin failed. Evidence suggests that GRK2 upregulation is associated with impaired PI3K/Akt/eNOS pathway and endothelial dysfunction (111,154). Apelin is known to activate P13K/Akt/eNOS pathway in endothelial cells. My results showed that apelin failed to increase Akt activity in SHR whereas CMF-019 increased Akt activity in WKY and SHR coronary endothelial cells. To my knowledge, this is the first report showing the apelin/APJ signaling mediated activation of the P13K/Akt/eNOS pathway in coronary arteries.

To further investigate the role of GRK2 in defective APJ receptor signaling in hypertensive coronary arteries, GRK2 inhibitor CMPD101 was used. GRK2 inhibition rescued the apelin-induced relaxation response in SHR coronary arteries. CMPD101 pretreatment resulted in increased eNOS and P13K/Akt activity in response to apelin in SHR coronary endothelial cells. Moreover, CMPD101 pretreatment nullified the inhibitory effects of apelin on ACh-induced relaxation which further confirmed the role of GRK2 in impaired apelin/APJ signaling. Similarly, ACh relaxation was unaffected by CMF-019 strongly suggests that GRK2 activation as a potential

mechanism responsible for impairment of APJ receptor signaling in hypertensive coronary arteries.

For the first time, present study showed that apelin signaling is impaired in hypertensive coronary arteries via GRK2 pathway activation. To explore whether apelin loss its beneficial effects only under hypertensive conditions or it is common to other disease conditions in coronary arteries, we explored the apelin/APJ signaling in CSE treated (second-hand smoke model) coronary arteries. In CSE exposed coronary arteries, apelin failed to cause relaxation. Moreover, apelin impaired the ACh relaxation response in CSE treated coronary arteries. GRK2 expression was upregulated in CSE exposed arteries. Unlike apelin, CMF-019 caused relaxation in CSE treated coronary arteries. Furthermore, CMPD101 pretreatment restored the apelin-induced relaxation in CSE treated arteries. My data suggests that apelin signaling is impaired in coronary arteries of secondhand smoke model via GRK2 pathway i.e., a mechanism similar to hypertension.

The present study shows that apelin behaves differently under physiological and pathological conditions (e.g., hypertension, SHS) (**Figure 26**). APJ receptor activation provides protective effects under normal conditions whereas apelin increases vasomotor tone in coronary circulation in disease conditions. Apelin/APJ receptor signaling via the GRK2 pathway may contribute to both the loss of relaxation to apelin itself as well as the ability of apelin to inhibit endothelium-dependent relaxation to ACh under pathological conditions in coronary arteries. Considering the fact that apelin and its analogs are currently under clinical trials, the results of the present study provide more relevant insight into the potential limitations of ongoing clinical trials. I anticipate that the results from my research will be very useful in improving the therapeutic strategies with apelin and other APJ receptor agonists that are aimed to alleviate different cardiovascular disorders.

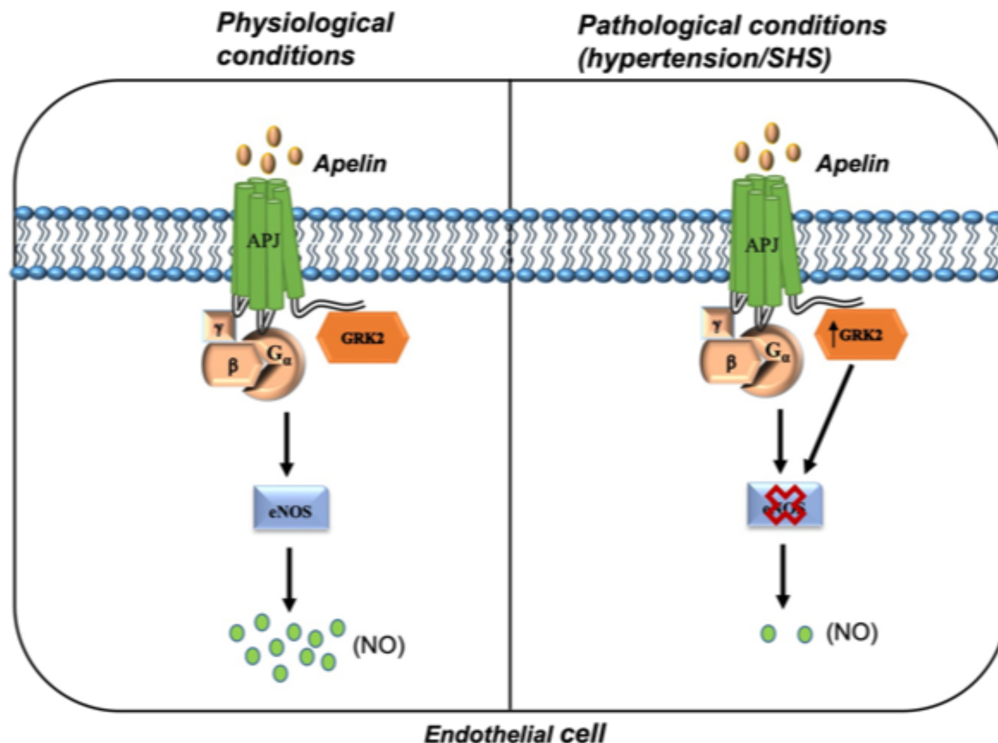

Figure 26. Proposed apelin/APJ signaling in coronary arteries under pathological conditions. Under physiological condition, binding of apelin to APJ receptors leads to eNOS activation and NO production in coronary endothelial cells. In contrast, under pathological conditions where GRK2 is upregulated, APJ receptor signaling via GRK2 pathway impairs the eNOS activity in coronary endothelial cells.

A novel finding of the present study is that APJ receptor biased agonist (CMF-019) provides beneficial vasodilatory effects even under pathological conditions in coronary arteries can benefit current apelin-based therapies as well as develop new therapeutic strategies based on the apelinergic system in cardiovascular diseases.

Limitations

The present study provides evidence for APJ receptor signaling via PI3K/Akt/eNOS pathway to cause vasodilatory effects in coronary arteries. However, apelin is also known to activate eNOS via IP3/Ca^{2+} -dependent pathway to cause relaxation (134). The binding of apelin to APJ receptors through the Gq-coupled signaling cascade can activate PLC/IP3 pathway and leads to increased cytosolic concentration of Ca^{2+}. Ca^{2+} forms a complex with calmodulin thereby

activating eNOS and increasing the production of NO in endothelial cells (155–157). The major limitation of the present study is that the role of Ca^{2+}-dependent relaxation in coronary arteries under pathological conditions was not explored. Furthermore, effect of the GRK2 upregulation under pathological conditions on Ca^{2+} mediated eNOS activation is needed to be studied.

APJ receptors undergo desensitization via the GRK2/β-arrestin -dependent pathway (31,158). In pathological conditions (hypertension, SHS) where GRK2 expression is upregulated, might result in increased recycling of APJ receptors. A limitation of the present study is that the effect of GRK2-mediated internalization of APJ receptors on altered apelin signaling under pathological conditions was not studied.

Future Directions

The present study provides several future directions. Since apelin causes relaxation in WKY coronary arteries, APJ receptor knockdown studies in endothelial cells will further help us to characterize the mechanism of APJ signaling under physiological conditions. eNOS protein phosphorylation and NO bioavailability studies in APJ receptor knocked down normotensive coronary endothelial cells will help us to better understand the apelin/APJ signaling in coronary arteries. Likewise, more research is required to understand the mechanism by which apelin exerts inhibitory effects on ACh-induced relaxation in hypertensive coronary arteries. Measurement of ACh-induced eNOS activity in apelin-treated APJ receptor knocked down SHR endothelial cells will provide us more clarity regarding the inhibitory effects of apelin on ACh response.

The present research provides evidence for the potential role of GRK2 in altered apelin/APJ signaling in hypertensive coronary arteries. eNOS and PI3K/Akt activity studies in GRK2 knocked down SHR coronary endothelial cells might provide more insight into the role of GRK2 in altered apelin response. Further research is required to understand how GRK2 interacts

with Akt thereby impairing its activity leading to defective eNOS activity and NO production in hypertensive endothelial cells. Consistent with published reports (108,121), the present study showed the upregulation of GRK2 in pathological conditions (hypertension, SHS). The future challenge will be understanding the mechanisms underlying the GRK2 upregulation under pathological conditions.

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