Interactions between Angiotensin II and Prostaglandin E2: Mechanisms for Regulation of Vascular Reactivity

By

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To my very first and most cherished mentors, Cindy and Joe Palazzo

&

To my best friend and enthusiast, Bradley Kraemer

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15-PGDH	15-hydroxyprostaglandin dehydrogenase
AA	Arachidonic acid
ACE	Angiotensin-converting enzyme
ADH	Anti-diuretic hormone
AGT	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
ARB	AT1 receptor blocker
AT1	Angiotensin II type-1 receptor
AT2	Angiotensin II type-2 receptor
AUC	Area under the curve
BP	Blood pressure
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
$cPLA_2$	Cytosolic phospholipase A2
CO	Cardiac output
COX	Cyclooxygenase enzyme
CRC	Concentration response curve
DMSO	Dimethyl sulfoxide
DP	Prostaglandin D receptor
EC	Effective concentration
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis tetraacetic acid
eNOS	Endothelium nitric oxide synthase
EP	E prostanoid
ER stress	Endoplasmic reticulum stress
EtOH	Ethanol

FAK	Focal adhesion kinase
FP	Prostaglandin F receptor
GFR	Glomerular filtration rate
GRK	G protein-coupled receptor kinase
ICBP	Intracarotid blood pressure
K_d	Dissociation constant
L-NAME	N^{ω}-Nitro-L-arginine methyl ester hydrochloride
LOX	Lipoxygenase
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NSAIDs	Non-steroidal anti-inflammatory drugs
NFA	Niflumic acid
PE	Phenylephrine
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E synthase
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PLA_2	Phospholipase A_2
PRR	(Pro)renin receptor
Pyk2	Proline-rich tyrosine kinase 2
RAG-1	Recombination activating gene 1
RAAS	Renin-Angiotensin-Aldosterone System
ROCK	Rho kinase

ROS	Reactive oxygen species
SBP	systolic blood pressure
SEM	Standard error of the mean
SFO	Subfornical organ
SHR	Spontaneously hypertensive rat
SII	Sar^{1},$Ile^{4,8}$-Angiotensin II
SMC	Smooth muscle cells
SNP	Single nucleotide polymorphism
SVR	Systemic vascular resistance
Tempol	4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl
TP	Thromboxane receptor
TUDCA	Tauroursodeoxycholic acid
TXA_2	Thromboxane

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CHAPTER I

INTRODUCTION

Historical Investigations of Blood Pressure Regulation

Currently, the prevalence of hypertension in the United States for individuals eighteen or older is 30.9%.[1] High blood pressure is a major risk factor for cardiovascular diseases including myocardial infarction, stroke, and renal failure.[2, 3] Since heart disease and stroke are among the leading causes of death not only in the United States but also around the world, it is critical that more research is conducted to reveal the multifaceted mechanisms resulting in onset of hypertension in order to prevent its deleterious effects on health. However, the importance of blood pressure-related research has not always been apparent and at one point was even discouraged.

The first mean arterial pressure reading is credited to Reverend Stephan Hales in 1733, who inserted a glass tube into an artery of a horse and measured the height to which the blood in the column rose.[4] By the late 1800s to- early 1900s more studies on blood pressure ensued leading to the invention of the instrument we use today to measure blood pressure, the sphygmomanometer.[5] Although the invention of the sphygmomanometer allow accurate measurements of blood pressure, there was still much debate regarding the clinical significance of hypertension.

Drs. Fred Mahomed and Otto Frank are credited for coining the term “essential hypertension” during the early 1900s which inferred that elevation of blood pressure was an *essential* compensatory response to overcome ischemia of tissues caused by constricted arterioles.[6, 7] Then in 1912, Dr. Sir William Osler addressed the Glasgow

Southern Medical Society in Great Britain, discouraging treatment of high blood pressure that accompanied atherosclerosis. Dr. Osler indicated that, "…the extra pressure is a necessity - as purely a mechanical affair as in any great irrigation system with old encrusted mains and weedy channels…".[8] This notion of high blood pressure being protective continued, and in 1931 in the *British Medical Journal*, Dr. John Hay notably wrote:

> "The greatest danger to a man with high blood pressure lies in its discovery, because then some fool is certain to try and reduce it."[9]

It wasn't until the late 1960s to -early 1970s that hypertension began to be considered a disorder that required treatment. The Veteran's Administration Cooperative Clinical Studies on hypertension were a major breakthrough yielding published results of studies that revealed reduced occurrence of cardiovascular events following drug therapy that lowered high blood pressure in patients with elevated diastolic blood pressure.[10, 11] These initial studies along with subsequent investigations have provided a basis of understanding that hypertension is a major modifiable risk factor for cardiovascular disease, and that treatment is associated with reduced risk of associated complications such as stroke, myocardial infarction and heart failure.[12, 13] Today the term essential hypertension refers to high blood pressure that has no known secondary cause and is often renamed primary hypertension.

Treatments for Hypertension

Although the clinical benefits of lowering blood pressure have only been widely appreciated within the last half-century, treatments for hypertension actually began in

ancient times. There are records, some dating as early as 2600 B.C., indicating the use of either phlebotomy, acupuncture, or bleeding by leeches as treatment for 'hard pulse disease'.[14] Treatment plans for arterial 'pulse' related diseases were suggested and used by a range of civilizations from the Yellow Emperor of China to the ancient Romans and Greeks.[15-17] One monumental Chinese text on early medicine, the *Yellow Emperor's Classic of Internal Medicine* (~2600 B.C.), indicated that people noticed how the heart influences pulse and even went so far as to note that eating too much salt resulted in a hardening of the pulse.[15] It wouldn't be until the mid-1940s until dietary restriction of salt was shown to reverse some pathophysiology of hypertension; however, a low salt diet is difficult for patients to maintain due to its monotonous flavoring.[18] The Greek scholar Herophilus seems to have been the first person to truly study pulse and link arterial pulse to functions of the heart.[19] Although early treatments seem barbaric, scholars from that time believed that if the patient survived treatment then their outcome would be promising.

Fortunately, in the 1900s, pharmacological treatments for essential hypertension were investigated and implemented, eventually progressing into the therapeutics that we know and use today. Prior to pharmacological treatments, patients were advised to get plenty of rest, eat simple diets, and sometimes physicians would administer sedatives.[20] One of the earliest drugs used to lower blood pressure was thiocyanate.[20] Although the mechanism for its beneficial effects is unclear, evidence suggests that thiocyanate may elicit depressant actions on nerves and smooth muscle.[21] However, the adverse side-effects such as fatigue, aching muscles and in some cases chest pain, along with the discovery of better anti-hypertensive drugs, lead to the discontinuation of its use.[20]

Surgical sympathectomy interventions suggested there were benefits from sympathetic nerve blockade in the most severe cases of hypertension; thus, ganglion-blockers were developed and prescribed in the 1950s.[22, 23] However, these drugs also had severe side effects, and their use was restricted to extreme cases of high blood pressure.[24] Eventually, as the benefits from treatment of hypertension became more widely accepted, so did the discovery of better treatment options. Thiazides (diuretics) and adrenergic blocking agents were discovered and used through the 1960s-1970s which allowed physicians to control hypertension better in their patients with only minor side-effects.[25, 26]

A major advance in treatment options came with the discovery of renin and angiotensin as potent systemic regulators of blood pressure, as discussed in greater detail below. Drugs targeting the renin-angiotensin system, including renin inhibitiors, angiotensin converting enzyme (ACE) inhibitors, and angiotensin II type 1 (AT1) receptor antagonists (ARB), have been effective for lowering high blood pressure. Around the same time frame came the discovery of calcium channel blockers, which further added to the possible treatment options. Although anti-hypertensive drugs are currently available to reduce morbidity and mortality associated with hypertension, no specific therapy will work well for every patient. Thus, the prevalence of end-organ damage and death resulting from chronic hypertension persists. Of the 70 million American adults with high blood pressure, only about half of these cases have their condition under control.[27] Research exploring novel pharmacological targets would therefore be beneficial for the development of better treatments for hypertension.

Components of Blood Pressure Regulation

There are many different physiological systems involved in blood pressure homeostasis since changes in blood flow results from specific tissue needs. In general, arterial pressure is a combination of blood volume, heart rate, and vascular resistance that can be defined in the simplest form as a derivation of Ohm's Law:

Blood pressure (BP) = cardiac output (CO) x systemic vascular resistance (SVR)[28]

Blood pressure is affected by changes in either the volume of blood pumped into the aorta with each minute, also known as cardiac output, and systemic vascular resistance. The ventricles fill with blood during a resting period known as diastole, followed by a ventricular contraction and ejection of blood into arteries known as systole. In the clinical setting blood pressure is reported as systolic versus diastolic pressure or the pressure in the arteries when the heart ventricles contract versus the pressure in the arteries between ventricular contractions. Normal blood pressure is considered as a systolic pressure reading of < 120 mm Hg and a diastolic pressure reading of < 80 mm Hg. Hypertension can be classified into various stages that are defined in Table 1 below.

Table 1 - High Blood Pressure Categories

Blood Pressure Category	Systolic mm Hg		Diastolic Mm Hg
Pre-hypertensive	120-139	or	80-89
Stage 1	140-159	or	90-99
Stage 2	≥160	or	≥100
Hypertensive Crisis	≥180	or	≥110

Based on American Heart Association recommendations (last updated 2014).

One key component in the regulation of blood pressure is peripheral vascular resistance. Many stimuli and hormones modulate blood vessel constriction and thereby regulate vascular resistance. Active contraction of vascular smooth muscle cells is facilitated by increases in intracellular calcium, leading to activation of the calcium-binding protein calmodulin. In turn calmodulin binds and activates myosin light chain kinase (MLCK).[29] MLCK is a serine/threonine kinase that phosphorylates myosin light chain (20 kD). Subsequently, phosphorylated myosin interacts with actin filaments and utilizes ATP hydrolysis to promote contraction. Additionally, Rho kinase has been shown to phosphorylate myosin light chain at the same phosphorylation site as MLCK, thus providing a mechanism of calcium-independent driven contraction.[30] There are also calcium sensitization pathways that induce vascular contraction through inhibition of myosin light chain phosphatase (MLCP). For example, activation of Rho kinase and/or PKC inhibits MLCP-mediated removal of phosphate from myosin.[31, 32] In contrast, smooth muscle relaxation reduces vascular resistance. Two common mechanisms of vasodilation

include dephosphorylation of myosin by MLCP, as previously mentioned, or release of nitric oxide from endothelial cells, which activates guanylyl cyclase in smooth muscle cells, thereby increasing intracellular levels of the secondary messenger cGMP. Consequently, cGMP mediates relaxation by inhibiting calcium entry into the cell, enhancing potassium channels conductance, and inducing kinases that activate MLCP.[29, 33, 34]

Although the vasculature has an important influence, there is still much debate about whether vascular dysfunction alone could lead to hypertension. Mean arterial pressure is a result of many factors such as renal and heart function, neuronal sympathetic drive, as well as central nervous system (CNS) activity. Historically, the primary regulator of long-term blood pressure has been credited to the functions of the kidneys.[35] However, a study by Dr. Thomas Coffman's lab demonstrated that mice deficient for the AT1 receptor, a receptor that mediates the blood pressure raising actions of the hormone angiotensin II, display reduced blood pressure even after undergoing a wildtype kidney transplantation.[36] This study implies that alterations in tissues other than the kidneys can in fact have blood pressure altering effects.

Blood supply is essential to virtually every organ, and therefore chronic hypertension can result in end-organ damage. Patients with untreated hypertension develop hypertrophy of the heart marked by enlargement of the left ventricle in response to mechanical stress from elevated blood pressure. This stiffness and enlargement of the heart results in elevated ventricle filling pressure and transmission of elevated pressure into the pulmonary vascular beds that can lead to angina, dyspnea, arrhythmia, and ultimately heart failure.[37] Patients can have increased blood volume. In turn, repeated

distension of the aorta and arteries from the larger blood volume promotes build-up of elastin and collagen, resulting in arterial stiffness. This stiffness provides more support to the vasculature; however, vascular resistance increases and further contributes to the higher pressure. Eventually, stiffness of the vasculature in combination with the high pressure results in endothelial damage and remodeling, leading to loss of compensatory endothelial-mediated vasodilation, arteriosclerotic stenosis, and potential aneurysms.[37] Damage to the vascular beds of many organs can result in critical functional damage to tissue. Hypertension is a significant risk factor for strokes and can cause retinopathy. The kidneys, which regulate blood volume, can undergo significant damage to the filtration components, or glomeruli, resulting in reduced glomerular filtration rates (GFR) and chronic renal failure.

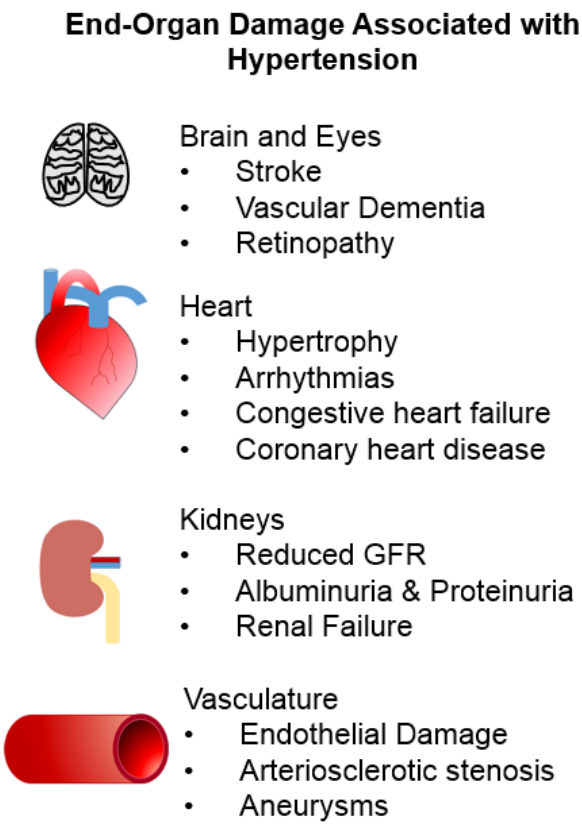

Figure 1.1 – Brief summary of end-organ damage resulting from chronic hypertension.

Renin-Angiotensin System

One key hormonal signaling pathway that is a critical regulator of blood pressure is the renin-angiotensin-aldosterone system (RAAS). Dysregulation of this pathway is often credited as a contributor to the pathophysiology of high blood pressure and thus is a common anti-hypertensive pharmacological target. This hormonal system produces a wide range of physiological effects. Historically, the RAAS was thought of as a systemic circulating pathway that is activated upon a drop in blood pressure. Briefly, this system involves the liver constantly producing an α-2-globulin protein angiotensinogen (AGT) into the plasma. Upon a reduction in blood pressure, the kidney secretes the active form of

the enzyme renin, which cleaves angiotensinogen into a smaller 10-amino'acid peptide angiotensin I (Ang I). Renin's conversion of angiotensinogen to Ang I is the rate-limiting step of the RAAS. Ang I is further cleaved in the plasma by ACE enzymes contained within the pulmonary blood vessel endothelium to create the 8-amino acid peptide angiotensin II (Ang II). Ang II is the principal effector of the RAAS that can cause a wide range of physiological effects through regulation of the vascular, renal, endocrine, and neural systems. Ang II increases water and salt reabsorption of the kidneys by directly activating specific G-protein coupled receptors and indirectly by causing secretion of anti-diuretic hormone (ADH) from pituitary glands and release of aldosterone from the adrenal cortex. Ang II increases activation of thirst centers in the CNS as well as sympathetic drive. Ang II also stimulates vasoconstriction by directly acting on smooth muscle cells (SMCs) of the vasculature (Figure 1.2).

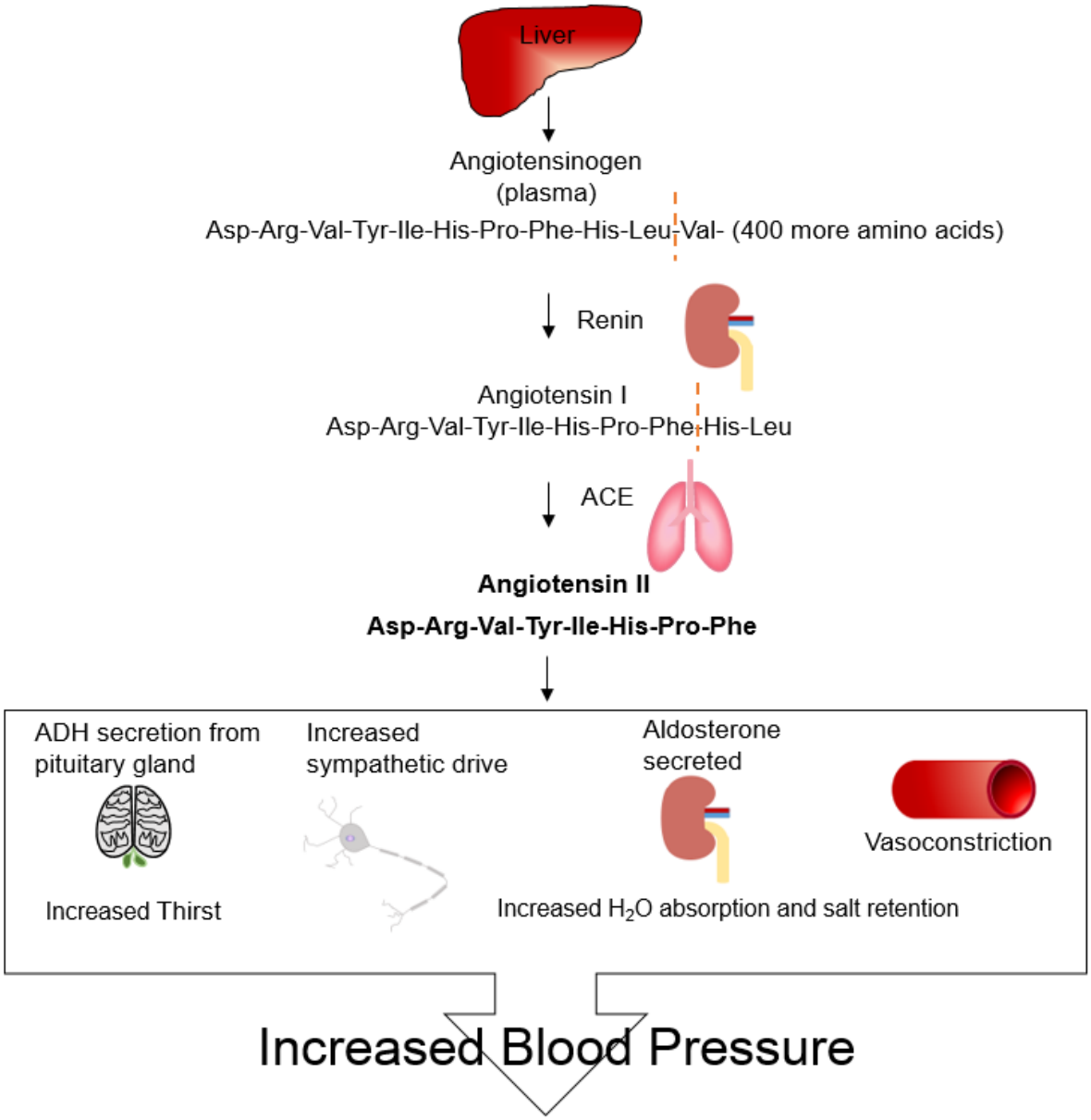

Figure 1.2 – Diagram of the classical RAAS system indicating angiotensin processing and key organ systems affected by Ang II signaling. Orange dotted lines indicate cleavage sites in the amino acid sequence.

Each component of the RAAS has grown more complex as research has unveiled the multifaceted regulation involved in each step of the pathway. Additionally, the discovery that target organs can have local synthesis of all necessary proteins of RAAS

has extended the understanding of RAAS from a systemic system to also include a local version of the RAAS. Several organs have been determined to express the necessary components of the RAAS including the heart, vasculature, brain, reproductive organs, skin, digestive organs, adipose, and eyes.[38]

Angiotensinogen. AGT has historically been described as a biologically inactive pre-cursor for the active angiotensin cleavage products of the RAAS; however, more recent studies are beginning to identify potential activity of AGT itself. There are several single nucleotide polymorphisms (SNPs) in *AGT* that have been linked to cardiovascular disease-related risks including M235T (rs699).[39-42] Particular SNPs, such as rs7079 occurring in the 3'-untranslated region, have been shown to correlate with increased effectiveness of ACE inhibitors in lowering high blood pressure in a subset of hypertensive patients.[43] Plasma levels of AGT are credited to hepatic synthesis, and various hormones and cytokines such as Ang II, glucocorticoids, estrogens, insulin, and IL-6 can affect plasma concentrations.[44-49] Recent discoveries have indicated synthesis of AGT is not limited to the liver, and AGT mRNA has been detected in many different tissues.[38] The structure of AGT indicates that it is a member of the non-inhibitory serpin (serine protease inhibitors) superfamily (SERPINA8) and of the ~453 amino acids the most conserved portion of the protein among species is the angiotensin cleavage product.[50] The angiotensin cleavage site of AGT seems to be buried inside the structure of the protein until bound to renin, which then allows conformational changes in AGT to move this cleavage site into the enzymatic active cleft of renin.[51]

Renin. Renin is an aspartic protease with high substrate specificity that hydrolyzes the N-terminus of AGT to form the decapeptide Ang I. Renin is one of the earliest studied proteins of the RAAS. Its discovery is credited to Robert Tigerstedt and his student at the time, Per Bergman, after they reportedly observed increased blood pressure in recipient rabbits after injecting them with rabbit kidney homogenate. This led to their further characterization of "...a blood-pressure raising substance…" that they then named renin.[52, 53] It was not until the 1970s that renin was purified and its structure determined by Tadashi Inagami.[54-57]

Renin expression and secretion are regulated by a complex network of cellular signaling cascades occurring in response to physiological changes. Plasma renin production is mostly localized to juxtaglomerular cells of the renal afferent arterioles. Renin begins as a precursor form of the protein called (pro)renin that becomes packaged into specific secretory pathway granules. Some of these granules secrete (pro)renin constitutively into the plasma, while a more regulated granule pathway will result in cleavage of (pro)renin by proteases, such as cathepsin B, that are localized in secretory granules to form active renin followed by controlled exocytosis.[58, 59] Secretion of active renin can be triggered by tubular chloride concentrations, sympathetic nerve activation of β-adrenergic receptors on juxtaglomerular cells, pressure-sensing mechanisms (baroreceptors), and cell membrane potentials.[60] A number of signaling events that regulate renin secretion also affect cAMP levels, such as activation of β-adrenergic receptors, suggesting cAMP regulates the rate of renin secretion. [61, 62]

More recently, the discovery of a receptor for renin, (pro)renin receptor (PRR), has further enhanced our understanding of renin's functions and regulation.[63] PRR is a single-

pass transmembrane protein that binds active renin or (pro)renin.[64] Binding of active renin to PRR enhances renin's catalytic conversion of AGT to Ang I. Furthermore, independent of the RAAS, PRR can activate mitogen-activated protein kinase (MAPK) signaling pathways when either prorenin or renin is bound. PRR can also serve as a subunit of the proton pump V-ATPase and contribute to Wnt/β-catenin signaling.[65, 66] PRR may also affect the pathophysiology of hypertension, as mice with a specific knockout of PRR in renal tubular cells are protected from Ang II-induced high blood pressure and exhibit reduced renal sodium retention.[67] Other studies have shown that when the aldosterone mimetic, DOCA-salt, is used as a model of hypertension, it leads to increased expression of PRR in the brain.[68] In this model, PRR expression is up-regulated by Ang II and required induction of cAMP response element-binding proteins.[68] The gene for renin, *REN*, has been reported to contain a cAMP response element in the promotor region, indicating that not only secretion of renin but also transcription of *REN* is modulated by cAMP.[69]

Renin is an attractive target for novel anti-hypertensive medications because of the potential to achieve complete blockade of the RAAS as compared to partial inhibition observed with ACE inhibitors and ARBs. Current renin inhibitors target the interaction of renin with AGT. In the 1970s, the peptide pepstatin was identified as a natural competitive renin inhibitor for the active site of renin.[70] However, pepstatin also inhibits other aspartic proteases; thus, a variety of pepstatin derivatives, commonly referred to as AGT analogues, have been developed.[71] Unfortunately, the benefits of AGT analogs toward hypertension and cardiovascular disease have mostly been observed only experimentally, and these types of inhibitors often have low bioavailability.[72] The first

direct renin inhibitor for use in humans was approved in 2007; the United States Food and Drug Administration approved the use of Aliskiren, a non-peptide inhibitor for the treatment of high blood pressure.[73-75] It is reported to be an effective oral monotherapy for lowering blood pressure; however, recent concerns have developed when considering Aliskiren for combinational drug therapies.[76] One clinical study testing the effectiveness of Aliskiren along with ACE inhibitors and/or ARBs in diabetic patients had to be terminated prematurely due to safety concerns.[77] Research continues to assess the extent of Aliskiren efficacy toward different disease states and explore the development of next generation renin inhibitors.

Angiotensin I & Angiotensin-converting enzyme. The cleavage product of AGT by renin is an inactive decapeptide, Ang I, which was formerly called hypertensin I/angiotonin I until a uniform nomenclature was later adopted. Ang I was found to have very minimal contractile or pressor effects on the isolated kidney; however, intravenous infusion of Ang I had similar blood pressure effects as Ang II.[78] Ang I was thus identified as simply a precursor that is quickly hydrolyzed by angiotensin-converting enzyme (ACE), a zinc metalloprotease, to form Ang II.

ACE was discovered by Leonard T. Skeggs Jr. who is also credited with determining the organization of substrates/enzymes of the RAAS.[78-81] Unlike renin, ACE does not have a strong substrate specificity. Although the most well-known substrate for ACE is Ang I, it can also hydrolyze bradykinin, substance P, neurotensin, angiotensin metabolites such as angiotensin(1-7), and other small peptides.[82, 83] ACE expression has been detected in the lung, vascular endothelium, renal proximal tubular epithelium,

intestinal epithelium, ovary, male germ cells, brain, and immune cells.[38] Additionally, a novel homolog of ACE termed ACE2 has been discovered in the last decade that seems to counter the functions of ACE by generating the metabolites Ang(1-7) and Ang(1-9) from Ang I and Ang II.[84, 85]

One of the first classes of drugs to clinically target the RAAS were ACE inhibitors. The first ACE inhibitor was identified by Sergio Ferreira, who noticed that snake venom peptides inhibited the conversion of Ang I to Ang II and the degradation of bradykinin.[86] The discovery of this inhibitory peptide eventually facilitated the development of orally active drugs with Captopril being the first ACE inhibitor used for the treatment of hypertension.[87] ACE inhibitors are recommended by the American Diabetes Association for patients with diabetes that also have hypertension as ACE inhibitors offer fewer side-effects in this population as compared to other anti-hypertensive drugs.[88-90] Yet their average effectiveness in lowering blood pressure in the general population is reported to be modest with a -8 mm Hg for systolic blood pressure and -5 mm Hg for diastolic blood pressure.[91]

Angiotensin II. The principal effector of the RAAS is Ang II, an octapeptide hormone product derived from ACE-mediated hydrolysis of Ang I. As depicted in Figure 1.2, Ang II can facilitate vasoconstriction, thirst, renal salt and water reabsorption, and increase sympathetic activity.[92]

In mammalian cells, Ang II activates cellular responses by signaling through two distinct G protein-coupled receptors designated angiotensin type 1 and type 2 receptors (AT1 and AT2). The widely expressed AT1 receptor mediates the classical actions of Ang

II and is inhibited by the commonly prescribed anti-hypertensive drugs, ARBs. There is at least one known human variant (A1166C) of the gene encoding the AT1 receptor (ATGER1) that has been implicated in essential hypertension.[93, 94] Uniquely, rodents express two isoforms of the AT1 receptor, $AT1_a$ and $AT1_b$, that share 95% of their amino acid sequence.[95] The $AT1_a$ receptor is significantly expressed in vascular smooth muscle cells, brain, kidney, and heart, whereas the $AT1_b$ receptor is primarily expressed in adrenal and pituitary glands.[96] Despite their similarity, *in vivo* studies have indicated that the $AT1_a$ receptor contributes more to Ang II-induced blood pressure effects as $AT1_b^{-/-}$ mice lack any compensatory phenotypes and display blood pressures similar to wildtype mice.[97, 98]

Ang II signaling through the AT1 receptor is tightly regulated which is evident by its ability to induce potent yet transient vasoconstriction. Upon activation, the AT1 receptor is quickly desensitized by β-arrestin-dependent receptor internalization.[99] The C-terminal tail is rich in serine/threonine phosphorylation sites that are targeted by G protein-coupled receptor kinases (GRKs) to initiate recruitment of β-arrestin.[100, 101] Interestingly, in addition to promoting receptor internalization, β-arrestin recruitment to AT1 receptors can activate MAPKs via β-arrestin scaffolding, which has been shown to have beneficial cardiovascular effects.[102-104] These findings have led to the development of biased agonists for the AT1 receptor that would provide anti-hypertensive effects from antagonizing the G-protein signaling cascades while maintaining the advantageous β-arrestin signaling. One of the leading biased agonists, TRV027, is currently in phase II clinical trials as a novel therapeutic for hypertensive acute heart failure.[105]

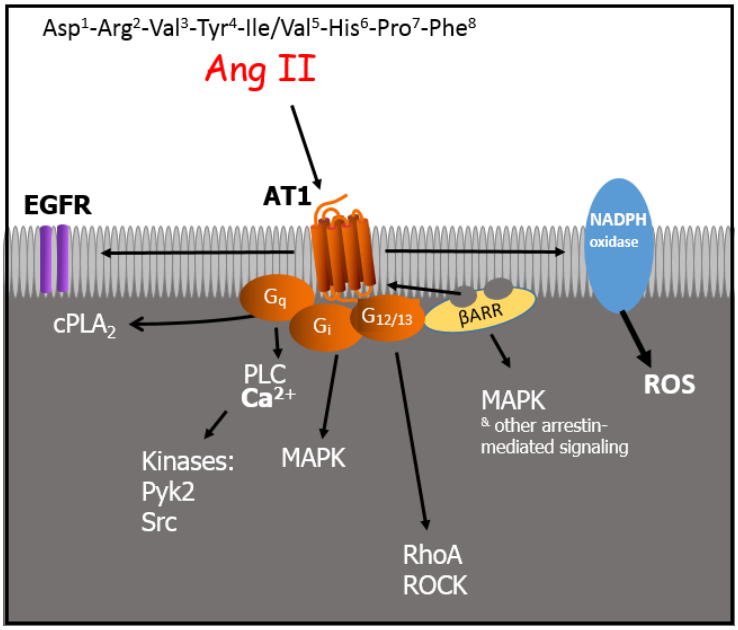

Figure 1.3 – The angiotensin II receptor 1 (AT1) signaling cascades and key proteins activated by the AT1 receptor.

The AT1 receptor couples to many different signaling cascades to mediate cellular responses. AT1 receptors can couple to G_q and activate phospholipase C, leading to production of inositol trisphosphate and increased intracellular calcium. Through coupling with $G_{12/13}$, AT1 can activate extracellular voltage-gated calcium channels and the Rho/ROCK cascade.[106, 107] AT1 has also been shown to activate Ras/Raf through coupling with G_i.[108] In addition to coupling to different G proteins, various downstream signaling cascades are activated by the AT1 receptor. Ang II via AT1 activates phospholipases A_2, which stimulates endogenous prostaglandin production and enhances inflammation.[109, 110] The AT1 receptor has been shown to transactivate the epidermal growth factor receptor (EGFR), which specifically has been linked to hypertension-associated hypertrophy of tissues.[111, 112] AT1 receptor activation can also

lead to reactive oxygen species production by activation of NADPH oxidase, which has been linked to endothelial cell dysfunction associated with hypertension.[113, 114] Numerous other kinases, such a Pyk2, Src, JAK, and MAPKs have been demonstrated to be regulated by AT1 signaling (Fig. 1.3).[115-118]

The characterization of and physiological implications for the presence of a second Ang II G-protein coupled receptor, the AT2 receptor, is currently an area of intense investigation. Some of the AT2 receptor's functions have been inferred from expression data. The AT2 receptor is ubiquitously expressed in fetal tissues; however, after birth, expression of AT2 is dramatically reduced in many regions to almost undetectable levels consistent with a role for AT2 in development.[119-121] In adults, the existence of the AT2 receptor has been detected in uterus, heart, lung, kidney, brain, and adrenals.[119-124] Despite low levels of expression in adult tissues, the AT2 receptor has been determined to counter-balance many of the actions resulting from Ang II signaling through the AT1 receptor. Some of the identified functions of the AT2 receptor include inhibiting cell growth, inducing apoptosis, and mediating vasodilation.[125-127] In the kidneys, AT2 stimulates the release of vasodilators such as bradykinin and nitric oxide.[128, 129] In neurons, the AT2 receptor has been found to inhibit norepinephrine release from catecholaminergic neurons and increase neurite outgrowth.[130] From studies investigating deletion of the AT2 receptor, the opposing function of AT2 receptors against Ang II-AT1 actions are apparent as $AT2^{-/-}$ mice exhibit elevated baseline blood pressures as well as exacerbated Ang II-induced pressor and antidiuretic effects.[131, 132] The AT2 receptor was found to signal through coupling to G_i.[133] Further studies have suggested that the AT2 receptor may dimerize and provide direct antagonism of the AT1 receptor.[134]

In healthy humans Ang II is cleared from circulation within 48 hours, this clearance can be altered in hypertensive patients.[135] Ang II was initially thought to be degraded into several different smaller inactive peptides; however, additional research has revealed that these metabolites are yet another way by which the RAAS affects physiology. One branch of angiotensin metabolism involves the above mentioned novel variant of ACE, ACE2, that can breakdown Ang I and Ang II to form Ang(1-7) and Ang(1-9). Ang(1-7) functionally opposes many AT1-mediated actions and has cardioprotective effects that are thought to be mediated through activation of the G protein–coupled Mas receptor.[136, 137] Evidence suggests Ang(1-9) either can be converted to Ang II or has Ang II-like functions.[138] Another branch of angiotensin metabolism involves the sequential cleavage of Ang II by aminopeptidases A and N to form Ang III and Ang IV, both of which have been found to have physiological functions.[139] Furthermore, a selective receptor for Ang IV, which was termed AT4 and later identified to be the insulin-regulated membrane aminopeptidase (IRAP), has been discovered that has been shown to have a significant role in neuronal plasticity.[140, 141] These metabolites of Ang II are processed further to smaller non-active peptides.[142] Despite the many investigations and accumulation of knowledge obtained regarding the RAAS pathway and the contributions of Ang II to hypertension, there remains an unmet need for novel pharmacological targets which may provide therapeutic options for the many unmanageable cases of essential hypertension.

Prostaglandins

Prostaglandins are potent lipid mediators that comprise a subclass of the eicosanoids, a family of signaling molecules derived from oxidation of 20-carbon

polyunsaturated fatty acids. Eicosanoids are synthesized from arachidonic acid (AA), eicosapentaenoic acid, or dihomo-gamma-linolenic acid, and can be further classified into two main groups, the prostanoids (including prostaglandins) and leukotrienes/lipoxins. Each group requires specific catalytic enzymes for their elaboration; cyclooxygenase (COX) enzymes generate the prostanoids, which include prostaglandins, thromboxanes, and prostacyclins, while lipoxygenase (LOX) enzymes generate the leukotrienes. Prostaglandins are produced upon the release of AA from phospholipids of the plasma membrane by phospholipase A_2 (PLA_2). AA is further processed by COX enzymes to form the prostaglandin precursors, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2), that are subsequently converted by specific prostaglandin synthases into the five bioactive prostanoids: prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane (TXA_2).

The term “prostaglandin” was first used by Ulf von Euler after he isolated a substance from sheep seminal vesicles that exerted both depressor and vasocontractile effects.[143] Believing its constituents to be secreted from the prostate gland, he named the substance prostaglandin. Following further purification, specific prostaglandins were eventually named based on the fraction from which they originated; prostaglandin E_2 was described as remaining in the ether fraction and prostaglandin F_2 in the phosphate fraction (the Swedish word for phosphate is “fosfat”, hence the “F”).[144-146] Later identified prostaglandins were termed to align within the initial alphabetical nomenclature, with the exception of thromboxane, which was termed after its role in clot formation. Subscripts are included in the name to indicate the number of double bonds in the molecule.

Structurally, prostaglandins are hydrophobic molecules that differ by slight variations in their cyclcopentanone ring and/or the two carbon side chains.

Prostaglandins are synthesized ubiquitously in response to various stimuli and released to induce autocrine and/or paracrine signaling events by binding to distinct cell surface G-protein coupled receptors. Prostaglandins have diverse physiological functions, from regulating fertility to modulating inflammation and blood pressure; however, the extent of their signaling is efficiently controlled by first-pass clearance from tissue and circulation initiated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH).[147-149]

Initial Indications of Prostaglandin Involvement in Blood Pressure

Non-steroidal anti-inflammatory drugs (NSAIDs) are over-the-counter analgesia medications that prevent the endogenous synthesis of prostaglandins by inhibiting cyclooxygenase (COX) enzymes. There are two isoforms of COX, and each is classically described as homodimers that catalyze the oxygenation and subsequent reduction of AA to form the prostaglandin precursors, although recent studies have suggested the existence of conformational heterodimers.[150] Preliminary findings suggested that COX-1 is constitutively expressed, whereas COX-2 expression is inducible and stimulated by pathophysiological conditions.[151] This initial evidence drove the formulation of selective COX-2 inhibitors to prevent the adverse gastrointestinal effects that were initially noted with non-selective NSAIDs.[152] However, this early understanding of COX isoforms may be outdated, since more recent studies have reported a role for COX-1 in inflammatory

states and determined that inhibition of COX-1 does not increase the risk of gastric complications.[153, 154]

Clinical studies investigating the effects of selective and non-selective COX inhibitors have revealed that prostaglandins affect cardiovascular homeostasis. Although the use of NSAIDs are well tolerated by many individuals, chronic use of primarily COX-2 selective inhibitors and of some non-selective NSAIDs have been shown to increase the risk of myocardial infarction, stroke, heart failure, arrhythmias, and hypertension.[155-157] This risk is most pertinent to patients with a pre-existing condition, potential drug-interaction, or a family history of cardiovascular complications.[155-158] Cardiovascular risks are also higher amongst the elderly population who are often prescribed NSAIDs more frequently. Rofecoxib (Vioxx, Merck), a COX-2 inhibitor that was prescribed for the alleviation of symptoms associated with osteoarthritis, was withdrawn from the market after it was reported that cardiovascular events occurred in 30,000 people taking the medication.[159] These unfortunate complications reveal that prostaglandins play an essential role in mediating cardiovascular health.

Prostaglandin E2

Prostaglandin E2 (PGE_2) is an abundant and extensively investigated prostanoid. PGE_2 is produced from the isomerization of PGH_2 by prostaglandin E synthases (PGES) and was initially described to facilitate systemic vasodepression as well as smooth muscle contraction.[160, 161] Many other biological functions have since been identified to be affected by PGE_2. Most notable are its roles in blood pressure regulation, neurogeneration, bone formation, inflammation, maintenance of gastrointestinal integrity,

patency of fetal ductus arteriosus, cervical ripening, and uterine contractions.[160-168] However, fully understanding the physiological roles of PGE_2 has often been challenging, since many opposing functions have been attributed to PGE_2. For example, PGE_2 has been shown to exhibit both vasodepressor and vasopressor effects.[169-175] In the immune system, PGE_2 is well-established to mediate pro-inflammatory effects, but some reports identify an immunosuppressor function.[165] These complex and counterregulatory actions of PGE_2 can be attributed to its ability to signal through four distinct G protein-coupled receptors, the E prostanoid (EP) receptors subtypes 1 – 4 (EP1 – EP4). While PGE_2 has the greatest affinity for the EP receptors (K_d values ranging from 1 – 40 nM), at higher concentrations PGE_2 can also activate the prostaglandin $F_{2\alpha}$ (FP), prostaglandin D_2 (DP), and thromboxane (TP) receptor (K_d = 100 nM, 300 nM, 30 µM respectively).[176-178] Different subtypes of EP receptors activate specific cellular transduction pathways and have unique tissue and cellular distribution, which collectively allows PGE_2 to facilitate a wide variety of cellular functions.

The EP Receptors and Their Role in Blood Pressure Regulation

PGE_2 has been shown to exhibit both vasodepressor and vasopressor effects indicating that PGE_2 has a counter-balancing role in maintaining blood pressure homeostasis. Much of the current understanding of PGE_2-mediated blood pressure effects originated from investigations of the specific EP subtypes. EP1 and EP3 receptors were recognized as mediating the vasopressor effects and the vasodepressor responses were attributed to activation of the EP2 and EP4 receptors.

The EP1 Vasopressor Receptor. The EP1 receptor is a smooth muscle contractile receptor that is thought to primarily couple with G_q leading to activation of PLC, production of inositol triphosphate, and increased intracellular calcium.[179, 180] However, in one study, EP1 was found to couple with G_i leading to increased expression of hypoxia-inducible factor-1α.[181] With the lack of specific antibodies for any of the EP receptors, expression patterns are limited to radioligand binding assays and quantification of mRNA. EP1 receptor mRNA is ubiquitously expressed with high transcript levels observed in blood vessels.[182] However, expression does not necessarily correlate with PGE_2 function. For example, the mRNA levels for the contractile EP3 receptor was found to be greater than the other EP receptors in the mouse aorta; however, PGE_2 on *ex vivo* aortic vessel rings exhibits vasodilation.[183, 184] In the rat, a variant form of the EP1 receptor has been cloned that is highly expressed in the kidney and has similar ligand specificity, yet exhibits reportedly lower calcium mobilization.[185] The EP receptors can also be differentiated by drug selectivity. Classically, 17-phenyl-PGE_2 has been used as an agonist for the EP1 receptor, but it, along with many of the EP1 agonists, has been shown to have activity at the EP3 receptor as well.[178] The most recent and selective EP1 agonist is ONO-D1-004 while the most used EP1 antagonists are SC-51322, ONO 8711 and ONO 8713.[177, 186-188]

Discovery of the EP1 receptor's role in blood pressure originated from investigations of EP1 receptor knockout mice. $EP1^{-/-}$ mice are essentially healthy, fertile, exhibit PGE_2-mediated vasodepressor responses, and have lower baseline MAP.[174, 189, 190] Additionally, $EP1^{-/-}$ mice from a DBA/1lacJ strain exhibited a reduction in pain sensitivity.[190] The EP1 receptor has been found in part to mediate Ang II-induced blood

pressure effects, as EP1$^{-/-}$ mice have a blunted response to acute intravenous infusion or chronic osmotic pump infusion of Ang II.[174] In a mouse model of end-organ damage (uninephrectomy/DOCA-NaCl/Ang II osmotic pump), EP1$^{-/-}$ mice displayed reduced mortality and organ damage as well as lower MAP.[191] The EP1 receptor seems to mediate Ang II-induced hypertension in the CNS.[192] Cerebrovascular dysfunction has been shown to involve ROS production and increased calcium influx; both of which have also been shown to require activation of central EP1 receptors.[192-194]

The EP3 Vasopressor Receptor. The other contractile EP receptor is the EP3 sub-type that is known to primarily couple to G_i leading to inhibition of adenylyl cyclase and reduced cAMP production.[180, 195-197] The EP3 receptor is widely expressed and contributes to diverse functions including regulation of the febrile response, kidney functions, maintenance of gastrointestinal health, and hepatic glucose metabolism.[173, 198-201] Abundant levels of (PTGER3) mRNA are found in metabolic and gastroenteric tissues including the kidneys, adrenal glands, pancreas, adipose tissue, and stomach.[182, 202, 203] Uniquely, multiple splice variants of the EP3 receptor have been identified that have similar ligand binding properties but differ by their C-terminal tail, enabling coupling to several different G proteins.[204] The number of EP3 variants differs with various species, with 9 identified in humans, 3 in mice, 4 in rats, and 5 in rabbits.[203, 205-209] The EP3 variants found in mice have been well investigated, and of the three variants, EP3γ and EP3α have been shown to have partial constitutive G_i activity, whereas EP3β can couple to G_i but does not exhibit constitutive activity.[210, 211] The EP3γ isoform has been demonstrated to couple to G_s as well.[209] Evidence also supports the EP3 receptor coupling to G_{13},

leading to activation of Rho kinase (ROCK).[212, 213] Additionally, the EP3 receptor has been shown to mediate calcium mobilization via the $G_{\beta\gamma}$ subunit of G proteins activating PLC.[214] More recent studies have suggested that the EP3 receptor may also couple to G_q since its calcium mobilization effects have been shown to be independent of G_i and is a result of PLC activation.[215] Despite what is known about the EP3 variants and their signal transduction properties, the physiological significance for each variant remains to be determined. There are selective agonists for the EP3 receptor with sulprostone (K_d = 0.6 nM) being the most used, although it has affinity for the EP1 receptor (K_d = 20 nM) as well.[178] ONO-AE-248 is a more recent and selective EP3 agonist.[216] There are selective antagonists for the EP3 receptor such as L-796,106 and DG-041; moreover, DG-041 has been tested in clinical trials for effectiveness as an antiplatelet drug.[217, 218]

Several studies have revealed that EP3 receptors regulate hemodynamic effects. In $EP3^{-/-}$ mice PGE_2 exhibits a slightly enhanced vasodepressor response when compared to wildtype mice indicating that the EP3 receptor counter-regulates the PGE_2-mediated vasodepressor response.[173] Furthermore, in knockout mice in which the primary EP vasodepressor receptor (EP2) is absent, PGE_2 exhibits vasopressor responses that are attributed to activation of the EP3 receptor as these responses can be reproduced with sulprostone in wildtype and $EP2^{-/-}$ mice.[169] The EP3 receptor also plays a role in regulating renal blood flow as $EP3^{-/-}$ mice have enhanced blood flow in response to PGE_2.[173] Similar to the $EP1^{-/-}$ mice, $EP3^{-/-}$ mice have been found to exhibit attenuated rises in blood pressure and vascular contractility in response to Ang II.[175] Furthermore, intracerebroventricular injection of PGE_2 in rats elicits an EP3-driven pressor response involving sympathetic nerve activation.[219, 220] The EP3 receptor has been shown to

mediate PGE_2-induced vasoconstriction in many different vascular beds including mesenteric, cerebral, intercostal, pulmonary, and femoral arteries.[180, 221-224] EP3-mediated vasoconstriction seems to in part require activation of protein kinase C δ and ROCK.[221, 222, 225]

Vasodepressor EP Receptors

EP2 receptor. The EP2 receptor is the primary vasodilator/vasodepressor EP receptor that couples to G_s leading to activation of adenylyl cyclase. This causes increases in cAMP that, in turn, induces vasodilation by inhibiting myosin light chain kinase.[226] Two different "EP2 receptors" were originally cloned by independent groups that claimed to have identified a G_s-coupled EP receptor; one clone was sensitive to butaprost and the other was not.[227, 228] Eventually, it was decided that the butaprost sensitive clone was the EP2 receptor and the insensitive clone was termed the EP4 receptor. The EP2 receptor has been found to be most abundantly expressed in bone marrow, spleen, ovary, adipose, lung, and olfactory epithelium.[182] Physiological functions regulated by the EP2 receptor include smooth muscle relaxation and stimulation of cell differentiation.[229-231] The EP2 receptor has been identified to be involved in cancer progression and tumorigenesis as a result of its identified role in stimulating cell growth.[232] The EP2 receptor may also contribute to fertility as female $EP2^{-/-}$ mice are infertile; however, human studies have yet to confirm the relevance of these findings.[169, 172]

As previously mentioned, the EP2 receptor is the primary receptor mediating PGE_2-induced vasodilator responses; thus, $EP2^{-/-}$ mice exhibit PGE_2-induced vasopressor responses upon intravenous infusion.[169] Mice lacking EP2 receptors are

more susceptible to salt-induced hypertension. Moreover, in a cohort of Japanese men, a SNP (rs17197) in the untranslated region of the PTGER2 gene has been correlated with essential hypertension.[169, 233] Although the EP2 receptor is not highly expressed in the kidney, intramedullary infusion of PGE_2 results in natriuresis and diuresis that is absent in $EP2^{-/-}$ mice.[234] Additionally, PGE_2 induces vasodilation of the afferent arterioles of wildtype mice that is switched to vasoconstriction in $EP2^{-/-}$ mice.[235] Interestingly, the renal afferent arteriolar vasoconstriction observed in $EP2^{-/-}$ mice can be inhibited with an ACE inhibitor indicating that this vasoconstriction is dependent on Ang II elaboration.[235] Collectively, these studies underscore a role for the EP2 receptor in regulating kidney function.

EP4 receptor. As previously specified, the EP4 relaxant receptor was originally designated as the EP2 receptor by initial cloning studies.[227] The EP4 receptor was also determined to couple to G_s and increase cAMP levels similar to the EP2 receptor.[195] The EP4 receptor is more abundantly expressed across tissue types when compared to the EP2 receptor; however, EP4 mRNA is enriched in the pancreas, large intestine, uterus, skin, ductus arteriosus, and thymus.[182, 236, 237] The EP4 receptor differs from the EP2 receptor by the presence of a longer C-terminal tail that includes six serine residues that are thought to allow increased phosphorylation leading to quick desensitization which is not observed with EP2 receptor activation.[238, 239] Additionally, agonists and antagonists of the EP4 receptor have been identified. The agonists include: ONO-AE1-329, TCS-2510, and L-902688.[240] The standard EP4 antagonist was previously AH-23848, which has since been superseded by more selective ligands.[240]

Investigating a role for the EP4 receptor in blood pressure regulation has been a challenge since global knockout of the EP4 receptor in mice from an inbred strain are perinatal lethal as a result of patent ductus arteriosus.[236, 237, 241] However, studies using mixed background $EP4^{-/-}$ mice and/or EP4 receptor agonists and antagonists have revealed the vasodilator actions of the EP4 receptor. The EP4 receptor has been shown to mediate endothelium-dependent vasodilation of isolated mouse aorta in response to PGE_2.[184] EP4-induced vasodilation is achieved by increased activation of endothelium nitric oxide synthase (eNOS) resulting in production of endothelium-derived nitric oxide that induces activation of guanylyl cyclase in smooth muscle cells and subsequent relaxation.[184] $EP4^{-/-}$ mice on a mixed background exhibit blunted depressor responses to PGE_2, and infusion of an EP4 agonist, ONO-AE1-329, into rats reveals a lowering of MAP.[189, 242] More recently, PGE_2 acting through the EP4 receptor has been shown to inhibit pig ciliary arterial contractions.[243] Additionally, intravenous infusion of ONO-AE1-329 into anesthetized dogs has been shown to reduce peripheral resistance, diastolic pressure and MAP.[244] Studies have indicated a role of the EP4 receptor in regulating kidney function which in turn could alter long-term blood pressure. In separate studies, the EP4 receptor has been shown to mediate renal activation of the RAAS under conditions of low salt diet and promote exocytosis of renin from juxtaglomerular cells in response to PGE_2.[245, 246]

Table 2 – Summary of cited EP receptor functions in regulating blood pressure and relevant phenotypes of EP receptor knockout mice. Commonly used agonists and antagonists are listed.

EP Receptor	Agonists	Antagonists	Vascular Functions	Knockout Mice Phenotypes
EP1	Sulprostone 17-phenyl-PGE_2 ONO-D1-004	SC-51322 ONO 8711 ONO 8713	Smooth muscle contraction Plays a major role in CNS in mediating Ang II-induced hypertension and cerebrovascular dysfunction	Lower baseline MAP Reduced response to Ang II-induced hypertension Reduced hypertensive end-organ damage Reduced pain sensitivity
EP2	Butaprost	PF-04418948 AH-6809	Smooth muscle relaxation Mediates PGE_2 -induced vasodepression	Female mice are infertile Exhibits vasopressor responses to intravenous infused PGE_2 Lack renal natriuresis and diuresis Afferent arterioles exhibit vasoconsitrction in response to PGE_2
EP3	17-phenyl-PGE_2 Sulprostone ONO-AE-248	L-796,106 DG-041	Smooth muscle contraction Mediates PGE_2 -induced vasopression Drives PGE_2-mediated intracerebroventricular-induced rise in blood pressure	Enhanced vasodepressor response to PGE_2 Enhanced renal blood flow Reduced response to Ang II-induced hypertension
EP4	ONO-AE1-329 TCS-2510 L-902688	AH-23848 CJ-023423 GW-627368 L-161982 ONO-AE3-20	Endothelium-dependent vasodilation Vasodepression Reduce peripheral resistence Promotes renin release and activation of RAAS	Global knockout is perinatal lethal

The Interplay of Prostaglandin E2 and Angiotensin II

An early indication that Ang II and PGE_2 signaling may interact came from the discovery that Ang II can activate PLA_2, leading to endogenous PGE_2 production via COX

and PGES enzymes.[247] Initial studies demonstrated that inhibition of PGE_2 with NSAIDs augmented the actions of Ang II in the kidney; thus PGE_2 was thought to negatively regulate the actions of Ang II.[248] Other studies demonstrated that that exogenous application of PGE_2 to the medullary vasa recta or renal artery generally provides negative feedback to the vasoconstrictor effects of Ang II.[249, 250] Although these early investigations led to the hypothesis that PGE_2 functions to buffer the actions of Ang II, more recent studies have revealed that the interaction between Ang II and PGE_2 is much more complex.[251] The notion that PGE_2 only counteracts Ang II's pro-hypertensive effects changed after PGE_2 was shown to increase the secretion of renin and subsequently increase levels of Ang II.[109, 170, 252, 253]

Since COX enzymes lead to the synthesis of PGE_2, many studies have been conducted investigating the effects of Ang II on these enzymes. Studies carried out by Dr. Matt Breyer's lab have indicated unique roles for each COX isoform in regulating the effects of Ang II on blood pressure and renal blood flow. Pharmacological or genetic inhibition of COX-2 was shown to potentiate Ang II-induced increase in blood pressure, as well as exacerbate Ang II-induced reduction in medullary blood flow.[254] Conversely, inhibition of COX-1 attenuated Ang II responses.[254] These data suggest that COX-2-derived products counteract Ang II while COX-1-derived products sustain the effects of Ang II. Additionally, Ang II has been demonstrated to differentially regulate COX-2 expression in the kidney with AT1 receptor activation leading to reduction in COX-2 expression and AT2 receptor activation leading to increases in COX-2.[255]

One caveat of investigations focused on COX enzymes is that COX enzymes lead to the synthesis of all prostanoids; thus, studies investigating COX enzyme expression

do not conclusively describe the contributions of PGE_2 specifically. Additional investigations of mice deficient for mPGES, the specific synthase that produces PGE_2, have demonstrated that these mice exhibit hypersensitivity to chronic low-doses of Ang.[256] However, even these studies over-generalize the role of PGE_2, as there are four EP receptors that can mediate diverse physiological effects upon activation by PGE_2. Depending on the specific tissue type and the EP receptors stimulated, concurrent Ang II and PGE_2 signaling can facilitate different outcomes.

Accumulating evidence indicates that Ang II is at least in part dependent on PGE_2 to facilitate its systemic blood pressure and vasculature responses. The EP contractile receptors, EP1 and EP3, have been identified to play a role in mediating Ang II-dependent hypertension. $EP1^{-/-}$ mice have a reduced rise in MAP in response to acute or chronic Ang II.[174] In isolated mesenteric arteries and preglomerular arterioles, treatment with the EP1 antagonist SC51322 significantly blunts Ang II-induced constriction.[174] Additional studies have indicated a significant role for the EP1 receptor in mediating the CNS effects of Ang II, including its pathological mechanisms. Ang II-induced cerebrovascular dysfunction requires PGE_2-induced activation of EP1.[194] More recently analyses conducted by Dr. Robin Davisson's lab have identified the requirement of PGE_2 signaling through EP1 receptors in the subfornical organ (SFO) of the brain for Ang II-induced hypertension.[192, 193] In these studies, mice that were given intracerebroventricular infusion of the EP1 antagonist, SC-51089, were protected from subcutaneous micro-osmotic pump Ang II-induced hypertension.[192] In follow-up studies using cultured SFO neurons, they showed that Ang II-induced ROS formation reportedly required AT1 activation of PLA_2 and COX-1 derived PGE_2 signaling through the EP1 receptor.[193] The EP3 receptor

has been indicated to play a role in Ang II-induced hypertension as well, as $EP3^{-/-}$ mice display a reduced rise in MAP in response to acute or chronic Ang II.[175] Additionally, EP3 receptor antagonists and $EP3^{-/-}$ mice have been shown to exhibit reduced Ang II-induced vasoconstriction of mouse mesenteric arteries.[175] Even though studies have indicated that PGE_2 signaling through the EP1 and EP3 receptors is to some extent necessary for the hypertensive and contractile effects of Ang II, it is still unclear how PGE_2 contributes to Ang II responses since systemic infusion of PGE_2 exhibits an EP2-induced vasodepressor effect. Further characterization of the complex balance of PGE_2 signaling through its four EP receptors and the mechanisms by which PGE_2 contributes to Ang II-induced hypertension is an active area of investigation that the work presented in this thesis elaborates upon.

Specific Aims

Hypertension is a major risk factor for renal damage and cardiovascular disease. Although anti-hypertensive drugs are available to reduce morbidity and mortality associated with hypertension, the prevalence of end organ damage and death resulting from chronic hypertension persists. Prostaglandins are potent lipid modulators of blood pressure. Prostaglandin E_2 (PGE_2), a prostanoid that normally acts as a systemic vasodepressor, has been shown to have vasopressor effects under certain circumstances. The objective of my doctoral research was to gain insights into the complex interaction of PGE_2 and Ang II in regulating vascular reactivity and blood pressure homeostasis. I hypothesized that Ang II, a principal effector of the renin-angiotensin-aldosterone system, potentiates PGE_2-mediated vasoconstriction.

Additionally, I hypothesized that angiotensin II priming could switch PGE_2 from its functional role as a vasodepressor to instead act as a vasopressor, ultimately contributing to high blood pressure. To investigate this interaction, I formulated the following aims:

Aim 1: Evaluate synergism between angiotensin II and PGE_2 pressor receptors in modulating vessel reactivity. To evaluate the ability of submaximal doses of angiotensin II to potentiate PGE_2-mediated vasoconstriction, vessel reactivity will be determined *ex vivo* by wire myography. Using receptor antagonists and knockout mice, the specific angiotensin receptor and EP receptors required for PGE_2-mediated vasoconstriction will be determined.

Aim 2: Determine the signaling pathways by which angiotensin II and PGE_2 synergize. Understanding the mechanism of Ang II and PGE_2 synergism will advance our knowledge of the complexity of vascular reactivity. In terms of therapy for hypertension, examining other key proteins besides the receptors themselves will allow the discovery of other, possibly novel, drug targets. Furthermore, the generation of biased agonists, drugs that exclusively stimulate a subset of beneficial pathways, may be possible.[257]

Aim 3: Determine PGE_2 vasopressor effects in a mouse model of hypertension. To investigate the relevance of this synergism to the physiology of the whole animal, *in vivo* experiments using a mouse model of chronic Ang II hypertension will be used to test PGE_2 blood pressure effects. These studies will investigate whether PGE_2 functionally behaves as a pressor under conditions of Ang II in wildtype mice and determine whether

this effect would be mediated by the EP3 receptor by incorporating $EP3^{-/-}$ mice into these experiments.

CHAPTER II

REGULATION OF ARTERIAL REACTIVITY BY CONCURRENT SIGNALING OF PGE_2 WITH ANGIOTENSIN II

Introduction

High blood pressure is a major risk factor for cardiovascular diseases, including myocardial infarction, stroke and renal failure.[2, 3] The renin-angiotensin-aldosterone-system (RAAS) has long been regarded as a major drug target for treatment of hypertension due to its critical role in maintaining blood pressure through regulation of the vascular, renal, endocrine, and neural systems.[258, 259] Angiotensin II (Ang II), a principal effector of the RAAS system, signals via angiotensin II type 1 and type 2 (AT1 and AT2) G-protein coupled receptors. AT1 receptors mediate classical Ang II pressor effects including smooth muscle contraction, increased sympathetic nerve activity, and renal tubular reabsorption of Na^+.[260] The AT2 receptor, although not fully characterized, has been shown to mediate opposing responses compared to AT1 function.[261, 262] The AT1 receptor induces vasoconstriction by coupling to G_q, thereby leading to activation of phospholipase C and increasing intracellular calcium.[262, 263] These resulting calcium increases then activate kinases, such as myosin light chain kinase and proline-rich tyrosine kinase 2, that promote an increase in myosin light chain phosphorylation and ultimately induce smooth muscle contraction. The AT1 receptor is also known to increase phospholipase A_2 activation, resulting in liberation of arachidonic acid and synthesis of prostaglandins.[264]

Prostaglandin E2 (PGE_2) is an oxygenated metabolite of arachidonic acid, which modulates blood pressure among many functions.[265, 266] PGE_2 acts primarily as a vasodilator and vasodepressor; however, under certain circumstances PGE_2 can induce vasopressor effects.[169, 170, 183, 267-269] These divergent functions are due in part to the ability of PGE_2 to activate four distinct E-prostanoid receptors, EP1 - EP4.[265, 270] EP1 and EP3 receptors facilitate vasoconstriction while the EP2 and EP4 receptors mediate vasodilation.[183, 267] As with the AT1 receptor, activation of the EP1 receptor leads to a rise in intracellular calcium via G_q signaling.[271] The EP3 receptor couples to G_i and $G_{12/13}$, which inhibits cAMP production, activates Rho GTPase, and elevates intracellular calcium levels through its $G_{\beta\gamma}$ subunit.[270] EP2 and EP4 receptors couple to G_s and upon activation increase cAMP.

The Breyer lab and Guan lab have previously demonstrated that blockade of the EP1 or EP3 receptor has salutary effects in mouse models of Ang II-mediated hypertension. EP1 receptor knockout mice display a reduced rise in mean arterial blood pressure in response to Ang II infusion and are protected against end-organ damage in hypertensive mouse models.[174, 191] Similarly, EP3 receptor knockout mice have a blunted blood pressure response to acute administration of Ang II.[175] Although these results indicate an interaction between Ang II and PGE_2 signaling, the molecular mechanisms through which these systems synergize to regulate vascular tone is incompletely characterized.

Previous studies have indicated a unique characteristic of PGE_2-mediated vessel reactivity in which, pre-stimulation of rat arterial tissue with a contractile agent followed by PGE_2 unmasks a significant vasoconstrictive response to PGE_2.[221] Thus, we

hypothesized that Ang II, a potent vasoconstrictor, could potentiate PGE_2-mediated vasoconstriction. Additionally, we postulated that this enhanced vasoconstriction would be mediated by potentiation of EP3 or EP1 receptor signaling rather than through desensitization of the vasodilator EP receptors. In the present study, we investigated the effects of low dose Ang II "priming" on PGE_2-induced contraction in isolated blood vessels to determine whether Ang II can alter PGE_2 vessel responses. We demonstrated that Ang II primes vessels to exhibit potentiated vasoconstriction in response to PGE_2. This effect is mediated by the AT1 and EP3 receptors and requires calcium-dependent mechanisms involving Rho-kinase (ROCK) and the proline-rich tyrosine kinase 2 (Pyk2).

Experimental Procedure

Animals

All studies were performed in accordance with the National Institutes of Health animal care standards and approved by the Vanderbilt University Institutional Animal Care and Use Committee. Male mice 9 to 15 weeks old on a C57BL/6 background were used for all studies unless noted otherwise. Generation of EP1 (*ptger1*), EP2 (*ptger2*), and EP3 (*ptger3*) receptor knockout mice has been previously described.[169, 174, 198] Mice deficient for the EP4 receptor (*ptger4*) have a lethal newborn phenotype when maintained on a C57BL/6 genetic background.[241] We bred C57BL/6 $EP4^{+/-}$ male mice to an outbred strain (CD-1, Charles River laboratories; 10-generation outcross) and observed $EP4^{-/-}$ mice viability into adulthood; thus, these mice were used for the EP4 studies.[236, 272]

Wire myography with isolated femoral arteries

Following euthanasia, femoral arteries were excised and placed into ice-cold Modified Krebs-Henseleit solution ($NaHCO_2$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, KCl 4.7, NaCl 118, $CaCl_2$ 2.5, Dextrose 10 mM). Vessel rings (~2 mm long) were cut and suspended between 40 µm stainless steel wire in a Danish Myo Technology Model 620M myograph system to record tension. Tissue baths contained 5.0 mL of Krebs-Henseleit at 37 ˚C aerated with 95% O_2, 5% CO_2 gas. Vascular preparations were allowed to stabilize for 45 minutes at a resting tension of 2.5 mN, which had been determined to induce about 90% of maximal response to 50 mM K^+ in mouse femoral arteries. Each preparation was initially challenged with 50 mM K^+, followed by 3 washes of Krebs-Henseleit solution before testing experimental reagents. Separate rings were used for each data point to avoid the rapid tachyphylaxis of the AT1 receptors. For Ang II priming, Ang II was added to vessel ring baths for 10 minutes followed by addition of PGE_2. At the end of each experiment, endothelium integrity was confirmed by reversal of 1 µM phenylephrine-evoked constriction with 10 µM acetylcholine. Rings were excluded that did not dilate at least 70% of the phenylephrine contraction (tension reduced to $\leq$ 30% of the phenylephrine response). Finally, rings were washed and retested with 50 mM K^+ to assure maintained responsiveness throughout the entire experiment. All drugs were dissolved in water, ethanol (PGE_2, nifedipine, ibuprofen, SC-236), or DMSO (DG-041, PF-431396) and then further diluted to ≥ 1:1000 or more in Krebs buffer before being added to the vessel bath. Data were collected and stored using ADInstuments LabChart 7 software.

Chemicals

Angiotensin II (human), acetylcholine, losartan, ibuprofen, nifedipine, PF-431396 hydrate, salicylic acid, and phenylephrine were purchased from Sigma (St. Louis, MO). Prostaglandin E_2 and SC-236 were purchased from Cayman Chemical (Ann Arbor, MI). PD123319 was purchased from Tocris (Bristol, UK). Y-27632 was purchased from Abcam Biochemicals (Cambridge, MA). EGTA was purchased from Thermo Fisher Scientific (Waltham, MA). FAK inhibitor 14 was purchased from Santa Cruz Biotechnology (Dallas, TX). (Sar^1,$Ile^{4,8}$)-Angiotensin II Trifluoroacetate salt was purchased from Bachem (Bubendorf, Switzerland). The Vanderbilt Chemical Synthesis Core synthesized DG-041. Salicylamine was a gift from L. Jackson Roberts (Vanderbilt University).

Competitive binding of [^{3}H]PGE_2

Total kidney membranes (100 µg) from wildtype C57BL/6 mice were incubated with [^{3}H]PGE_2 (2 nM) in binding buffer (25 mM potassium phosphate, 1mM EDTA, and 10mM $MgCl_2$, pH 6.2) at a total volume of 200 µl for 1 hr at 30 ^oC. Nonspecific binding was determined in the presence of excess unlabeled PGE_2 (1 µM). EP1/3 selective agonist sulprostone (1 µM) was used as a positive control. Competitive binding experiments were performed in the presence of 2 nM [^{3}H]PGE_2 and 1 µM of competing ligands, either Losartan or PD123319. Reactions were terminated by vacuum filtration and radioactivity bound was quantified.[273, 274]

Data analysis

Vascular responses were quantified as a change in force (maximal contraction-baseline tension) normalized to the first 50 mM K^+ response (K-standard) of each ring. All data were graphed as means ± SEM using GraphPad Prism 5 software. Data were fitted with variable slope sigmoidal curves using GraphPad Prism. Area under the curve (AUC) (% contraction/Log[treatment]) values were used to statistically compare concentration response curves (CRC) of PGE_2 with or without priming agents. Student's t-test was used to determine significance between AUCs as well as for the [^{3}H]PGE_2 binding data. Ang II CRCs were fitted to a variable-slope sigmoidal curve. Comparison of single dose PGE_2 to various conditions was performed using one-way ANOVAs with a Bonferroni post-hoc test. For all studies, $P \leq 0.05$ was considered statistically significant. Significance was represented throughout each figure as the following: *= $P \leq 0.05$, **= $P \leq 0.01$, and ***= $P \leq 0.001$.

Results

Ang II potentiation of PGE_2-mediated vasoconstriction

To investigate the effect of Ang II on PGE_2-mediated vasoconstriction, mouse femoral arteries (I.D. ~0.25-0.3 mm) were studied *ex vivo* using a wire myograph. Femoral arteries were selected for our studies as a model of peripheral vasculature in order to compare to previous studies conducted using rat femoral arteries.[221] To begin investigating the vascular responsiveness of arteries to PGE_2, the optimal resting tension for mouse femoral arteries was determined. This was conducted by recording the arterial force exhibited in response to 50 mM K^+ at different manually set basal tensions. From

these results (Fig. 2.1), the resting tension selected for use in all myography experiments using mouse femoral arteries was 2.5 mN. This basal tension resulted in 90% of maximal force in response to K^+ standard (50 mM).

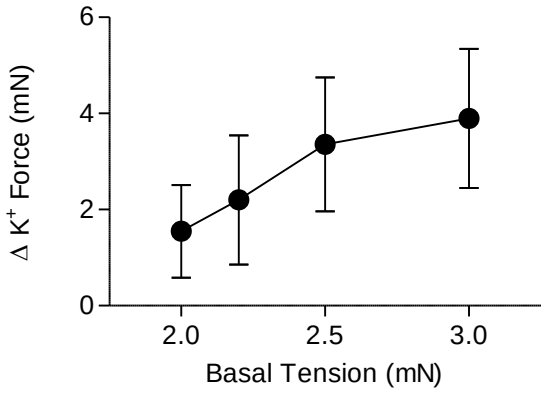

Figure 2.1 – Determination of optimal wire myograph basal tension setting for mouse femoral arteries. Graph represents the change in force resulting from vessel ring contraction in response to 50 mM K^+ at various tensions (2.0 - 3.0 mN) in wildtype mice. Vertical bars indicate SEM. (N = 3)

Using the determined optimal baseline tension, arterial rings were assayed for responsiveness to a range of single Ang II concentrations to formulate a concentration response curve (CRC) (Fig. 2.2A). Arterial rings were then primed with a sub-threshold constrictor concentration of Ang II (1 nM, ~ EC_{15}) followed by a single concentration of PGE_2 at varying doses to construct a PGE_2 CRC. Exposure of arterial rings to PGE_2 alone, even at a high concentration of 1 µM (3.326 ± 0.647 % contraction of K-standard, N = 5) did not cause significant vasoconstriction. However, priming of arterial rings with a submaximal concentration of Ang II (1 nM) resulted in robust PGE_2-mediated vessel contraction (Fig. 2.2B, C). Ang II priming facilitated arterial contraction following exposure to doses of 2 nM PGE_2 or greater ($AUC_{untreated}$ 2.778 ± 2.091, $AUC_{Ang\ II\ primed}$ 22.830 ± 8.560, t test ***P<0.001) (Fig. 2.2, C).

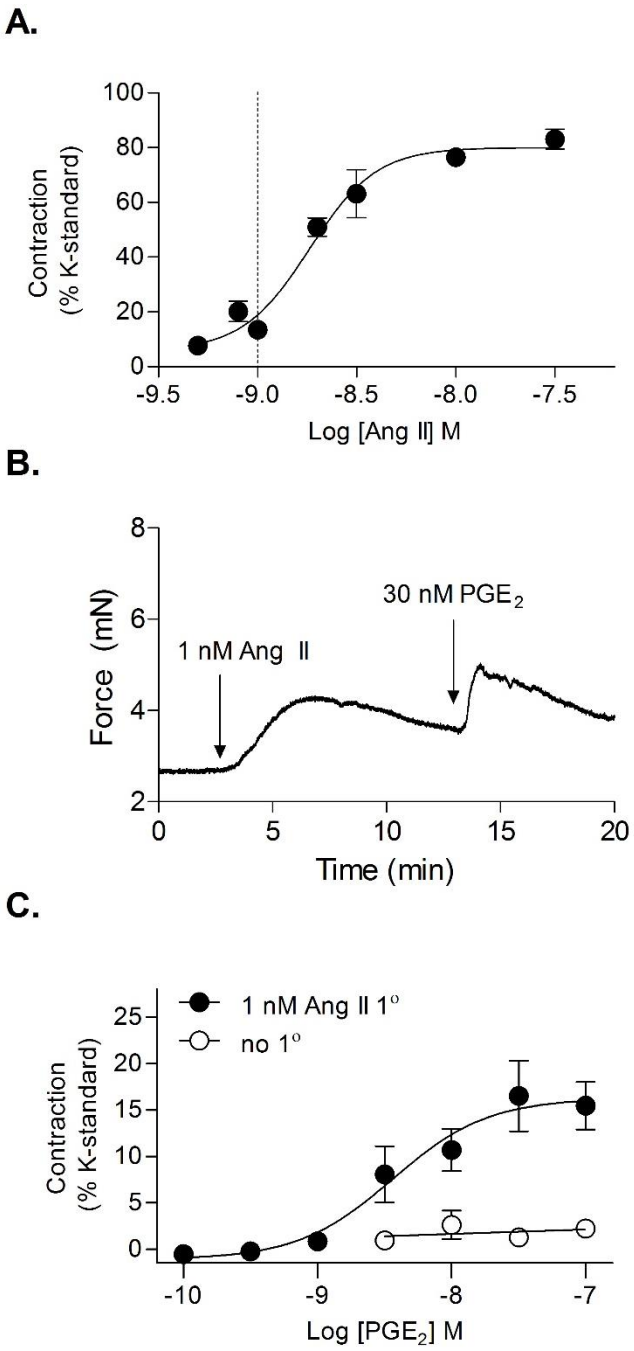

Figure 2.2 - Ang II priming enhances PGE_2-mediated vasoconstriction. (A) Ang II CRC in wildtype mice. (N = 5-7) Dotted line indicates concentration of Ang II used to prime vessels in B and C. (B) Representative trace of 30 nM PGE_2-induced contraction of vessels primed with 1 nM Ang II. (C) Comparison of PGE_2 concentration-response curve with (filled circles, N = 5-6) or without (open circles, N = 5) 1 nM Ang II priming. Data for each concentration was collected on separate rings. Results were normalized to each ring's response to 50 mM KCl. Vertical bars indicate SEM.[275]

Additional studies were performed to test the time duration that PGE_2-mediated vasoconstriction would occur after Ang II pre-treatment. Vessels rings were treated with 1 nM Ang II followed by addition of 100 nM PGE_2 at varying time points (10 – 60 minutes). PGE_2-induced contraction was greatest when PGE_2 was added at approximately 10 minutes after Ang II and was significantly reduced at 30 minutes after Ang II (Fig. 2.3). It was also of interest to determine whether the presence of or continuous stimulation by Ang II was necessary for the ability of PGE_2 to result in contractile responses or whether Ang II would predispose the vessel to PGE_2-mediated vasoconstriction even after Ang II was removed from the bath. In experiments in which 1 nM Ang II was incubatd (5 minutes) and then quickly washed from the vessel bath followed by immediate addition of 100 nM PGE_2, contractile responses induced by PGE_2 were significantly attenuated (Fig. 2.3, right bar) when compared to contractile responses observed at the 10-minute time point.

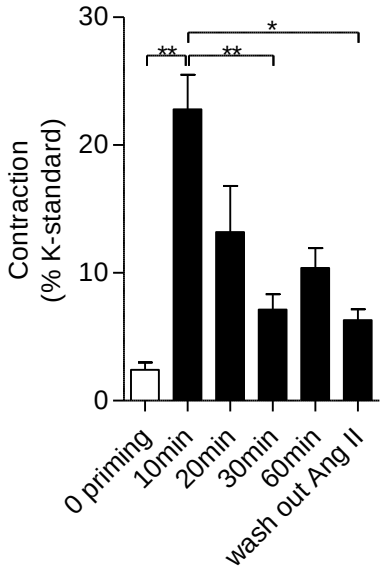

Figure 2.3 – PGE_2-facilitated contraction at varying times after Ang II pretreatment. 100 nM PGE_2 responses with (black bars) or without (white bar) 1 nM Ang II priming. Minutes indicate time after Ang II that PGE_2 was added to vessel bath. The far right bar indicates treatment of vessel rings with Ang II for 5 minutes then immediately removing Ang II from the bath prior to PGE_2 addition. Vertical bars indicate SEM. (N = 3-5) *= P<0.05, **= P<0.01

Reversal effects: PGE_2 potentiation of Ang II-mediated vasoconstriction

To further characterize vascular interactions between Ang II and PGE_2, studies were conducted to determine whether priming femoral arteries with PGE_2 for 10 minutes could potentiate Ang II-mediated vasoconstriction. Priming of femoral arteries with 30 nM PGE_2 resulted in a slight leftward shift in the Ang II CRC (Fig. 2.4, Ang II EC_{50} = 1.8 nM, Ang II_{+PGE2} EC_{50} = 1 nM). These results indicated that PGE_2 is able to modestly potentiate Ang II-mediated vasoconstriction.

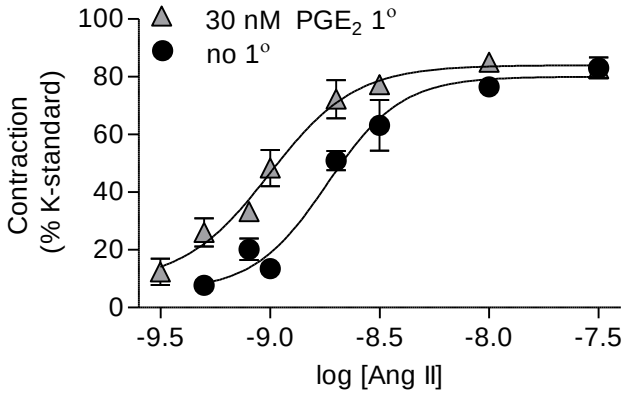

Figure 2.4 – PGE_2 pre-treatment enhances Ang II-induced vasoconstriction. Ang II CRC with (grey triangles) or without (black circles) 30 nM PGE_2 priming (Ang II_{-PGE2} data from Fig. 2.2). Ang II_{-PGE2} $LogEC_{50}$ = -8.74 ± 0.05 and Ang II_{+PGE2} $LogEC_{50}$ = -9.00 ± 0.06 (N = 5) The EC_{50} values are significant with a $P < 0.01$.

Mesenteric vessels exhibited PGE_2-mediated vasoconstriction after Ang II priming

Femoral arteries were selected in order to compare to previous studies conducted using rat femoral arteries.[221] Femoral arteries are a model for peripheral vasculature, but in other studies we also investigated whether different vascular beds would exhibit Ang II-induced potentiation of PGE_2-mediated vasoconstriction. PGE_2 facilitated vasoconstriction in response to Ang II priming of rings from 1st order mesenteric (Fig. 2.5A), but induced apparent vasodilation in aortic vessel rings (Fig. 2.5B). PGE_2-mediated vasodilation of the aorta was also observed when priming arterial rings with K^+ or phenylephrine (Fig. 2.5C, D). All other wire myography studies were conducted using mouse femoral arteries.

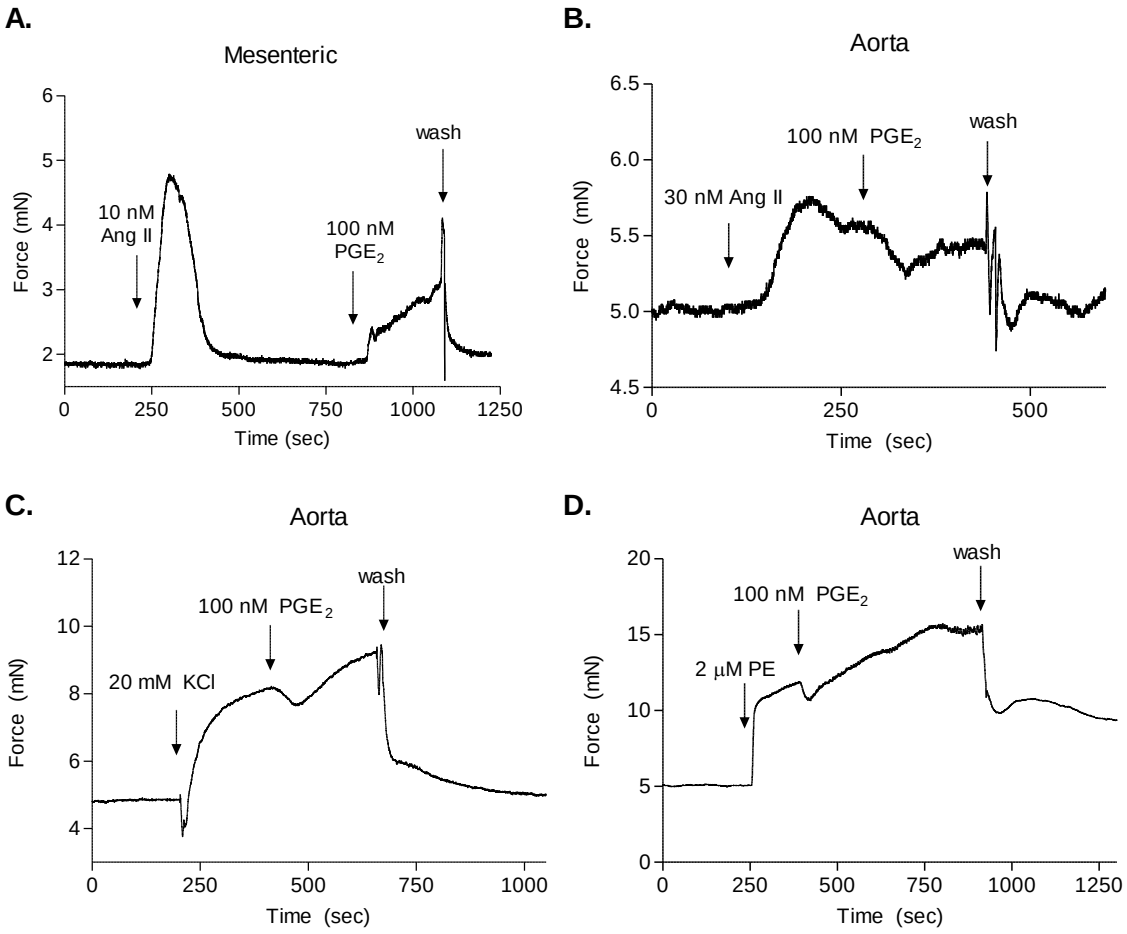

Figure 2.5 – Mesenteric vascular beds but not aorta exhibit PGE_2-mediated contraction after Ang II pre-treatment. (A) Wire myography trace from a 1st order mesenteric vessel ring showing PGE_2-mediated contraction after 10 nM Ang II pre-treatment. (B) Wire myography trace from an aortic vessel ring showing PGE_2-mediated vascular response after 30 nM Ang II pre-treatment. (C) Wire myography trace from an aortic vessel ring showing PGE_2-mediated vascular response after 20 mM K^+ pre-treatment. (D) Wire myography trace from an aortic vessel ring showing PGE_2-mediated vascular response after 1 μM PE pre-treatment. Drug additions are indicated with arrows. (N = 1)

Other contractile agents potentiate PGE_2-mediated vasoconstriction

Additional studies were conducted to test whether other known contractile agents could enhance PGE_2-mediated vasoconstriction of mouse femoral arteries. Indeed, sub-maximal concentrations of K^+ (20 mM), a smooth muscle cell depolarizing agent; phenylephrine (100 nM), an adrenergic receptor agonist; or vasopressin (10 nM), a hormone also referred to as anti-diuretic hormone (ADH), were also able to reveal PGE_2-mediated contraction (Fig. 2.6). These studies indicate that other contractile agents can potentiate PGE_2-induced vasoconstriction of the mouse femoral artery.

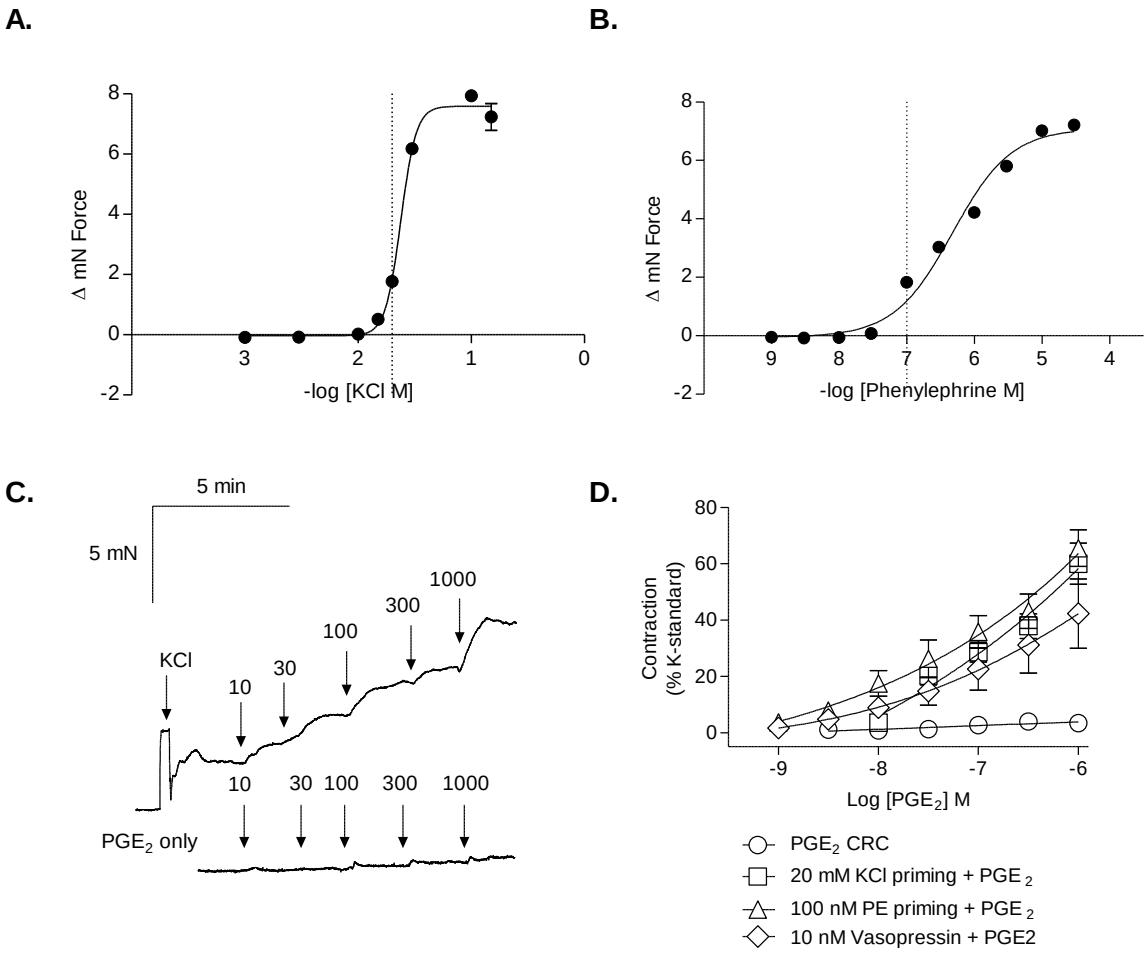

Figure 2.6 - Vascular reactivity of PGE_2 on mouse femoral arteries. (A) K^+ CRC and (B) PE CRC with dotted lines indicating doses used for priming experiments in C & D (N = 1). (C) Example traces from wire myography studies showing effects of PGE_2 alone and PGE_2 with 20 mM K^+ priming. Arrows indicate when drugs were added, and values are in units of nM. (D) PGE_2 CRC with or without K^+, PE, or vasopressin priming. (N = 2-5). Vertical bars indicate SEM. Data are represented as the change in force from the maximum response minus the baseline tension. Baseline for the primed vessels was set at the response of the priming agent.

EP3 receptor signaling primed by AT1 receptor activation

To determine which prostaglandin receptor mediates the PGE_2-induced vasoconstriction of Ang II-primed vessels, wire myography studies were performed utilizing a combination of pharmacological antagonists and genetic deletion models. Femoral arteries isolated from $EP3^{-/-}$ mice displayed a lack of PGE_2-mediated vasoconstriction even with Ang II priming (Fig. 2.7). PGE_2-mediated contraction was then assessed using wildtype femoral arteries treated with the EP3 receptor antagonist, DG-041. Pre-treatment with 1 μM DG-041 had no effect on resting tone or Ang II-mediated constriction: however, Ang II failed to potentiate PGE_2-mediated vasoconstriction in DG-041 pretreated vessels (Fig. 2.7B). The apparent PGE_2-induced vasodilation of vessels primed with Ang II in the presence of DG-041 (Fig. 2.7B, far right bar) was not statistically significant compared to zero priming. By contrast, in femoral arteries isolated from $EP1^{-/-}$ mice, Ang II was able to potentiate PGE_2-mediated vasoconstriction to a similar level as observed with wildtype mice (Fig. 2.7B). The contributions of the FP receptor was also investigated since this receptor has been shown to mediate vasoconstriction as well and has an affinity for PGE_2 (pK_i = 7.0).[178] Additionally, EP3 and FP are thought to have evolved from a gene duplication event.[265] Despite similarities between the EP3 and FP receptors, femoral arteries isolated from $FP^{-/-}$ mice exhibited similar PGE_2 responsiveness compared to wildtype vessels (Fig. 2.7B).

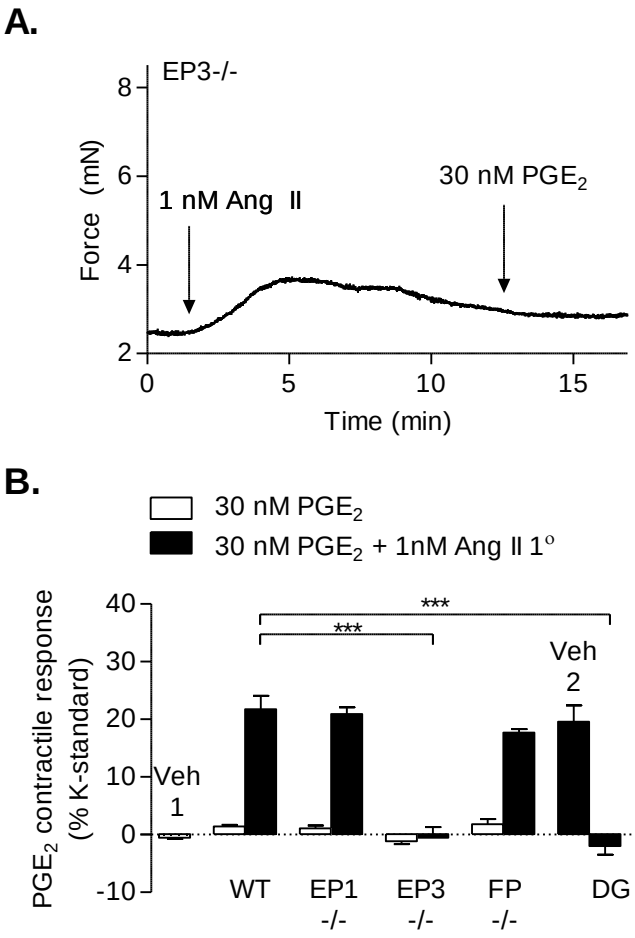

Figure 2.7 - Effects of constrictor EP-receptors on PGE_2-induced contraction of Ang II-primed arteries. (A) Representative wire myography trace of an $EP3^{-/-}$ femoral artery that shows the lack of PGE_2 response when primed with Ang II. (B) Comparison of % contraction during 30 nM PGE_2-induced vasoconstriction with or without 1 nM Ang II priming in $EP1^{-/-}$, $EP3^{-/-}$, or $FP^{-/-}$ vessels or in wildtype vessels treated with or without the EP3 antagonist DG-041 (DG) (N = 4-5 for $EP1^{-/-}$, N = 3 for $FP^{-/-}$, all other data sets N = 5). PGE_2 alone did not cause significant vasoconstriction in isolated femoral arteries from any of the mouse strains (wildtype, $EP1^{-/-}$, $EP3^{-/-}$, $FP^{-/-}$). Veh1 indicates Ang II priming of PGE_2 solvent (ethanol). Veh2 indicates pre-treatment with DG-041 solvent (DMSO). Wildtype data with or without Ang II are taken from Fig 2.2. Vertical bars indicate SEM.[275] ***= $P < 0.001$

Although these data indicated that upon Ang II priming the subsequent PGE_2-induced contraction was EP3 receptor-mediated, Ang II promoting desensitization of either vasodilator receptor, EP2 or EP4, could also explain the PGE_2-induced contraction. Thus, to test the contribution of the EP2 or EP4 receptor, we assessed the effect of PGE_2 on $EP2^{-/-}$ and $EP4^{-/-}$ femoral arteries. In the absence of Ang II priming, deletion of either EP2 or EP4 was insufficient to facilitate comparable PGE_2-induced vasoconstriction to that observed in wildtype vessels primed with Ang II (Fig. 2.8, $EP2^{-/-}$ & $EP4^{-/-}$ open bars). PGE_2-mediated contraction of Ang II-primed vessels was notably greater in $EP2^{-/-}$ vessels, presumably from loss of the EP2 receptor vasodilator function (Fig. 2.8, $EP2^{-/-}$ black bar, $P \leq 0.01$). Arterial rings isolated from $EP4^{-/-}$ mice exhibited responses that were indistinguishable from the responses observed in wildtype vessels.

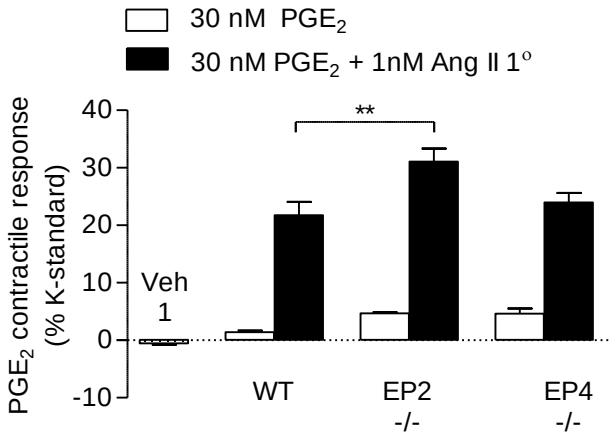

Figure 2.8 - Effects of EP-receptors on PGE_2-induced contraction of Ang II-primed arteries. Comparison of % contraction during 30 nM PGE_2-induced vasoconstriction with or without 1 nM Ang II priming in $EP2^{-/-}$ or $EP4^{-/-}$ vessels or in wildtype vessels (N = 5; two $EP2^{-/-}$ mice were one year old). PGE_2 alone did not cause significant vasoconstriction in any of the mouse strains (wildtype, $EP2^{-/-}$, $EP4^{-/-}$). Veh1 indicates Ang II priming of PGE_2 solvent (ethanol). Wildtype data with or without Ang II and Veh1 are taken from Fig 2.7. Vertical bars indicate SEM.[275] **= P<0.01

Ang II vascular responsiveness in EP1$^{-/-}$ and EP3$^{-/-}$ mice is similar to wildtype

To investigate whether EP3$^{-/-}$ mice have altered contractile responses to Ang II, concentration-response relationships for wildtype or EP3$^{-/-}$ vessels were compared. The EC_{50} and E_{max} for Ang II-induced vasoconstriction were not different between wildtype and EP3$^{-/-}$ mice (Fig. 2.9A). Interestingly, the CRC to Ang II exhibited a steeper slope in wildtype vessels (Hill coefficient of 2.581) as compared to that observed in vessels isolated from EP3$^{-/-}$ mice (Hill coefficient of 1.198). In conducting these studies, I also wanted to determine whether vessels from EP1$^{-/-}$ mice have altered Ang II-mediated vasoconstriction since previous studies had indicated that EP1$^{-/-}$ mice were protected from Ang II-mediated hypertension.[174, 191] Ang II vascular reactivity was essentially unchanged in EP1$^{-/-}$ mice compared to wildtype Ang II CRC (Fig. 2.9B). PGE_2 alone did not cause significant vasoconstriction in any of the mice (wildtype, EP1$^{-/-}$, EP2$^{-/-}$, EP3$^{-/-}$, EP4$^{-/-}$). Taken together, these results indicate the EP3 receptor is necessary for PGE_2-induced vasoconstriction potentiated by Ang II.

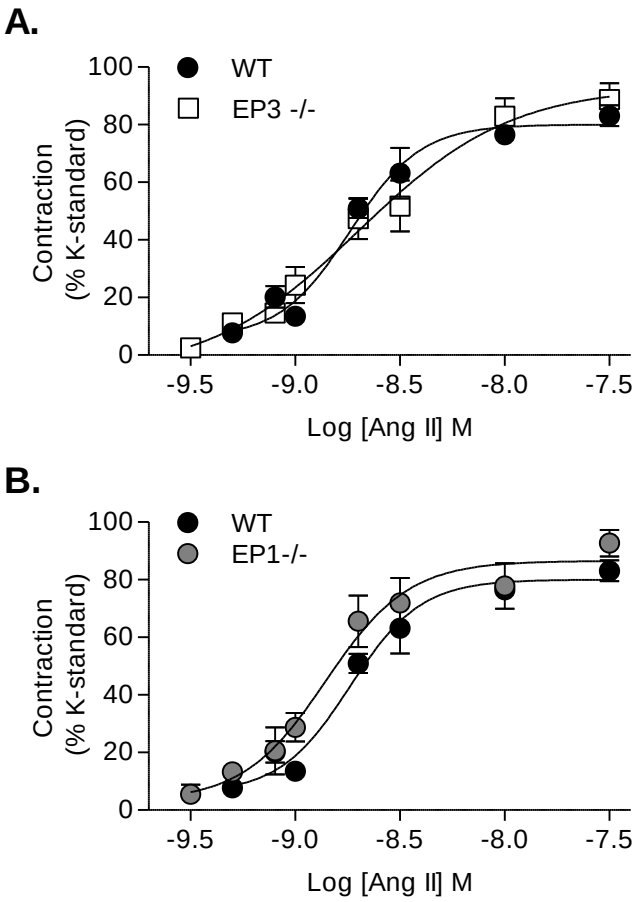

Figure 2.9 - Effects of EP-receptors on Ang II-induced vasoconstriction. (A) Ang II concentration-response curves comparing contraction of wildtype (taken from Fig 2.2, N = 5-7) and $EP3^{-/-}$ vessels (N = 5-6). $LogEC_{50}$ for each was -8.74 and -8.70, respectively. (B) Ang II concentration-response curves comparing contraction of wildtype (taken from Fig 2.2, N = 5-7) and $EP1^{-/-}$ vessels (N = 3-8). $LogEC_{50}$ for each was -8.74 and -8.86. Vertical bars indicate SEM.[275]

AT1 receptors mediate the Ang II priming effect

To identify the Ang II receptor responsible for potentiation of PGE_2-mediated vasoconstriction by Ang II, vessel contraction was measured in arterial rings pre-treated for 30 minutes with the AT1 antagonist losartan or the AT2 antagonist PD123319 prior to Ang II priming and PGE_2 addition. Losartan blocked the ability of Ang II to potentiate PGE_2-induced vasoconstriction, while PD123319 had no significant effect. (Fig. 2.10A, B, C) To verify that the effect of losartan was not due to off target competitive inhibition of the prostanoid receptors, the ability of either losartan or PD123319 to compete with [^{3}H]PGE_2 binding in mouse kidney membrane preparations was assessed. In contrast to the EP1/3 selective agonist, sulprostone, neither losartan nor PD123319 competed with [^{3}H]PGE_2 binding (Fig. 2.11). Together, these results indicate that the AT1 receptor facilitates the Ang II priming effect on EP3 receptor-mediated contraction.

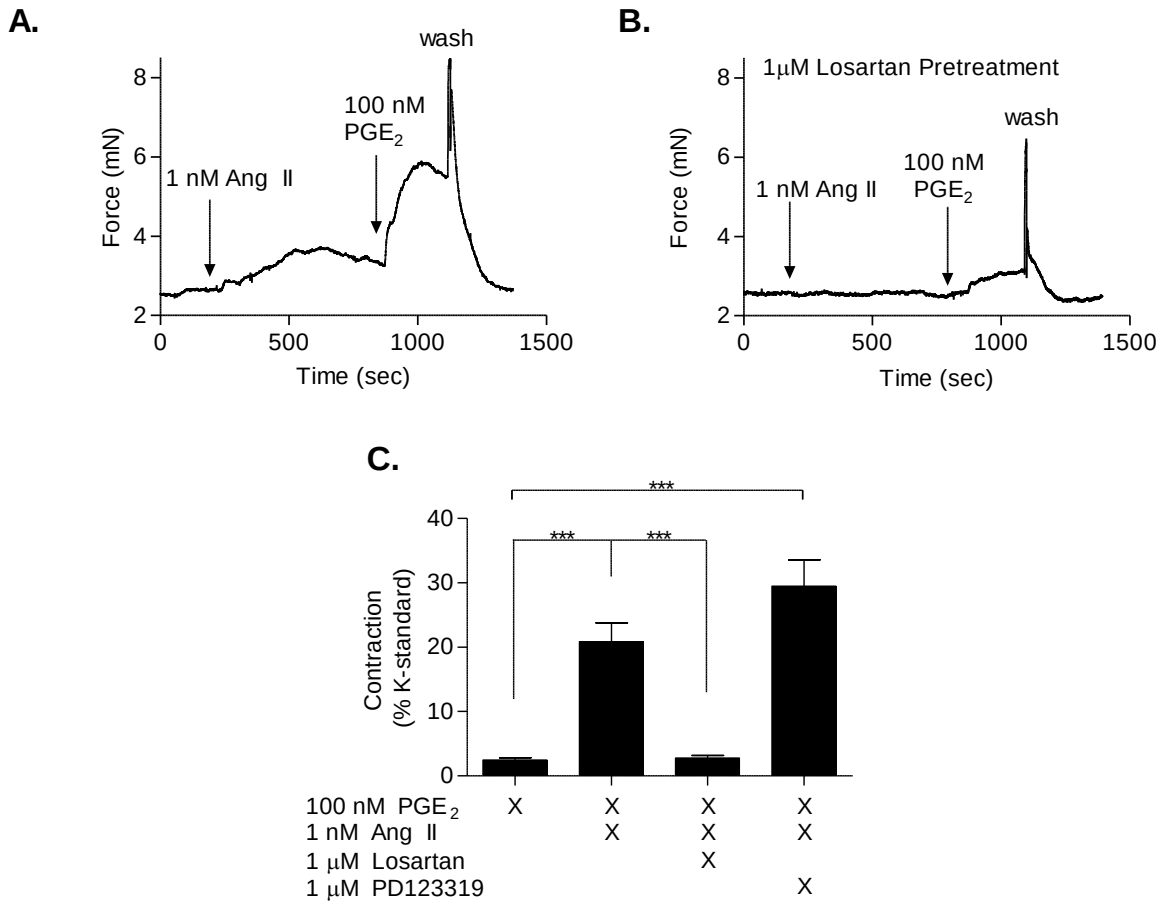

Figure 2.10 - AT1 antagonist inhibits Ang II priming of PGE_2. (A) Representative wire myography trace of femoral arterial rings primed with 1 nM Ang II, followed by addition of 100 nM PGE_2. (B) Representative trace of femoral artery with 30-minutes pretreatment with 1 μM losartan followed by addition of 1 nM Ang II priming of 100 nM PGE_2. (C) Comparison between femoral arterial ring responses to 100 nM PGE_2 with and without 1 nM Ang II priming, as well as with priming in the presence of an AT1 (losartan) or AT2 antagonist (PD123319) (N = 5). [275] ***= P<0.001

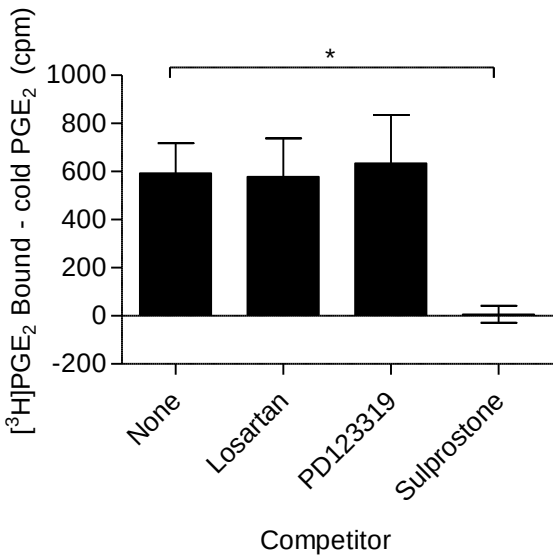

Figure 2.11 – AT1 and AT2 antagonists do not compete for EP receptor ligand binding. Competition binding in wildtype mouse kidney membranes; displacement of [^{3}H]PGE_2 by the EP3 agonist sulprostone, losartan, and PD123319. Nonspecific binding was determined in the presence of excess unlabeled PGE_2. (N = 3). * = $P < 0.05$. [275]

AT1-mediated β-arrestin pathways are not sufficient to potentiate PGE_2-mediated contraction

AT1 has been identified to mediate several physiological responses mainly through activation of G_q; however, some AT1-mediated effects can be attributed to activation of G-protein independent pathways via GRK/β-arrestin/MAPK signaling. To determine whether Ang II-induced potentiation of PGE_2-mediated vasoconstriction involved activation of β-arrestin pathways, 30 μM of the biased agonist $Sar^1,Ile^{4,8}$-Angiotensin II (SII) (AT1a K_D = 300 nM, [276]) was used in wire myography studies. SII has been previously shown to activate β-arrestin signaling pathways via AT1 while not stimulating the G_q-associated calcium cascade.[277] SII did not evoke vasoconstriction at doses up to 30 μM. SII also did not exhibit a potentiation effect on PGE_2-mediated constriction of femoral arteries (Fig. 2.12A, C). Furthermore, SII blocked Ang II-induced contraction as well as Ang II priming of PGE_2-mediated contraction, presumably by acting as a competitive antagonist for Ang II-AT1 priming (Fig. 2.12B, C).

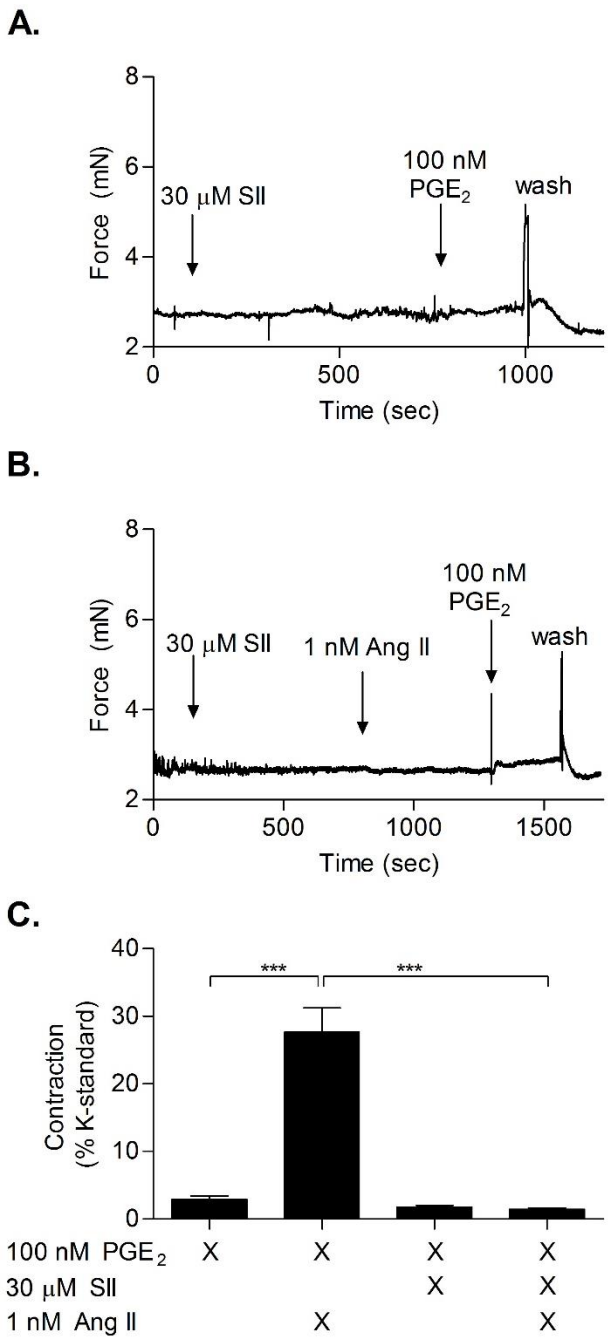

Figure 2.12 - SII does not potentiate PGE_2-mediated contraction. (A) Representative wire myography trace showing that 10-minute pre-treatment with 30 µM SII did not potentiate 100 nM PGE_2-mediated contraction. (B) Representative trace of arterial rings preincubated with 30 µM SII for 10 minutes, followed by priming with 1 nM Ang II and addition of 100 nM PGE_2. (C) Quantification of SII results, showing a lack of potentiation by SII on PGE_2-induced contraction (3rd bar from left), and SII blockade of PGE_2-mediated contraction of Ang II-primed vessels (4th bar from left) (N = 5).[275] ***= P<0.001

A role for reactive oxygen species in Ang II potentiated PGE_2-induced contraction

Ang II has been shown to induce production of reactive oxygen species (ROS) via NADPH oxidases as well as increase expression of several NADPH oxidase subunits.[278, 113] To determine whether Ang II-induced potentiation of PGE_2-mediated vasoconstriction involved the elaboration of different sources of ROS, several different studies were conducted. First, 1 mM Tempol, a superoxide dismutase mimetic, was added to the bath 20 minutes before Ang II priming of PGE_2.[279] Pre-treatment with Tempol resulted in significant reduction in 100 nM PGE_2-mediated vasoconstriction (Fig. 2.13A, B). 1 mM Tempol had no effect on 30 nM Ang II-mediated vasoconstriction (Fig. 2.13A, C). These data suggest that Ang II mediates PGE_2-induced vasoconstriction through a mechanism involving ROS.

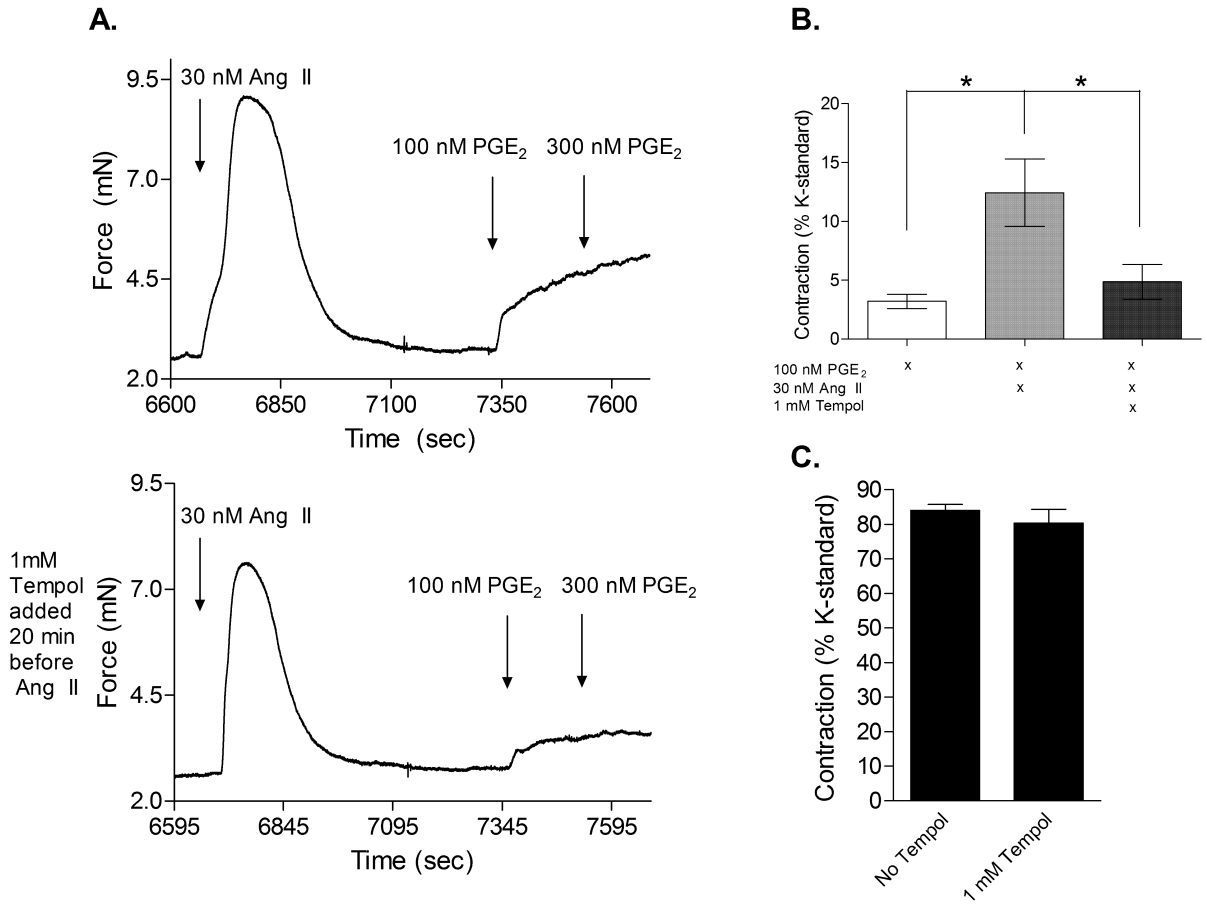

Figure 2.13 - Tempol reduces Ang II potentiation of PGE_2-mediated vasoconstriction. (A) Example traces of Ang II priming in the presence of 1 mM 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol), a superoxide dismutase mimetic. (B) PGE_2 responses with or without Ang II priming as compared to priming in the presence of 1 mM Tempol. (N = 3) (C) Ang II-mediated vasoconstriction with or without 1 mM Tempol. No significant differences. (N = 3) Vertical bars indicate SEM. *= $P<0.05$

To further investigate the contribution of ROS in PGE_2-mediated vasoconstriction vessels rings were treated with pharmacological inhibitors or stimulators of ROS. Since Tempol is known to metabolize superoxide anions, I was interested in determining whether the source of superoxide contributing to PGE_2-mediated contraction was from Ang II activation of NADPH oxidase.[279] In vessels pre-treated with apocynin, an NADPH oxidase inhibitor, PGE_2-mediated contraction following Ang II priming was unaffected (Fig. 2.14A). To investigate whether ROS are sufficient to induce PGE_2-mediated vasoconstriction, vessels were primed with menadione, which generates ROS through redox cycling.[280] Menadione pretreatment (10 µM) did not result in enhanced PGE_2-mediated vasoconstriction comparable to that observed with Ang II priming. Moreover, a higher dose (50 µM) resulted in no PGE_2-induced contraction (Fig. 2.14A), likely from cell death induced by high concentrations of menadione since these rings did not respond to either KCl or phenylephrine (data not shown). In other studies, we tested whether inhibition of endoplasmic reticulum stress (ER stress) via pre-treatment with tauroursodeoxycholic acid (TUDCA) would affect PGE_2-induced contraction since Ang II is reported to cause ER stress.[281, 282] TUDCA blunted Ang II potentiation of PGE_2-mediated contraction without affecting Ang II-induced contraction (Fig. 2.14A, B).

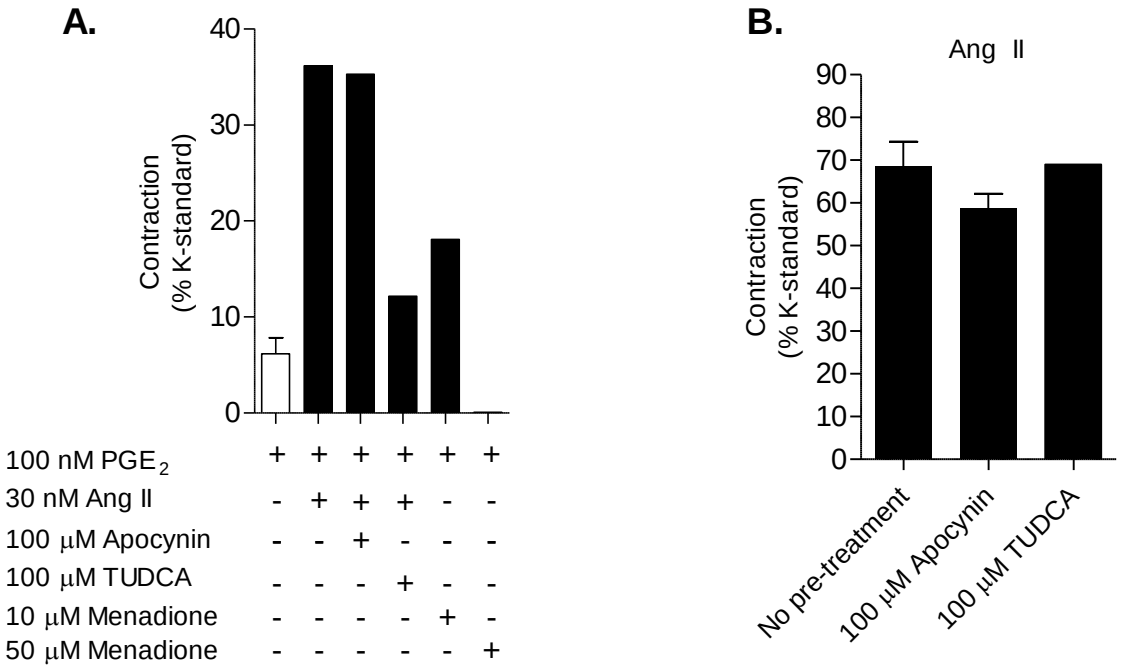

Figure 2.14 - Role of reactive oxygen species and ER stress in Ang II priming of PGE_2. (A) PGE_2 responses with or without 30 nM Ang II priming as compared to priming in the presence of apocynin or TUDCA. The last two bars on the right depict 100 nM PGE_2 response on vessels primed with menadione instead of Ang II. (N = 1-2) (B) Ang II-mediated vasoconstriction with or without apocynin or TUDCA. (N = 1-4)

EGFR activation does not contribute to PGE_2-mediated vasoconstriction

Ang II transactivation of epidermal growth factor receptors (EGFR) is a well identified signaling mechanism involved in Ang II-mediated physiological responses such as kidney fibrosis and cardiac hypertrophy.[283-285] There is evidence to support a role for EGFR in mediating vasoconstrictor responses such as contributing to Ang II-mediated contraction of rat renal artery or aorta and contributing to endothelin-1-induced contractions of rabbit basilar artery.[286-288] Furthermore, studies have indicated a link between ROS and EGFR in mediating vascular contraction of ductus arteriosus.[289] Thus, we performed an experiment to determine whether inhibition of EGFR would affect PGE_2-mediated vasoconstriction. Vessels were pretreated with 1 μM erlotinib to inhibit EGFR activation; however, erlotinib had no significant effects on PGE_2-induced contraction (Fig. 2.15A).

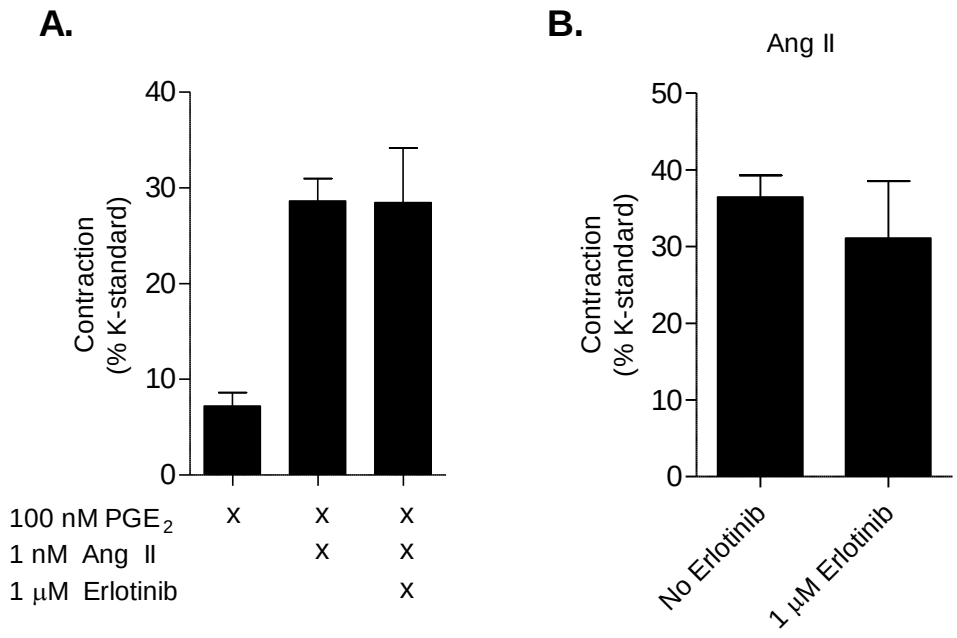

Figure 2.15 - EGFR does not contribute to Ang II potentiation of PGE_2-mediated vasoconstriction. (A) PGE_2 responses with or without 1 nM Ang II priming as compared to priming in the presence of erlotinib. (N = 3) (B) Ang II-mediated vasoconstriction with or without erlotinib. No significant differences. (N = 3)

Endothelial dysfunction does not explain Ang II potentiation of PGE_2-mediated contraction

One of the detrimental effects of Ang II on the vasculature *in vivo* is endothelial cell dysfunction that results in a loss of endothelium facilitated vasodilation. Because this vasodilation is often a counter balance for vascular contractions, I was interested in determining whether endothelial dysfunction was sufficient to unmask PGE_2-mediated vasocontraction. For these experiments femoral arterial rings were primed with 100 μM L-NAME for 20 minutes prior to PGE_2 addition. L-NAME is a nonspecific nitric oxide synthase inhibitor that reversibly prevents endothelial cell mediated vasodilation. PGE_2 did not invoke vasoconstriction in the presence of L-NAME without Ang II priming (Fig. 2.16). Notably, Ang II potentiation of PGE_2-mediated vasoconstriction was also not significantly affected by L-NAME treatment (Fig. 2.16). These results indicated that in our studies endothelial cells do not affect the ability of PGE_2 to mediate contraction.

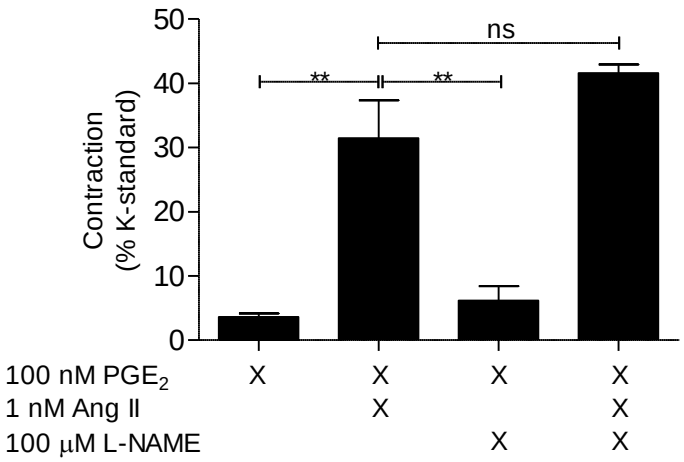

Figure 2.16 – Endothelial dysfunction does not affect Ang II potentiation of PGE_2-mediated vasoconstriction. 100 nM PGE_2 responses with or without 1 nM Ang II priming as compared to Ang II priming in the presence of 100 µM L-NAME or to PGE_2 responses from arterial rings primed with L-NAME instead of Ang II. (N = 3) ns = not significant, **= P<0.01

Extracellular calcium stores and Rho-kinase are required for contraction

The contribution of extracellular stores of calcium to Ang II-induced potentiation of PGE_2-mediated vasoconstriction was assessed by analyzing vessels in Ca^{2+} free buffer. Arterial rings were incubated in calcium free Krebs buffer containing 1 mM ethylene glycol-bis tetraacetic acid (EGTA). EGTA was subsequently removed from the bath, and the contractile response of Ang II-primed vessels to PGE_2 was determined. In control vessels without Ang II pre-treatment, 100 nM PGE_2 did not cause significant contraction in normal Krebs or upon addition of higher calcium concentrations to the bath (3.5 or 4.5 mM), demonstrating that increasing extracellular calcium concentration is not sufficient to evoke potentiated PGE_2-mediated contraction (Fig. 2.17A). In the absence of extracellular Ca^{2+}, femoral arteries exhibited minimal responsiveness to Ang II. Furthermore, Ang II did not potentiate PGE_2-induced contraction in Ca^{2+} free media. However, re-addition of Ca^{2+} into the bath restored PGE_2-mediated contraction (Fig. 2.17B, C). Pre-treatment (30 minutes) of arteries with the L-type calcium channel blocker, nifedipine, inhibited the PGE_2-mediated contraction (Fig. 2.17C, far right bar). Collectively, these studies indicate a significant contribution of extracellular sources of calcium, particularly from calcium influx by L-type channels, in mediating PGE_2-induced contraction after priming with Ang II. Due to its known role in sensitizing smooth muscle cells to calcium-induced contraction, ROCK was evaluated for its effect on PGE_2-induced vasoconstriction of Ang II-primed vessels. Addition of the ROCK inhibitor Y-27638 to PGE_2-induced vasoconstriction returned vascular tone to baseline (Fig. 2.17D & E). These data indicate that ROCK is required for EP3-induced arterial contraction.

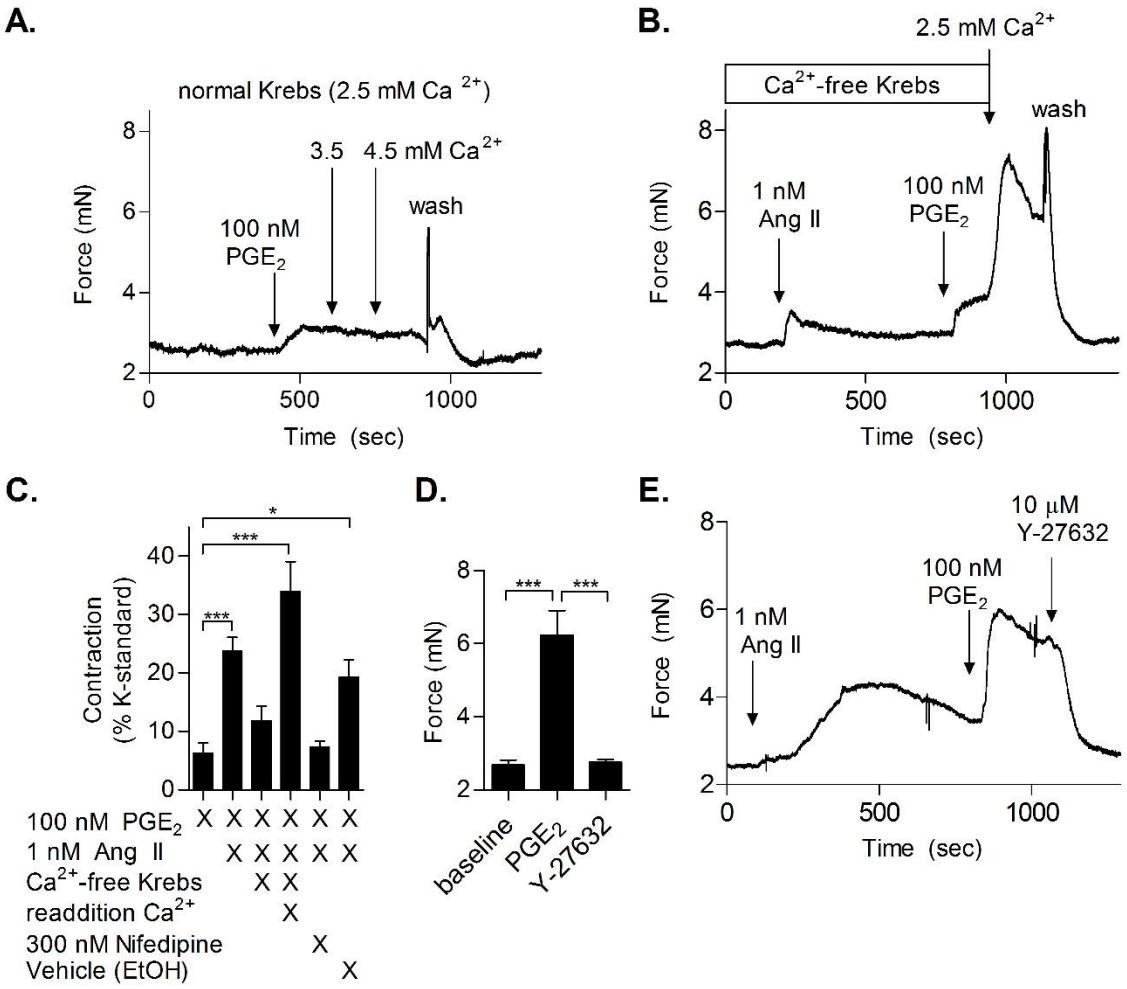

Figure 2.17 - Contribution of extracellular calcium and Rho-kinase to PGE_2 primed vasoconstriction**.** (A) Representative wire myography trace (N = 5) of PGE_2-mediated contraction with additional calcium added (normal Krebs 2.5 mM, additional calcium 3.5 or 4.5 mM). (B) Example trace of femoral arterial ring maintained in calcium-free Krebs. Vessels were briefly treated with EGTA, washed, and subsequently administered Ang II, followed by PGE_2. Calcium was then added back to the bath to restore contraction. (C) Quantification of PGE_2 response with and without Ang II priming, in the presence or absence of calcium, compared to vessels pre-incubated with 300 nM nifedipine or vehicle control (ethanol) (N = 5-8). (D) Quantification of the effects of the ROCK inhibitor Y-27632 on PGE_2-mediated contraction (N = 5). (E) Example trace of Ang II priming of PGE_2-induced contraction with ROCK inhibitor, Y-27632, addition during PGE_2 response.[275] * = $P < 0.05$, **= $P < 0.01$, ***= $P < 0.001$

Chloride channels are involved in contraction induced by Ang II and PGE_2

Along with determining the contributions of calcium to vasoconstriction, further studies were completed to assess the contributions of intracellular chloride. Chloride levels within smooth muscle cells (SMC) are typically higher than extracellular levels; consequently, SMCs depolarize upon efflux of chloride through chloride channels resulting in activation of voltage-dependent calcium channels resulting in contraction.[290] To assess the contribution of different chloride channels in Ang II potentiation of PGE_2-mediated vasoconstriction, vessel rings were pre-treated with either DCPIB, an inhibitor of volume-sensitive anion channels, or niflumic acid (NFA), an inhibitor of calcium-activated chloride channels.[291] NFA inhibited PGE_2-mediated contraction potentiated by Ang II ($P < 0.05$, Fig. 2.18A). Ang II-mediated contraction was not observed with vessel rings treated with NFA ($P < 0.1$, Fig. 2.18B). DCPIB treatment also blunted Ang II potentiation of PGE_2-mediated vasoconstriction ($P < 0.1$, Fig.2.18A). Although reporting these results with a 95% confidence interval would require increasing the sample size, these preliminary studies (N = 3-4) indicated that chloride channels may contribute to the enhanced PGE_2-mediated vasoconstriction along with the calcium response.

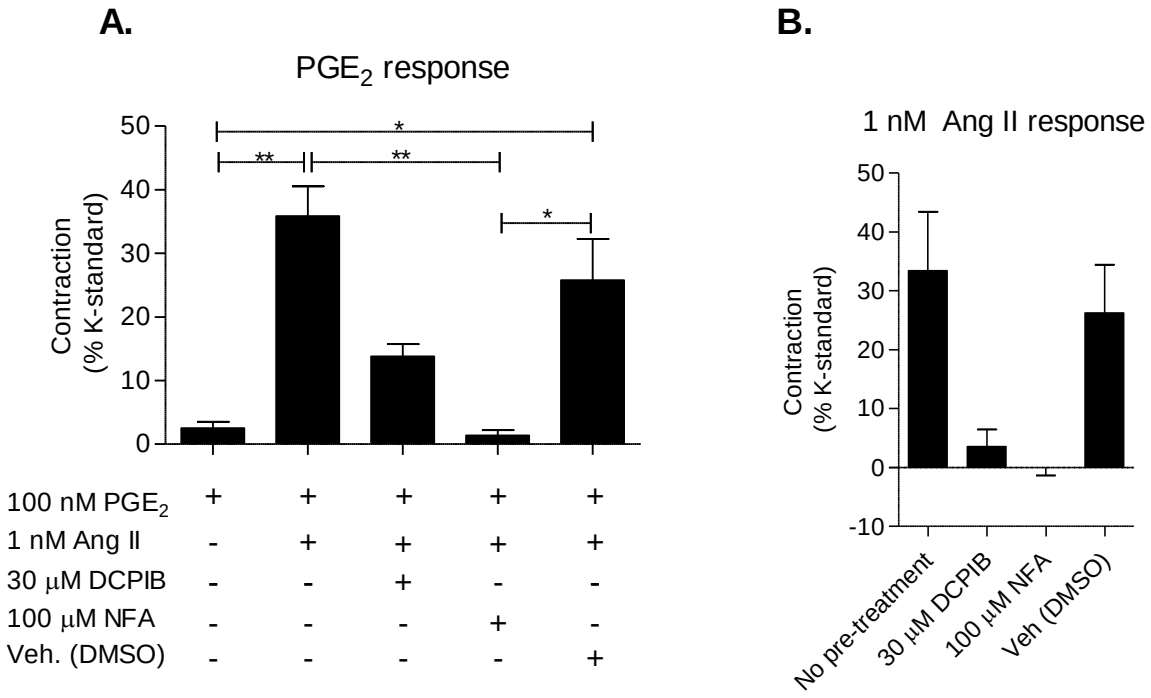

Figure 2.18 - Chloride channel contributions to vasoconstriction induced by PGE_2 and Ang II. (A) PGE_2 responses with or without 1 nM Ang II priming as compared to priming in the presence of DCPIB or NFA. (N = 3-4) (B) Ang II-mediated vasoconstriction with or without DCPIB or NFA. Asterisks indicate significance of at least $P < 0.05$ (N = 3-4, ns) *= $P < 0.05$, **= $P < 0.01$

Salicylate diminishes Ang II potentiation of PGE_2-mediated contraction

To determine whether Ang II potentiates PGE_2-mediated vasoconstriction through its action on $cPLA_2$, resulting in increased prostaglandin biosynthesis, femoral arterial rings were pre-incubated with nonsteroidal anti-inflammatory drugs (NSAID). 100 µM ibuprofen, a potent COX inhibitor (COX-1 IC_{50} = 7.6 µM, COX-2 IC_{50} = 7.2 µM) [292], did not affect PGE_2-induced constriction of Ang II-primed vessels (Fig. 2.19 A, $P>0.05$), nor did 100 nM SC-236 (COX-1 IC_{50} = 17.8 µM, COX-2 IC_{50} = 10 nM) [293], a COX-2 specific inhibitor. However, 10 mM salicylate, an active metabolite of aspirin and poor COX enzyme inhibitor (COX-1 IC_{50} = 5 mM, COX-2 IC_{50} = 0.5-34 mM) [292], significantly attenuated PGE_2-mediated vasoconstriction of Ang II-primed vessels (Fig. 2.19 A, $P<0.01$). Pretreatment with 100 µM – 10 mM salicylamine, a structurally similar compound to salicylate and identified isoketal scavenger, did not inhibit PGE_2-mediated vasoconstriction (Fig. 2.19 A & B, $P>0.05$).[294] Inhibition of contraction by salicylate but not ibuprofen or SC-236, suggests that the salicylate effect was a result of a non-COX mechanism.

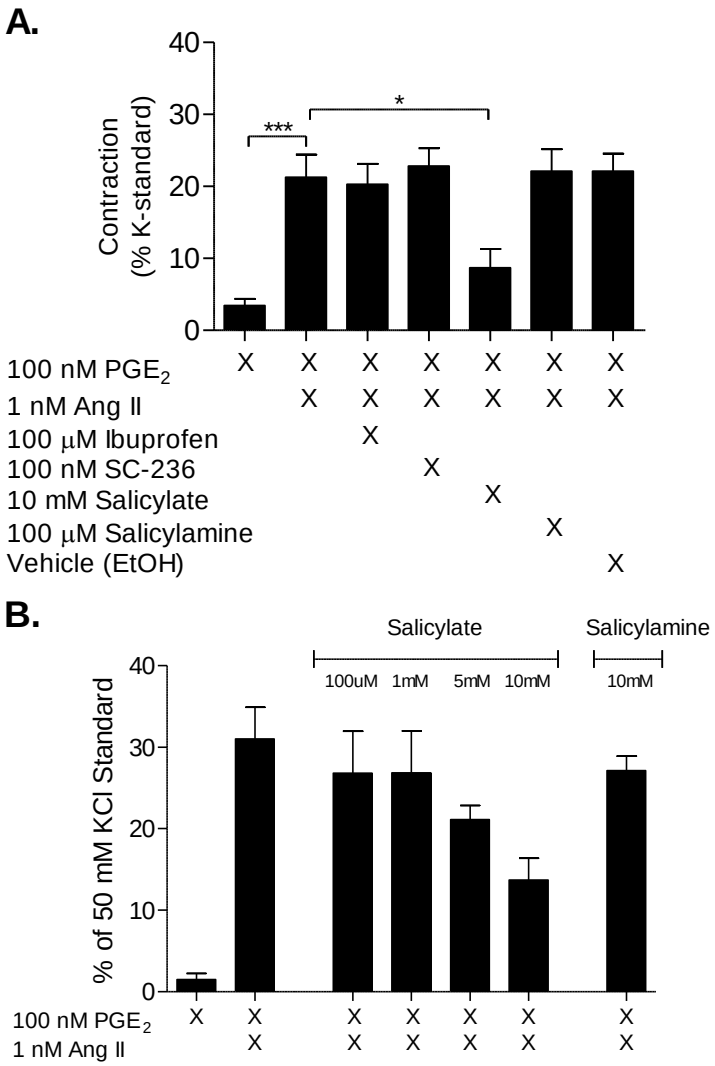

Figure 2.19 – Contribution of endogenous prostaglandin production. (A) PGE_2-induced contraction of femoral arterial rings pretreated for 30 minutes with the indicated concentrations of ibuprofen, SC-236, salicylate, salicylamine, or control solvent for ibuprofen/SC-236 (EtOH) followed by priming with Ang II and subsequent addition of PGE_2 (N = 7-10). Salicylate attenuated PGE_2-induced constriction of vessels primed with Ang II ($P<0.05$ 1way ANOVA).[275] (B) Varying doses of salicylate and salicylamine to determine whether concentration differences had an effect on vascular function. Salicylamine did not inhibit PGE_2-induced contraction even at 10 mM (far right bar). * = $P < 0.05$, ***= $P < 0.001$

Effects of proline-rich tyrosine kinase 2 inhibitors

Salicylate has been shown to inhibit the non-receptor tyrosine kinase Pyk2. [115, 295] To test whether salicylate exerted its effect by inhibition of Pyk2, the Pyk2 inhibitor PF-431396 was tested. PF-431396 abolished PGE_2-induced contraction of Ang II-primed vessels (Fig 2.20). Since Pyk2 is structurally similar to focal adhesion kinase (FAK), arteries were pre-treated with a FAK specific inhibitor, 10 μM FAK inhibitor 14, to determine the contribution of FAK (Fig 2.20). The FAK inhibitor did not affect Ang II priming of PGE_2, indicating that Pyk2 underlies PGE_2-mediated contraction. Collectively, these findings demonstrate that Ang II potentiates vasoconstriction induced by PGE_2 via convergent signaling between the AT1 and EP3 receptors. These convergent events occur through a Ca^{2+}-Pyk2-ROCK mechanism and are independent of β-arrestin signaling and AT1-mediated activation of PLA_2 (Fig. 2.21).

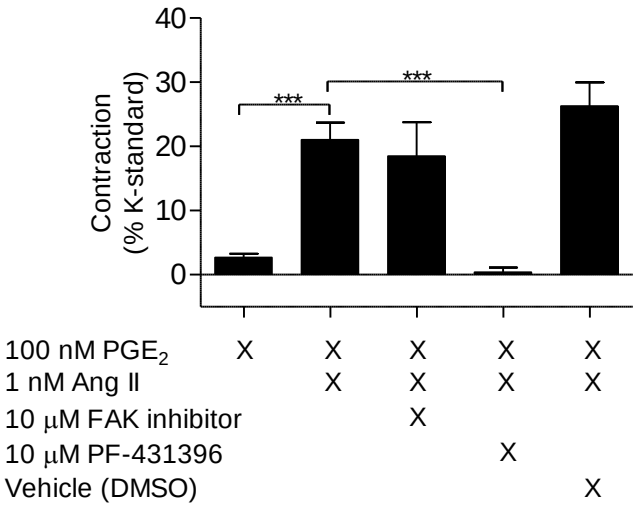

Figure 2.20 - Pyk2 is necessary for PGE_2-mediated contraction of Ang II-primed vessels. Preincubation with the Pyk2 inhibitor PF-431396 blocked PGE_2-induced contraction of Ang II-primed vessels, while inhibiting FAK with FAK inhibitor 14 did not affect contraction (N = 5). Vehicle represents control solvent for PF-431396 (N = 5). Vertical bars indicate SEM.[275] ***= $P < 0.001$

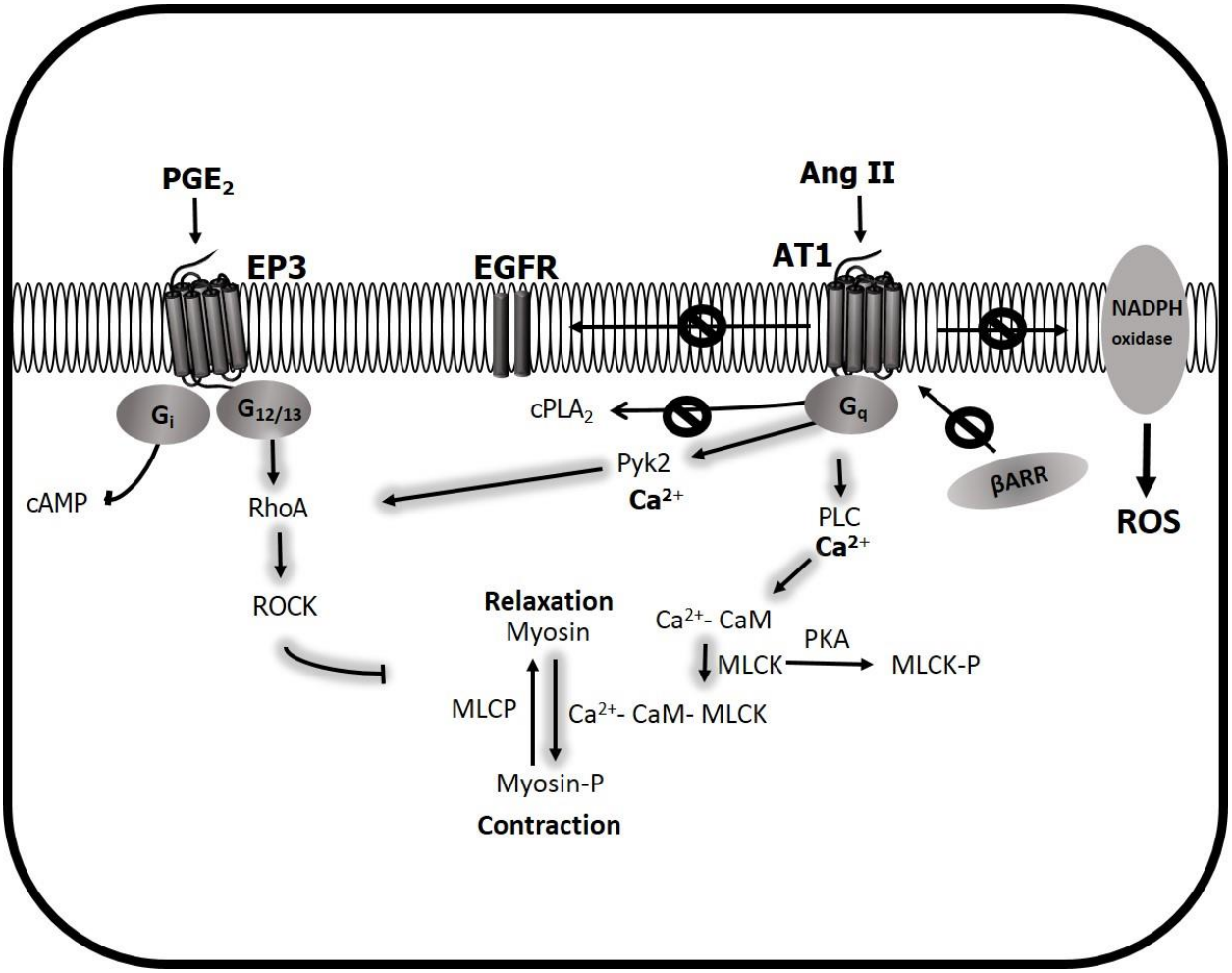

Figure 2.21 - Schematic of key proteins involved in PGE_2-induced contractile responses. Prior activation of the AT1a receptor by Ang II facilitates vasoconstriction via a PGE_2 - EP3 receptor pathway. PGE_2-induced vasoconstriction is dependent upon signaling through a Ca^{2+}-Pyk2-ROCK cascade, as opposed to other defined pathways such as activation of phospholipase A_2, EGFR, NADPH oxidase, or signaling via β-arrestin. EP3 and AT1 signaling converges through Pyk2-ROCK to sensitize smooth muscle cells to calcium and thus trigger a significant contractile response.

Discussion

In the present study, we demonstrated that PGE_2 functions as a vasoconstrictor in femoral arteries pretreated with low-dose Ang II. In contrast, PGE_2 was shown to have no detectable effect on vascular tone in the absence of priming by Ang II. These findings reveal a novel interaction between Ang II and PGE_2 in regulating peripheral vascular reactivity. Other constrictors may also potentiate EP3 receptor-induced contraction, indicated by published results with rat femoral arteries primed with thromboxane or phenylephrine [221], Ang II is of particular interest due to its central role in the development of cardiovascular disease. One contributing factor of high blood pressure is increased peripheral vascular resistance.[296] Increased formation of vasoconstrictors and altered effectiveness of vasodilators may underlie such impairments. PGE_2 has been demonstrated to function primarily as a vasodilator and vasodepressor when intravenously infused into many species, including humans, dogs, rabbits, mice and rats; however, in certain physiologic settings PGE_2 acts as a vasoconstrictor.[161, 169, 170, 183, 225, 297-300] Our studies indicate that in the presence of Ang II, PGE_2 exhibits vasoconstrictor effects in mouse femoral arteries. Because Ang II and PGE_2 are well known to regulate peripheral vasculature, contractile responses mediated by concurrent signaling by Ang II and PGE_2 may contribute to vascular dysfunction associated with hypertension.

The Ang II concentration of 1 nM used for priming femoral arteries in our studies is greater than typical reported plasma concentrations of Ang II, which have been measured to be in the pM range [301, 302]. However, evidence indicates that tissue sources of Ang II may contribute more to vascular contractions than circulating Ang II, and interstitial levels of Ang II have been detected in the nM range in femoral arteries and

kidneys.[303, 304] Therefore, our priming concentration of Ang II, which produced only a modest contractile response prior to PGE_2 administration (Fig. 2.2), may approximate levels observed by AT1 receptors *in vivo* and could thus be relevant to vascular responsiveness.

Studies presented here demonstrate that the EP3 receptor is required for PGE_2-mediated vasoconstriction. We found that PGE_2-induced vasoconstriction following Ang II priming was lost upon loss or blockade of the EP3 receptor. Previous studies indicate that $EP3^{-/-}$ mice have lower mean arterial pressure (MAP) in response to acute or chronic Ang II infusions.[175] The observed changes in vascular reactivity described in the present studies may contribute to the reduction in MAP in response to Ang II observed in $EP3^{-/-}$ mice.[175] Of note, femoral arteries from $EP3^{-/-}$ mice exhibited Ang II concentration response curves similar to wildtype femoral arteries, suggesting the alterations in vascular reactivity to PGE_2 observed in $EP3^{-/-}$ femoral vessels are not due to a loss of Ang II responsiveness. In contrast, a previously reported study investigating the effect of EP3 receptor signaling on mouse mesenteric vasculature demonstrates that Ang II-mediated contraction was diminished by knockout of the EP3 receptor.[175] Considering these findings, our data suggest differences in the interactions between Ang II and PGE_2 signaling that are specific to particular vascular beds. Additionally, differences in the steepness of the Ang II dose response curve was noted in the $EP3^{-/-}$ vessels indicating that the EP3 receptor may contribute to Ang II binding to the AT1 receptor. More studies would need to be done to determine the effects of the EP3 receptor in regulating vascular sensitivity to Ang II.

Our findings support a model, diagrammed in Figure 2.21, in which activation of the AT1 receptor by Ang II sensitizes smooth muscles cells to calcium, thereby facilitating a contractile response to PGE_2. This vasoconstriction does not require AT1-mediated production of endogenous prostaglandins or β-arrestin scaffolding. Importantly, our studies identified Pyk2 as a tyrosine kinase crucial for PGE_2-induced contraction. This finding aligns well with previous investigations of Pyk2 function. Pyk2 is a calcium-dependent tyrosine kinase that acts upstream of the RhoA/ROCK pathway.[115, 295] Pyk2 can be activated by Ang II and plays a role in the ability of Ang II to mediate many cellular functions, including vasoconstriction.[115, 305] The EP3 receptor has been identified to mediate contractile responses through activation of ROCK.[175, 221, 222] Therefore, Ang II signaling may activate Pyk2, thereby sensitizing the ROCK pathway to EP3 signaling (depicted in Figure 2.21); alternatively, EP3 and AT1 signaling may concurrently converge through a Pyk2-RhoA-ROCK pathway. Activation of ROCK would ultimately lead to inhibition of myosin phosphatase and enhance Ca^{2+} sensitivity of myosin filaments. The inhibition of Ang II priming of PGE_2-mediated contraction observed with salicylate and the specific Pyk2 inhibitor (PF-431396) may also explain some of the beneficial effects of salicylate on the cardiovascular system.

Although the present studies focused on peripheral vascular reactivity, the observed interaction between the EP3 and AT1 receptors may be important in other vascular beds (Fig. 2.5), tissue types, and physiologic events associated with hypertension. For example, studies using rats have indicated that intracerebroventricular infusion of PGE_2 results in a rise in blood pressure and heart rate.[306] Interestingly, these responses were exacerbated in spontaneously hypertensive rats (SHRs), a hypertension

model known to have elevated brain levels of Ang II.[307] These findings suggest a potential interaction of Ang II and PGE_2 in the central nervous system that might affect blood pressure. Future studies investigating additional mechanisms through which the EP3 and AT1 receptors produce synergistic responses and how these interactions relate to the pathology of hypertension may reveal novel therapeutic strategies for treatment.

CHAPTER III

ANG II EFFECTS ON PGE_2-MEDIATED BLOOD PRESSURE RESPONSES

Introduction

Prostaglandin E2 (PGE_2) is a prostanoid that normally acts as a systemic vasodepressor when infused into mice. In wildtype mice a 100 µg/kg intravenous bolus of PGE_2 exhibits a transient depressor effect that last several minutes before blood pressure is fully restored to baseline mean arterial pressure (MAP). This vasodepressor effect can be attributed primarily to PGE_2-mediated activation of EP2 receptors. PGE_2 intravenous infusion into mice lacking the EP2 receptor induces a pressor response.[169, 298] This pressor response is attributed to activation of EP3 receptors.[183] From studies conducted using EP receptor knockout mice, complex blood pressure regulating effects of PGE_2 have been revealed. For example, $EP2^{-/-}$ mice are more susceptible to dietary salt induced hypertension[169], and knockout of either vasopressor receptor, EP1 or EP3, results in salutary effects against angiotensin II (Ang II)-induced increases in blood pressure.[174, 175] Although studies of EP receptor knockout mice have revealed some of the physiological and pathophysiological roles of these receptors in blood pressure regulation, there are no studies that have examined altered PGE_2 responses in wildtype mice under different physiological conditions.

The vessel reactivity studies presented in Chapter II indicated that PGE_2 exhibits contractile responses in wildtype mice when the arterial tissue is primed with Ang II. However, the relevance of Ang II synergism with PGE_2 regulating vascular reactivity to

the physiology of the whole animal remains incompletely characterized. Thus, it was hypothesized that Ang II in wildtype mice would alter the vascular responsiveness to PGE_2 resulting in a vasopressor effect upon PGE_2 intravenous infusion. Furthermore, since *ex vivo* vascular results presented in Chapter II indicated the involvement of the EP3 receptor in PGE_2-induced contraction, it was hypothesized that PGE_2 vasopressor effects would be dependent on the EP3 receptor.

Experimental Procedure

Animals

All studies were performed in accordance with the National Institutes of Health animal care standards and approved by the Vanderbilt University Institutional Animal Care and Use Committee. Male C57BL/6 strain wildtype or $EP3^{-/-}$ mice, 12 to 15 weeks old, were used for all studies.

Intracarotid Blood Pressure Measurements

Mice were anesthetized using ketamine (100 mg/kg) and inactin (100 mg/kg) via intraperitoneal injections. Mice were positioned onto a heating pad and a PE10 indwelling catheter was secured into the left carotid artery. The catheter was connected to a pressure transducer (Digi-Med TXD-310, Micro-Med) and blood pressure was measured using a blood pressure analyzer (Digi-Med BPA 400, Micro-Med). For drug infusions, a PE50 indwelling catheter was secured into the right jugular vein and attached to a blunt needle of a 1 mL saline syringe. Drug infusion rates were controlled by a Genie Plus Syringe Pump (Kent Scientific). For experiments in which two drugs were simultaneously

infused, a bifurcated indwelling catheter attached to separate syringes regulated by individual syringe pumps was used. To improve respiration and survival during blood pressure recordings, tracheotomy was performed by inserting a 1 inch in length PE60 tube into an incision between two superior tracheal cartilages down into the trachea. Immediately after surgeries, mice were equilibrated for 45 minutes until stable MAP values were attained. For continuous intravenous infusions, drugs were injected at a rate of 5 µl/min. For bolus injections, the jugular catheter was able to hold 50 µl without going into the vein. Drugs were slowly injected into the catheter by hand and then attached to a continuous pump of saline at a controlled rate of 50 µl/min to infuse the bolus into the mouse. All drugs were diluted into sterile saline.

Mouse model of chronic Ang II hypertension

Micro-osmotic pumps (alzet, model 1002) filled with either saline or Ang II (300ng/kg/min) were inserted subcutanously into wildtype mice 14 days prior to intracarotid blood pressure (ICBP) recordings. Tail cuff blood pressure measurements were collected throughout the 14 days leading up to ICBP experiments. Two different protocols were used for ICBP on the osmotic pump treated mice. Protocol 1 was conducted as described above (*Intracarotid Blood Pressure Measurements*). Protocol 2 included additional steps to ensure mice did not experience fluid loss during ICBP surgery. To prevent fluid loss, mice were administered saline (5 µl/min) as a continuous intravenous infusion through the jugular catheter during the 45-minute post-surgical MAP equilibration period, and saline was added to the exposed tissue at the surgical site of the throat and covered with Parafilm in which an opening was cut to allow for projection of the tracheostomy tube.

Wire myography with isolated femoral arteries

Methods were performed as described in Chapter II.

Results

PGE_2 blood pressure effects are unaltered by acute continuous infusion of Ang II

In order to determine whether Ang II would alter PGE_2-mediated vasodepressor effects *in vivo*, we first determined whether intravenous infusion of PGE_2 along with Ang II would alter PGE_2-induced blood pressure changes. Figure 3.1 depicts the experimental protocol used for these studies. Briefly, continuous infusion of Ang II or saline was administered for 30 minutes prior to infusing a bolus of vehicle control (saline) followed by a bolus of 100 µg/kg PGE_2. Infusion of the vehicle control did not alter blood pressure responses in any of the mice; however, infusion of Ang II did raise the baseline blood pressure (Fig. 3.2 A, B). PGE_2 typically induces a transient vasodepressor response lasting about 180 seconds that is similar to responses observed with the vehicle control mice (Fig. 3.2A).[169] In mice that received continuous infusion of Ang II (75 pmol/kg/min), PGE_2 also induced a vasodepressor response (Fig. 3.2B). Of note, the PGE_2 response in combination with Ang II exhibited a quicker recovery to baseline that was followed by a gradual decline in blood pressure over time (Fig. 3.2B). For comparison, in an additional cohort of wildtype mice, the α-adrengeric receptor agonist phenylephrine (100 µg/kg/min) was continuously infused to determine whether another contractile agent, which was previously shown in *ex vivo* studies to prime the vasculature to PGE_2-induced vasoconstriction, would alter PGE_2 blood pressure effects. Although phenylephrine did

raise baseline blood pressure (MAP = 120 mmHg, Fig. 3.2, C), it did not cause PGE_2 to exhibit vasopressor responses either (Fig. 3.2C).

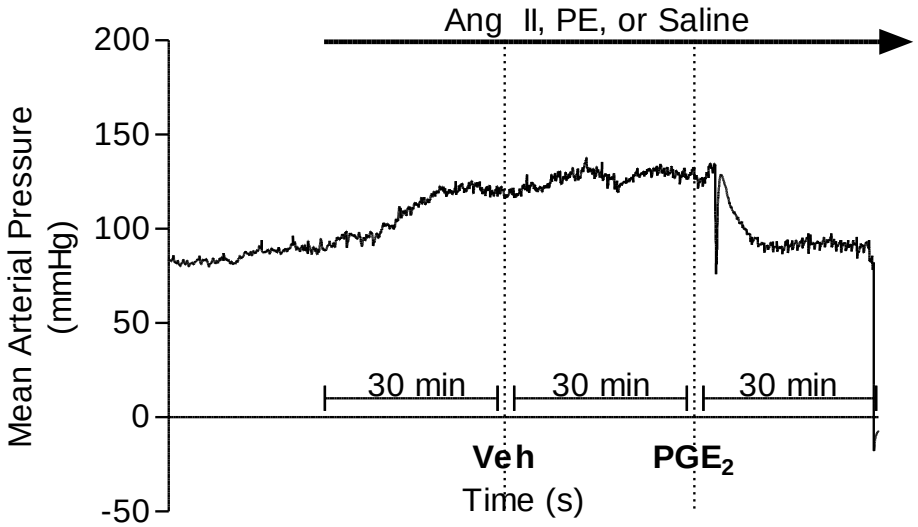

Figure 3.1 – Experimental design of ICBP studies investigating the effects of acute intravenous infusion of PGE_2 in combination with other vasoconstrictors. Wildtype mice were given intravenous continuous infusion of either saline (5 µl/min), Ang II (75 pmol/kg/min), or phenylephrine (PE) (100 µg/kg/min). After 30 minutes of continuous infusion, a bolus of vehicle (saline) followed by a bolus of PGE_2 (100 µg/kg) was infused without interfering with the continuously infused drug. Arrow indicates continuous infusion time and dotted lines indicate bolus infusion time-points. (N = 3)

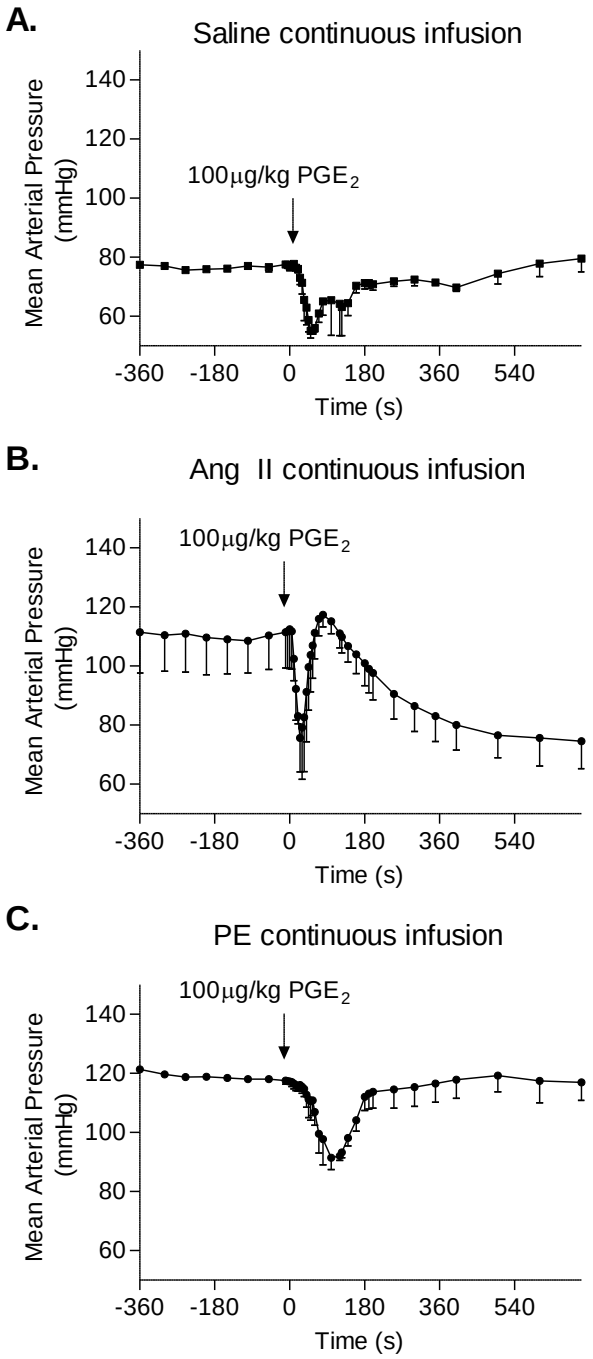

Figure 3.2 – PGE_2 acts a transient vasodepressor when infused during continuous infusion of other vasoconstrictors in wildtype mice. MAP recording after PGE_2 (100 µg/kg) bolus intravenous infusion during (A) saline (5 µl/min), (B) Ang II (75 pmol/kg/min), or (C) PE (100 µg/kg/min) continuous infusion. Arrows indicate PGE_2 infusion time. Vertical bars below each data point indicate SEM. (N = 3)

PGE_2 is a vasodepressor in a chronic Ang II mouse model of hypertension

Since acute Ang II treatment did not appear to alter PGE_2 blood pressure responses, I sought to determine whether a longer exposure to Ang II would produce an effect. For these studies, a chronic Ang II pump model of hypertension was used in which wildtype mice were implanted with a micro-osmotic pump that slowly diffused Ang II subcutaneously over two weeks to induce hypertension. This Ang II model of hypertension has been reported to closely simulate the onset of human hypertension.[308, 309] Mice that were implanted with an Ang II pump develop a slow rise in baseline blood pressure that was observed to reach a maximum (30 % rise in SBP) by 9 days after pump implantation whereas baseline blood pressure in vehicle control mice displayed modest changes (< 10 % change in SBP, Fig. 3.3). After 14 days of Ang II or saline infusion, ICBP was recorded while infusing an intravenous bolus of PGE_2. PGE_2-evoked an immediate and sustained pressor response in mice that had an Ang II pump implanted (Fig. 3.4A) and a rapid (60 seconds) depressor effect in saline pump treated mice (Fig. 3.4B).

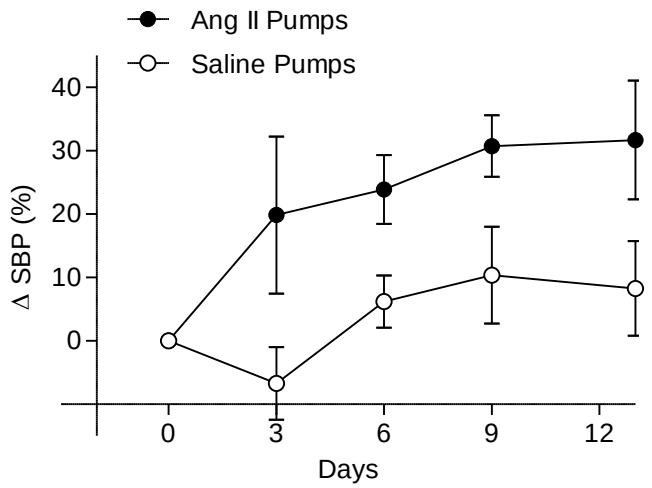

Figure 3.3 – Mice given subcutaneous slow-pressor doses of Ang II develop high blood pressure. Systolic blood pressure (SBP) over time (days) in wildtype mice implanted with micro-osmotic pumps filled with either saline (white circles) or 300 ng/kg/min of Ang II (black circles). (N = 7) t test of means = *P < 0.05. Vertical bars represent SEM.

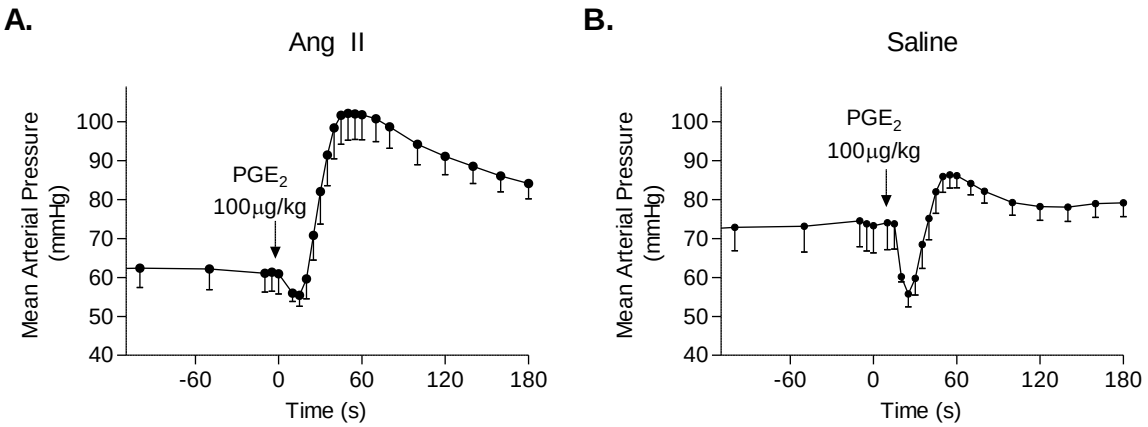

Figure 3.4 – Chronic Ang II effects on PGE_2-mediated blood pressure responses. (A) MAP recording during an intravenous bolus of PGE_2 (100 µg/kg) into mice that previously were implanted with a subcutaneous micro-osmotic pump filled with either Ang II (300ng/kg/min) or (B) vehicle control (saline) for 2 weeks. Arrows indicate PGE_2 infusion time. Vertical bars below data points indicate SEM. (N = 5-7)

Due to significant mortality during the initial studies, optimization of the ICBP surgical method (protocol 1) was performed in an attempt to obtain more consistent, reliable results. The average baseline MAP for mice in our initial experiments was 66 mmHg; thus, mice appeared to be hypovolemic and/or dehydrated after surgical procedures. To ensure the mice were euvolemic and healthy after surgery, mice were administered a continuous intravenous infusion of saline (5 µl/min) throughout the 45-minute MAP equilibration period, and the surgical site was coated in saline to prevent dehydration from the exposed tissue. Another set of experiments were conducted using the new and improved protocol (protocol 2). The average baseline MAP for mice in these studies was 77 mmHg, and more mice survived the surgical procedure. However, the pressor response to PGE_2 originally observed in chronic Ang II mice was no longer present. Ang II mice that had undergone this new protocol exhibited a vasodepressor response to PGE_2 that resembled that of wildtype mice with the saline pump (lasting ~180 seconds) (Fig. 3.5A, B).

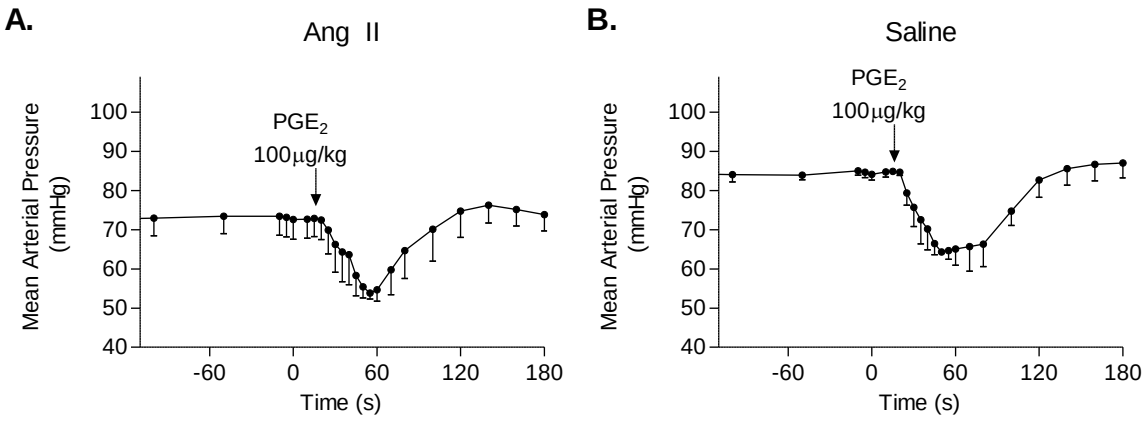

Figure 3.5 – ICBP experiments using the euvolemic protocol used to investigate chronic Ang II effects on PGE_2-mediated blood pressure responses. (A) MAP recording during an intravenous bolus of PGE_2 (100 µg/kg) into mice that were previously implanted with a subcutaneous micro-osmotic pump filled with either Ang II (300ng/kg/min) or (B) vehicle control (saline) for 2 weeks. Arrows indicate PGE_2 infusion time. Vertical bars below data points indicate SEM. (N = 4-5)

EP3$^{-/-}$ mice exhibit sustained vasodepressor effects to intravenously infused PGE_2

Contribution of the EP3 receptor was determined by testing PGE_2 blood pressure effects in EP3$^{-/-}$ mice. Ang II micro-osmotic pumps (300 ng/kg/min) were subcutaneously implanted into mice lacking the EP3 receptor. After 14 days of chronic infusion, MAP was recorded during infusion of an intravenous bolus of PGE_2. Using either ICBP protocols with or without saline infusion to prevent volume depletion on the EP3$^{-/-}$ mice, PGE_2 infusion resulted in a sustained vasodepressor response (Fig 3.6A, B).

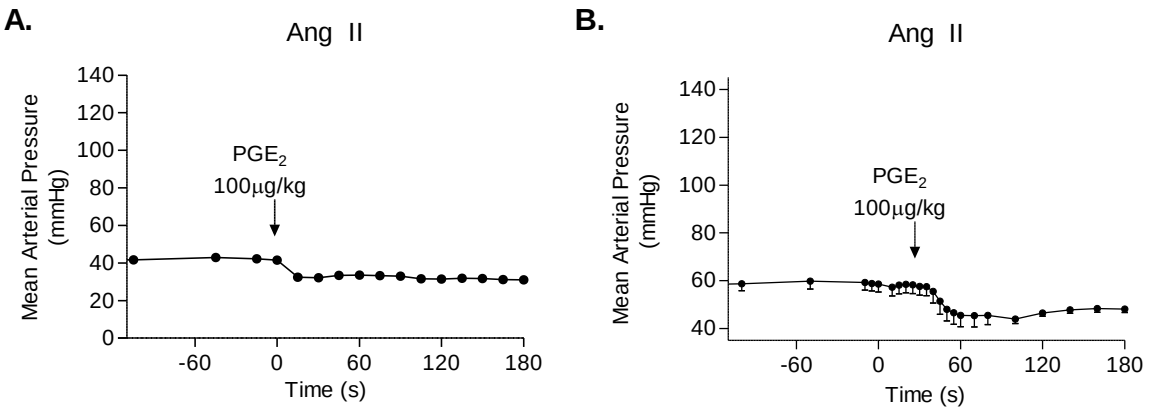

Figure 3.6 – PGE_2 is a vasodepressor in $EP3^{-/-}$ mice. (A) MAP reading during a bolus of PGE_2 (100 µg/kg) in $EP3^{-/-}$ mice that had a micro-osmotic pump of Ang II (300ng/kg/min) for 14 days and had either under gone protocol 1 or (B) protocol 2 for ICBP readings. (N = 1 for A, N = 4 for B)

Enhanced sympathetic tone in combination with Ang II may unmask PGE_2-mediated pressor responses

To investigate the basis of the different responses observed in the two protocols, I compared the heart rate data acquired during PGE_2 infusion between the two different protocols. Interestingly, Ang II osmotic-pump mice that had undergone protocol 1 responded with a significantly sustained elevation in heart rate concurrent with the rise in MAP observed upon PGE_2 infusion (Fig. 3.7A, B). However, mice that had undergone protocol 2 responded with a rise in heart rate that was only transient and coordinated with the depressor response induced by PGE_2 (Fig. 3.7C, D). In general, when an abrupt drop in arterial pressure is encountered by the cardiovascular system the autonomic reflex is stimulated that increases sympathetic activity and ultimately drives increases in heart rate. The heart rate response that occurred concomitantly with the MAP readings from mice that had undergone protocol 2, demonstrated a typical autonomic response (Fig. 3.7C, D). However, the significant rise in heart rate observed in mice that had undergone protocol 1 suggested that sympathetic tone may be amplified in these mice.

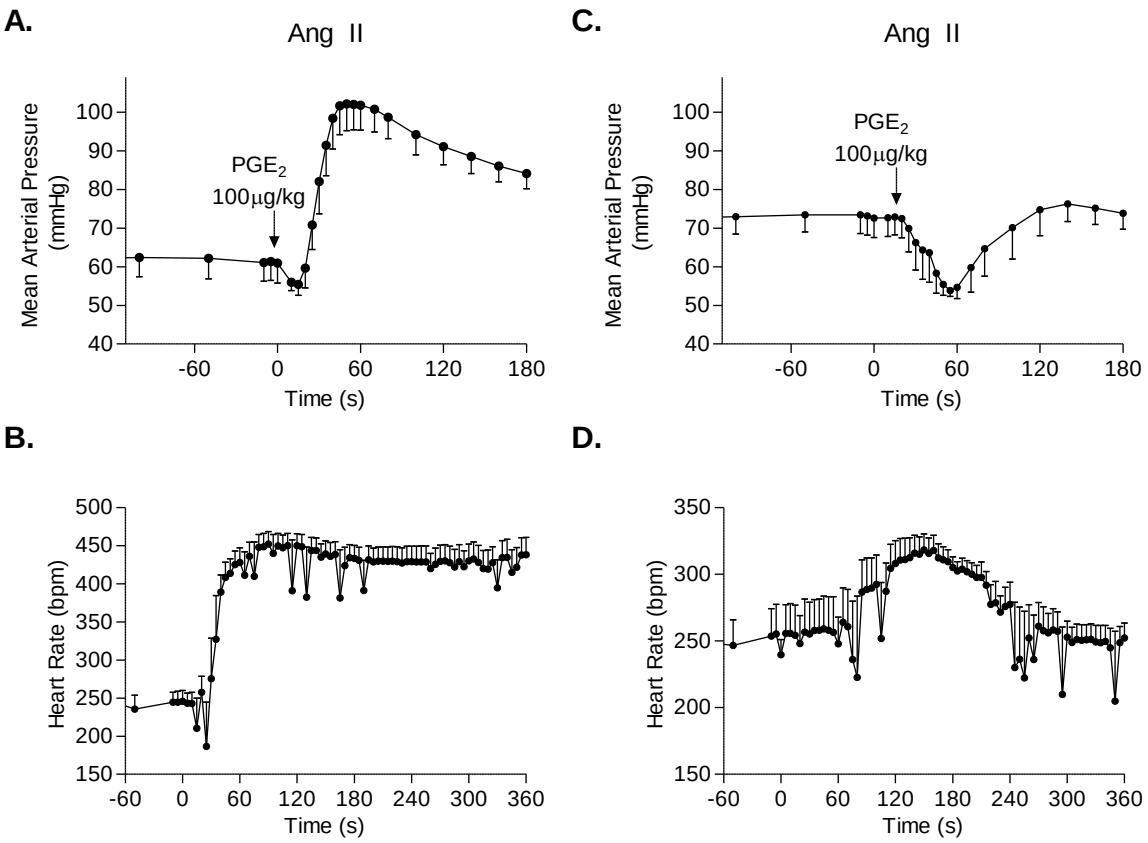

Figure 3.7 – Comparison of heart rate and MAP during PGE_2 bolus in mice undergoing continuous Ang II intravenous infusion between two different experimental ICBP methods. (A) Graph shown is the same depicted as Fig 3.3B in order to compare to (B) heart rate over time (N = 5-7). Data shown in A and B are from the same mice that had under gone protocol 1, where mice were thought to be dehydrated and volume depleted. (C) Graph shown is the same depicted as Fig 3.4B in order to compare to (D) heart rate over time (N = 4-5). Data shown in C and D are from the same mice that had under gone protocol 2, where mice were thought to be hydrated and had replete blood volume. Vertical bars indicate SEM (below data points for MAP data and above data points for heart rate data).

Endogenous catecholamine release does not contribute to Ang II potentiation of PGE_2-mediated vasoconstriction

Since the *in vivo* heart rate data suggested that PGE_2 vasodepressor responses could be altered to exhibit pressor effects under assumed amplification of autonomic tone, preliminary studies were conducted to analyze whether sympathetic activity contributed to Ang II potentiation of PGE_2-mediated vasoconstriction observed in experiments presented in Chapter II. Although wire myography studies are *ex vivo* experiments, femoral arteries excised from mice still retain nerve terminals embedded into the tissue. To determine whether release of catecholamines from these nerve terminals could explain PGE_2-mediated vasoconstriction, femoral arteries were pre-treated with 100 µM tyramine, a catecholamine releasing agent, to rid the vessel of residual vesicular catecholamines. The vessel bath was then washed several times before priming with 1 nM Ang II followed by addition of 100 nM PGE_2. Tyramine induces a slight contractile response presumably from catecholamine activation of adrenergic receptors; however, removal of tyramine and of released catecholamines from the bath did not inhibit Ang II potentiation of PGE_2-mediated vasoconstriction (Fig. 3.8A). In additional experiments, the irreversible adrenergic receptor antagonist, phenoxybenzamine (100 µM), was added to vessels during PGE_2-mediated vasoconstriction to which no effect on contraction was observed (Fig. 3.8B). Collectively, these preliminary results indicated that catecholamine release by Ang II or PGE_2 did not contribute to PGE_2-mediated vasoconstriction.

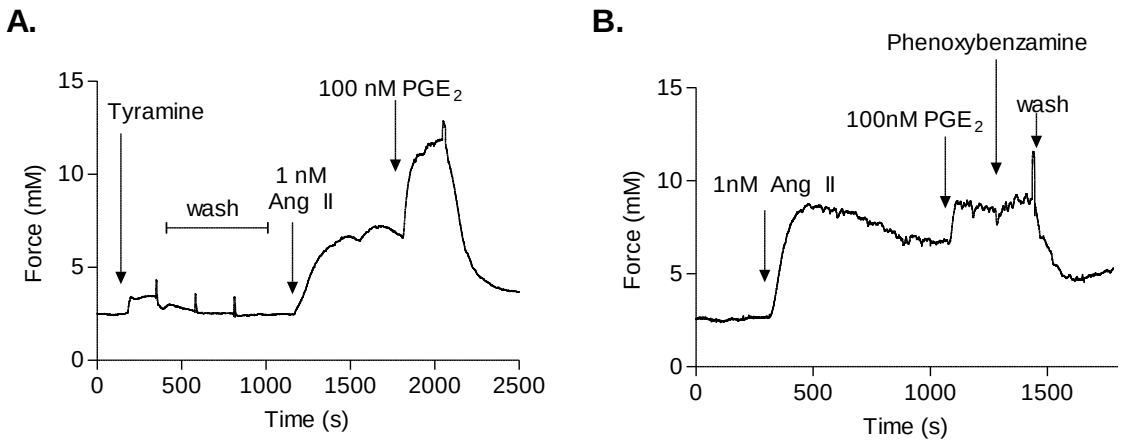

Figure 3.8 – Catecholamine signaling does not contribute to Ang II potentiation of PGE_2-mediated vasoconstriction in isolated femoral arteries. (A) Representative wire myography trace (N = 1) of PGE_2-mediated contraction on a femoral arterial ring pre-treated with 100 µM tyramine to release catecholamines and washed prior to 1 nM Ang II priming. (B) Example trace (N = 1) of a femoral arterial ring treated with 100 µM phenoxybenzamine during PGE_2-mediated vasoconstriction. Arrows indicate time at which drugs were added.

Discussion

While previous investigations have alluded to a balance between EP receptor signaling in facilitating subsequent hemodynamic physiological responses induced by PGE_2, in general, these conclusions have come from studies using EP receptor knockout mice. Here we investigated whether Ang II could alter the PGE_2 vasodepressor response to a vasopressor response in wildtype mice. Although in *ex vivo* femoral arteries Ang II was able to prime smooth muscle cells to PGE_2-induced vasoconstriction, *in vivo* studies did not indicate that acute or chronic Ang II caused PGE_2 to be a vasopressor in healthy male mice (Fig. 3.2 & 3.5). Ultimately, these studies were found to be more complex under *in vivo* conditions in which additional blood pressure regulatory mechanisms are intact. Ang II potentiation of PGE_2-mediated vasoconstriction may still occur *in vivo* and may contribute to blood pressure; however, other inputs such as the sympathetic nervous system, other circulating hormones, and sodium balance may have affected the overall blood pressure response observed in the presented studies.

Another complication with the blood pressure readings of the chronic Ang II model was that even though Ang II osmotic-pump treated mice exhibited hypertension via tail cuff readings, the baseline blood pressure was the same for mice that were treated with either an Ang II or saline osmotic pump once they had undergone anesthsia and surgery for intracartotid blood pressure readings. This loss of elevated blood pressure during/after surgery could be a result of the blood pressure lowering effects of anesthsia. Additional studies using mice that are fitted with a telemeter device and indwelling jugular catheters prior to PGE_2 infusions would be nessary to test whether unanesthetized mice would exhibit altered PGE_2 responses in the presence to Ang II.

Interestingly, PGE_2 did facilitate a robust pressor response in mice that were exposed to chronic Ang II whose cardiovascular system was compromised after ICBP surgical procedures (Fig. 3.4). Preliminary studies indicated that this pressor response was mediated by the EP3 receptor (Fig. 3.6). In initial experiments using protocol 1 mice were thought to be in a hypovolemic state since these mice exhibited tachycardia, tachypnea, low baseline MAP, and intense pressor responses to PGE_2. Hypovolemia was thought to develop as a result of blood loss during cannulation of the carotid artery and dehydration through exposed tissues after surgery. Although this was corrected in subsequent studies by giving mice intravenous saline supplementation, these earlier experiments provided a unique physiological state in which PGE_2 exhibited pressor responses. Although further studies would be required to confirm blood volume levels in these mice, interestingly, these findings suggest that during a state of low blood volume, PGE_2 could exhibit vasopressor qualities.

Hypovolemia is an emergency condition that is often present after trauma, severe burns, and hemorrhage. Clinical treatment involves stopping the source of blood volume loss along with transfusion of blood/fluids to regain homeostasis. The body's initial physiological response to hypovolemia is enhanced sympathetic activation to increase cardiac output and vasoconstriction in maintaining core pressure; however, with continued volume loss this mechanism is not enough to maintain pressure to vital organs leading to hypotension and cardiac arrest.[28] During these late phases of hypovolemic shock, vasopressor therapy may be beneficial to avert cardiac arrest and avoid reperfusion injury by lowering the amount of transfusion fluid required to restore blood pressure homeostasis.[310] Although this therapy is heavily debated in the clinic,

norepinephrine is commonly used and current investigations into more efficient pressor agents such as vasopressin are being conducted (NIH funded Clinical Trial NCT00379522).[311, 312] Based on our findings in chronic Ang II mice (Fig. 3.4), exogenous PGE_2 in combination with Ang II may provide beneficial vasopressor effects during life-threatening hypotension stages of hypovolemia. Certainly, our studies suggest that there may be physiological differences in hypertensive patients experiencing hypovolemic shock and that these findings merit further investigation.

Ang II and PGE_2 are known to play a role in the physiological response to hypovolemia. Previous studies investigating the effects of AT1 antagonists in experimental central hypovolemia have indicated that Ang II via AT1 receptor aides in the maintenance of MAP and vascular resistance.[313-315] In other studies using dogs with hemorrhagic-induced shock, intravenous infusion of arachidonic acid, the precursor to prostaglandins, led to attenuated lowering in MAP and increased prostaglandin levels.[316] In rats, PGE_2 and COX-2 have been identified as essential components to mediating intestinal ischemia during hemorrhagic shock, a compensatory vasoconstriction of the mesenteric and surrounding circulation to maintain central blood pressure.[317] The present study provides further evidence that PGE_2 may be a vasoconstrictor/pressor during conditions of shock. Additional investigations into the interaction of Ang II and PGE_2 in hypovolemic shock and into the beneficial versus detrimental effects of this interaction may unveil novel therapeutic strategies.

CHAPTER IV

FUTURE DIRECTIONS AND CONCLUSIONS

Future Directions

Although studies presented in Chapter II have indicated that Ang II and PGE_2 synergize to enhance contractile responses of peripheral vasculature, other tissue types where this synergism may be important are yet to be fully characterized. One region where such interactions may be particularly important for the regulation of blood pressure is in the central nervous system. Intracerebroventricular infusion of PGE_2 results in a rise in blood pressure that is exacerbated in spontaneously hypertensive rats.[306, 307] Additionally, a combination of Ang II and PGE_2 has been shown to reduce cerebral blood flow.[194] The receptors mediating these central cardiovascular effects are incompletely understood. EP1 receptors in the brain have been identified as crucial for Ang II-dependent hypertension.[192] However, the EP3 receptor is also expressed in the brain, and further studies are needed to determine its role in regulating central cardiovascular effects.[318]

Another current area of research is investigating the contributions of the immune system to the etiology of hypertension. Studies have indicated that hypertension is associated with immune cell infiltration into the vasculature, kidney, and heart.[319, 320] Additionally, immunosuppressants are protective against salt-sensitive hypertension in rats acutely pre-treated with Ang II.[319] In particular, T cells seem to be key to mediating Ang II-induced hypertension. RAG-$1^{-/-}$ mice, which lack B and T cells, exhibit blunted

hypertension in response to chronic Ang II infusion; furthermore, adoptive transfer of wildtype T cells into RAG-1$^{-/-}$ mice restores Ang II-sensitivity in these mice.[320] While these studies highlight the contribution of T cells to Ang II-induced hypertension, the signaling mechanisms which underlie such contributions are incompletely understood. In this regard, PGE_2, which has long been known to regulate inflammatory responses, may be critical. Preliminary studies done in collaboration with Dr. David Harrison's lab have shown that adoptive transfer of EP1/EP3 double knockout T cells into RAG-1$^{-/-}$ mice did not restore Ang II-sensitivity (data not shown). These results indicate that the EP1 and/or EP3 receptors may be involved in T cell-driven hypertensive effects in response to Ang II. Further investigations are needed to understand the role of EP receptor signaling in regulating T cell function and the effects of these functions on the pathophysiology of hypertension.

Although our *ex vivo* analyses indicated that concurrent Ang II and PGE_2 signaling induces peripheral vasoconstriction, simultaneous infusion of PGE_2 with Ang II *in vivo* resulted in vasodepression. One explanation for the discordance between our *ex vivo* and *in vivo* findings could be that the *in vivo* experiments were limited to studying the effects of an intravenous infusion of PGE_2. Ang II has long been known to have systemic effects; however, prostaglandins are primarily local autocrine and paracrine signaling effectors. Due to its quick first-pass metabolism, PGE_2 is not believed to be a physiological effector in the systemic circulation. Thus, while measuring the *ex vivo* response of specific tissues to PGE_2 and Ang II may be feasible, determining how such stimulation affects the contribution of individual tissue types to blood pressure *in vivo* remains a current challenge.

One approach to specifically study the potential vasopressor effects of PGE_2 *in vivo* may be to circumvent the PGE_2-mediated vasodepressor effects by measuring the blood pressure responses to infusion of PGE_2 with Ang II in $EP2^{-/-}$ mice, a model in which PGE_2 is known to induce vasopressor effects via the EP3 receptor.[169] Interestingly, PGE_2-mediated vasoconstriction in the afferent arteriole of $EP2^{-/-}$ mice is prevented by treatment with an ACE inhibitor.[235] This result indicates that Ang II may affect PGE_2-induced vasopressor responses in $EP2^{-/-}$ mice. Future studies using $EP2^{-/-}$ mice may provide an experimental model to characterize the role of Ang II in modulating PGE_2-mediated vasoconstrictor and vasopressor effects in specific tissue regions.

Despite the complications noted with our *in vivo* studies, one interesting finding was that in mice that were likely hypovolemic, PGE_2 administration after chronic Ang II infusion resulted in vasopressor responses (Fig. 3.4). Additional studies would be needed to further characterize the physiological state of these mice, including confirming the blood volume levels, blood osmolality state, and plasma levels of norepinephrine to determine whether these mice had enhanced sympathetic activation. However, these studies suggest that it would of interest to assess PGE_2-responses in an experimental model of hypovolemia, such as a hemorrhagic shock model, in which blood is withdrawn until MAP approaches 40 mmHg.[317] Since the heart rate data indicated that these mice may have enhanced sympathetic activation, future studies analyzing the contribution of sympathetic activity to PGE_2-induced vasopressor responses would be of interest. Studies comparing the effects of PGE_2 in hypovolemic mice treated with or without a ganglionic blocker, alternatively in a mouse model of overactive sympathetic activity, such as the hypertensive inbred mouse strain BPH/2J, could reveal the contribution of

sympathetic activity to PGE_2-mediated blood pressure effects.[321, 322] Collectively, these studies could have implications for a variety of disorders and complications, including with hypovolemia, hyperadrengeric postural orthostatic tachycardia syndrome, and sympathetically driven hypertension.[323-325]

One interesting condition that is marked by increased expression of mPGES resulting in elevated levels of PGE_2 and by high levels of renin and Ang II is hyperprostaglandin E syndrome (Barter syndrome).[330] Despite increased levels of both Ang II and PGE_2, patients with Barter syndrome lack a high blood pressure phenotype.[330] This lack of increased blood pressure is contrary to what would be predicted from studies described in Chapter II that indicated Ang II-priming of *ex vivo* femoral arteries potentiated PGE_2-induced contractions. However, these patients have also been shown to have a continuous loss of calcium that is partialy independent of elevated PGE_2.[331] Loss of calcium may affect the vascular responsiveness to concurrent Ang II and PGE_2. Although the blood pressure phenotype is absent, this rare and unique condition could provide a model for studying other key physiological functions affected by concurrent Ang II and PGE_2.

In Chapter II my studies indicated that PGE_2-induced vasoconstriction potentiated by Ang II priming occurs through concurrent signaling by the AT1 and EP3 receptors, resulting in activation of a Ca^{2+}/ROCK/Pyk2 calcium sensitization pathway. However, the relative contributions of AT1 signaling and EP3 signaling to induction of downstream mediators in this pathway remain an important topic of investigation. One limitation of our vascular reactivity studies was that many of the inhibitors (L-type Ca^{2+} channel blockers, ROCK inhibitors, and Pyk2 inhibitors) that blocked EP3-mediated contraction also

affected Ang II-mediated contractions. In attempts to study each response individually, experiments were proposed to add inhibitors either before or after Ang II priming but before PGE_2 addition. However, since the time after Ang II that PGE_2 exhibits contraction is short (10-20 min, Fig. 2.3) many of the drugs would not have enough incubation time on the vessel to confirm appropriate inhibition of specific targets.

It is also unlikely that the signaling mechanism (Ca^{2+}/ROCK/Pyk2) described in Chapter II is specific to Ang II. Many other vasoconstrictors have been identified by other investigators, as well as from our studies, to potentiate PGE_2-mediated contraction, including phenylephrine, vasopressin, KCl, and thromboxane receptor agonists (Fig. 2.6).[221] Additionally, Pyk2 has been identified to enhance contractile responses induced by a variety of G-protein coupled receptors, such as adrenergic receptors and vasopressin receptors.[326, 327] The evidence that a variety of contractile agents could affect PGE_2-mediated contraction implies that PGE_2 may contribute to vasoconstriction through this mechanism in numerous different physiological scenarios. More investigations would be necessary to determine whether Ca^{2+}/ROCK/Pyk2 signaling transduction mediates the PGE_2-induced vasoconstriction when vessels are primed by other contractile agents.

One commonality between all of the contractile agents identified thus far that potentiate PGE_2-mediated contraction, with the exception of KCl, is that they all activate G-protein coupled receptors known to couple to G_q. Other published work has described the convergence of the H_1 histamine G_q-coupled receptor with the $mGlu_4$ metabotropic glutamate G_i-coupled receptor through activation of phospholipase C leading to increased intracellular calcium mobilization.[328] A similar signaling mechanism may apply to the convergence of AT1 (G_q-coupled receptor) with EP3 (G_i-coupled receptor) and would

explain why many other G_q-coupled receptors can potentiate EP3-mediated contractions. Indeed, there is evidence that this mechanism may be involved in AT1/EP3 signaling: in studies conducted using human erythroleukaemic cells, the EP3-mediated signaling was shown to converge with thromboxane-induced signaling via this mechanism.[329] Further *in vitro* investigations into alterations in calcium mobilization occurring by Ang II or PGE_2 independently as compared to concurrent stimulation with both ligands, as well as distinguishing this calcium pathway from EP3-mediated cAMP inhibition and determining differences in phosphatidylinositol hydrolysis, would provide more information about whether the H_1/$mGlu_4$ mechanism would apply to AT1/EP3 signaling.[328]

Conclusions

This dissertation project sought to understand the regulation of PGE_2-mediated hemodynamic effects by Ang II. The major findings from this work were that a sub-contractile dose of Ang II can sensitize peripheral vasculature to PGE_2-mediated vasoconstriction. Potentiation of PGE_2-mediated vasoconstriction by Ang II was found to occur through convergent signaling by the AT1 and EP3 receptors of smooth muscle cells, resulting in calcium sensitization via a mechanism involving ROCK and Pyk2 kinases. These findings reveal a novel interaction between the EP3 and AT1 receptor in regulating vascular reactivity. Other contractile agents were also found to facilitate PGE_2-mediated contraction, indicating that this may be a common mechanism through which PGE_2 signaling may be regulated in many different physiological settings. Acute intravenous or chronic subcutaneous infusion of Ang II *in vivo* did not substantially alter PGE_2-mediated vasodepressor responses. However, additional preliminary studies

indicated that enhanced sympathetic tone in combination with Ang II may switch PGE_2 blood pressure responses from vasodepressor to vasopressor effects. Future studies investigating calcium mobilization and phospholipase C activity in vascular smooth muscle cells would further elucidate the molecular mechanisms mediating the interaction of AT1 and EP3 receptors. Additional investigations evaluating AT1 and EP3 synergism in other tissue types may also clarify the extent by which the interaction of Ang II with PGE_2 can affect overall physiology.

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