# ROLE OF THE PROSTAGLANDIN $E_2$ RECEPTOR EP1 IN HYPERTENSIVE END-ORGAN DAMAGE

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

In

Pharmacology

December, 2012

Nashville, Tennessee

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# ACKNOWLEDGEMENTS

Completion of my doctoral work would not have been possible without the help of so many people. First, I must thank my advisor and mentor, Dr. Richard Breyer. Rich has always offered encouragement and advice on both a professional and personal level. I am grateful for his patience and guidance throughout the last six years and serving as a great scientific role model. I would also like to thank all the current and past members of the Breyer lab who have been instrumental in making my experience in the lab enjoyable. In particular I am grateful to Jason Downey, who has been a sounding board of all crazy ideas and become a good friend.

I would like to acknowledge the Pharmacology Department and Division of Nephrology and Hypertension of Vanderbilt University for administrative and/or financial support. Additionally, I would like to acknowledge the remaining members of my committee Drs. Brian Wadzinski (chair), Seva Gurevich, Ambra Pozzi and Al George for making my committee meetings a valuable and pleasurable experience. I would also like to acknowledge Dr. Jason Morrow, who was a member of my committee from 2007-2008.

There are a number of other people who have provided support for various aspects of my doctoral training. I am grateful to Leslie Gewin for teaching me to perform uninephrectomies and all the members of the MMPC, especially Lianli Ma, for teaching me to perform intracarotid blood pressure measurements and help with various experiments. Dr. Jeff Reese

enthusiastically welcomed me into his lab space and taught me myography studies. I am also grateful to the wonderful collaborators I have had the pleasure to work with; Drs. Roy Zent, Kelli Boyd, and Ray Harris. Thank you all!

Finally, I would like to thank my friends and family; both in and out of Nashville, for helping me emotionally survive my graduate experience. I am grateful for my parents who have given me unconditional love and support, and my best friend and sister, who I can always depend on to lend an ear. There is no one who deserves more credit for this work than my husband, Robert. Thank you for being my rock through all the ups and downs. You always manage to bring a smile to my face and provide motivation and encouragement. I am indebted to you.

PUBLICATIONS

**Bartlett CS**, Boyd KL, Harris RC, Zent R, Breyer RM. EP1 disruption attenuates end-organ damage in a mouse model of hypertension. *Hypertension*. 2012 Nov; 60(5): 1184-91.

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| | |
|---|---|
| AA | Arachidonic acid |
| AAALAC | Association for accreditation and assessment of laboratory animal care |
| ACEi | Angiotensin converting enzyme inhibitor |
| ACR | Albumin:creatinine ratio |
| ALT | Alanine transaminase |
| Ang II | Angiotensin II |
| ARB | Angiotensin receptor blocker |
| AT | Angiotensin II receptor |
| BP | Blood pressure |
| BUN | Blood urea nitrogen |
| $[cAMP]_i$ | Intracellular cyclic adenosine monophosphate |
| CHF | Congestive heart failure |
| CNS | Central nervous system |
| COX | Cyclooxygenase |
| $cPLA_2$ | Cytosolic phospholipase $A_2$ |
| CVD | Cardiovascular disease |
| DGLA | Dihomo-gamma-linolenic acid |
| DN | Diabetic nephropathy |
| DOCA | Deoxycorticosterone acetate |
| EF | Ejection fraction |
| EGFR | Epidermal growth factor receptor |

| | |
|---|---|
| eNOS | Endothelial nitric oxide synthase |
| EP | E-prostanoid receptor |
| EPA | Eicosapentaenoic acid |
| FS | Fractional shortening |
| ESRD | End-stage renal disease |
| GBM | Glomerular basement membrane |
| GFR | Glomerular filtration rate |
| GPCR | G protein-coupled receptor |
| H/E | Hematoxylin and eosin |
| ICV | Intracerebroventricular |
| IVS | Intraventricular septum |
| JNC | Joint National Committee |
| Kim-1 | Kidney injury molecule-1 |
| LVID | Left ventricular interior diameter |
| LVPW | Left ventricular posterior wall |
| LTs | Leukotrienes |
| MAP | Mean arterial pressure |
| mPGES | Microsomal prostaglandin E synthase |
| mRNA | Messenger ribonucleic acid |
| Ngal | Neutrophil gelatinase-associated lipocalin |
| Nphx | Uninephrectomy |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| PGs | Prostaglandins |

| | |
|---|---|
| $PGE_2$ | Prostaglandin $E_2$ |
| PGIs | Prostacyclins |
| PTX | Pertussis toxin |
| RAAS | Renin-angiotensin-aldosterone system |
| SBP | Systolic blood pressure |
| STZ | Streptozotocin |
| TX | Thromboxanes |

# CHAPTER I

# INTRODUCTION

## History of Hypertension

In 1733, the idea of blood pressure (BP) was first credited to British veterinarian Stephen Hales who observed the pulsation of blood when inserting a pipe into the artery of a horse. Hales concluded that pressure must be pushing the blood. While blood pressure was an accepted idea, it was not until 1847 when the first recording was made using a kymograph by Carl Ludwig. Many years later, Samuel Karl Ritter von Basch invented the sphygmomanometer, which was further improved to a mercury filled sphygmomanometer with an inflatable cuff in 1896 by Scipione Riva-Rocci. Massachusetts General Hospital was among the first to incorporate its use into routine checkups, courtesy of neurosurgeon Harvey Cushing. Although the risks of high blood pressure were acknowledged by insurance companies, it was not until the 1960s that the medical community had wide acceptance of these dangers. Until this time many experienced and prominent cardiologists considered high blood pressure to be an important compensatory phenomenon that should be left alone.

*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*. – J.H. Hay, 1931

*Hypertension may be an important compensatory mechanism which should not be tampered with, even were it certain that we could control it.*

– Paul Dudley White, 1937

President Franklin Delano Roosevelt suffered and eventually died as a result of high blood pressure, and the progression of his disease is an eloquent example of the natural history of untreated hypertension (1). In 1937, 54 year old Roosevelt had a BP of 162/98 mm Hg, though received no treatment to reduce his hypertension. Within three years, his blood pressure had risen to 180/88 mm Hg. In 1941, his physician prescribed phenobarbital and massages, as his blood pressure had risen to 188/105 mm Hg. Over the next three years, President Roosevelt suffered shortness of breath, drowsiness, and lethargy. At the request of Roosevelt's daughter, cardiologist Howard G. Bruenn examined President Roosevelt finding strong evidence of congestive heart failure (CHF) including pulmonary edema, an enlarged heart, left ventricular hypertrophy and proteinuria. He treated Roosevelt by reducing his alcohol and cigarette use, limiting his activity, administering digitalis and implementing a low salt diet which helped to improve his symptoms. That year Roosevelt's blood pressure was as high as 230/140 mm Hg. In 1945, his blood pressure had risen to 260/150 and on April 12, 1945 while sitting for a portrait President Roosevelt lost consciousness. His blood pressure had risen to > 300/190 mm Hg and he died of a cerebral hemorrhage.

At this time there were very few options with regards to the treatment of hypertension and all had severe side effects. The low salt diet had been suggested as a treatment in 1922 by Allen and Sherrill, though this received very little attention in the United States (2). The Kempner rice diet published in 1944 was demonstrated to significantly impact BP although this diet had very low compliance (3). Other more radical therapies included inoculation with fever producting agents such as typhoid bacilli, or surgical procedures like sympathectomy and adrenalectomy (4). The herb veratrum viride showed great promise for lowering blood pressure but had a very narrow therapeutic window. Dr. Edward Freis, a leader in antihypertensive treatment, began using the antimalarial drug pentaquine as a hypotensive drug (1).

The 1950s were a time of great struggle with regards to acceptance of the dangers and need to treat hypertension in the medical community. In 1955, Perera of Columbia Univerisity published conclusions from a study of 300 patients suggesting hypertension is relatively benign and the label overused (5). Meanwhile, clinical evidence began accumulating demonstrating the benefit of lowering blood pressure, and the search for novel therapeutic agents boomed. Alternatives to the dangerous surgical procedures were discovered including sympathetic nerve blockers (phenoxybenzamine), ganglionic blockers (hexamethonium, pentolinium), peripheral adrenergic inhibitors (guanethidine), catecholamine reduction (reserpine) and vasodilators (hydralazine). In 1957, a clinical trial began for a major antihypertensive agent still used today, chlorothiazide (6).

In the 1960s, the Framingham Heart Study showed a correlation between blood pressure and heart attacks, congestive heart failure, stroke and kidney damage (7). The first Veterans Administration study showed antihypertensive therapy in patients with diastolic BP > 105 mm Hg was able to reduce the incidence of stroke, CHF, and kidney damage (8). Propranolol began its clinical use in the early 1960s as the first of the beta blocker family. The National High Blood Pressure Education Program was started as a way of informing the medical community and public of the dangers of high blood pressure and methods to treat it. In 1977, the first report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC) was published, setting the standards for blood pressure diagnosis and treatment (9). These standards are updated regularly and JNC 8 is currently in development.

## Pathophysiology and Consequences of Hypertension

Still today, hypertension is a prevalent disease which causes substantial morbidity and mortality in humans, and affects approximately 33 % of adults in the United States (10). Roughly one-third of afflicted individuals are receiving treatment for hypertension, though only one-half of treated patients have their blood pressure under control. Thus, prevention and treatment of hypertension is an important public health goal.

Hypertension can be classified into several categories based on the degree of blood pressure elevation (Table 1.1) ranging from prehypertension (systolic 120-139 mm Hg/ diastolic 80-90 mm Hg) to Stage 2 hypertension (systolic ≥ 160 mm Hg/ diastolic ≥100 mm Hg) (11). Alternatively, hypertension can be designated either essential or secondary, based on the root cause of the high blood pressure. About 95 % of hypertensive adults have essential hypertension, meaning no secondary cause was discovered. Secondary causes of hypertension can include renal, vascular, endocrine, neurogenic and drug or toxin induced hypertension.

Following chronic elevated blood pressure, target end-organ damage to the aorta and small arteries, heart, kidneys, and retina can be observed. Blood pressure is the product of cardiac output (stroke volume x heart rate) and systemic vascular resistance. Thus, it is reasonable to conclude that patients

Table 1.1 Classification of Hypertension

| | Systolic BP (mm Hg) | Diastolic BP (mm Hg) |
|---|---|---|
| Normal | < 120 | < 80 |
| Prehypertension | 120-139 | 80-90 |
| Stage 1 | 140-159 | 90-99 |
| Stage 2 | ≥ 160 | ≥ 100 |

For adults 18 years or older, based on recommendation of the JNC VII.

with hypertension may have increased cardiac output, increased vascular resistance, or both. Early in the course of disease, cardiac output is often elevated. Over time, vascular stiffness and increased systemic vascular resistance plays a greater role (12). This increases the afterload on the left ventricle, inducing left ventricular hypertrophy and dysfunction, which is an independent risk factor for sudden death and stroke. Coronary artery disease is another cardiac consequence of hypertension. At higher MAP the heart requires more oxygen to constrict, and lipid plaques in the arteries decrease coronary oxygen supply. This combination leads to myocardial ischemia and infarction. Congestive heart failure also occurs with chronic pressure overload. Often patients present with diastolic dysfunction, which eventually leads to systolic failure and cardiac congestion.

Renal disease is an additional complication of hypertension. Hypertension-induced vascular lesions can also result in ischemia to the nephrons, or hyperperfusion of the glomerulus can damage the glomerular capillaries themselves. Patients initially present with microalbuminuria, and pathology reveals glomerulosclerosis, which eventually leads to ischemic renal tubules. The risk of end-stage renal disease (ESRD) is greater in hypertensive black patients than white patients, even when blood pressure is properly regulated. Hypertension also increases the risk of ESRD in patients with diabetic nephropathy.

Hypertension-induced damage to the vasculature is a common feature observed in many complications of the disease. In addition to coronary artery

disease, stroke and renal disease, the damage to the vasculature of the eye or aorta can also have serious consequences. Increased pressure in the aorta can cause a weakening of the vessel wall leading to the formation of an aneurysm. Most patients with aortic aneurysms are asymptomatic until the aneurysm ruptures. This is a life-threatening event, and only 10-25 % of patients survive. The vasculature of the retina of the eye is also uniquely sensitive to damage. Chronic elevated pressure can result in narrowing of the arterioles with presentation of microaneurysm or macroaneurysm formation. These hypertensive retinopathies can result in loss of vision.

The regulation of blood pressure is vastly intricate and multifactorial. Many risk factors - both environmental and genetic - have been identified to increase BP including obesity, insulin resistance, high salt diet, aging, sedentary lifestyle, stress, and low potassium and calcium intake (13),(14). On a whole body-level, many more factors can influence blood pressure regulation including neurogenic control, vascular reactivity, the renin-angiotensin system, sodium and water retention, nitric oxide, adrenal steroids and eicosanoids – including prostaglandins (PGs).

## Prostaglandins: Biosynthesis and Catabolism

Prostaglandins are biologically active lipid-derived autacoids generated by prostaglandin H synthase, commonly referred to as cyclooxygenase (COX). The COX enzymes are the target of non-steroidal anti-inflammatory drugs (NSAIDs).

PGs are part of the family of eicosanoids which includes prostaglandins, prostacylins (PGIs), thromboxanes (TXs), and leukotrienes (LTs). Eicosanoids are derived from the 20-carbon essential fatty acids arachidonic acid (AA), eicosapentaenoic acid (EPA), or dihomo-gamma-linolenic acid (DGLA). Specifically, PGs are derived from AA which is cleaved from the plasma membrane by phospholipase $A_2$, the rate limiting step in PG biosynthesis. COX catalyzes the oxidation of AA to $PGG_2$ and reduction to $PGH_2$. The tissue-specific prostaglandin synthase enzymes then convert $PGH_2$ into one of the five principal prostanoids – $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGI_2$ and $TXA_2$ (Figure 1.1).

Prostaglandins are ubiquitously produced and act in an autacrine or juxtacrine manner. Their actions can be halted by efficient catabolic pathways. When $PGE_2$ is administered intravenously, 95 % of $PGE_2$ is inactivated within the first pass through the lungs. This is achieved by β-oxidation, ω-oxidation by cytochrome P-450s, and PG-15-keto-reductase (15). $PGE_2$ is primarily inactivated by the conversion of the C-15 hydroxyl group into a ketone by $NAD^+$ dependent 15-hydroxyprostaglandin dehydrogenase (16).

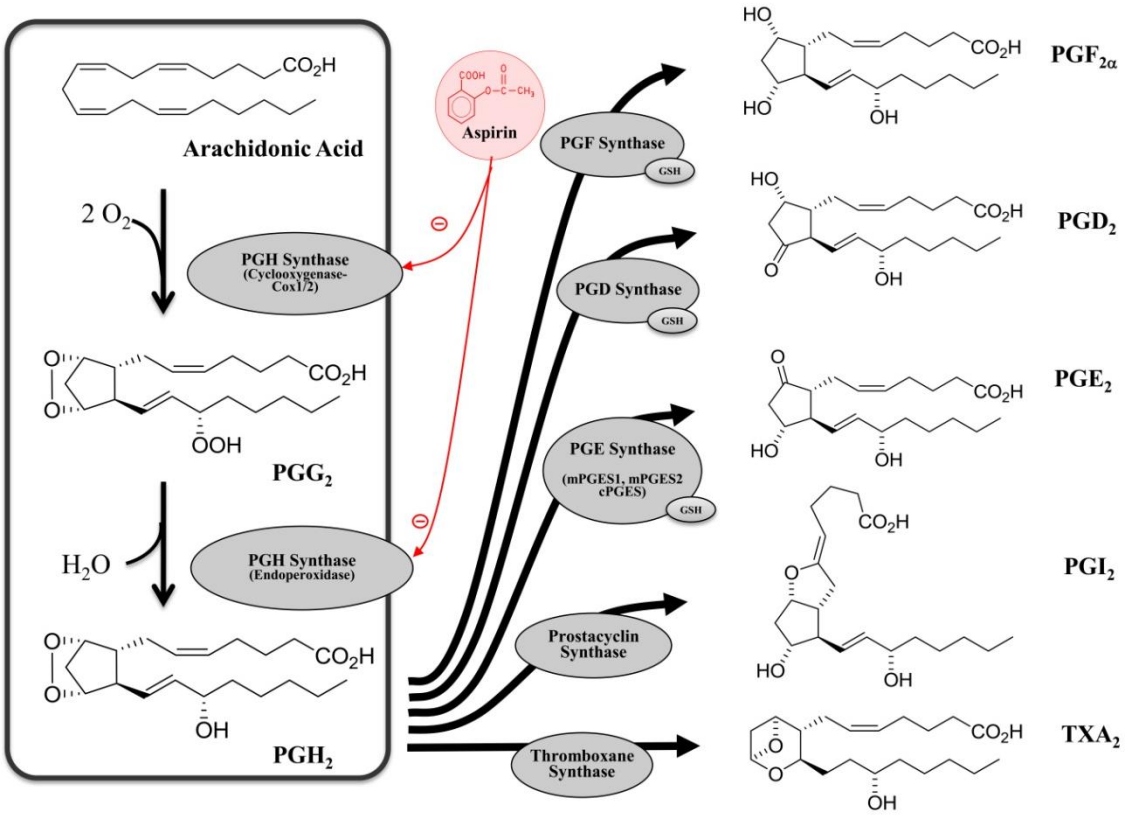



Figure 1.1 Biosynthesis of prostaglandins. This figure illustrates the formation of the five principal prostanoids from arachidonic acid.

## $PGE_2$ Receptors: Pharmacological properties and signaling

$PGE_2$ mediates diverse and at times physiologically opposing effects, as is the case with regard to blood pressure regulation (17-19). This unique feature can be explained in part by the existence of four $PGE_2$ receptors, designated the E-Prostanoid (EP) receptors EP1 through EP4 which were cloned in the early 1990s (20-23). EP1-EP4 are G-protein coupled Family A receptors with seven transmembrane domains. The EP1 and EP3 receptors are unique among $PGE_2$ receptors in that splice isoforms exist (24-27). The splice isoforms for the EP3 receptor alter the C-terminal tail and each isoform has been shown to have similar binding properties but activate different signal transduction pathways. $PGE_2$ binds with highest affinity to the EP3 and EP4 receptors ($K_D$ < 1 nM), and with lower affinity to the EP1 and EP2 receptors ($K_D$ > 10 nM)(28). Even though each EP receptor binds the same endogenous ligand, the amino acid identity conserved across receptors is only 28-33 % (29). Thus, selective compounds for the EP subtypes have been highly pursued.

### *EP1 receptors*

The EP1 receptor was cloned in 1993 from a mouse lung cDNA library and later from human erythroleukemia cells (21,30). EP1 was first characterized based on its ability to induce smooth muscle constriction. Signaling by the EP1 receptor is primarily transduced by increasing intracellular calcium, most likely mediated by $G_q$ family G-proteins (31). However, it was reported that the calcium

flux weakly induces phosphoinositide turnover and generation of $IP_3$ and the calcium flux is partially dependent on extracellular calcium, suggesting a non-$G_q$ dependent pathway may also exist (24). Additionally, the EP1 receptor has been demonstrated to couple to $G_{i/o}$ –type G proteins, inducing the up-regulation of HIF-1α (32).

An EP1 splice variant has been identified in rats (27). The rEP1-variant mRNA was found to be highly expressed in the rat kidney, in a proportion roughly equal to that of rEP1. This variant lacks the intracellular C-terminal tail of the receptor. It is able to bind ligand similar to rEP1, but unable to transduce signals. When co-expressed in CHO-cells with rEP1 or rEP4, rEP1-variant significantly suppresses signaling through the rEP1 and rEP4 receptors, suggesting it may be physiologically relevant for attenuating the actions of $PGE_2$ (27). However, no splice variants have been identified in other species to date.

Northern blot analysis of mouse EP1 mRNA demonstrated that the kidney has the highest expression of EP1, followed by lung, spleen and skeletal muscle, and low expression in the testes (21,30). More recently, utilizing quantitative PCR analysis Regard et al. showed ubiquitous expression of EP1, present in all 41 tissues tested, suggesting that EP1 is likely expressed in the vasculature (33). Unfortunately, due to the lack of well-characterized antibodies for EP receptors and the lower affinity of EP1 for $PGE_2$, the only radiolabeled ligand available, protein distribution remains unknown.

Selective agonists and antagonists have been essential for characterization of the individual EP receptor subtypes (Table 1.2). 17-phenyl-ω-

trinor-$PGE_2$ is the frequently utilized agonist with high affinity for mouse EP1 ($pK_i$ = 7.9) (34,35). Sulprostone and partial agonist iloprost have the next highest affinities for mouse EP1 ($pK_i \approx 7.7$), but are less selective (34,36). ONO-DI-004 is by far the most selective agonist for EP1, but has lower affinity than other agonists ($pK_i$ = 6.8)(37). AH6809 was frequently used in the past as an EP1 antagonist, although its affinity for the mouse EP1 receptor is very poor, and much lower than its affinity for EP2. ONO-8713, ONO-8711 and SC-51322 are currently used antagonists for EP1. ONO-8713 is the most selective antagonist with a $pK_i$ of 9.5 at the mouse EP1 receptor (38). ONO-8711 also has high affinity ($pK_i$ = 8.8) for mouse EP1, although it blocks EP3 receptor with reasonable affinity ($pK_i$ = 7.2) (39). SC-51322 is by far the most commonly used antagonist due to its commercial availability, however, its pharmacological properties have only been reported in humans ($pK_i$ = 7.9) and not in mice (28). In humans, SC51322 is also reported to block the EP3 receptor ($pK_i$ = 6.2).

### *EP2 receptors*

EP receptors were initially classified based on their function on smooth muscle (40). The EP2 and EP4 receptors induce smooth muscle relaxation. Initially, there were two cloned receptor subtypes called EP2 (22,23). Based on pharmacological selectivity, it was later shown that the first cloned EP2 receptor was actually EP4; the latter representing EP2.

Table 1.2 Pharmacological agents targeting mouse $PGE_2$ receptors

| Receptor | Signaling | Selective Drugs |
|---|---|---|
| EP1 | $G_q$, $G_{i/o}$, ? | Agonists: 17-phenyl-$\omega$-trinor-$PGE_2$ > sulprostone ≈ iloprost > M&B 28767 ≈ ONO-DI-004<br>Antagonists: ONO-8713 > ONO-8711 >> SC-51322 > AH6809 |
| EP2 | $G_s$, Arrestin | Agonists: ONO-AE1-259 >> butaprost ≈ AH13205 ≈ 11-deoxy-$PGE_1$<br>Antagonists: PF-04418948 >>> AH6809 |
| EP3 | $G_{i/o}$, $G_q$, $G_s$, $G_{12}$ | Agonists: M&B 28767 ≈ sulprostone ≈ SC-46275 > GR63799 ≈ ONO-AE-248<br>Antagonists: ONO-AE3-240 ≈ L-798,106 ≈ DG-041 >> ONO-8711 > SC-51322 |
| EP4 | $G_s$, $G_i$, Arrestin | Agonists: ONO-4819 > ONO-AE-329 ≈ 11-deoxy-$PGE_1$ > 1-OH-$PGE_1$<br>Antagonists: ONO-AE3-208 ≈ MF-498 >> GW 627368 >> AH23848 |

Both EP2 and EP4 receptors couple to $G_s$-type G proteins which activate adenylate cyclase and increase intracellular cAMP ($[cAMP]_i$) (22,23,41,42), however EP2 appears to increase $[cAMP]_i$ with greater efficacy than EP4 (43). Additionally, EP2 has also been demonstrated to couple to arrestin-mediated signaling pathways (44-46). The EP2-beta-arrestin1-p-Src complex plays a significant role in the development and progression of skin cancer by activating EGFR (46). Although EP2 has the least abundant mRNA expression of the EP receptors, EP2 mRNA is expressed in the lung, bone marrow and ovary (33,47), however its expression is known to be altered in response to stimuli. Therefore, mRNA quantitation in healthy mice may not portray an accurate picture of EP2 expression in a disease setting.

The EP2 receptor is classified due to its ability to be activated by the agonist butaprost. In the mouse, butaprost, AH13205 and 11-deoxy-$PGE_1$ all have similar affinity for the receptor ($pK_i$ = 6.6-7.3), although 11-deoxy-$PGE_1$ also binds to the EP3 and EP4 receptors (34). ONO-AE1-259 has improved potency over the previously named agonists ($pK_i$ = 8.5) and is highly selective for the EP2 receptor in mice (37). However, there is no reported data regarding its selectivity or affinity against human receptors. Until recently, a quality EP2 antagonist did not exist. AH6809 was shown to have moderate affinity for the EP2 receptor (mouse $pK_i$ = 6.5), however it also blocked the EP1 and DP receptors with high affinity in human tissue (28,34,48). Recently, Pfizer reported development of a highly selective, potent EP2 receptor antagonist, PF-04418948 (49-51), which

should prove very useful for the study and validation of EP2 as a therapeutic target.

### *EP3 receptors*

The EP3 receptor is known as the "inhibitory" $PGE_2$ receptor and induces smooth muscle contraction. EP3 primarily couples to a $G_i$-type family G proteins and inhibits adenylate cyclase and decreases $[cAMP]_i$ (20,41,52). The EP3 receptor has been demonstrated to increase intracellular calcium (52,53), and also to couple to $G_{12}$-type G proteins, inducing the activation of RhoA (54). EP3 receptors are unique among the EP receptors in that several splice variants have been identified: at least 7 variants in humans, 5 in rabbits, 4 in rats, and 3 in mice (55-57). The splice variants of EP3 have been demonstrated to alter constitutive activity, agonist-induced desensitization, trafficking, and signal transduction (26,58-60). For example, of the three splice variants found in mice, alpha and beta isoforms couple to $G_i$ G-protein, while the gamma isoform can couple to both $G_i$ and $G_s$ G-proteins (26).

EP3 receptor expression is widespread, and it is found in the kidney, uterus, pancreas, stomach, thymus, spleen, smooth muscle of the gastrointestinal tract, vasculature, and CNS (33,61). In the brain, EP3 receptor mRNA has been observed in the hippocampus, preoptic area, hypothalamus, locus coeruleus and raphe nuclei (62).

Several selective agonists and antagonists exist for the EP3 receptor. The most commonly used agonist for EP3 is sulprostone, which has a $pK_i$ at mouse EP3 of 9.2 (34). However, sulprostone is not entirely selective for EP3,

and has a $pK_i$ at mouse EP1 of 7.7. Therefore interpretation of studies utilizing high concentrations must be carefully considered. Sulprostone has a similar affinity for EP3 as M&B 28767 and SC-46275. GR-63799 and ONO-AE-248 are more selective for mouse EP3 and have $pK_i$'s of 8.7 and 8.1 respectively (34,37,63). Multiple antagonists exist for the EP3 receptor as well, although not all have been characterized with mouse receptors. ONO-AE3-240, L-798,106 and DG041 have the highest affinities for the EP3 receptor and are selective for the human EP3 receptor (64-67). ONO-8711 is also a selective antagonist with a $pK_i$ of 7.6. Some of the effects of SC-51322, a commonly used antagonist for EP1, may be explained by the blockade of EP3 ($pK_i$ = 6.2), especially when high concentrations are used (28).

### *EP4 receptors*

The EP4 receptor is an inducer of smooth muscle relaxation. Initially, it was identified as the EP2 receptor but later determined to be the fourth subtype of EP receptors (22). EP4 mRNA is predominantly localized the uterus, thymus, ileum, lung, spleen, adrenal gland, and kidney (33). EP4 signals through $G_s$-type G protein, which activates adenylate cyclase and increases $[cAMP]_i$. The EP4 receptor can also activate PI 3-kinase signaling pathways, leading to activation of extracellular signal-regulated kinases 1 and 2 (68). The activation of PI-3K was demonstrated to be pertussis toxin (PTX) sensitive, and therefore mediated by $G_i$ G-protein (69). PTX reduced $PGE_2$ stimulated $[cAMP]_i$ in cells expressing EP4 receptors, suggesting EP4-$G_i$ signaling both inhibits adenylate cyclase and

activates PI-3K. EP4 was also demonstrated to couple to arrestin-mediated signaling pathways (45,46).

EP4 was distinguished from EP2 due to its lack of activation in response to butaprost. The EP4 receptor is preferentially activated by ONO-4819 ($pK_i$ = 9.2) and ONO-AE-329 ($pK_i$ = 8.0), whereas these compounds' affinities for the EP2 receptor are 100-1000 times lower (37,63,70). In humans, 11-deoxy-$PGE_1$ is selective for EP4, however in mouse and rat 11-deoxy-$PGE_1$ is able to bind EP2 and EP3 receptors in addition to EP4 (34,71,72). In mice, 1-OH-$PGE_1$ is a selective agonist but has lower affinity for EP4 ($pK_i$ = 6.7) (34). In addition to agonists there are several antagonists available for blockade of the EP4 receptor. AH23848 was the most commonly used antagonist, however it has low affinity for EP4 (pKi = 5.3), and although selective against mouse receptors, has higher affinity for the human $TXA_2$ receptor than human EP4 (28,71,73). Among the newer antagonists, ONO-AE3-208 has the highest affinity for the mouse EP4 receptor, although it also blocks the EP3 receptor at slightly higher concentrations despite being selective for the human EP4 receptor (74). Fortunately Merck compound MF-498 has both high affinity and selectivity for the EP4 receptor (75,76). Additionally, GW627368 is selective and has reasonable affinity for EP4 ($pK_i$ = 7.1) (77). These EP4 antagonists have proven to be particularly useful for the study of EP4 in disease given 95 % of EP4-/- mice die as a result of patent ductus arteriosis shortly after birth (78).

## $PGE_2$ receptors as therapeutic targets

$PGE_2$ mediates a wide range of physiological conditions including modulation of salt and water transport in the kidney, maintenance of blood pressure, preservation of the gastric mucosa, tumor growth and angiogenesis, and modulation of immune responses. The COX/$PGE_2$ pathway has long been a target of therapeutic benefit. Some of the most commonly used over-the-counter medications - NSAIDs - block this pathway by inhibiting the COX enzymes. These drugs are very valuable for the treatment of pain, inflammation, and fever; however chronic use can cause serious side effects including ulcers and gastrointestinal bleeding, hypertension, heart attack or stroke. In an effort to eliminate the side effects, subtype selective inhibition of COX-2, while maintaining the function of the "housekeeping" isoform COX-1, was pursued. The drugs produced include rofecoxib (Vioxx) and celecoxib (Celebrex). Unfortunately, rofecoxib was withdrawn from the market due to an increased risk of myocardial infarction and stroke, despite showing efficacy against cancer progression and reduced incidence of gastrointestinal bleeding. These unexpected side effects were a result of an imbalance in thromboxane and prostacylin action. Celecoxib has remained on the market and is currently prescribed for rheumatoid and osteoarthritis, acute pain and familial adenomatous polyposis.

Attention has since focused on alternate ways to modulate the COX/$PGE_2$ pathway. These include targeting the catabolic enzyme for $PGE_2$, prostaglandin dehydrogenase, targeting the synthases responsible for production of the $PGE_2$,

targeting the transporter which moves the intracellular $PGE_2$ across the plasma membrane, or targeting the $PGE_2$ receptors themselves. Therefore, selective agents and knockout mice have been used to investigate the subtype specific roles of the EP receptors in health and disease.

### *EP1 receptors*

Although EP1-/- mice have no gross dysfunctions, they have been used to support the role of EP1 in impulse activity, pain, hypertension, urine concentration and cancer. Most notably, the EP1 receptor is actively being pursued as a therapeutic for pain. In two acute pain models, EP1-/- mice have been shown to have reduced nociceptive responses (79). The reduction in pain phenocopied the effect observed by treatment with a COX-2 inhibitor, suggesting the EP1 receptor is the major mediator of COX/$PGE_2$ pain (79). $PGE_2$-dependent mechanical allodynia, or enhanced sensitivity to a stimulus which normally does not cause pain, is prevented in EP1-/- mice (80). Furthermore, anti-nociceptive and anti-inflammatory responses have been observed as a result of administration of an EP1 antagonist in several studies (67,81-84).

EP1-/- mice exhibit modestly reduced systolic blood pressure (79). This effect was also observed in a setting of hypertension. EP1-/- mice have a blunted response to acute and chronic angiotensin II-dependent hypertension (85). Additionally, EP1 antagonists significantly reduced hypertension in diabetic mice and spontaneously hypertensive rats (85,86). EP1 antagonists have also

been used for the prevention of hypertensive renal damage and diabetic nephropathy (87,88).

In the lung, EP1 receptors constrict the airway of mice and mediate surfactant secretion from rat alveolar cells (89,90). In the stomach, EP1 plays both beneficial and harmful roles. EP1 enhances histamine-induced gastric injury while also conferring cytoprotection against hemorrhagic lesions and is essential for $HCO_3^-$ secretion (91-94). EP1 receptors have also been shown to play a critical role in carcinogenesis. EP1-/- mice have reduced aberrant crypt foci and tumor formation in the azoxymathane colon cancer model (39,95). EP1 antagonist ONO-8711 was demonstrated to reduced tumor burden in a model of breast cancer (96). Therefore, EP1 antagonists may be of therapeutic importance for pain, hypertension, and cancer.

There are few indications when EP1 agonism would be beneficial. EP1-/- mice exhibit behavioral deficits including impaired social interaction, aggression, diminished cliff avoidance and exaggerated acoustic startle response (97). Therefore, agonism of EP1 receptors could prove to be useful for control of impulsive behavior, although major unwanted side effects would be expected.

### *EP2 receptors*

EP2 has been demonstrated to be involved in asthma, blood pressure homeostasis, uterine contraction, fertility, maintenance of bone, and cancer. EP2 is generally considered to have anti-inflammatory roles (98). EP2-/- mice have reduced fertility (17,78,99). Although female EP2-/- mice are able to become

pregnant, there is a reduction in litter size. This is a result of reduced fertilization of the oocyte due to incomplete expansion of the cumulus cells required for oocyte maturation (78,99,100). The EP2 receptor mediates the vasodilator responses of $PGE_2$ (101). During high salt intake, deletion of EP2 results in salt-sensitive hypertension (17,99). One mechanism by which EP2 can modulate blood pressure is through $PGE_2$-induced renin release. EP2 and EP4 activate renin exocytosis at the juxtaglomerular cell, eventually resulting in vasoconstriction of the afferent arteriole (102-104). EP2 also mediates sodium excretion, suggesting that in a high salt setting EP2 stimulates natriuresis and maintenance of normal blood pressure (105).

The EP2 receptor mediates the $PGE_2$ bronchodilator response, suggesting agonism of EP2 may be beneficial for the treatment of asthma. This has been demonstrated in both human and mouse (89,106-108). Agonism of EP2 receptors could prove to be more beneficial than commonly used treatments which relieve bronchospasm, because EP2 receptors also inhibit lung mast cell degranulation (109). AH13205 was tested clinically with no success, however it should be noted that the potency to this EP2 agonist is weak, and there may be great benefit from a more potent compound if it becomes available (110).

The EP2 receptor can positively and negatively contribute to cancer. In skin cancer, overexpression of EP2 enhances chemically induced skin cancer and EP2-/- mice are resistant to the cancer (111,112). In intestinal and prostate cancers, EP2 receptors contribute to endothelial cell motility and mediate angiogenesis (113). In mammary cancer, EP2 deficiency reduces tumor growth,

angiogenesis, and metastasis, while over expression of EP2 enhances tumor burden (114). This supports a pro-tumor role of EP2. In contrast, loss of EP2 receptors in keratinocytes has been suggested as a mechanism for neoplastic progression by increasing invasiveness. Furthermore, EP2 receptors are protective in UV-induced carcinogenesis (115,116), supporting an anti-tumor role of EP2. It is essential to appreciate the complexity of the EP2 receptor in cancer progression. Therapeutic manipulation of EP2 for the treatment of cancer would require determining whether EP2 is acting in a pro- or anti-tumorigenic fashion prior to treatment.

### *EP3 receptors*

EP3 receptors have been implicated in many physiologic events including fever, pain, platelet aggregation, myocardial infarction, bladder hyperactivity, cancer, obesity, and Alzheimer's disease. Upon gross observation, EP3-/- mice appear to be normal (117,118). However, EP3-/- mice have been shown to have an impaired febrile response (118), suggesting EP3 antagonists could be effective antipyretic agents. Analgesic effects are also produced by EP3 antagonists, or the use of EP3-/- mice, in a model of acute herpetic pain (119). EP3 antagonism has been clinically pursued for treatment of thrombotic disorders. $PGE_2$ inhibits platetet aggregation at high dose, but also can potentiate other proaggregatory agents (120). EP3 receptors have been demonstrated to mediate this proaggretory effect (64,121-124). Furthermore,

EP3-/- mice have increased bleeding and reduced susceptibility to thromboembolism (123).

With the use of EP3 antagonists, the receptor has been identified as a target for treatment of bladder hyperactivity disorders. Treatment with DG041 inhibited responses to bladder distension and reduced bladder motility (125). Furthermore, EP3-/- mice have been reported to have increased bladder capacity, while infusion of an EP3 agonist reduces bladder capacity in wildtype mice (126).

EP3 has also been implicated in metabolic regulation. EP3-/- mice had increased frequency of feeding during the light cycle and became obese on a normal fat diet (127). Increased leptin and insulin levels were also observed with the obesity (127). In a model of subacute neuroinflammation, deletion of EP3 reduced pro-inflammatory gene expression, cytokine production, oxidative stress, and reversed the decline in presynaptic proteins, suggesting a possible role for EP3 in Alzheimer's disease (128,129).

Similar to the EP2 receptor, in cancer EP3 can have both pro- and anti-tumorigenic effects. In human colon cancer EP3 mRNA expression is reduced. Agonism of EP3 in a colon cancer cell line reduced cell viability, supporting the theory that downregulation of EP3 allows for enhanced colon carcinogenesis (130). Overexpression of individual splice variants decreased tumorigenic potential in cell lines and reduced tumor burden in vivo (54). EP3-/- mice also exhibited reduced tumor burden and angiogenesis in an implanted tumor model (65). Similar reductions in tumor burden were observed upon treatment with an

EP3 antagonist (65). In contrast, EP3 decreases aromatase activity, suggesting a possible inhibitory role in breast cancer (131).

### *EP4 receptors*

EP4 receptors have been proposed to mediate vasodepression, closure of the ductus arteriosus, renin secretion,bone homeostasis and intestinal homeostasis. EP4-/- mice have been demonstrated to have persistent patent ductus arteriosus, and most mice die within 3 days of birth due to pulmonary congestion and congestive heart failure (132,133). The ductus arteriosus is the fetal shunt between the pulmonary artery and aorta, and its closure at birth is essential. Indomethacin can be given late in pregnancy to induce closure of the ductus in wildtype, but not EP4-/- mice (132).

EP2 and EP4 receptors have also been shown to mediate $PGE_2$ stimulated renin secretion. $PGE_2$ is a well established stimulator of renin (19,134). It is also known that $PGE_2$ is produced locally at the macula densa in response to relevant concentrations of NaCl (20-40 mM) (135). Using isolated, perfused kidneys for EP1, EP2, EP3 and EP4 knockout mice, it was demonstrated that EP4 stimulates renin secretion at low $PGE_2$ concentrations, regulating basal activity, while both EP4 and EP2 stimulate renin at higher concentrations of $PGE_2$ (102). However, EP receptors do not appear to mediate COX-2 dependent production of renin at the macula densa in low-NaCl settings, since no differences in renin secretion were observed between genotypes when

loop diuretics, which impair the ability of the macula densa to sense NaCl, were administered (102).

EP4 is the primary $PGE_2$ receptor supporting bone maintenance, and is able to mediate both bone formation and bone reabsorption. EP4-/- mice have been shown to have reduced bone mass and an inability to properly repair fracture damage (136). Despite having similarly sized bones, EP4-/- mice had reduced structural and apparent material strength in the femur and vertebrae compared to wildtype mice (137). Administration of an EP4 agonist has been shown to restore bone mass and strength in ovariectomized or immobilized rats, suggesting activation of EP4 could be of great therapeutic benefit for conditions with reduced bone mass (70).

It has been established that treatment with NSAIDs can trigger or worsen inflammatory bowel diseases. This phenomenon is due to reduced activation of EP4. In a dextran sodium sulfate–induced colitis, disruption of EP4 and not EP1, EP2, EP3, FP, TP or IP resulted in enhanced colitis in wildtype mice (74). Administration of an EP4 antagonist to wildtype mice was able to mimic this effect, while treatment with an EP4 agonist ameliorated severe colitis (74). The anti-inflammatory EP4 receptor was critical to mucosal barrier function and maintenance of epithelial cells, and suggests EP4 agonism may be of therapeutic value for treatment of inflammatory bowel diseases such as Crohn disease and ulcerative colitis.

## $PGE_2$ receptors in blood pressure regulation

$PGE_2$ is a major prostanoid contributing to the regulation of blood pressure, where it can exert either vasopressor or vasodepressor effects depending upon the setting (17-19). Previous studies have determined that the EP1 and EP3 receptors primarily mediate the pressor response, while the EP2 and EP4 receptors mediate the depressor response (17,85,99,134,138-140)(Figure 1.2).

The role of PGs in the regulation of blood pressure is highlighted by the pro-hypertensive action of non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit cyclooxygenase mediated prostanoid production, suggesting that prostaglandins have an anti-hypertensive role (141,142). However, under certain conditions NSAIDs can also have hypotensive effects supporting a prohypertensive role for PGs (143). Of note, $PGE_2$ is a well established stimulator of renin (19,102,135,144). Experiments in conscious dogs demonstrated that intrarenal infusion of $PGE_2$ results in a sustained rise in mean arterial pressure, which highly correlates with plasma renin activity (19). This suggests the rise in blood pressure was related to the increased renin activity. Futhermore, 20 – 40 mM NaCl concentrations have to shown to induce local $PGE_2$ production in the macula densa (135). Overall, the balance of functionally antagonistic prostaglandin action fine-tunes blood pressure homeostasis.

Isomerization of $PGH_2$ to $PGE_2$ by microsomal PGE synthase (mPGES) plays a key role in the maintenance of blood pressure homeostasis by regulating

vascular tone, sodium balance and/or renin release (145). Inhibitors of mPGES are currently being pursued for treatment of cancer, pain and inflammation; however, unwanted pro-hypertensive effects may result from this strategy. In rodents, deletion of mPGES-1 has been shown to further increase blood pressure in several models of hypertension, including deoxycorticosterone-salt water-induced hypertension and acute or chronic treatment with angiotensin II (146-148). In humans, where there is a great deal of phenotypic heterogeneity, the contribution of $PGE_2$ towards hypertension is more controversial. In some cases, patients with essential hypertension have been shown to have low urinary excretion of $PGE_2$; however, in other cases patients have been shown to have high urinary $PGE_2$ excretion (149). This inter-patient variability may result in a wide range of untoward side effects for mPGES inhibitors. In order to gain a better understanding of the effects of $PGE_2$ on blood pressure homeostasis, attention has been focused on the actions of its receptors using knockout mouse models.

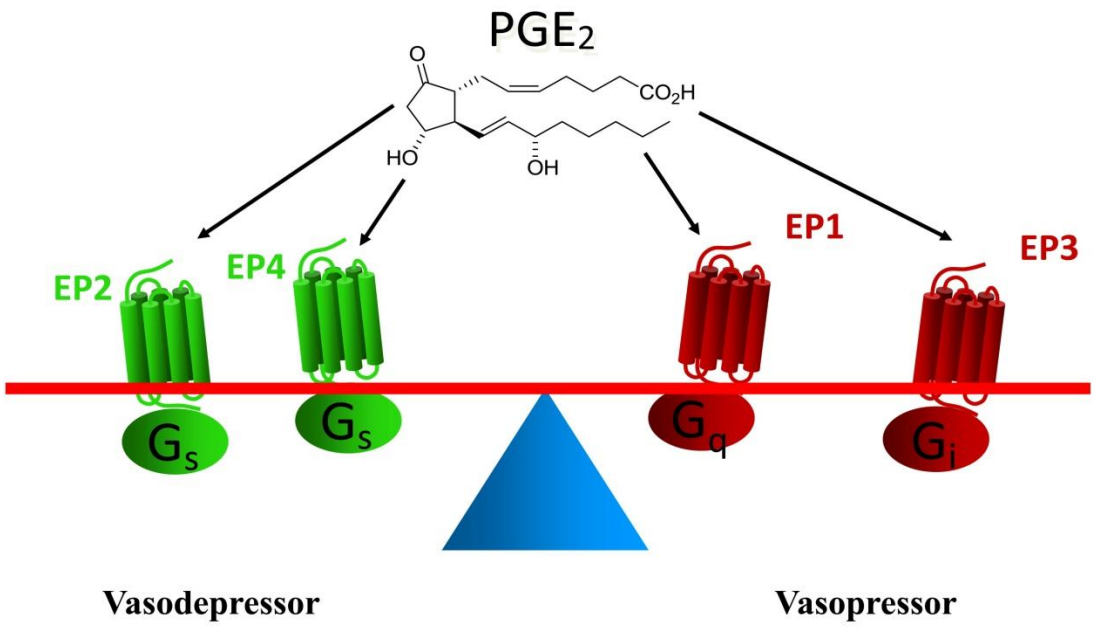



Figure 1.2 $PGE_2$ receptors in blood pressure regulation. Upon $PGE_2$-induced activation EP2 and EP4 function as vasodepressor receptors and EP3 and EP1 function as vasopressor receptors

### *Vasodepressor Receptors*

Upon acute infusion, $PGE_2$ is a vasodepressor in both humans and mice (17,138,150). This observation underscores the pro-hypertensive effects of blockade of all prostaglandin production and subsequent receptor activation by NSAIDs (141,151-154), which is consistent with the loss of a tonic vasodepressor PG effect. It has been shown that this depressor effect is primarily due to the activation of the EP2 receptor; however, when the EP2 receptor is deleted, the loss of the depressor response unmasks a $PGE_2$ pressor response (17). Moreover, EP2-/- mice fed a high-salt diet experienced an increase in blood pressure consistent with a protective role for EP2 activation in salt-sensitive hypertension (17). Intravenous infusion of the EP2 agonist ONO-AE1-259 into

Wistar rats increases retinal arteriolar and venous diameter and substantially reduces mean arterial pressure (155). EP2 and EP4 receptors evoke an increase in $[cAMP]_i$ through a $G_s$ coupled pathway, a classical mechanism for smooth muscle relaxation. Hristovska et al. demonstrated a dose-dependent relaxation in response to $PGE_2$ in aortic rings which was lost in tissue from EP4-/- mice but remained intact in EP2 -/- tissue. The EP4 dilator effect was dependent upon endothelium-derived nitric oxide production via eNOS (156). Acute blood pressure studies are challenging in EP4 -/- mice because the mice exhibit near complete perinatal lethality in inbred strains as a result of persistent patent ductus arteriosus (132). Analysis of studies performed with surviving EP4-/- animals on a mixed-strain background may not be straightforward as their survival may be dependent on modifier genes. Nonetheless, deletion of EP4 resulted in a diminished vasodepressor response to $PGE_2$ (138). In rats, infusion of the EP4 selective agonist ONO-AE1-329 significantly reduces blood pressure; it does not alter retinal vessel diameter (155). Taken together is consistent with EP4 vasodilator action in a subset of vascular beds.

### *Vasopressor Receptors*

As described above, the pressor effects of systemic $PGE_2$ infusion are only observed in the absence of the predominant depressor receptor EP2 (17). In contrast, infusion of EP3 receptor selective agonists such as sulprostone, M&B28767 or SC46275 in wildtype mice results in an acute and substantial rise in mean arterial pressure (139). The EP3 mediated pressor effect undergoes

desensitization with repeated administration of EP3 agonists. In EP2-/- mice after desensitization of EP3 responses, the depressor action of EP4 in response to $PGE_2$ infusion is then apparent (139). Thus, upon systemic infusion of $PGE_2$ in mice the depressor action of EP2 predominates, followed by the pressor action of EP3, and then the depressor action of EP4. Importantly, the order of expression of EP receptor mRNA does not mirror the phenotypic effects of the EP receptors. RNA levels determined by RNAse protection identified expression levels of EP3>>EP4>EP1≥EP2 in both renal resistance vessels and the aorta (139). It is unclear whether changes in EP receptor density underlie changes in vascular tone in the hypertensive state. Because messenger RNA levels do not correlate with receptor function, and anti-receptor antibodies are of questionable value, this remains an important unanswered question.

In contrast to the depressor effects of systemic administration, when $PGE_2$ is administered intracerebroventricularly (ICV) a rise in mean arterial pressure occurs, accompanied with tachycardia and enhanced renal sympathetic nerve activity (157). These effects were ascribed to the EP3 receptor using ICV infusion of subtype selective EP receptor agonists (157). Taken together, these data demonstrate that the centrally mediated pressor actions of $PGE_2$ are EP3-mediated.

Although the EP1 receptor does not appear to play a significant role in the blood pressure effects of systemically administered $PGE_2$, it has been shown to be a significant contributor to hypertension, particularly in cases with enhanced renin-angiotensin system activity. Genetic deletion of the EP1 receptor in mice

has been shown to significantly decrease systolic blood pressure, an effect amplified when mice are fed a low sodium diet (79). Importantly, EP1-/- mice have blunted pressor responses to both acute and chronic angiotensin II administration (85). In isolated vascular preparations of preglomerular arterioles and mesenteric arteries, pre-treatment with SC51322, an EP1/3 antagonist, was able to abolish any angiotensin II-mediated vasoconstriction (85). Furthermore, treatment of spontaneously hypertensive rats, a multifactorial model of essential hypertension, with SC51322 significantly reduces blood pressure (85), indicating the EP1 receptor and/or EP3 receptor may be novel targets for the treatment of hypertension.

### *$PGE_2$ and the consequences of hypertension*

Hypertension is an established risk factor for cardiovascular diseases including stroke, myocardial infarction, heart failure, arterial aneurysm and is the leading cause of chronic kidney failure. Current anti-hypertensive therapies reduce the risk of the related cardiovascular sequelae, though not to baseline risk observed in normotensive subjects. There is an unmet need for treatment which will reduce blood pressure and maximize target organ protection (158). In considering whether $PGE_2$ and its receptors make viable drug targets for hypertension, determination of their ability to reduce end-organ damage will be important. It has been shown that genetic deletion of mPGES-1 in mice results in increased blood pressure (146-148). Despite this, deletion of mPGES-1 has also

been shown to protect against aortic aneurysm formation and vascular injury (159,160). Angiotensin II infusion into hyperlipidemic mice produced fewer and less severe aneurysms, and reduced oxidative stress on a mPGES-1-/- background compared to wildtype mice (160). However, these results were complicated by the observed increase in $PGI_2$ and $PGD_2$ production accompanying the reduction in $PGE_2$ (160). It is yet to be determined whether potentially beneficial substrate diversion is a consequence specific to genetic mPGES-1 deletion, or would be recapitulated with chronic use of an mPGES inhibitor.

Blockade of individual $PGE_2$ receptors might result in a reduction in end-organ damage while being less likely to produce unwanted side effects. In addition, GPCRs are demonstrably “druggable” and are one of the most common targets of currently developed therapeutic agents. Antagonism of EP1 receptors has been shown to preserve renal function, and reduce tubulointerstitial damage, proliferative lesions, fibrotic area and proteinuria in stroke-prone spontaneously hypertensive rats (87), as well as cerebrovascular dysfunction induced by angiotensin II (161). In the study performed in stroke-prone hypertensive rats, tail cuff blood pressure was modestly reduced two weeks post-treatment with an EP1 antagonist, but this reduction was not maintained past five weeks of treatment. Nonetheless treatment with the EP1 antagonist provided end-organ protection. In contrast to the deleterious actions of the EP1 receptor, EP2 and EP4 receptors have been shown to be cardioprotective; it would seem important to maintain function of these receptors. For example, deletion of the EP4

receptor in a mouse model of ischemia reperfusion of the heart significantly increased infarct size, while treatment of wildtype mice with an EP4 agonist, 4819-CD, reduced infarct size (162). Therefore, EP4 agonists could be useful for reducing blood pressure and afford cardioprotective benefits. Selective blockade of EP1 and/or EP3 receptors, while EP2 and EP4 signaling remains intact may be preferable to the loss of signaling in all four receptors resulting from inhibition of $PGE_2$ ligand production.

$PGE_2$ plays a dynamic role in regulation of blood pressure homeostasis. The existence of multiple receptors with diverse signaling abilities allows for modulation both positively and negatively. The development and availability of additional highly selective agonists and antagonists for EP receptors is fundamental to the advancement of the field. Unwanted side effects resulting from inhibition of the cyclooxygenase enzymes upstream of prostanoid production demonstrated the value of selective targeting as proximal to the pathophysiological action as possible. Development of new therapeutics targeting specific $PGE_2$ receptors could reduce blood pressure and provide end-organ protection, while minimizing side effects.

## Objective

As summarized above, $PGE_2$ and its receptors mediate a wide range of physiologic processes. With regards to blood pressure regulation, $PGE_2$ can act in a pro-hypertensive or anti-hypertensive manner. While it has been demonstrated that EP2 and EP4 receptors mediate the vasodepressor actions of $PGE_2$ and the EP1 and EP3 receptors mediate the vasopressor actions, the role of the EP receptors in modulating downstream end-organ damage in incompletely characterized. Current therapies for cardiovascular and renal complications associated with hypertension and diabetes are largely focused on reduction of mean arterial pressure. However, pharmacologic reduction of blood pressure in patients with hypertension – even to the same blood pressure as normotensive individuals – does not reduce the risk of CVD and renal events to an incidence similar to that of normotensive individuals. This highlights the need for development of novel therapeutic agents which will not only lower blood pressure but also reduce end-organ damage.

Drugs which block the renin-angiotensin-aldosterone pathway are considered superior to other anti-hypertensive treatments due to their beneficial actions directly on the kidney. Furthermore, $PGE_2$ and the EP1 receptor have been demonstrated to mediate at least part of the actions of angiotensin II. Therefore, I sought to determine the role of the vasopressor $PGE_2$ receptors in the development of hypertensive end-organ damage. Specifically, I sought to address the following questions: What are the consequences of genetic

disruption of EP1 or EP3 receptors in development of Ang II- mediated hypertension? Do EP1 or EP3 receptors contribute to end-organ damage in a setting of hypertension of diabetes? Are beneficial effects on end-organ damage dependent of blood pressure reduction? Does the EP1 receptor contribute to Ang II independent hypertension? This thesis describes studies utilizing EP1 and EP3 receptor knockout mice which are designed to answer these questions and advance our knowledge of the role of EP receptors in settings of disease.

CHAPTER II

# DISRUPTION OF EP1 ATTENUATES END-ORGAN DAMAGE IN A MOUSE MODEL OF HYPERTENSION

## Introduction

Hypertension is a major risk factor for cardiovascular diseases (CVD), increasing the risk of stroke, myocardial infarction, arterial aneurysms, and heart failure. Approximately 30 % of adults in the US have hypertension and the incidence of CVD remains greater in hypertensive patients than normotensive patients, highlighting the need for novel therapeutic agents (158).

$PGE_2$ is a major prostanoid found in the mouse kidney and vasculature contributing to the regulation of blood pressure, where it can exert either vasopressor or vasodepressor effects (17-19). Four $PGE_2$ receptors (EP1 through EP4) mediate these effects with the EP1 and EP3 receptors primarily mediating the pressor response of $PGE_2$, while the EP2 and EP4 receptors mediate the depressor response (17,85,99,134,138-140).

Each $PGE_2$ receptor has distinct tissue localization and elicits characteristic signal transduction pathways (61). EP1 couples to $G_q$-proteins, resulting in mobilization of intracellular calcium, and stimulation of phosphoinositide turnover activating protein kinase C. The EP2 and EP4 receptors couple to $G_s$-proteins, which increase intracellular cAMP. The EP3

receptor couples to $G_i$-proteins, decreasing intracellular cAMP (For reviews, see (29,61)). Receptors couple to alternative signal transduction pathways as well, including arrestin-mediated signaling pathways (45,46).

Systemic infusion of $PGE_2$ results in a vasodepressor response (17,138,150), primarily through EP2 activation. In the absence of EP2, a $PGE_2$ pressor response is unmasked (17), mediated by the EP3 receptor (139). Agonist induced EP3 tachyphylaxis in the background of EP2 -/- mice uncovers a depressor action of EP4 (139). EP1 does not appear to play a significant role in the blood pressure effects of systemically administered $PGE_2$, however, it does contribute to hypertension. Genetic disruption of the EP1 receptor in mice has been shown to decrease blood pressure, particularly when mice are fed a low sodium diet (79). Furthermore, EP1-/- mice have blunted pressor responses to both acute and chronic angiotensin II (Ang II) administration (85). In isolated vessel preparations, pre-treatment with the EP1 selective antagonist SC51322 reduced Ang II mediated vasoconstriction (85). Treatment of spontaneously hypertensive rats with SC51322 significantly reduces blood pressure (85), indicating blockade of the EP1 receptor may be a target for the treatment of hypertension.

EP1 blockade has been shown to positively affect renal function in stroke-prone spontaneously hypertensive rats (87), as well as cerebrovascular dysfunction induced by Ang II (161), implicating the EP1 receptor in hypertension and resultant end-organ damage. This has yet to be investigated in detail and in

the context of other organ damage. Therefore, EP1+/+ and EP1-/- mice were studied in a model of severe hypertension.

## Experimental Procedures

### *Animal procedures*

The hypertension model was carried out as described by Kirchhoff and co-workers (163). Ten-to-16 week old male C57BL/6J (EP1+/+, Jackson Labs, USA) and EP1-/- mice (85) backcrossed at least ten generations onto C57BL/6J were uninephrectomized (Nphx) under ketamine/xylazine (100 mg/kg and 15 mg/kg) anesthesia. Two weeks later, a subcutaneous 50 mg deoxycorticosterone acetate (DOCA) pellet (Innovative Research of America, USA) was implanted and drinking water supplemented with 1 % NaCl. After an additional week, a subcutaneous osmotic mini-pump was implanted (Alzet model 1002; Durect Corporation, USA) delivering 1.5 ng angiotensin II (Calbiochem, USA) per minute per gram body weight. Mice were followed for two more weeks when tissues were collected for histology. Additional studies eliminating one of the three elements from the protocol were performed, while keeping the time frame between surgeries consistent. Animals were maintained in an AAALAC accredited rodent facility in individually ventilated microisolator cages on a 12:12 light dark cycle. All procedures were done in accordance with the policies of the Institutional Animal Care and Use Committee at Vanderbilt University.

### *Examination of aortic aneurysm and dissection*

Upon necropsy aortic aneurysms and dissections were observed in both the thoracic and abdominal aorta, and severity was scored visually using the following scale adapted from Manning et al., 2002 (164). Type 0: no aneurysm, Type 1: dilated aorta with no thrombus, Type 2: remodeled tissue that frequently contains thrombus, Type 3: a pronounced bulbous form of type 2, Type 4: multiple overlapping aneurysms, or a dissection extending the length of the aorta, Type 5: ruptured aorta. Hematoxylin and eosin (H/E) and Masson's Trichrome stains was used on formalin-fixed, paraffin-embedded aorta sections. Immunohistochemistry was performed for macrophage (CD11b and F4/80, Novus Biologicals cat# NB600-1327 and NB600-404), neutrophils (myeloperoxidase, Dako cat# A0398), T-cells (CD3, Santa Cruz cat# sc-1127), and B-cells (CD45R/B220, BD Pharmigen cat# 553084). Inflammation, collagen organization and all immunohistochemistry sections were scored at the site of the lesion in a blinded fashion by a comparative veterinary pathologist.

### *Fraction water weight determination*

Fractional water weight was determined as previously described (165). Eight EP1+/+ and seven EP1-/- mice were sacrificed 5 days post-Ang II administration, their carcasses weighed (wet weight) and incubated at 60°C. Body weight was measured daily until it remained unchanged for 3 days, indicating dry body weight. Fractional water weight was determined using the equation: Fractional water weight = 1 - (dry weight/wet weight).

***Determination of urinary albumin/creatinine ratios***

Albumin/Creatinine ratios (ACR; expressed as mg albumin/mg creatinine) were measured from 20-200 µL volumes of spot urine using Albuwell M ELISA kit, and urinary creatinine was measured using the Creatinine Companion (Exocell, Philadelphia, USA).

***Determination of Blood Urea Nitrogen levels***

To assess the renal function, blood urea nitrogen (BUN) was determined using an iSTAT-1 analyzer (Abbott Point of Care Inc., New Jersey, USA). Whole blood was obtained from saphenous vein and immediately assayed utilizing Chem8+ cartridges.

***Assessment of Renal Histopathology***

Tissues were fixed overnight in 10 % neutral buffered formalin. Kidneys were processed routinely, embedded in paraffin, sectioned at 5 microns, stained with H/E or Masson's Trichrome and evaluated by light microscopy.

***Quantitative PCR***

Total RNA from kidneys and aortae was isolated using the TRIzol reagent followed by RNA cleanup with a Qiagen RNeasy kit. cDNA was made using the high-capacity cDNA archive kit (Applied Biosystems). Quantitative PCR was performed using Applied Biosystems 7900HT Fast Real-time PCR system with Taqman gene expression assays for Ngal (Mm01324470_m1), Kim-1

(Mm00506686_m1), Cyclooxygenase-2 (COX-2) (Mm00478374_m1), and 18S rRNA (4319413E). Fold difference in mRNA expression is plotted relative to a normal EP1+/+ kidney sample.

***Echocardiography***

Transthoracic echocardiography was performed on lightly-anesthetized (isoflurane) mice using the VisualSonics VEVO2100 system (30 MHz transducer). Left ventricular posterior wall dimensions (LVPW), intraventricular septum dimensions (IVS), left ventricular interior diameter (LVID), fractional shortening (FS) and ejection fraction (EF) were measured for analysis of cardiac structure and function at baseline, prior to uninephrectomy, and five days post-Ang II administration.

***Intracarotid blood pressure measurement***

Intracarotid blood pressure was measured under ketamine (25 mg/kg) and inactin (100 mg/kg) anesthesia delivered intraperitoneally. Mice were placed on a thermal pad and a PE-10 catheter was inserted into the left carotid artery. The catheter was connected to a TXD-310 transducer and blood pressure was measured using a Digi-Med BPA 400 (Micromed). Mice were equilibrated 30-60 minutes until stable values were attained. Ten minute blood pressure measurements were collected and average mean arterial pressure (MAP) is plotted.

### *Antihypertensive Treatment*

Blood pressure reduction was achieved by adding hydralazine (200 mg/L) to the 1 % NaCl drinking water beginning three days prior to angiotensin II mini-pump implantation. Treatment was continued for the duration of the experiment, or a total of 17 days.

### *Statistical Analysis*

Data are means ± SEM, using GraphPad Prism software (GraphPad Software Inc., USA). Analysis utilized Student's t test and Fisher's exact test. Kaplan Meier survival curves were evaluated with the Log-rank (Mantel-COX) test. $P < 0.05$ was considered statistically significant for all studies.

## Results

### *EP1-/- mice were protected against Nphx/DOCA-NaCl/Ang II mortality*

We employed a model of severe hypertension to investigate the contribution of the EP1 receptor in hypertensive end-organ damage (163). Unexpected mortality was observed following implantation of the Ang II osmotic pump (Figure 2.1A). Of 58 EP1+/+ mice, 60 % died within 14 days. EP1-/- mice were significantly protected against mortality; of 35 EP1-/- mice only 24 % died ($P = 0.0044$). Modified protocols omitting one of the three components (Nphx, DOCA-NaCl, or Ang II) demonstrated all components of the model played an essential role in causing mortality (Figure 2.1B, Nphx/DOCA-NaCl/Ang II vs. Nphx/DOCA-

NaCl, Nphx/Ang II or DOCA-NaCl/Ang II, N = 10 per group, P = 0.011, < 0.005, or < 0.005 respectively).

***EP1-/- mice had reduced aortic aneurysm rupture, but comparable aortic histopathology***

Post-mortem analysis of EP1+/+ and EP1-/- mice in the full model indicated a significant portion of the mice died as a result of rupture of the aorta. Aneurysms and dissections were observed in both the thoracic and abdominal aorta. 37 % of EP1+/+ and 13 % of EP1-/- mice died due to aortic rupture. Aortic aneurysms were present in 67 % of EP1+/+ mice and 40 % of EP1-/- mice. Aneurysm severity was scored at death on a scale of 0-5 (Figure 2.2A). A reduction in aneurysm severity was observed in EP1-/- mice compared to EP1+/+ mice (Figure 2.2B, P = 0.049). H/E staining revealed aneurysms and dissections in the wall of the thoracic and abdominal aorta were accompanied by inflammation in both EP1+/+ and EP1-/- mice (Figure 2.3A-E, P = 0.3975). Analysis of aortic sections from both thoracic and abdominal lesions of each genotype demonstrated macrophage and neutrophils were most abundant, with no differences observed between the genotypes in any immune cell component (Figure 2.3F). COX-2 has been shown to play a significant role in aortic aneurysm formation and macrophage infiltration (166,167). COX-2 mRNA was elevated in abdominal aortae 2-5 days post Ang II administration, though differences between genotypes were not observed (Figure 2.3G, P = 0.774). Aortic sections stained with Masson's Trichrome showed that regardless of genotype there was less fibrillar collagen present in vessels that ruptured, and

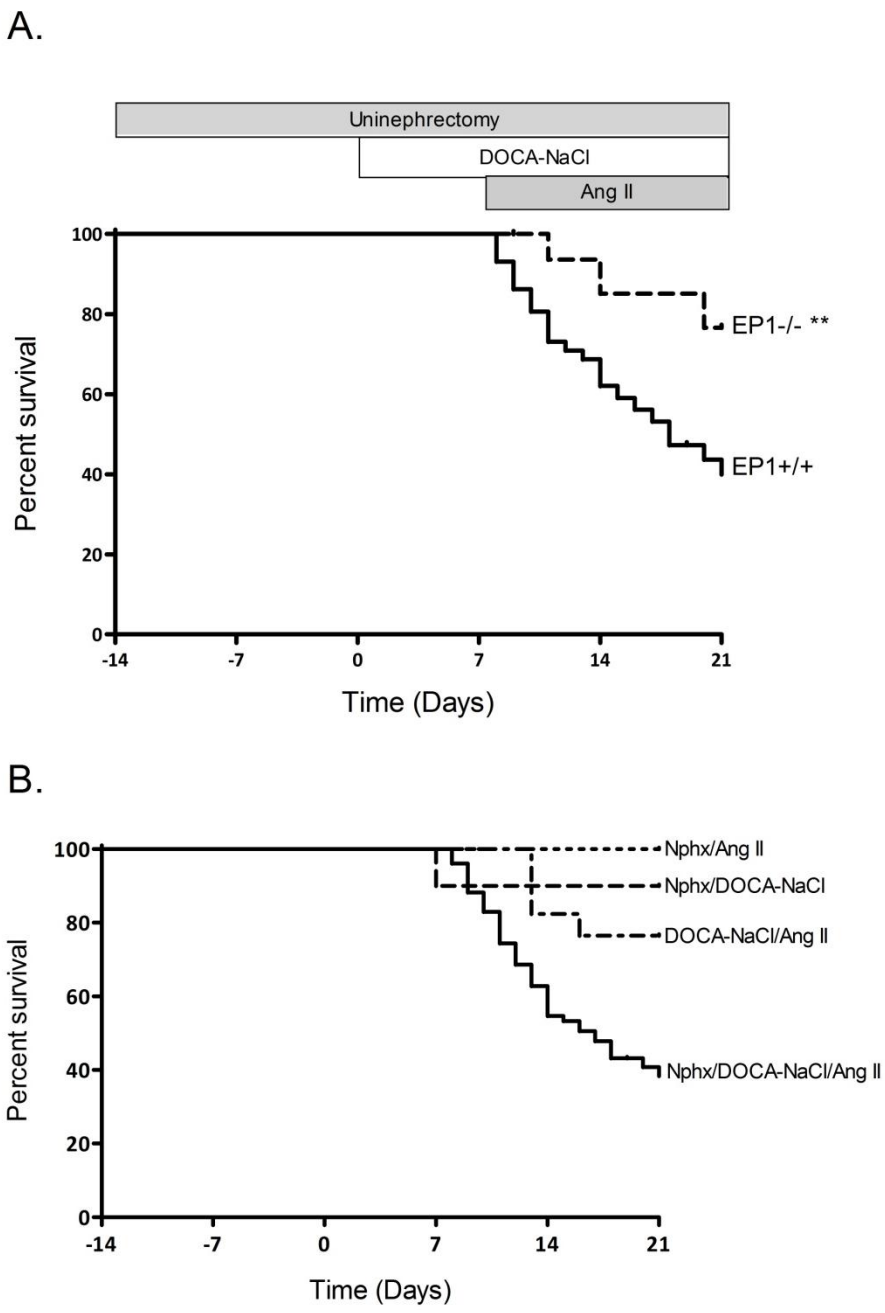


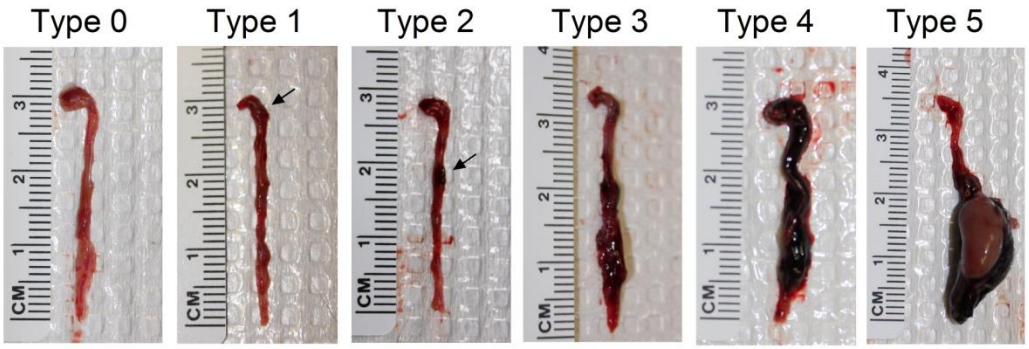

Figure 2.1. Nphx/DOCA-NaCl/Ang II induced substantial mortality in EP1+/+ mice. A. Survival of EP1+/+ and EP1-/- mice. EP1+/+ mice experience a high mortality rate after implantation of the Ang II minipump (60 %, N=58) which is significantly reduced in EP1-/- mice (25 %, N=35). Kaplan Meier survival curves for each genotype are plotted, **P = 0.004. B. Survival of EP1+/+ modified protocol groups. Survival curves were plotted indicating reduced mortality in all modified protocol groups. Nphx/DOCA-NaCl/Ang II (data replotted from Figure 1A) vs. Nphx/DOCA-NaCl, Nphx/Ang II or DOCA-NaCl/Ang II, P = 0.011, <0.005, or < 0.005.

the amount and organization of collagen surrounding the lesion was not significantly different in EP1+/+ and EP1-/- intact aneurysms (Figure 2.4, P = 0.1925).

***Anasarca was observed in EP1+/+ mice***

A subset of EP1+/+ mice appeared to have substantial edema and displayed an increase in body weight, peaking approximately five days post-Ang II administration (Figure 2.5A). Average body weight in EP1 +/+ cohort subsequently decreased due to mortality in the animals with the largest weight gain. At baseline, EP1+/+ mice weighed more than EP1-/- mice though this difference was modest (EP1+/+ 26.6 ± 0.39 grams, EP1-/- 24.9 ± 0.68 grams, P = 0.024). Body weight of EP1-/- mice was unchanged over the course of the study. EP1+/+ mice had a significantly greater fraction water weight as compared to EP1-/- mice (Figure 2.5B, P = 0.0138), and aneurysm incidence was lower in mice which developed anasarca compared to mice without anasarca (33 % vs 76 %). Anasarca is commonly a result of liver failure, nephrotic syndrome or heart failure (168,169). Plasma alanine transaminase (ALT) activity was analyzed as a marker of liver function in Nphx/DOCA-NaCl/Ang II treated EP1+/+ mice. ALT activity was not elevated above baseline values and no correlation with body weight was observed (Figure 2.6).

A.




B.

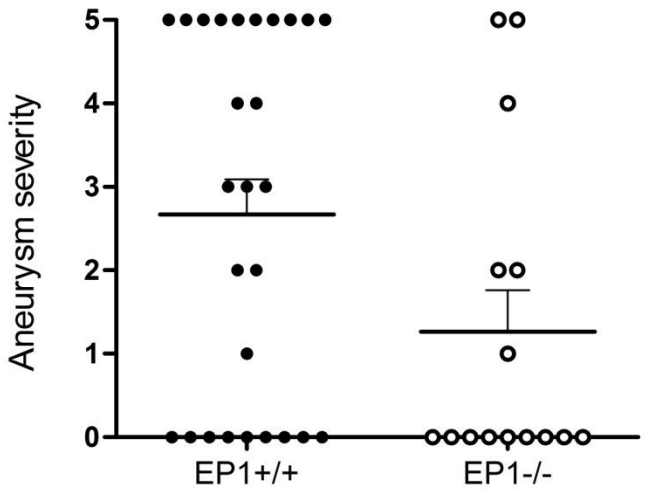



Figure 2.2. Nphx/DOCA-NaCl/Ang II model induces aortic aneurysm formation. A. Nphx/DOCA-NaCl/Ang II treatment resulted in formation of aneurysms and dissections in the thoracic and abdominal aorta. Aortae were scored visually upon necropsy and representative images are shown. B. Post-mortem examination revealed 18 of 27 EP1+/+ mice and 6 of 15 EP1-/- mice developed aneurysms as a result of the Nphx/DOCA-NaCl/Ang II model. Aneurysm severity was significantly reduced in EP1-/- mice compared to EP1+/+ mice. Fisher's exact test, Severity < 3 vs. ≥ 3, P = 0.0344.

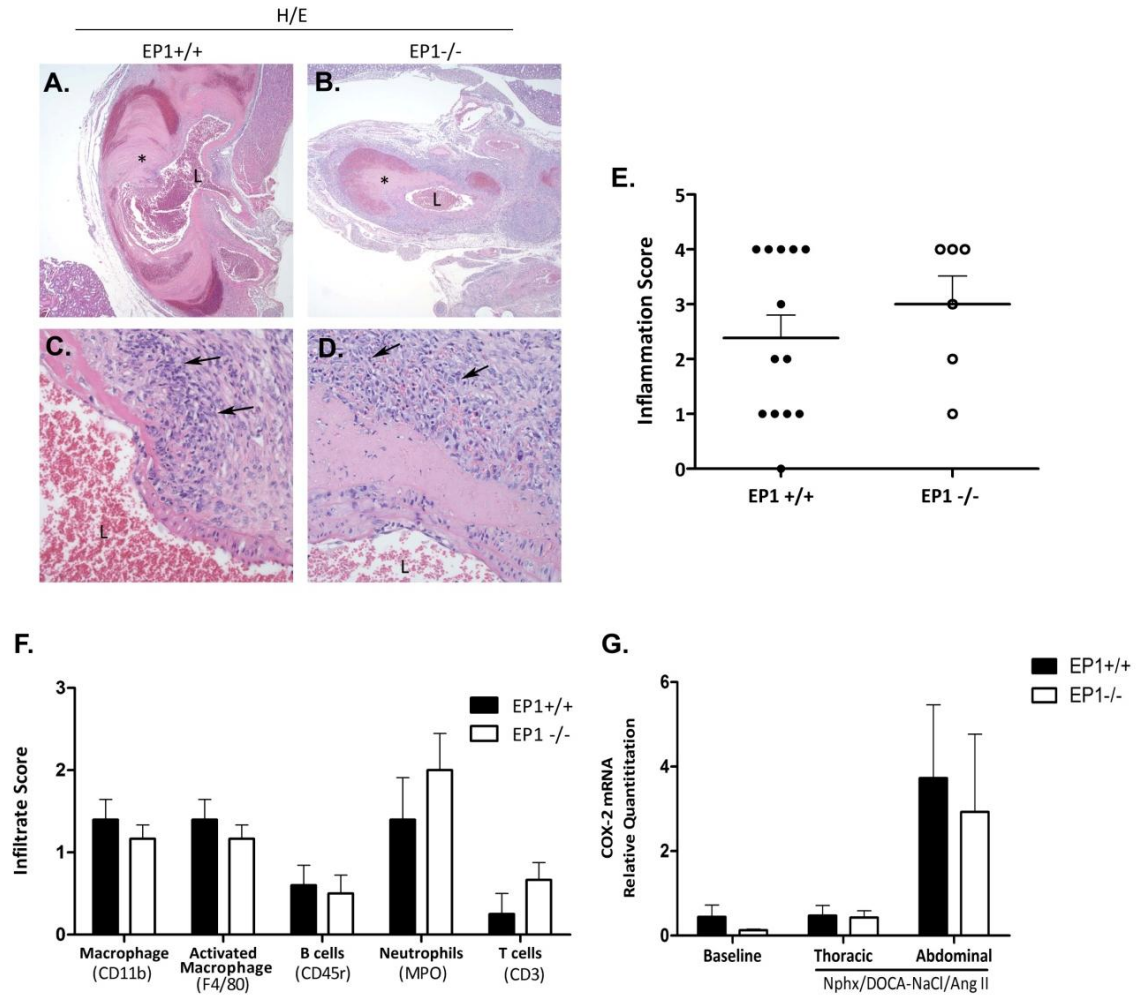



Figure 2.3. Aortic inflammation is observed in EP1+/+ and EP1-/- mice. A-D. Hematoxylin and eosin stained aortae (40X and 400X magnification). A ruptured aneurysm in an EP1+/+ mouse (A, C) and a large aneurysm without rupture in an EP1-/- mouse (B, D). Vessel necrosis and perivascular inflammatory infiltration of macrophages and neutrophils are observed under high magnification (arrows, C and D). L = vessel lumen, * = aneurysm. E. Overall inflammation was scored based on H/E stained aortic sections. No differences in inflammation were observed between EP1+/+ and EP1-/- aortas ($P = 0.3975$). F. Immunohistochemistry for detection of macrophage, B cells, T cells and neutrophils in aortae was performed and scored in a blinded fashion by a comparative veterinary pathologist. No differences in infiltrate were observed ($P > 0.05$). G. COX-2 mRNA in the thoracic and abdominal aortae 2-5 days post Ang II administration revealed a trend in increased COX-2 expression in the abdominal aorta of both EP1+/+ and EP1-/- mice post treatment. No significant differences were observed by treatment or between genotypes ($P > 0.05$).

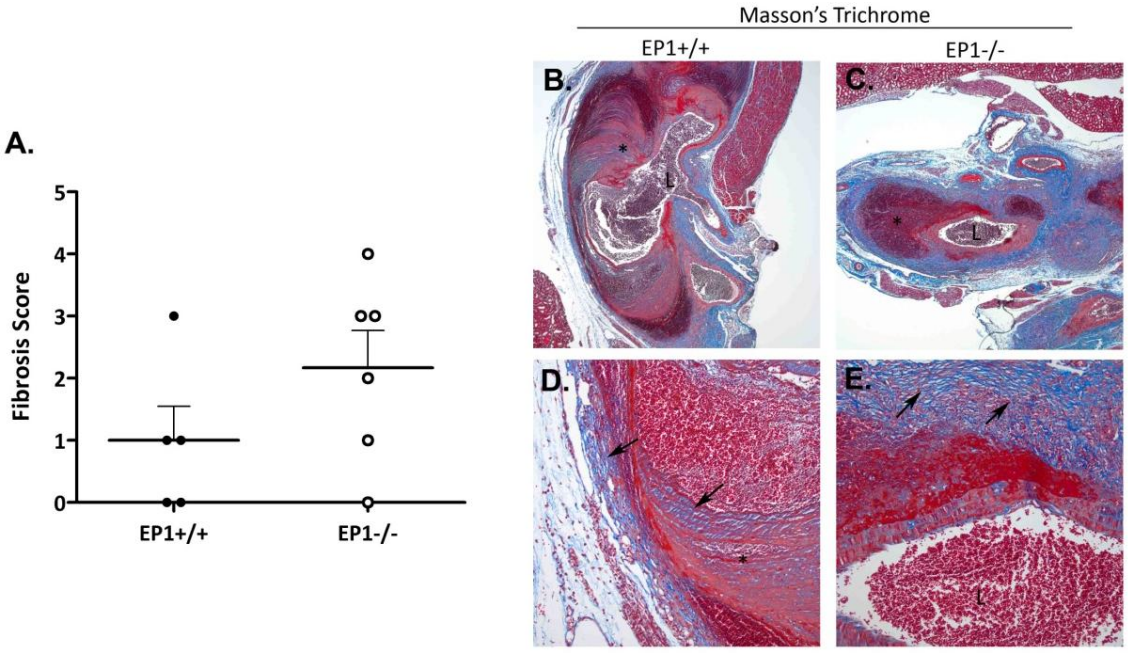



Figure 2.4. Aortic fibrosis is observed in EP1+/+ and EP1-/- mice. A. Extracellular matrix deposition was determined using Masson's Trichrome stain (P = 0.1925). B-E. Masson's trichrome stained aortae. EP1+/+ aorta (B, D) with rupture displays less collagen organization. EP1-/- aorta (C, E) with an intact aneurysm shows well developed and organized collagen (arrows) surrounding the aneurysm. L = vessel lumen, * = aneurysm.

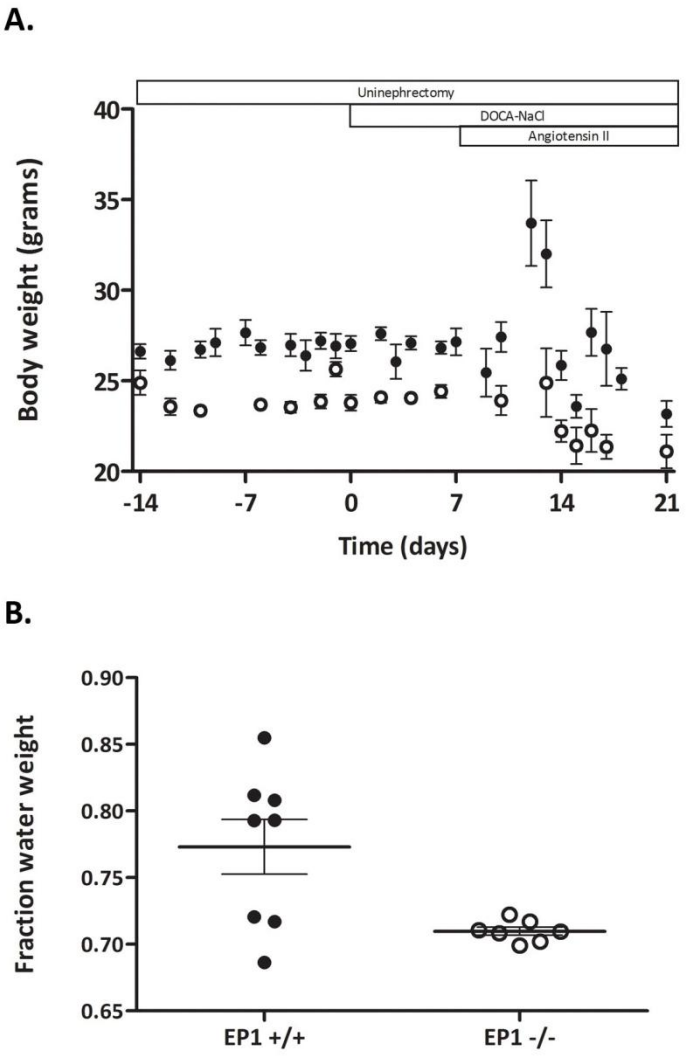



Figure 2.5. Body weight increases during Nphx/DOCA-NaCl/Ang II treatment in EP1+/+ mice. A. Average body weight of EP1+/+ (N=50) and EP1-/- mice (N=22) throughout the duration of the experiment. Body weight increased in EP1+/+ mice, but not EP1-/- mice, following Ang II administration. Average body weight declined at approximately five days post-Ang II due to mortality in the subset of mice which gained the most weight (open symbols, EP1-/-; closed symbols, EP1+/+). B. Fraction water weight 5 days post-angiotensin II administration. The fraction water weight was significantly greater in EP1+/+ mice, as compared to EP1-/- mice, P = 0.0138.

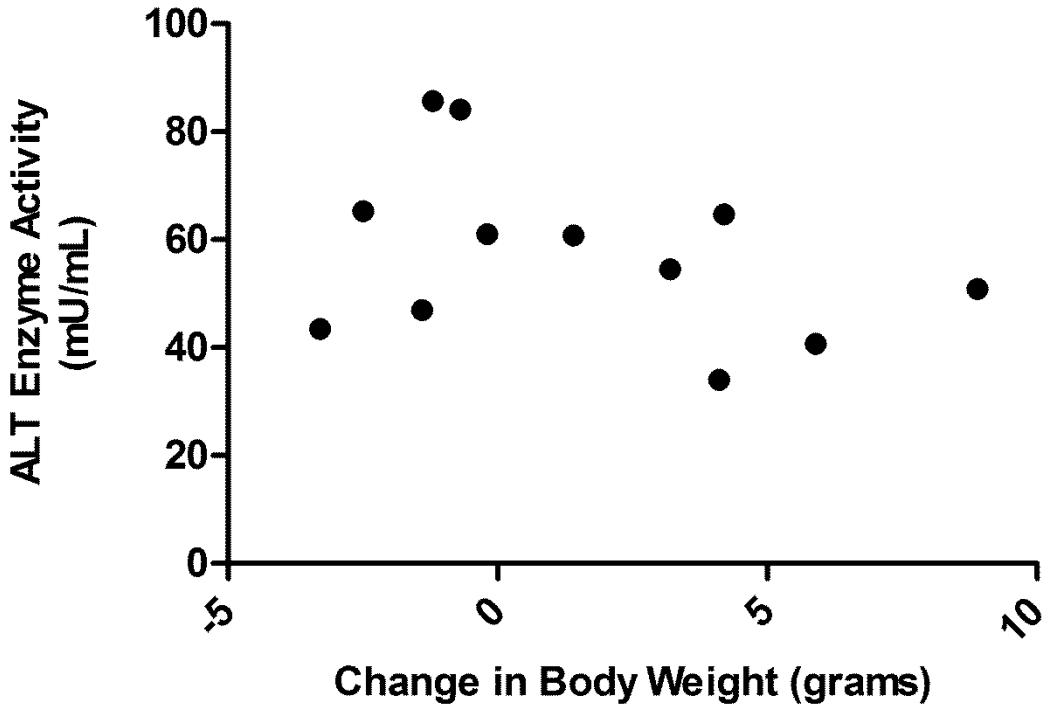
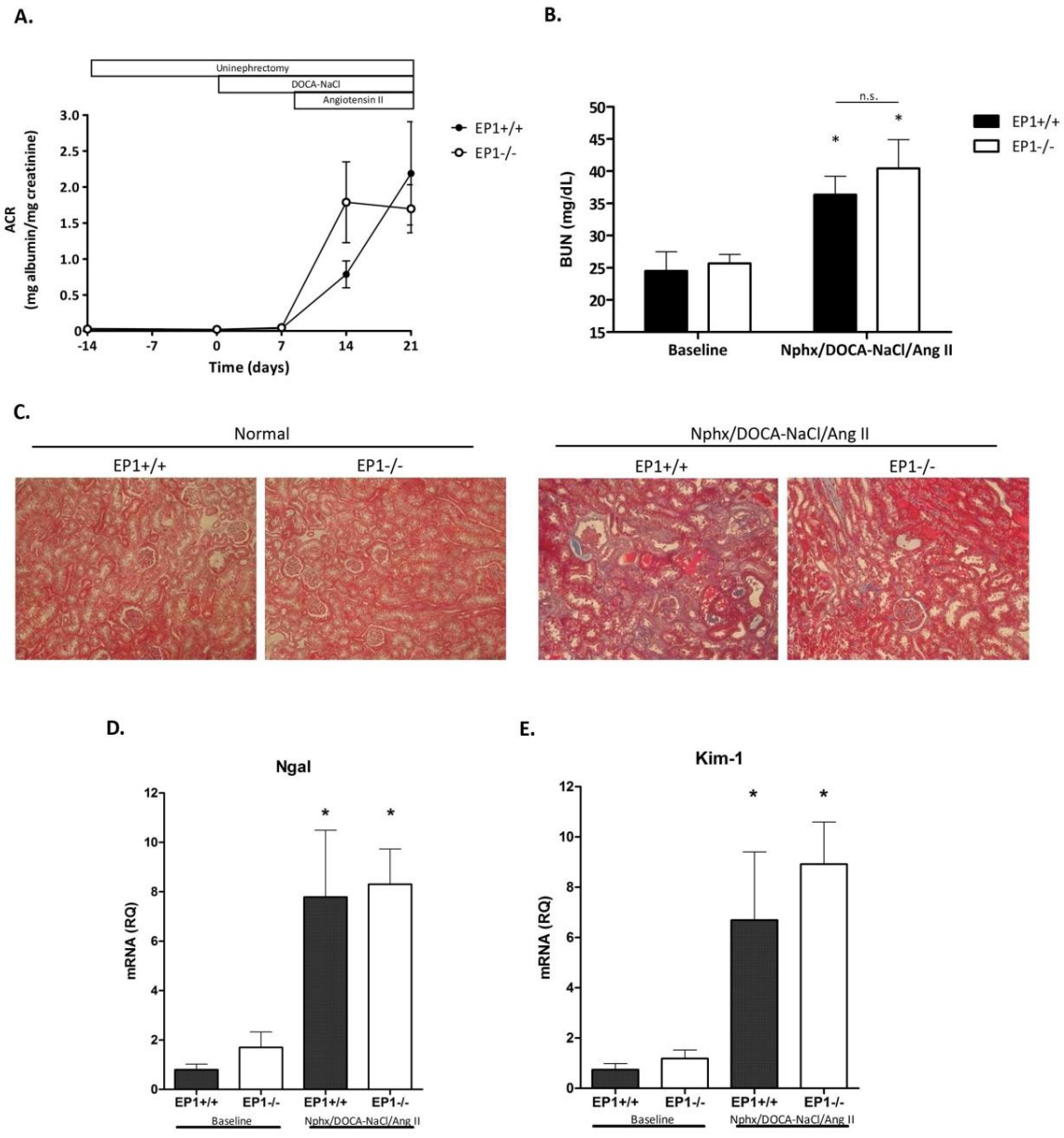



Figure 2.6. Plasma alanine transaminase activity and body weight showed no correlation in EP1+/+ mice. ALT activity was not elevated above expected baseline values (< 100 mU per mL) and no correlation with body weight was observed ($R^2$ = 0.1368).

***Modest renal injury was induced in EP1+/+ and EP1-/- mice***

The Nphx/DOCA-NaCl/Ang II model was initially developed to induce hypertensive renal damage on the C57BL/6 background (163). To quantify renal damage in the EP1+/+ and EP1-/- mice, we monitored urinary albumin excretion, blood urea nitrogen, renal histopathology, and biomarkers of acute kidney injury, neutrophil gelatinase-associated lipocalin (Ngal) and kidney injury molecule-1 (Kim-1) mRNA expression (Figure 2.7). ACR and BUN were elevated but no significant differences were observed between genotypes (Figure 2.7A, B). Renal histopathology showed modest hypertensive renal damage compared to the contralateral kidney removed at time of uninephrectomy (Figure 2.7C). Dilated tubules with moderate glomerulosclerosis and tubulointerstitial fibrosis were observed. Significant increases in Ngal and Kim-1 mRNA expression in the kidney of EP1+/+ and EP1-/- mice were observed, though no differences were detected between genotypes (Figure 2.7D-E).

Figure 2.7. Modest renal damage is observed in EP1+/+ and EP1-/- mice. A. Urinary albumin excretion. ACR was determined on spot urine collected throughout the duration of the experiment. High albumin levels were detected in the urine after the addition of Ang II and no significant differences were observed in ACR between the genotypes. Fisher's Exact test, P = 0.4559. B. Blood urea nitrogen levels were modestly increased by the end of the full model as compared to baseline in both genotypes (EP1+/+ P = 0.0382, EP1-/- P = 0.0132), though no differences were observed in the degree of BUN levels between the genotypes (P = 0.5867). C. Renal histopathology. Masson's trichrome stain revealed modest hypertensive renal damage observed in both genotypes following full model treatment (right panels) and compared to normal kidneys removed at time of uninephrectomy (left panels). Specifically, dilated tubules with moderate glomerular sclerosis and tubulointerstitial fibrosis were observed. D and E. Quantification of Ngal and Kim-1 mRNA expression in whole kidneys. Expression of clinically used renal injury biomarkers, Ngal and Kim-1, revealed significant increases in both genotypes treated with the full model (Ngal: EP1+/+ P = 0.0029, EP1-/- P = 0.0008; Kim-1: EP1+/+ P = 0.007, EP1-/- P = 0.0002), though no significant differences between EP1+/+ and EP1-/- mRNA levels were observed (Ngal P = 0.8609, Kim-1 P = 0.4931).


A.
Uninephrectomy
DOCA-NaCl
Angiotensin II
ACR
(mg albumin/mg creatinine)
3.0
2.5
2.0
1.5
1.0
0.5
0
-14
-7
0
7
14
21
Time (days)
EP1+/+
EP1-/-
B.
n.s.
*
*
BUN (mg/dL)
50
45
40
35
30
25
20
15
Baseline
Nphx/DOCA-NaCl/Ang II
EP1+/+
EP1-/-
C.
Normal
Nphx/DOCA-NaCl/Ang II
EP1+/+
EP1-/-
EP1+/+
EP1-/-
D.
Ngal
mRNA (RQ)
12
10
8
6
4
2
0
*
*
EP1+/+
EP1-/-
EP1+/+
EP1-/-
Baseline
Nphx/DOCA-NaCl/Ang II
E.
Kim-1
mRNA (RQ)
12
10
8
6
4
2
0
*
*
EP1+/+
EP1-/-
EP1+/+
EP1-/-
Baseline
Nphx/DOCA-NaCl/Ang II

***Cardiac function is reduced in EP1+/+ and EP1-/- mice***

No structural differences in the heart were observed by echocardiography between EP1+/+ and EP1-/- mice at baseline. A modest increase in ejection fraction and fractional shortening was observed in EP1-/- mice at baseline. At five days post-Ang II administration, EP1+/+ had increased left ventricular posterior wall (LVPWd) and interventricular septum diastolic diameters (IVSd). EP1-/- mice had increased IVSd, though no significant change in LVPWd was observed. EP1+/+ and EP1-/- mice displayed increased left ventricular interior diameter, though no differences were observed between genotypes. Cardiac function was significantly reduced in both genotypes upon treatment with Nphx/DOCA-NaCl/Ang II, as demonstrated by decreased fractional shortening and ejection fraction (Table 2.1). Additionally, heart weights of EP1+/+ and EP1-/- mice after treatment with Nphx/DOCA-NaCl/Ang II showed no significant difference between the genotypes (EP1+/+: 198.9 ± 9.6 grams N = 21, EP1-/-: 190.0 ± 4.3 grams N = 17, P = 0.461).

Table 1. Echocardiography data at baseline and 5 days post Ang II treatment.

| | Baseline | | Nphx/DOCA-NaCl/Ang II | |
|---|---|---|---|---|
| Parameter | EP1+/+ (N = 9) | EP1-/- (N = 8) | EP1+/+ (N = 6) | EP1-/- (N = 8) |
| LVPWd (mm) | 0.760 ± 0.015 | 0.772 ± 0.021 | 0.773 ± 0.021 † | 0.794 ± 0.028 |
| IVSd (mm) | 0.812 ± 0.011 | 0.790 ± 0.019 | 0.921 ± 0.038 † | 0.895 ± 0.010 † |
| LVIDd (mm) | 3.058 ± 0.043 | 2.985 ± 0.040 | 3.551 ± 0.24 † | 3.482 ± 0.13 † |
| FS (%) | 49.225 ± 0.28 | 50.847 ± 0.13 ‡ | 31.784 ± 2.98 † | 33.268 ± 2.31 † |
| EF (%) | 81.917 ± 0.27 | 83.462 ± 0.15 ‡ | 60.228 ± 4.22 † | 62.467 ± 3.20 † |

† $P < 0.05$, Baseline vs Nphx/DOCA-NaCl/Ang II

‡ $P < 0.05$, EP1+/+ vs EP1-/-

***Hypertension was less severe in EP1-/- mice than EP1+/+ mice***

To determine the effect of disruption of the EP1 receptor on blood pressure in Nphx/DOCA-NaCl/Ang II treated animals, intracarotid blood pressure was determined in EP1+/+ and EP1-/- mice two days post-Ang II administration (Figure 2.8). MAP was significantly increased compared to untreated animals in both EP1+/+ (76.79 +/- 5.33 mm Hg baseline vs. 128.8 +/- 5.08 mm Hg, P = 0.0004) and EP1-/- mice (74.36 +/- 5.61 mm Hg baseline vs. 102.4 +/- 7.77 mm Hg, P = 0.0423). However, the rise in MAP was significantly lower in EP1-/- compared to EP1+/+ mice (P = 0.0295).

***Reduction of blood pressure protected against mortality***

Fifteen EP1+/+ and EP1-/- mice treated with Nphx/DOCA-NaCl/Ang II were administered the antihypertensive agent hydralazine. Hydralazine treatment significantly reduced MAP in EP1+/+ mice (106.1 +/- 5.76 mm Hg vs. 128.8 +/- 5.08 mm Hg, P = 0.024), and but had no significant effect on EP1-/- mice (Figure 2.9A, 99.47 +/- 4.82 mm Hg vs. 102.4 +/- 7.77 mm Hg, P = 0.762). A significant decrease in the incidence of mortality was observed in EP1+/+ but not EP1-/- mice (Figure 2.9B, P = 0.007 and P = 0.642 respectively). Hydralazine treatment reduced aneurysm incidence and severity in EP1+/+ though this did not achieve statistical significance (Figure 2.9C, P > 0.05). Anasarca was not observed in hydralazine treated EP1+/+ (Body weight 5 days post-Ang II: 21.40 +/- 0.65 grams) as compared to untreated EP1+/+ mice (Body weight 5 days post-Ang II: 32.28 +/- 1.59 grams, P < 0.0001).

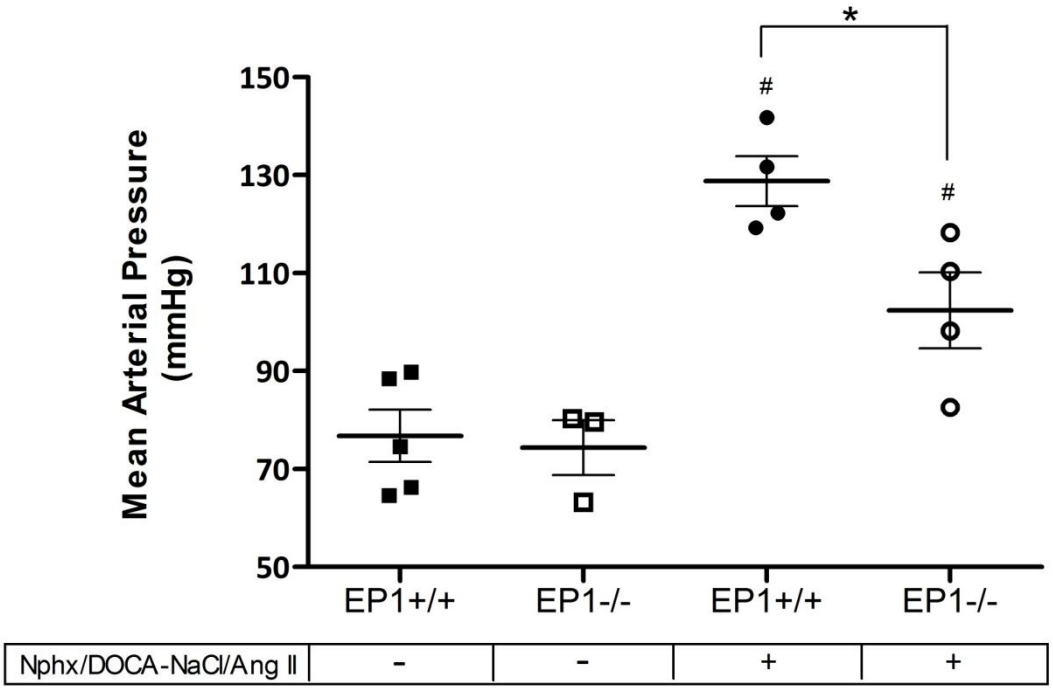
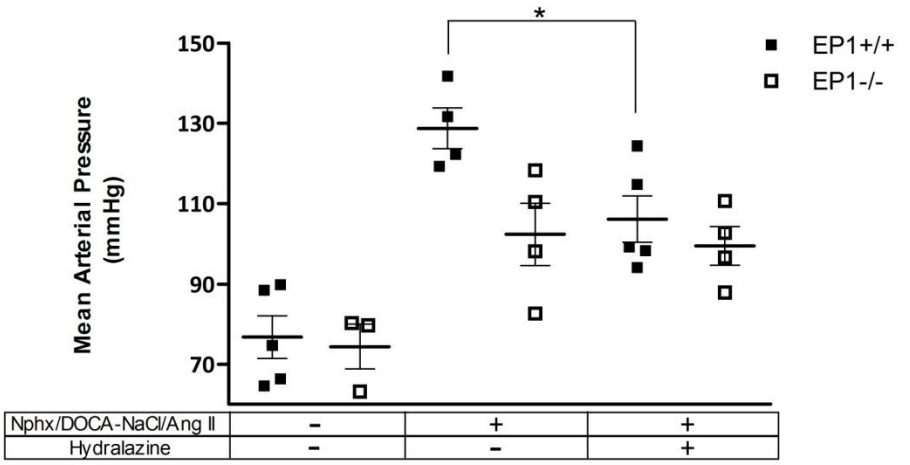


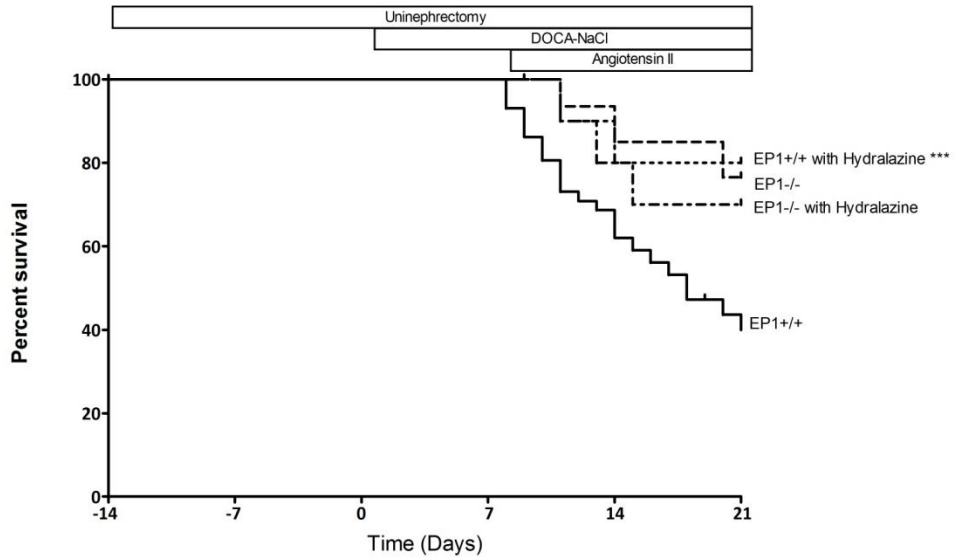

Figure 2.8. Mean Arterial Pressure is increased in EP1+/+, and blunted in EP1-/-, mice. Intracarotid blood pressure was measured in EP1+/+ and EP1-/- mice two days post-Ang II administration under ketamine and inactin anesthesia. MAP was elevated in both groups compared to baseline (EP1+/+ $^{\#\#}P = 0.0004$, EP1-/- $^{\#}P = 0.0423$) but was significantly lower in EP1-/- compared to EP1+/+ two days post-Ang II ($^{*}P = 0.0295$).

Figure 2.9. Hydralazine treatment reduced mortality in EP1+/+ but not EP1-/- mice. A. Mean arterial pressure was measured in EP1+/+ and EP1-/- mice treated with hydralazine at 2 days after Ang II administration and compared to existing data shown in figure 2.7. EP1+/+ with and without hydralazine, P = 0.024. EP1-/- with and without hydralazine, P = 0.762. B. Mortality observed in EP1+/+ mice was significantly reduced by treatment with hydralazine, ***P = 0.007. No change was observed in EP1-/- mice, P = 0.642. C. Aortic aneurysm severity. Aneurysm severity was decreased in EP1+/+ mice treated with hydralazine (P > 0.05).

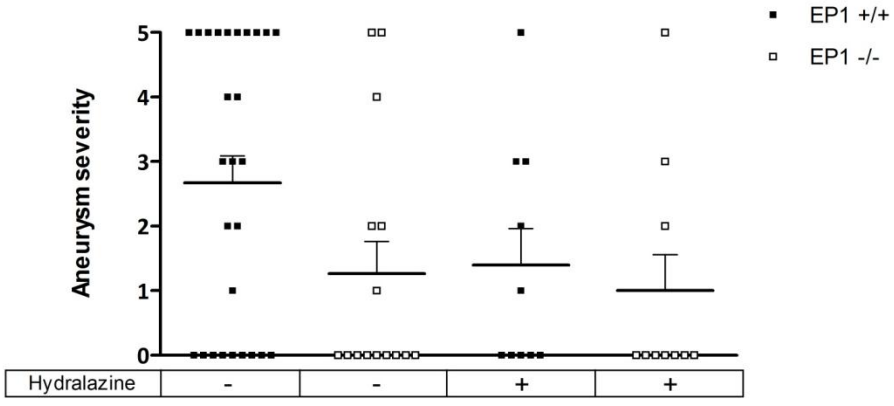
A.


*
150
130
110
90
70
Mean Arterial Pressure (mmHg)
EP1+/+
EP1-/-
Nphx/DOCA-NaCl/Ang II
Hydralazine
-
+
-
-
+
+

B.


Uninephrectomy
DOCA-NaCl
Angiotensin II
100
80
60
40
20
0
Percent survival
-14
-7
0
7
14
21
Time (Days)
EP1+/+ with Hydralazine ***
EP1-/-
EP1-/- with Hydralazine
EP1+/+

C.


EP1 +/+
EP1 -/-
5
4
3
2
1
0
Aneurysm severity
Hydralazine
-
-
+
+

## Discussion

Disruption of the EP1 receptor affords substantial protection in the Nphx/DOCA-NaCl/Ang II evoked hypertension. The incidence of mortality was significantly decreased and appeared to result from reduction in MAP. Mortality was a result of ruptured aortic aneurysm or occurred after developing anasarca. The results presented here are consistent with previous studies demonstrating the role of EP1 in modulating the rise in MAP in response to Ang II (85), and further reveal the protective effect disruption of the EP1 receptor has on end-organ damage.

There are several limitations to these studies which deserve mention. First, high mortality observed in EP1+/+ mice confounds the analysis of measurements taken after implantation of Ang II, such as cardiac and renal function, since the analyses are only performed on surviving mice. Second, there was a modest (<10 %), though statistically significant difference in body weight observed between EP1+/+ and EP1-/- mice. Although the dosage of Ang II was adjusted by weight, the dosage of the DOCA pellet was not. However, one would predict the genotype receiving the greater dose/weight (EP1-/-) would have the worse phenotype, and this is opposite what we observed. Lastly, EP1-/- mice were observed to have lower blood pressure than EP1+/+ mice following treatment with the Nphx/DOCA-NaCl/ Ang II model. This is consistent with our previously published data (170) suggesting EP1 mediates part of Ang II-induced hypertension. In this model we measured MAP at baseline or after

treatment with all three model components; it is possible that EP1 also contributes to hypertension induced by Nphx or DOCA-NaCl as well.

Current models of aortic aneurysm include a combination of hyperlipidemic mice or high fat diet with modulation of the renin-angiotensin-aldosterone axis, or aberrant production of extracellular matrix components (171). Ang II-induced aortic aneurysms are characterized by accumulation of macrophages in the adventia and media, disruption of elastin fibers, expansion of the lumen, thrombus formation and disordered extracellular matrix deposition (172). These characteristics were also observed in the Nphx/DOCA-NaCl/Ang II model, although no significant differences in macrophage accumulation, matrix deposition, or COX-2 mRNA expression were detected between the two genotypes. It should be noted that the aneurysms and dissections observed in this model occur after acute severe hypertension and although the pathology appears similar to that observed in human disease, the disease genesis may not be. In humans, development of a true aneurysm is a slowly progressing disease initiating with local inflammation, disruption of the connective tissue matrix, and is often associated with atherosclerosis. In contrast, development of false aneurysm, or dissection as a result of a tear in the intima, can occur more acutely by a sudden large rise in blood pressure or direct injury and may be more representative of the damage induced by the Nphx/DOCA-NaCl/Ang II model. Our data demonstrate that protection observed when EP1 is disrupted is likely due to the prevention of a large rise in blood pressure, since treatment with

hydralazine phenocopied EP1-/- mice. This does not eliminate the possibility that EP1 receptors might also provide protection directly at the target tissue.

Data exist suggesting a role for prostaglandins, in particular $PGE_2$, in aortic aneurysm formation. COX-2 initiates the production of prostaglandins, and its expression is induced by infusion of angiotensin II in the smooth muscle of the aorta surrounding aneurysms (166). Furthermore, either selective inhibition of COX-2 or genetic deletion of COX-2 significantly reduced aortic aneurysm formation and macrophage infiltration (166,167). Deletion of microsomal PGE synthase-1, which transforms the product of COX-2 metabolism into $PGE_2$, has also been demonstrated to reduce aortic aneurysm formation and oxidative stress in LDLR-/- mice with an angiotensin II infusion (160), suggesting $PGE_2$ plays an important role in development of aneurysms and the EP receptors may be viable targets for treatment of aneurysm progression.

Previous reports of the role of EP1 in renal injury are contradictory. In spontaneously hypertensive rats, treatment with an EP1 antagonist reduced proteinuria and tubulointerstitial damage (87), while in anti-GBM nephrotoxic serum nephritis genetic deletion of EP1-/- in mice resulted in enhanced mesangial expansion and tubular dilation and increased blood urea nitrogen and serum creatinine (173). In our studies, modest hypertensive renal damage was observed, although no significant differences in renal function were detected between genotypes. However, our interpretation was confounded by the differential mortalities in EP1+/+ and EP1-/- mice, potentially biasing our results. Examination of renal histopathology at time points prior to significant mortality

failed to detect any severe renal damage or differences between the genotypes. This suggests the role of EP1 in renal damage is highly context dependent.

Anasarca, or extreme generalized edema, can occur in many disease settings. It is commonly a result of liver failure, nephrotic syndrome or heart failure (168,169). In our Nphx/DOCA-NaCl/Ang II model, a subset of EP1+/+ mice developed severe anasarca prior to mortality, while EP1-/- mice were protected. The EP1 receptor has previously been shown to be natriuretic (85). With this paradigm, one might predict EP1-/- mice would retain more salt and water; however in our results we demonstrate that EP1+/+ mice gain excessive fluid volume that is not observed in EP1-/- mice. This contradiction leads us to conclude that alterations in kidney function by disruption of EP1 do not play a dominant role in development of the observed edema. Additionally, cardiac function was reduced to similar degrees in EP1+/+ and EP1-/- mice. Edema was prevented by treatment with hydralazine, suggesting elevation in blood pressure was responsible for development of edema. We hypothesize that hypertension induced by DOCA-NaCl and Ang II results in volume loading and enhanced vasoconstriction, which places excessive stress on the vascular wall leading to enhanced permeability, resulting in edema and susceptibility to dissections and rupture. Future experiments will be required to identify whether vascular permeability differences are observed between EP1+/+ and EP1-/-mice.

The EP1 receptor plays an important role in the development of hypertensive damage. In the Nphx/DOCA-NaCl/Ang II model, disruption of EP1 results in increased incidence of survival, lessened aneurysm severity and the

absence of anasarca. This effect is a result of a reduced rise in blood pressure observed in EP1-/- mice, and suggests the EP1 receptor may be a viable pharmaceutical target for the treatment of hypertension and subsequent organ damage. Furthermore the Nphx/DOCA-NaCl/Ang II model may prove to be a useful tool for studying the pathology of aortic aneurysm and dissection formation in a setting of acute severe hypertension.

CHAPTER III

# DISRUPTION OF EP3 IS PROTECTIVE AGAINST MORTALITY IN A MOUSE MODEL OF HYPERTENSION

## Introduction

There is a strong association between hypertension and progressive renal failure, and mitigation of hypertension is a major therapeutic goal for the prevention of end-stage renal disease. A number of pharmacologic agents are available for the treatment of hypertension including those that affect the renin-angiotensin aldosterone system such as angiotensin converting enzyme inhibitors, angiotensin receptor blockers and more recently, the introduction of renin inhibitors (11,174-178). Angiotensin II mediates its effects via two GPCRs, designated AT1 and AT2, which are distinguished by their pharmacology and the signal transduction pathways that they activate (175,176,179). The AT1 receptor is the target for the anti-hypertensive receptor antagonist ARBs (180). AT1 receptor activation leads to a number of signal transduction pathways including increases in intracellular $Ca^{++}$ and activation of $cPLA_2$ (180-188), a critical regulatory step in the formation of PGs. PGs, which are oxygenated metabolites of the essential fatty acid arachidonic acid, are themselves modulators of blood pressure and evidence suggests that blockade of one or more prostaglandin receptors may be useful for the treatment of hypertension. Recent evidence

suggests that at least one $PGE_2$ receptor may play a role in the actions of the RAAS on blood pressure (85).

PGs are potent mediators of a wide range of physiological actions including inflammation, modulation of smooth muscle tone and water and ion transport in the kidney (98,189). The five primary bio-active prostanoids $PGE_2$, $PGF_{2\alpha}$, $PGD_2$, $PGI_2$, and $TXA_2$, activate a family of specific GPCRs, EP for E-prostanoid receptors, FP, DP, IP and TP receptors respectively (98). The EP receptors are unique among the PG receptors, in that four receptors, designated EP1 through EP4, have been described for $PGE_2$ each encoded by a distinct gene. $PGE_2$ has been demonstrated to act as either a pressor or depressor depending upon the EP receptor activated (17,19,79,99,157,190-193). Upon acute infusion, $PGE_2$ is a vasodepressor in both humans and mice (17,138,150). Previous studies with knockout mice have shown that this depressor effect is primarily due to the activation of the EP2 receptor (139). When the EP2 receptor is deleted, the loss of the depressor response unmasks a $PGE_2$ pressor response, suggesting a balance of pressor and depressor receptors activated by $PGE_2$. The pressor response is mediated by the EP3 and EP1 receptors (85,139).

Development of models for the study of hypertension and renal damage that recapitulate human disease have been challenging, particularly on the C57BL/6 background (194,195). A model of hypertension that causes renal damage on the C57BL/6 background has been recently reported (163). This model which employs a combination of uninephrectomy, deoxycorticosterone

acetate (DOCA), high sodium intake and Ang II, was reported to initiate renal damage over a relatively short time course of five weeks. Using this model, the effects of the EP3 receptor on hypertension and renal damage were evaluated.

## Experimental Procedures

### *Materials*

Angiotensin II was purchased from EMD Gibbstown, NJ, and DOCA was purchased from Innovative Research of America, Sarasota, FL, USA. Osmotic minipumps were purchased from DURECT Corporation, USA.

### *Induction of Hypertension*

EP3-/- or EP3+/+ mice were uninephrectomized under ketamine/xylazine anesthesia two weeks before the start of the study. At day 0, mice received subcutaneous implantation of a 50 mg DOCA pellet and were given ad libitum access drinking water containing 1 % NaCl. At day 7, an osmotic mini-pump (Alzet model 1002) delivering 1.5 ng/min/gram body weight angiotensin II was implanted subcutaneously. At day 19 or 21, the animals were euthanized. Tissues were removed for examination by histological study or snap frozen for RNA and protein analysis. Four separate studies were performed with six to eight EP3-/- animals in each study and seven or eight EP3+/+ littermates or commercially obtained C57BL/6J mice (The Jackson Laboratory, Bar Harbor,

ME). A total of 34 EP3+/+ mice and 27 EP3-/- mice were used. All experimental studies were approved by the IACUC of Vanderbilt University Medical Center.

***Measurement of Systolic Blood Pressure***

Systolic BP was determined in conscious mice using a computerized tail-cuff system (Visitech systems BP-2000 Blood Pressure Analysis System, Apex NC, USA) in the Mouse Metabolic Phenotyping Core at Vanderbilt University Medical Center. Mice were trained for four days minimizing physiologically apparent stress. Each measurement is the average of at least 10 consecutive readings after stabilization of blood pressure.

***Intracarotid blood pressure measurement***

Intracarotid blood pressure was measured under ketamine (25 mg/kg) and inactin (100 mg/kg) anesthesia delivered intraperitoneally. Mice were placed on a thermal pad and a PE-10 catheter was inserted into the left carotid artery. The catheter was connected to a TXD-310 transducer and blood pressure was measured using a Digi-Med BPA 400 (Micromed). Mice were equilibrated 30-60 minutes until stable values were attained. Ten minute blood pressure measurements were collected and average mean arterial pressure (MAP) is plotted.

### *Determination of Blood Urea Nitrogen levels*

To assess renal function, BUN levels were determined using Infinity Urea liquid stable reagent (Thermo scientific, USA). Heparinized blood was obtained from saphenous vein and plasma was stored at -80 °C until assayed.

### *Assessment of Renal Histopathology*

Mice were humanely euthanized and tissues fixed overnight in 10 % neutral buffered formalin. Tissues were then processed routinely, embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin and evaluated by light microscopy. Kidney injury was scored by a renal pathologist who calculated the percent of tubules with cell necrosis, loss of brush border, cast formation, and tubular dilation as follows: 0, none; 1, <10 %; 2, 11-25 %; 3, 26-45 %; 4, 46-75 %; 5, >76 %. At least 10 fields (x200) were reviewed for each slide in a blinded fashion.

### *Statistical Analysis*

Data were analyses using ANOVA or Student's *t* test were performed with GraphPad Prism software (GraphPad Software Inc., CA, USA) and are shown as means ± SEM. Survival curves were evaluated by the Kaplan-Meier method. $P < 0.05$ was considered statistically significant for all studies.

## Results

***Uninephrectomy/DOCA-NaCl/Ang II model of Hypertension caused significant mortality.***

C57BL/6 animals were treated using the recently reported uninephrectomy/DOCA-NaCl/Ang II model of hypertension (163). In contrast to the published report, this model resulted in significant mortality after implantation of the Ang II minipumps. Deletion of the EP3 receptor was protective; EP3+/+ mice having 36 % survival while EP3-/- mice had 53% survival (P =0.037; Fig. 3.1).

In the EP3+/+ group, mice displayed severe anasarca, while EP3-/- rarely developed anasarca even in the subset of mice that died. This is reflected in the change in body weight of mice from the initiation of the study to time of death, or the end of the study (Fig. 3.2A). While EP3+/+ had a marked increase in body weight, the EP3-/- mice had no significant change in body weight. This difference in body weight was transient over the course of the study (Fig 3.2B). The transient change in body weight observed in the EP3+/+ group was a result of higher mortality observed in mice with the greatest increase in body weight (Figs. 3.2).

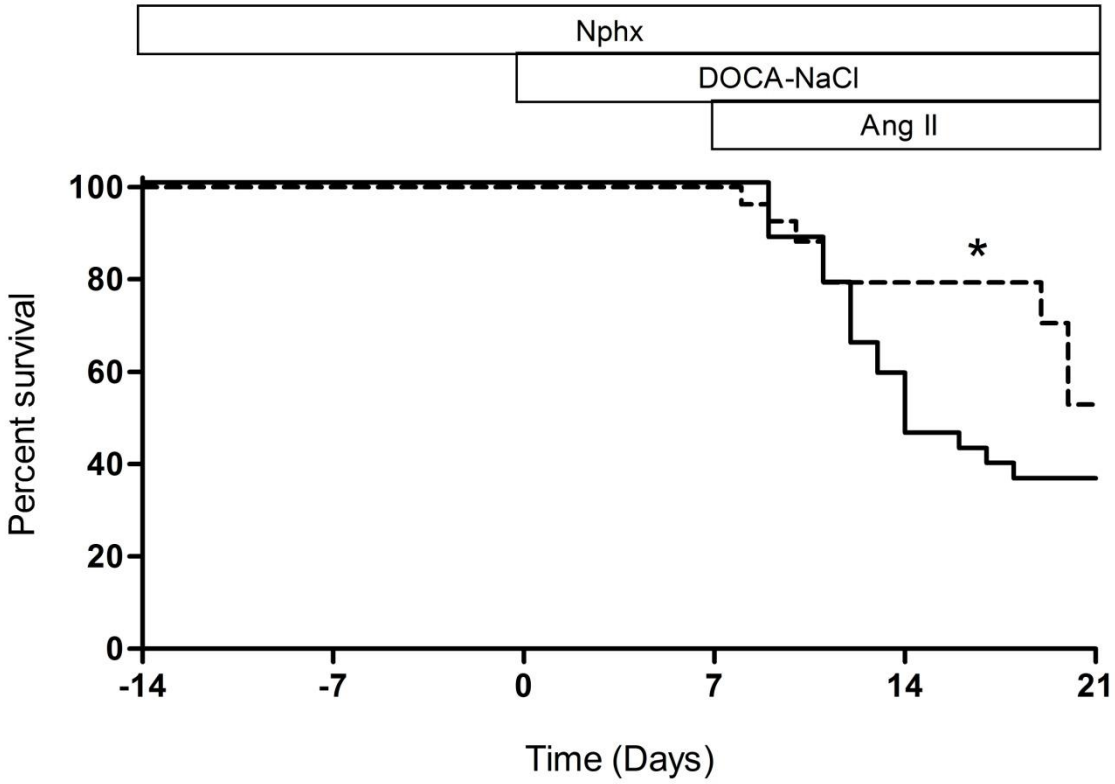



Figure 3.1. The uninephrectomy/DOCA-NaCl/Ang II model resulted in significant mortality. Significant mortality was observed in both EP3+/+ (solid line), and EP3-/- genotypes (broken line). The survival curves of two groups were compared using Kaplan-Meier analysis. Initial EP3+/+, N = 34, EP3-/-, N = 27, P = 0.037.

***Blood pressure was elevated in both genotypes, but less in EP3-/- mice***

The EP3 receptor has vasopressor action, and therefore we hypothesized that significant changes in blood pressure between the two groups might underlie the decreased mortality in the EP3-/- mice. Baseline blood pressure was not significantly different between genotypes in untreated animals (EP3+/+ 108.8 ± 1.7 mm Hg, EP3-/- 107.1 ± 1.6 mm Hg P = 0.47, Figure 3.3A). Concurrent treatment with DOCA salt water and Ang II for two weeks resulted in elevated blood pressure as reported previously in this model. Although there was a dramatic increase in systemic blood pressure over time in both experimental groups terminal SBP was not different between the two groups (EP3+/+ 182.6 ± 5.0 mm Hg, EP3-/- 193.1 ± 4.5 mm Hg P = 0.131; Fig. 3.3B). However, measurement of blood pressure by direct carotid catheterization under anesthesia at 2 or 14 days post Ang II revealed a significantly lower MAP in EP3-/- mice as compared to EP3+/+ mice (EP3+/+ 129.7 ± 2.8 mm Hg, EP3-/- 116.6 ± 5.4 mm Hg, P = 0.0314, Figure 3.3C).

***EP3+/+ and EP3-/- mice have modest renal damage***

The uninephrectomy/DOCA-NaCl/Ang II model was developed to induce hypertensive renal damage on the C57BL/6 background. Histologic analyses indicated similar degrees of renal injury in the EP3+/+ and the EP3-/- mice. The kidneys were characterized by multifocal, segmental to global glomerular microangiopathy and acute renal tubular necrosis with protein casts. Distinction between the EP3+/+ and EP3-/- renal damage was limited to the renal tubules,

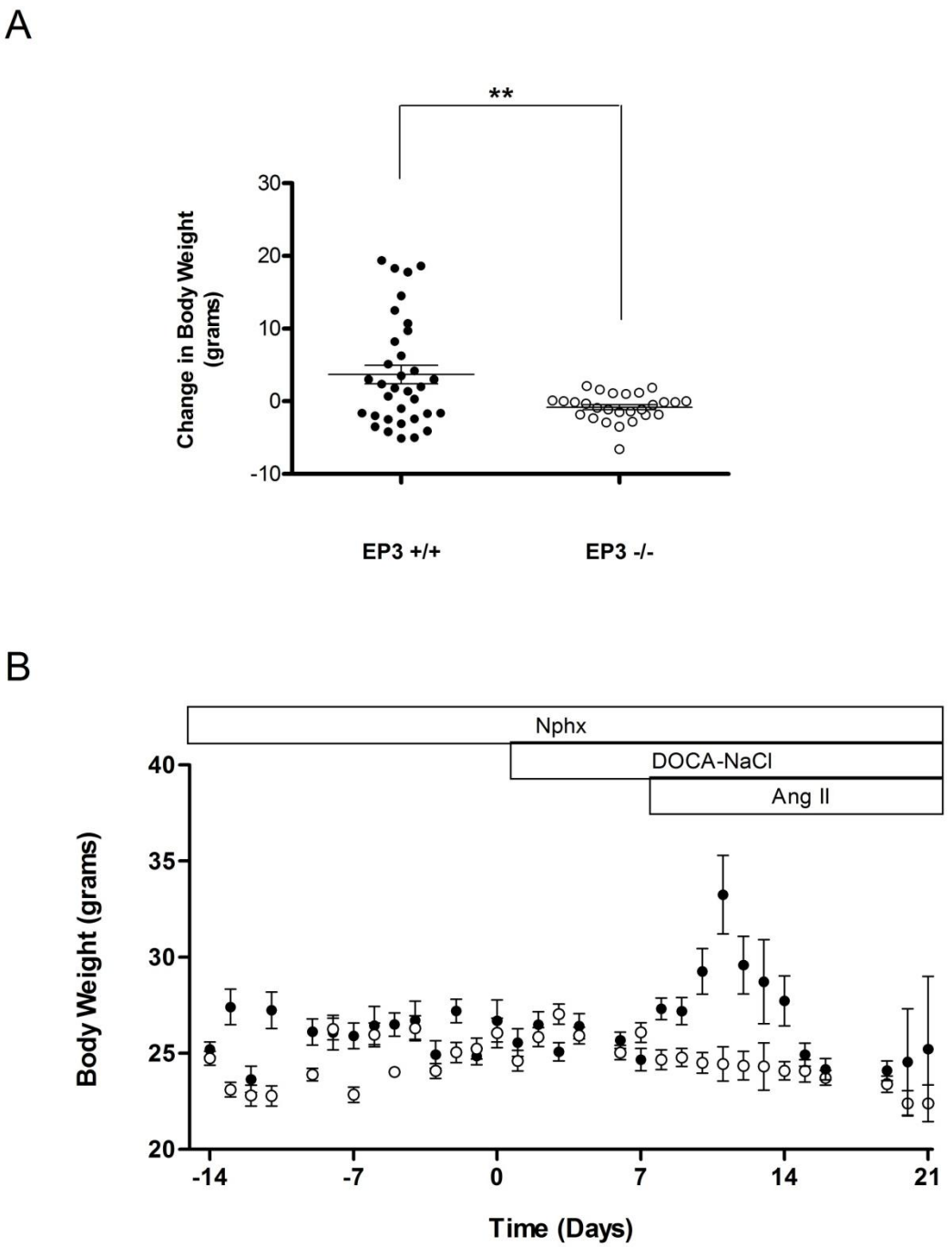
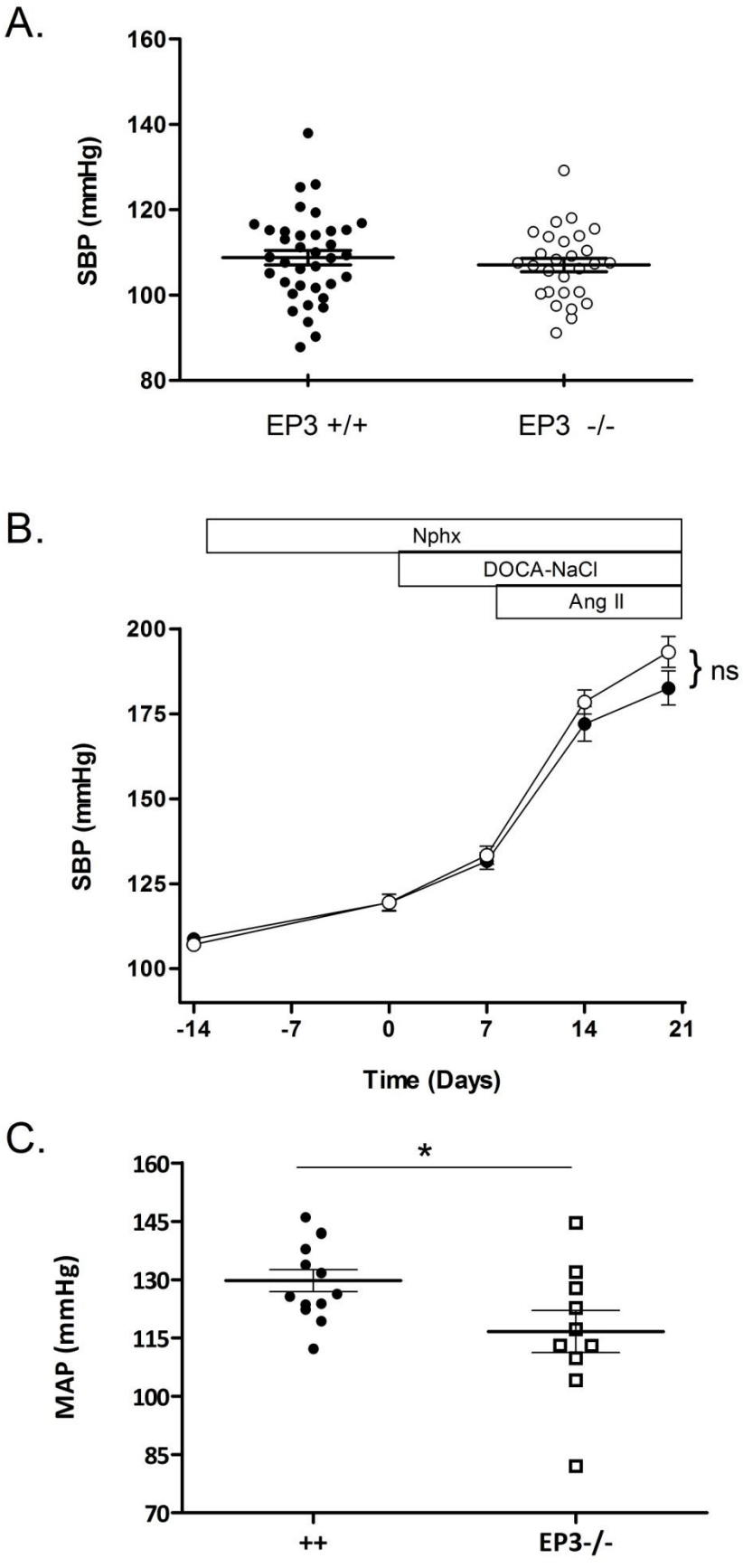



Figure 3.2. Body weight increased in EP3+/+ mice but not EP3-/- mice. A. The difference in terminal body weight and initial body weight was determined. EP3+/+ gained significantly more weight than EP3-/- mice. EP3 +/+, n = 34, EP3-/- n= 27 ** P = 0.0032. B. After implantation of the Ang II pump EP3+/+ mice increased body weight. Initial EP3+/+, n = 34, EP3-/-, N = 27.

Figure 3.3. Both EP3+/+ and EP3-/- animals had significant increases in systolic blood pressure (SBP). A. Baseline SBP was measured in untreated EP3+/+ or EP3-/- mice by the tail cuff method (EP3+/+, n = 37, EP3-/-, n= 28, P = 0.47). B. SBP for the two groups over the course of the hypertension study are shown. Data were analyzed by two-way ANOVA (genotype and time). Initial EP3+/+, n = 34, EP3-/-, n= 27, all surviving mice were analyzed at each time point. There was a statistically significant change in blood pressure over baseline with time in each genotype ($P < 0.0001$). There was no effect of the genotype on SBP over time ($P = 0.29$). C. Intracarotid blood pressure was measured at 2 and 14 days post Ang II. MAP was significantly lower in EP3-/- mice as compared to EP3+/+ mice ($P = 0.0314$). Wildtype data from figure 2.7 are replotted as EP3+/+ MAP.


A.
160
140
120
100
80
SBP (mmHg)
EP3 +/+
EP3 -/-
B.
Nphx
DOCA-NaCl
Ang II
200
175
150
125
100
SBP (mmHg)
} ns
-14
-7
0
7
14
21
Time (Days)
C.
*
160
145
130
115
100
85
70
MAP (mmHg)
++
EP3-/-

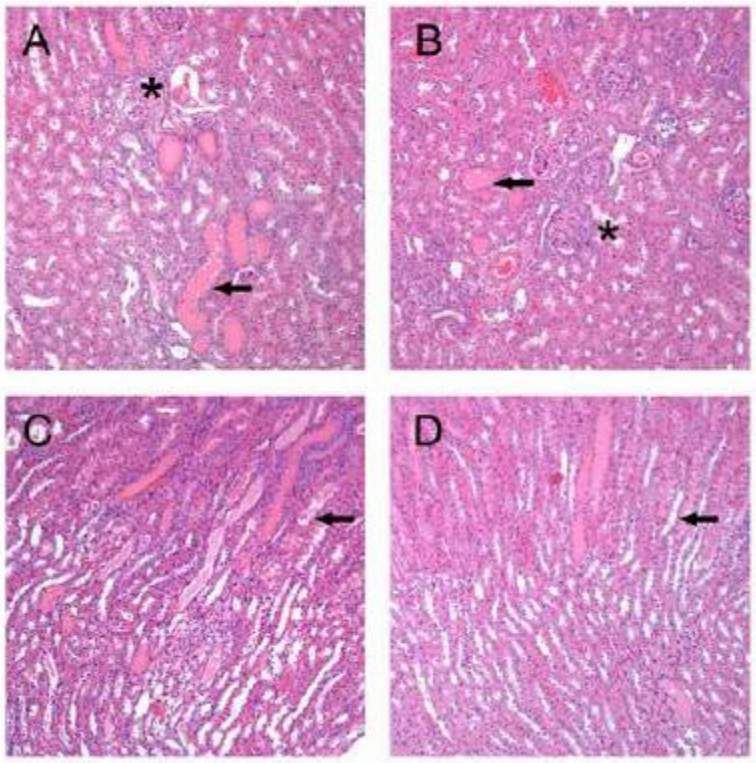



Figure 3.4. Modest end organ renal damage is observed in EP1+/+ and EP1-/- mice. A. H/E staining reveals glomerular microangiopathy and proteinaceous casts in the EP3+/+ and B. EP3-/- renal cortex (200x). *Asterisks* indicate glomeruli with segmental microangiopathies. *Arrows* indicate proteinaceous tubular casts. There is a mild decrease in tubular disease in the EP3-/- as compared to EP3+/+ kidneys. C. H/E staining of the corticomedullary junction in the EP3+/+ mouse kidney shows acute tubular necrosis and proteinaceous cast formation. In the more severely affected areas, tubular epithelial cells are swollen and vacuolated and lose intercellular and basement membrane attachments (*Arrows)*. D. There is a modest decrease in damage seen in the EP3-/- mice (200x). Kidney tissue from eight EP3+/+ and ten EP3-/- mice were examined.

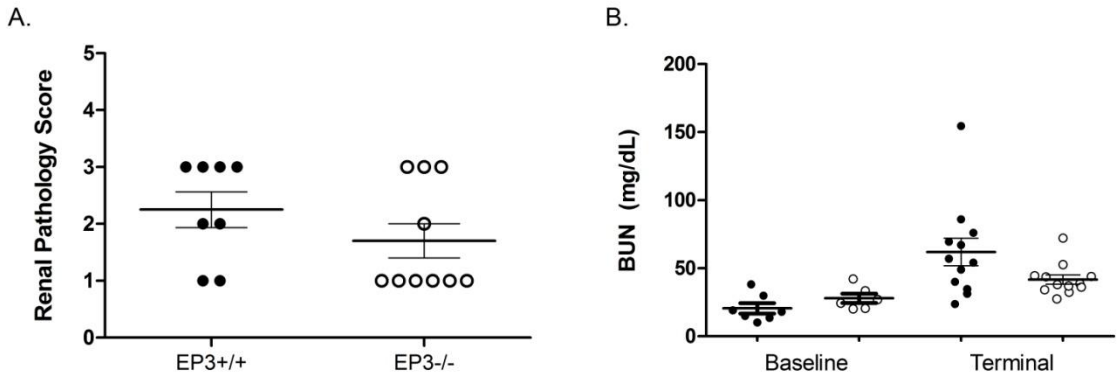



Figure 3.5. Quantitation of renal damage. A. Kidney injury was scored by pathologist Kelli Boyd, DVM as described in the Methods section. At least 10 fields (x200) were reviewed for each slide in a blinded fashion. No difference was seen between EP3+/+ and EP3-/-. B. Blood Urea Nitrogen (BUN) was determined as described in the Methods section. A significant increase in BUN was observed for both genotypes, comparing terminal BUN (n = 11 or 12 in each group) to baseline BUN values (n = 6 or 7 in each group, 2 way ANOVA $P < 0.0001$). The contribution of genotype to the BUN value was not significant.

where the tubular injury was slightly decreased in the EP3-/- mice as compared to EP3+/+ mice (Fig. 3.4). Overall renal damage was quantitated as described in the methods section, and no significant differences were observed in renal histopathological score (Fig 3.5A). Similarly, although both EP3+/+ and EP3-/- genotypes exhibited elevated BUN levels when subjected to the Nphx/DOCA-NaCl/AngII model ($P < 0.0001$) no statistically significant increase in terminal BUN of EP3+/+ as compared to EP3-/- animals was observed (baseline: EP3+/+ BUN 20.6 ± 3.8 mg/dL, EP3-/- 28.0 ± 3.5 mg/dL; terminal: EP3+/+ BUN = 53.7 ± 6.0 mg/dL, EP3-/- 41.8 ± 3.4 mg/dL 2 way ANOVA $P > 0.05$; Fig. 3.5B).

## Discussion

These studies demonstrated that deletion of the EP3 receptor resulted in decreased mortality and modestly reduced hypertension in a mouse model of severe hypertension. Although both EP3+/+ and EP3-/- animals displayed increased BUN and pathological changes in the kidney, these changes were not significantly different between the genotypes. These studies were carried out on the C57BL/6 background, which is normally resistant to renal damage. A recent report described this model to induce hypertensive end organ damage on the C57BL/6 background strain (163). We were able to induce renal damage in the present studies, although we observed substantial differences compared to the reported findings. Most strikingly the EP3+/+ mice developed severe edema and mice in both groups experienced significant mortality. This was surprising as no

mortality was reported in the original description of the model, nor was a gain in weight reported. The EP3 receptor opposes the diuretic action of vasopressin in the renal cortical collecting duct (196,197). Loss of EP3 receptor might be expected to affect water balance, but in this case it should increase water retention in the EP3-/- animals, the opposite of the observed effect of anasarca in the EP3+/+ mice. Previous studies have shown that mice with a disruption of the EP3 receptor concentrated urine normally in response to a range of physiologic stimuli, again suggesting that renal effects of the loss of the EP3 receptor are not playing a critical role in the phenotypic differences observed here (117).

Using tail cuff measurements, we observed SBP of near 200 mm Hg, almost 60 mm Hg higher than observed in the previous report. No deaths were observed in our study until after implantation of the Ang II pump, suggesting that the presence of exogenous Ang II is critical to the cause of death. Deletion of the EP3 receptor was associated with increased survival from 36 % in the EP3+/+ group to 53 % in the EP3-/- group. The EP3 receptor has been implicated in the pressor effect of $PGE_2$ (139). It might be anticipated that deletion of this receptor would lead to lower blood pressure, and this would underlie its protective effect. In the present studies we noted that the EP3-/- mice had modestly reduced hypertension compared to EP3+/+ mice. This suggests the protective effect of EP3 may be due to changes in blood pressure. These results are consistent with studies presented in Chapter II. Futhermore, the degree of hypertension correlates with protection against mortality. Given these results, these studies

that blockade of the EP3 and/or EP1 receptor would be protective from hypertension and its downstream consequences.

# CHAPTER IV

# CONTRIBUTION OF THE EP1 RECEPTOR IN HYPERTENSIVE RENAL DAMAGE

## Introduction

Chronic kidney disease (CKD) is a major public health concern. The 2010 annual report from the United States Renal Data Systems estimated approximately 600,000 patients had end-stage renal disease (ESRD) in 2008, which cost the US $39.5 billion/year. Additionally, the incidence and prevalence of CKD continues to rise in the United States creating a need for novel therapeutic agents (198).

Development of new onset kidney disease is strongly associated with hypertension, diabetes, smoking and low HDL cholesterol (199). In men, baseline blood pressure ≥ 120/80 mm Hg strongly increased the development of ESRD as demonstrated by the Multiple Risk Factor Intervention Trial (200). Several other randomized clinical trials have also demonstrated correlations between hypertension and CKD (201-208), including the RENAAL (Reduction of Endpoints in NIDDM with Angiotension II Antagonist Losartan) trial which showed that each 10 mm Hg rise in systolic blood pressure resulted in an increased risk of ESRD or death of 11 % (209). Therefore, the treatment of renal disease is very closely tied to the treatment of hypertension.

Kidney disease is characterized by changes that involve renal inflammation followed by interstitial fibrosis, atrophy of the renal tubules, and glomerulosclerosis (210). Agents that block the renin-angiotensin-aldosterone system, such as ARBs, ACEi, and renin inhibitors, are among the most commonly used therapeutics for treatment of hypertension and renal damage. AT1 receptors mediate the majority of Ang II induced effects. AT1 is a G protein coupled receptor, which canonically couples to $G_q$ G-proteins, but is also known to activate multiple second messenger signal transduction pathways including extracellular signal-regulated kinases 1 and 2, activation of phospholipases, inhibition of adenylate cyclase, and stimulation of tyrosine phosphorylation and Akt (211). Although classically Ang II has been thought to primarly modulate blood pressure and renal damage through vasoconstriction and aldosterone-induced sodium retention, it is now appreciated to also have affects on proteinuria, inflammation, proliferation, apoptosis, and fibrosis (212,213), thus the benefits of blockade of RAAS often extend beyond that of just blood pressure control (178,214-222). In these studies, the ARBs and ACEi had effects that occur in the absence of a blood pressure change or had a greater change than observed with a drug which reduced blood pressure comparably. One such example utilized a 5/6$^{th}$ nephrectomy model on male Munich-Wistar rats. Rats were treated with either an ACEi, enalapril, or a triple therapy of reserpine, hydralazine and hydrochlorothiazide (RAAS independent). Both treatments normalized blood pressure, although only enalapril was able to reduce the development of proteinuria and glomerulosclerosis, suggesting the

renoprotective effects of enalapril are independent of blood pressure reduction (216).

As mentioned previously, agonism of AT1 receptors can activate phospholipases, which yields products such as prostaglandins. $PGE_2$ is a known modulator of blood pressure and renal disease. $PGE_2$ can act as either a vasopressor or a vasodepressor (17-19,138). This antagonistic property of $PGE_2$ can be explained by the existence of four $PGE_2$ receptors, designated EP1 through EP4, each with distinct tissue localization and characteristic signal transduction pathways. Using pharmacologic agents and genetics, it has been demonstrated that the EP1 and EP3 receptors primarily mediate the pressor response of $PGE_2$, while the EP2 and EP4 receptors mediate the depressor response (17,79,85,99,134,138-140). Of most interest, the EP1 receptor has been shown to mediate Ang II-induced hypertension (85). EP1-/- mice have blunted pressor responses to both acute and chronic Ang II administration (85). Furthermore, in isolated vessel preparations pre-treatment with the EP1 selective antagonist SC51322 reduced Ang II mediated vasoconstriction (85). Given that EP1 mediates some of the blood pressure and vasoconstrictor effects of Ang II and RAAS, it is of great interest whether EP1 contributes to renal fibrosis and CKD, possibly in a blood pressure independent manner similar to that of AT1.

EP1 blockade has been shown to positively affect renal function in a rodent model of human malignant hypertension (87). Stroke-prone spontaneously hypertensive rats were treated with vehicle or a selective EP1 antagonist. Although no differences were observed in blood pressure, treatment

with the EP1 antagonist reduced tubulointerstitial fibrosis, lessened urinary protein excretion, and blunted the drop in plasma creatinine levels. This suggests blockade of the EP1 receptor has a positive effect on renal function and may be a therapeutically relevant target (87). Nonetheless, it is imperative to continue investigating the contribution of EP1 to renal damage in order to demonstrate whether this is model specific and can be observed with both pharmacologic as well as genetic manipulation. Therefore, EP1+/+ and EP1-/- mice were studied in a model hypertensive renal damage.

## Experimental Procedures

### *Animal procedures*

Hypertension-induced renal damage was obtained utilizing a previously published model involving uninephrectomy and Ang II administration (214), with the exception of using the 129S6 background instead of FVB/N. Twelve week old 129S6/SvEvTac (EP1+/+, Taconic, USA) and EP1-/- mice (85) underwent unilateral nephrectomy and implantation of a subcutaneous osmotic minipump delivering Ang II (1.4 mg/kg/day) for 6 weeks as illustrated in Figure 4.1. In some cases, uninephrectomy was performed in the absence of Ang II administration. Blood was collected at baseline and 6 weeks post-treatment for measurement of BUN and GFR. Every two weeks, systolic blood pressure was measured and urine collected. Animals were maintained in an AAALAC accredited rodent facility in individually ventilated microisolator cages on a 12:12 light dark cycle.

All procedures were done in accordance with the policies of the Institutional Animal Care and Use Committee at Vanderbilt University.

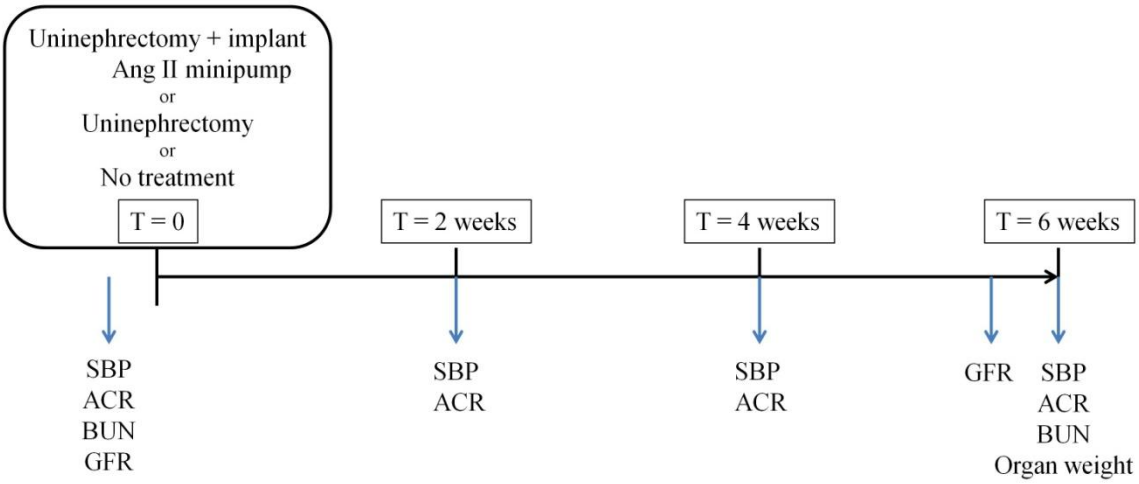



Figure 4.1 Experimental Design of Nphx + Ang II induced renal damage. EP1+/+ and EP1-/- mice underwent uninephrectomy + implantation of an Ang II osmotic minipump delivering 1.4 mg/kg/day, uninephrectomy only, or no treatment and followed for 6 weeks.

***Measurement of Systolic Blood Pressure***

Systolic BP was determined in conscious mice using a computerized tail-cuff system (Visitech systems BP-2000 Blood Pressure Analysis System, Apex NC, USA) in the Mouse Metabolic Phenotyping Core at Vanderbilt University Medical Center. Mice were trained for four days minimizing physiologically apparent stress. Each measurement is the average of at least 10 consecutive readings after stabilization of blood pressure.

***Determination of Blood Urea Nitrogen levels***

To assess the renal function, blood urea nitrogen (BUN) was determined using an iSTAT-1 analyzer (Abbott Point of Care Inc., New Jersey, USA). Whole blood was obtained from saphenous vein and immediately assayed utilizing Chem8+ cartridges.

***Determination of urinary albumin/creatinine ratios***

Albumin/Creatinine ratios (ACR; expressed as mg albumin/mg creatinine) were measured from 20-200 µL volumes of spot urine using Albuwell M ELISA kit, and urinary creatinine was measured using the Creatinine Companion (Exocell, Philadelphia, USA).

***Glomerular filtration rate determination***

GFR was determined based on plasma FITC–inulin clearance following a single bolus injection (223). Briefly, dialyzed FITC-inulin solution was injected into tail

vein. Approximately 20 uL of blood was collected via saphenous venesection at 3, 7, 10, 15, 35, 55 and 75 minutes post injection for measurement of FITC concentration. Plasma samples were buffered to pH 7.4 with 500 mM HEPES and fluorescence was determined using 485-nm excitation and read at 538-nm emission. Parameters were estimated by two-phase exponential decay nonlinear regression of plasma fluorescence data (GraphPad Prism). GFR was calculated using the equation GFR = I/($A$/α + B/β).

### *Statistical Analysis*

Data are presented as means ± SEM, using GraphPad Prism software (GraphPad Software Inc., USA). Analysis utilized Student's t test and two-way ANOVA. $P < 0.05$ was considered statistically significant for all studies.

## Results

### *Hypertension was induced by uninephrectomy and Ang II*

We employed a model of hypertension (214) to investigate the contribution of EP1 receptors to the pathogenesis of hypertension-induced renal damage. At baseline, SBP in EP1-/- mice was comparable to that of EP1+/+ mice (EP1+/+: 117.8 ± 2.485 mm Hg, EP1-/-: 114.3 ± 3.853 mm Hg, Figure 4.2A). Following uninephrectomy and Ang II administration, SBP increased in both EP1+/+ and EP1-/- mice, though no differences were detected between the two genotypes at any time point (Figure 4.2B).

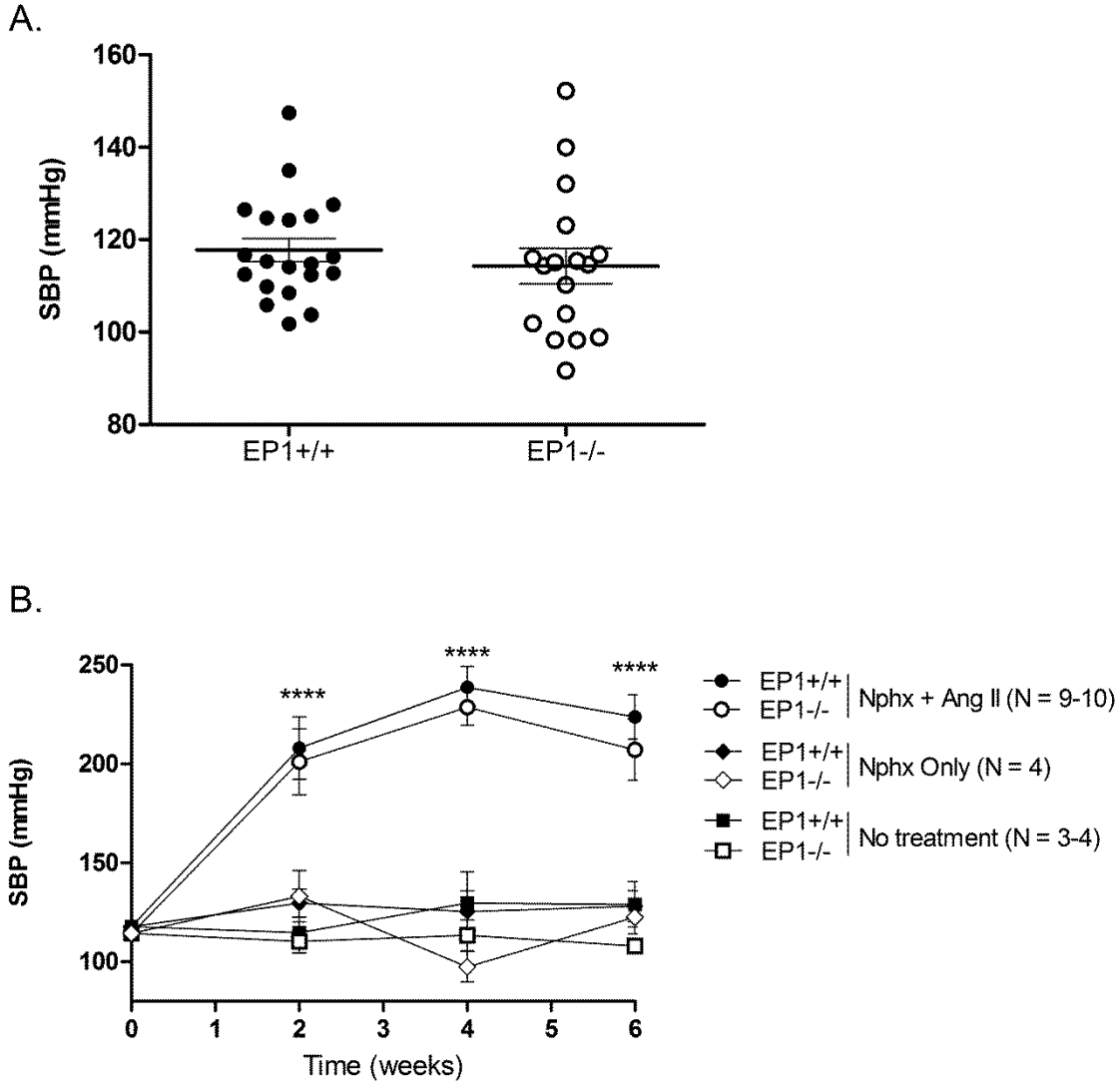



Figure 4.2 Hypertension was induced by Nphx + Ang II administration. A. Baseline systolic blood pressure was similar in EP1+/+ and EP1-/- mice (P = 0.44, N = 17 or 20 mice). B. Treatment with the Nphx + Ang II model induced comparable hypertension in both EP1+/+ (solid symbols) and EP1-/- (open symbols) mice. (P < 0.0001 vs baseline). No differences were detected between EP1+/+ and EP1-/- SBP.

### *Organ weight was increased in EP1+/+ and EP1-/- mice*

Heart and kidney weight were increased in both EP1+/+ and EP1-/- mice after 6 weeks treatment with Nphx + Ang II. Kidney weight was modestly increased by treatment with Nphx + Ang II in EP1+/+ and EP1-/- mice compared to baseline (Figure 4.3A). Uninephrectomy alone decreased kidney weight in EP1+/+ mice, though no difference was observed in EP1-/- mice. Heart weight was also significantly greater in Nphx + Ang II treated animals as compared to Nphx only, or untreated animals (Figure 4.3B). No significant differences in heart weight were observed between EP1+/+ and EP1-/- mice, indicative of a similar degree of hypertension.

### *Renal damage was induced by Nphx + Ang II treatment and reduced in EP1-/- mice*

Blood urea nitrogen was significantly elevated in EP1+/+ and EP1-/- mice with Nphx + Ang II (Figure 4.4A). Interestingly, EP1-/- mice had modestly elevated BUN at baseline as compared to EP1+/+ mice. Over the 6 week treatment period, EP1+/+ mice experienced a rise in BUN, even in the absence of Nphx or Ang II, while EP1-/- mouse BUN remained consistent unchanged. The difference between genotypes at baseline complicates the interpretation of the data. Therefore, change in BUN was determined using paired data from mice treated with Nphx + Ang II. Compared to baseline, six weeks post treatment EP1+/+ mice had a rise in BUN of 37 mg/dL, whereas EP1-/- mouse BUN rose only 17 mg/dL ($P = 0.007$, Figure 4.4B). Urinary ACR was measured every other week throughout the experiment, and revealed significantly lower urinary protein

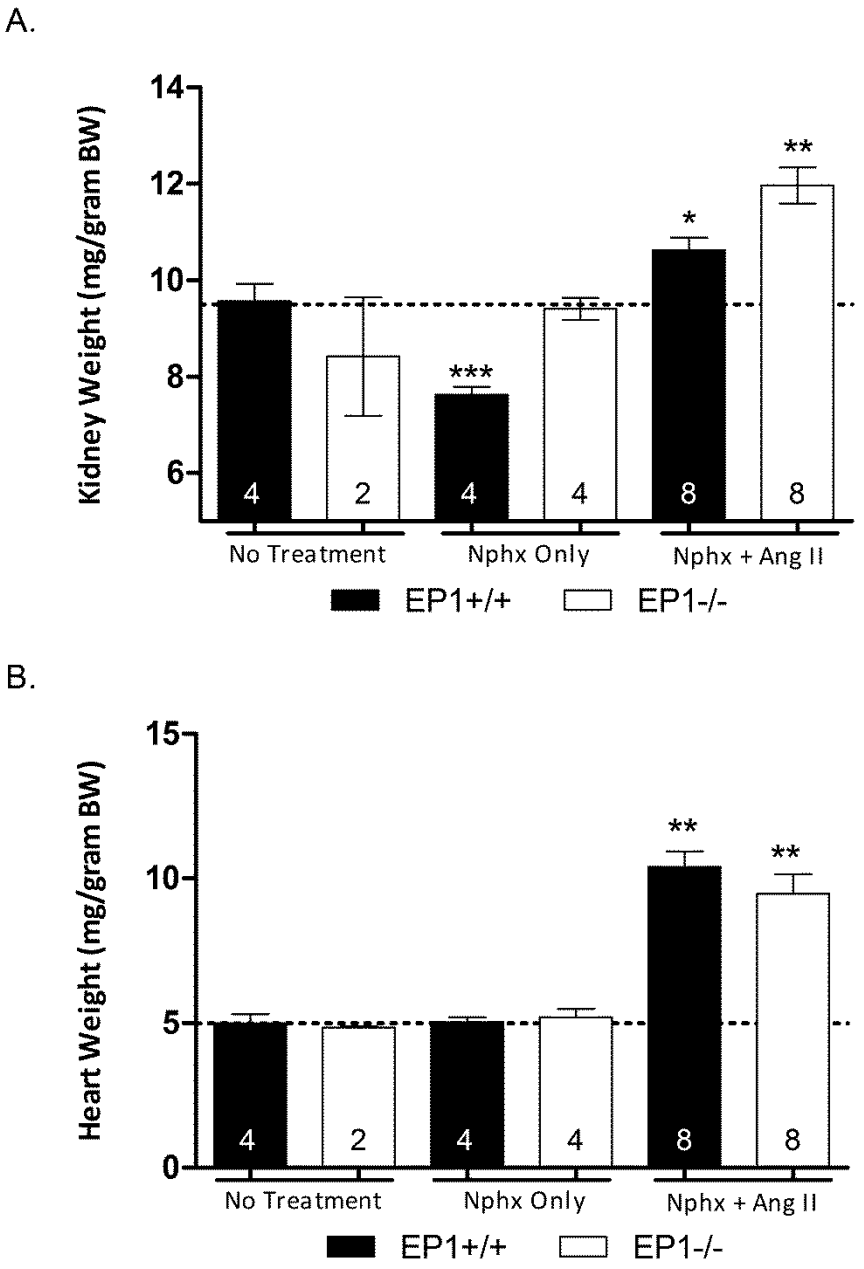



Figure 4.3 Heart and kidney weight were increased in both EP1+/+ and EP1-/- mice after 6 weeks treatment with Nphx + Ang II. A. Kidney weight, expressed mg/gram body weight, was increased in EP1+/+ and EP1-/- mice compared to baseline by treatment with Nphx + Ang II (EP1+/+ $P = 0.04$, EP1-/- $P = 0.001$). Nphx alone decreased kidney weight in EP1+/+ mice ($P = 0.005$), though no difference was observed in EP1-/- mice. B. Heart weight, expressed mg/gram body weight, was significantly greater in Nphx + Ang II treated animals as compared to Nphx only, or untreated animals ($P < 0.01$ for both genotypes). No significant differences were observed between EP1+/+ and EP1-/- mice ($P > 0.05$).

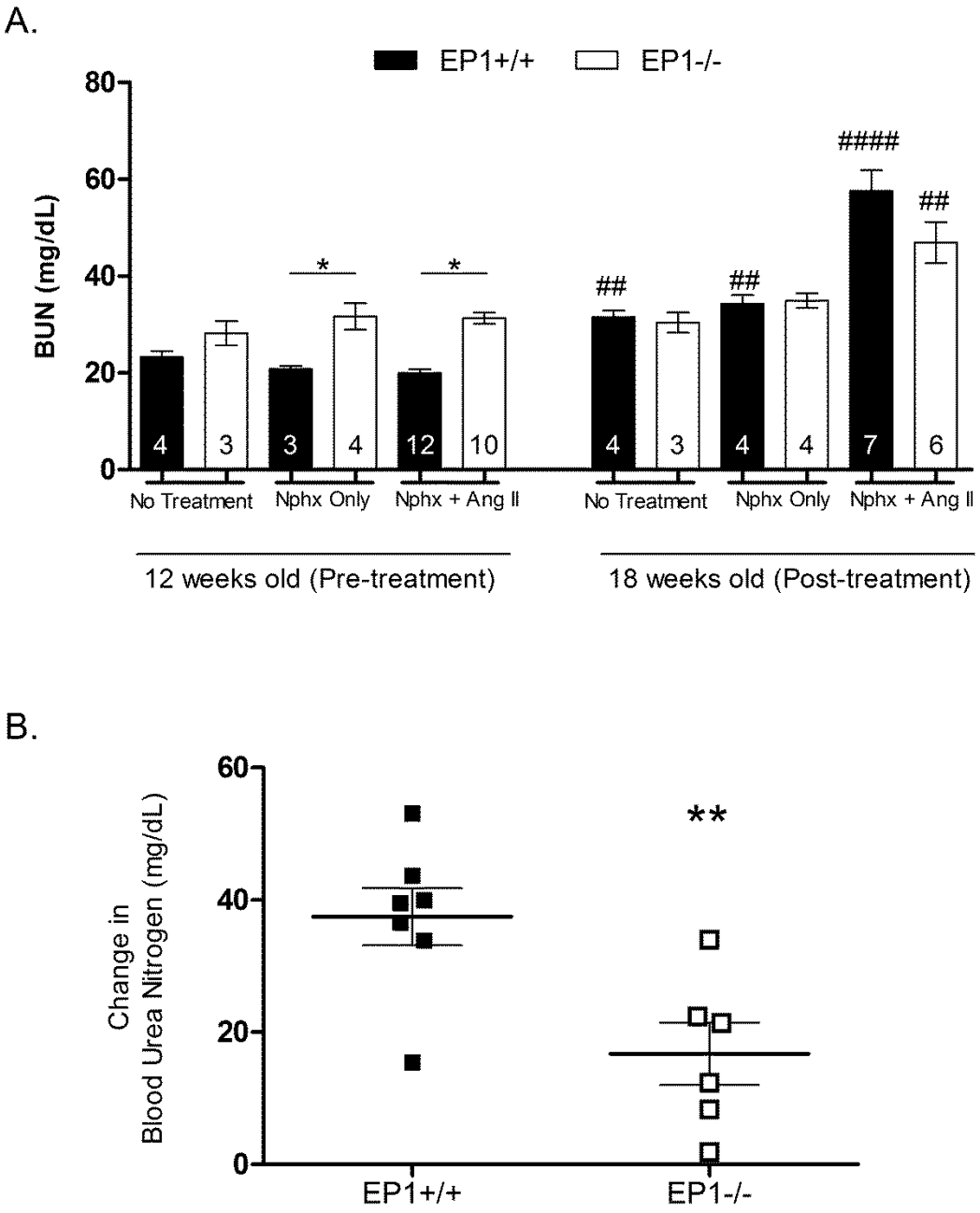



Figure 4.4 BUN was increased by Nphx + Ang II treatment. A. Blood urea nitrogen was measured prior to start of the model or six weeks after treatment. At baseline EP1-/- mice had elevated BUN compared to EP1+/+ mice (*P < 0.05). Six weeks later, BUN was elevated over baseline in all treatment groups of EP1+/+, and Nphx + Ang II treated EP1-/- mice (## P < 0.004, #### P < 0.0001). B. The change in BUN from baseline to six weeks treatment with Nphx + Ang II revealed EP1-/- mice had an attenuated rise in BUN compared to EP1+/+ mice (**P = 0.007)

in EP1-/- mice compared to EP1+/+ mice ($P < 0.0001$, Figure 4.5A). GFR was decreased in EP1+/+ and EP-/- mice treated with Nphx + Ang II, compared to untreated 18 week old mice, dropping 60 % in EP1+/+ mice and 40 % in EP1-/- mice (Figure 4.5B). However, aging from 12 to 18 weeks of age resulted in a reduction in GFR in EP1-/- mice only.

## Discussion

In the present study we examined whether genetic ablation of the EP1 receptor affords renal protection in a model of hypertensive end-organ damage. The uninephrectomy and Ang II model resulted in functional renal damage including a rise in BUN, elevated ACR and reduced GFR, while genetic ablation of EP1 reduced the rise in BUN and decreased ACR. These studies suggest the EP1 receptor plays an important role in hypertensive renal disease.

Our results are consistent with Suganami et al., who showed that pharmacological blockade of EP1 reduced proteinuria and tubulointerstitial damage in stroke prone spontaneously hypertensive rats (87). However, in anti-GBM nephrotoxic serum nephritis, which occurs in the absence of elevated blood pressure, genetic deletion of EP1-/- in mice resulted in increased mesangial expansion, tubular dilation, BUN and serum creatinine (173). Furthermore, no differences were observed in renal damage with genetic ablation of EP1 in the Nphx/DOCA-NaCl/Ang II model, although in this case only modest renal damage was induced and ablation of EP1 did substantially increase survival and protect

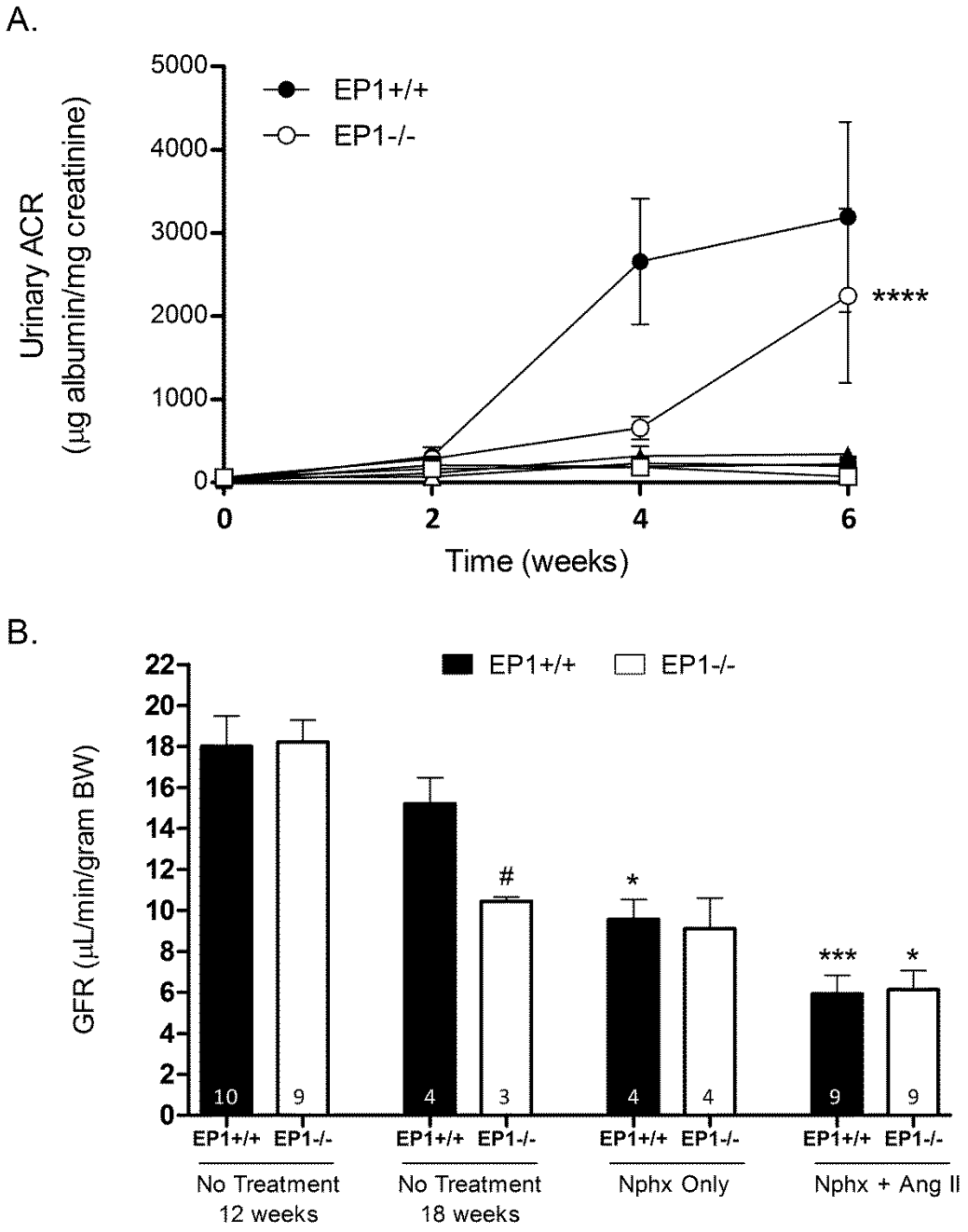



Figure 4.5 ACR and GFR following treatment with Nphx + Ang II. A. Urinary ACR was measured every other week throughout the experiment, revealing significantly lower urinary protein in EP1-/- mice compared to EP1+/+ mice. (2 way ANOVA, ****P < 0.0001, N = 3-12 samples per point). B. GFR was decreased in EP1+/+ and EP-/- mice treated with Nphx + Ang II, compared to untreated 18 week old mice (EP1+/+ P = 0.001, EP1-/- P = 0.028). Aging from 12 to 18 weeks of age resulted in a reduction in GFR in EP1-/- mice only ($^{\#}$P = 0.002).

against vascular defects (224). This suggests the role of EP1 in renal damage is highly context dependent.

Throughout these studies blood pressure was measured by tail cuff plethysmography. Treatment with uninephrectomy and Ang II raised SBP as compared to uninephrectomy alone or no treatment. However, EP1-/- mice had a similar degree of hypertension compared to EP1+/+ mice. This is inconsistent with previous reports demonstrating that pharmacological blockade and genetic ablation of EP1 reduces blood pressure, modestly at baseline and exaggerated in a setting of hypertension, and administration of EP1 agonists results in an increase in blood pressure (79,85,224). It is possible that blood pressure differences were not observed in our studies due to the lack of sensitivity of the tail cuff technique. To address this, more accurate blood pressure measurements would need to be made utilizing a direct arterial catherization either anesthetized or by telemetry.

EP1-/- mice had a reduction in the rise in BUN from baseline vs 6 weeks post uninephrectomy and Ang II, as compared to EP1+/+ mice. However, the raw values for BUN at 6 weeks post treatment failed to reach significance. Change in BUN reached significance because EP1-/- began the study with significantly higher BUN as compared to EP1+/+ mice. Similarly GFR declined with age in untreated EP1-/- mice and not EP1+/+ mice although the sample size was small in this case. This effect has not been observed previously, and it would be important to determine if this trend holds true in repeated experiments or is observed with prolonged treatment by pharmacological blockade in a

healthy animal. This could indicate that treatment with EP1 antagonists would be better utilized for slowing the progression of renal damage and not advised for use in patients without renal damage.

In summary, genetic ablation of the $PGE_2$ receptor EP1 affords protection against renal function decline as a result of hypertension. This protection may be independent of blood pressure reduction. These results suggest the EP1 receptor may be a viable target for the treatment of renal damage.

# CHAPTER V

# CONTRIBUTION OF THE EP1 RECEPTOR IN DIABETIC RENAL DAMAGE

## Introduction

Diabetes is a disease characterized by high circulating blood glucose levels. This can occur as a result of inadequate insulin production, the inability to properly respond to insulin, or both. The prevalence of diabetes increases with age. In 2010, approximately 11.3 % of adults over 20 years of age had diabetes, while 26.9 % of adults over 65 years of age were diabetic. According to the CDC, in 2007 diabetes cost the US $174 billion, $116 billion of that composed of direct medical costs. Diabetes is the leading cause of blindness, lower-limb amputation, and kidney failure, and also increases the risk of coronary heart disease, peripheral vascular disease and stroke (225).

Diabetic nephropathy (DN) is a progressive disease which results in irreversible loss of kidney function. The initial mechanism of damage occurs due to adaptive hyperfiltration which eventually leads to long term damage of the nephrons. In type I diabetes, progression of DN begins within 5 years of onset of diabetes with glomerular hyperfiltration, which transitions into the presence of glomerular lesions without clinical disease or urinary albumin excretion (226-228). These lesions include thickening of the glomerular basement membrane and expansion of the mesangial cells. After about 10-15 years, incipient diabetic

nephropathy develops. The patient now has microalbuminuria along with the glomerular lesions. This is an important stage of DN because therapeutic intervention is started with the goal of preventing progression to overt DN. In overt DN there is worsening proteinuria, a drop in glomerular filtration rate and eventual progression to ESRD. The pathology of overt DN usually consists of glomerulosclerosis, fibrinoid caps and arteriolar hyalinosis (226-228).

In 2008, 44 % of all new cases of kidney failure were a result of diabetes. It was estimated over 200,000 people with ESRD due to diabetes were on chronic dialysis or living with a transplant in 2008, even though the majority of diabetic patients with DN die of cardiovascular causes before progression to ESRD. Treatments for DN are aimed at controlling blood glucose and blood pressure (11,201,229). 75 % of adults with diabetes have blood pressure ≥ 130/80 mm Hg. Treatment with ACEi or ARBs is more effective at reducing the decline in renal function than other blood pressure lowering drugs (217,218). According to the CDC, they reduce proteinuria, a risk factor for developing kidney disease, by 35 %. The AT1 receptor is the principal receptor mediating angiotensin II pressor effects and is the direct target of ARBs. AT1 receptor activation leads to a number of signal transduction pathways including increases in $[Ca^{++}]_i$ and activation of $cPLA_2$ (180,181,184-187). cPLA2 is the rate limiting step in prostaglandin synthesis, freeing arachidonic acid from the plasma membrane. COX enzymes catalyze the oxidation and reduction of AA into $PGH_2$, which is rapidly converted into one of the principal prostanoids by tissue specific synthases. COX-2 expression has been shown to be upregulated in the thick

ascending limb and macula densa of both type I and type II diabetic rats (230-233). Increased COX-2 expression in the macula densa has also been seen in human diabetic kidneys (234). One product of COX-2 produced $PGH_2$ is the prostanoid $PGE_2$, which is of particular interest in diabetes.

$PGE_2$ has been shown to be elevated in the urine of diabetics, suggesting they have increased renal $PGE_2$ production (235). In STZ and Akita diabetes models, renal EP1 and EP3 mRNA expression is increased, suggesting these receptors may play an important role in the pathogenesis of DN (236).

In the $Lepr^{db/db}$ model of diabetes, a homozygous mutation of the leptin receptor, treatment with antagonist AH6809 reduced SBP and blocked the vasoconstriction of $PGE_2$ and 17-phenyl trinor $PGE_2$, which was enhanced by the diabetic phenotype (86). Furthermore, pharmacologic blockade of EP1 was able to ameliorate DN in a rat model of STZ induced diabetes (88). Treatment with the EP1 antagonist was able to reduce renal and glomerular hypertrophy, reduce mesangial expansion, and suppress proteinuria (88). Therefore, we sought to determine the contribution of EP1 to DN utilizing a genetic disruption of EP1 in mice.

## Experimental Procedures

### *Animal procedures*

Diabetes-induced renal damage was obtained utilizing a previously published model involving low dose streptozotocin (STZ) treatment on an eNOS-/-

background (195). Eight week old eNOS-/- mice (EP1+/+, eNOS-/- Jackson Labs, USA), EP1-/- , eNOS-/- mice, EP1+/+, eNOS+/+ mice and EP1-/-, eNOS-/- mice underwent five consecutive daily i.p. doses of STZ (50 mg/kg). Two weeks after STZ treatment blood glucose was measured by saphenous venesection. Diabetic mice with blood glucose greater than 300 mg/dL at 10 weeks of age were included in the studies. Blood glucose, blood pressure, urinary albumin excretion, and glomerular filtration rate were assessed 20 weeks after the onset of diabetes as illustrated in Figure 5.1. Animals were maintained in an AAALAC accredited rodent facility in individually ventilated microisolator cages on a 12:12 light dark cycle. All procedures were done in accordance with the policies of the Institutional Animal Care and Use Committee at Vanderbilt University.

***Intracarotid blood pressure measurement***

Intracarotid blood pressure was measured under ketamine (25 mg/kg) and inactin (100 mg/kg) anesthesia delivered intraperitoneally. Mice were placed on a thermal pad and a PE-10 catheter was inserted into the left carotid artery. The catheter was connected to a TXD-310 transducer and blood pressure was measured using a Digi-Med BPA 400 (Micromed). Mice were equilibrated 30-60 minutes until stable values were attained. Ten minute blood pressure measurements were collected and average mean arterial pressure (MAP) is plotted.

***Determination of urinary albumin/creatinine ratios***

Albumin/Creatinine ratios (ACR; expressed as mg albumin/mg creatinine) were measured from 20-200 µL volumes of spot urine using Albuwell M ELISA kit, and urinary creatinine was measured using the Creatinine Companion (Exocell, Philadelphia, USA).

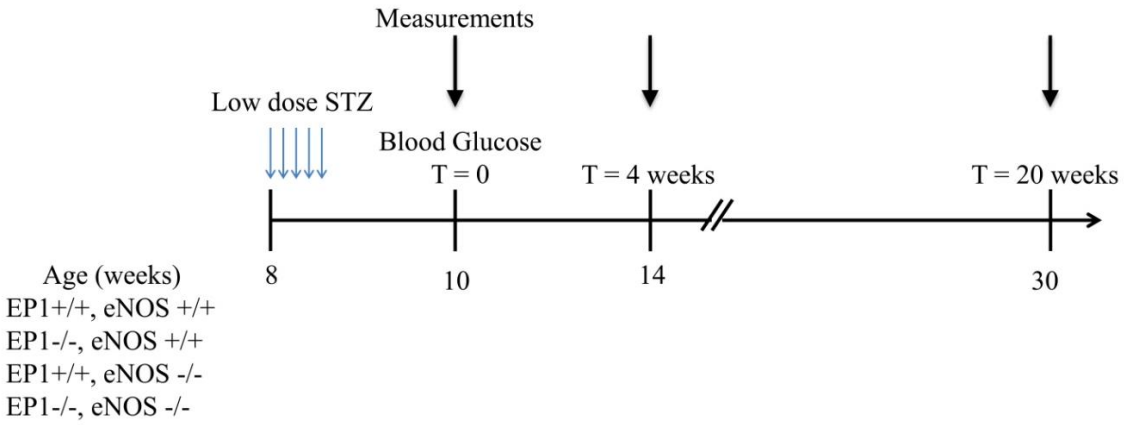



Figure 5.1 Induction of diabetes in mice. Eight week old EP1+/+, eNOS-/- mice, EP1-/- , eNOS-/- mice, EP1+/+, eNOS+/+ mice and EP1-/-, eNOS+/+ mice underwent five consecutive daily i.p.doses of STZ (50 mg/kg). Diabetic mice with blood glucose greater than 300 mg/dL at 10 weeks of age were included in the studies. Blood glucose, blood pressure, urinary albumin excretion, and glomerular filtration rate were assessed 20 weeks after the onset of diabetes.

### *Glomerular filtration rate determination*

GFR was determined based on plasma FITC–inulin clearance following a single bolus injection (223). Briefly, dialyzed FITC-inulin solution was injected into tail vein. Approximately 20 µL of blood was collected via saphenous venesection at 3, 7, 10, 15, 35, 55 and 75 minutes post injection for measurement of FITC concentration. Plasma samples were buffered to pH 7.4 with 500 mM HEPES and fluorescence was determined using 485-nm excitation and read at 538-nm emission. Parameters were estimated by two-phase exponential decay nonlinear regression of plasma fluorescence data (GraphPad Prism). GFR was calculated using the equation GFR = I/(*A*/α + B/β).

### *Statistical Analysis*

Data are means ± SEM, using GraphPad Prism software (GraphPad Software Inc., USA). Analysis utilized Student's t test. $P < 0.05$ was considered statistically significant for all studies.

## Results

### *Diabetes was induced by low dose STZ treatment*

Diabetic nephropathy was induced in EP1+/+, eNOS-/- mice, EP1-/-, eNOS-/- mice, EP1+/+, eNOS+/+ mice and EP1-/-, eNOS+/+ mice by five consecutive daily administrations of low dose STZ. Two weeks after STZ treatment, blood glucose was measured and diabetic mice with blood glucose greater than 300

mg/dL at 10 weeks of age were included in the studies. Four and 20 weeks after onset of diabetes, blood glucose was measured. In all genotypes blood glucose increased over time, and no differences were observed between the four genotypes (Figure 5.2).

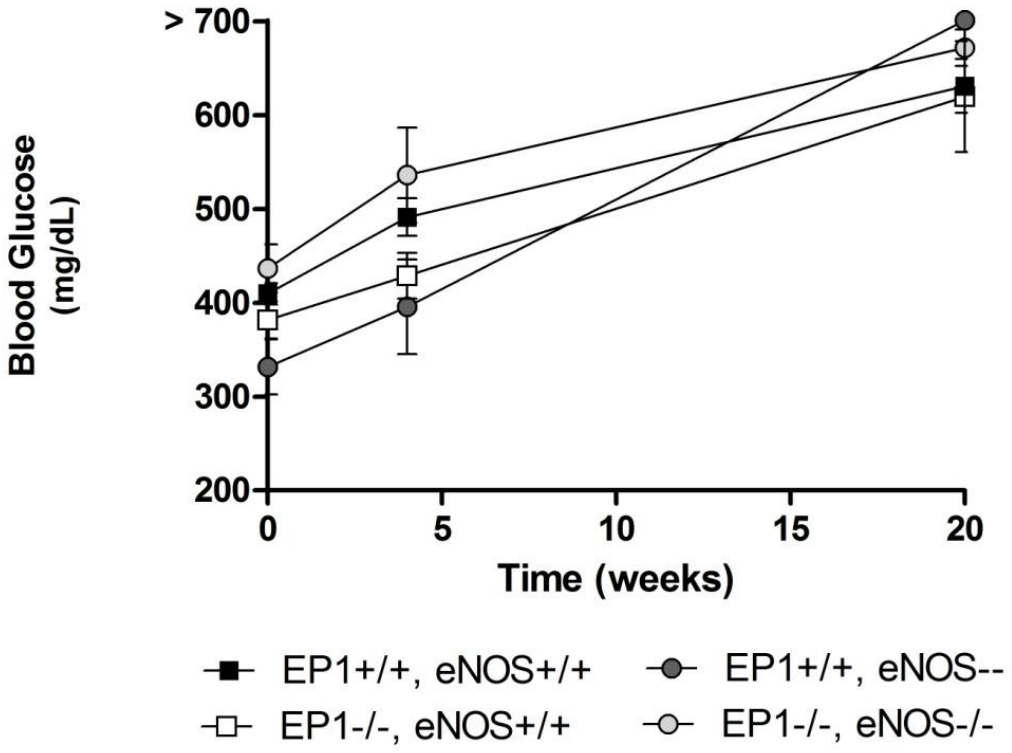



Figure 5.2 Diabetes was induced in all genotypes with low dose STZ treatment. Blood glucose was measured at onset of diabetes, four weeks diabetic and twenty weeks diabetic. Blood glucose increased with time in all groups, and no differences were observed between genotypes (EP1+/+, eNOS+/+ N = 5; EP1-/-, eNOS+/+ N = 7; EP1+/+, eNOS-/- N = 3; EP1-/-, eNOS-/- N = 10).

***MAP was altered by genetic disruption of eNOS and not EP1***

Anesthetized intracarotid blood pressure was measured at 20 weeks post onset of diabetes. No significant differences were observed between any of the genotypes (Figure 5.3A). Genetic deletion of eNOS has been well characterized to modestly increase MAP. When MAP was stratified based on eNOS genotype, regardless of the status of the EP1 gene, a significant increase in MAP was observed ($P = 0.027$, Figure 5.3B).

***Urinary albumin excretion was reduced by genetic ablation of EP1***

Low dose STZ has been demonstrated to induce diabetic nephropathy in C57BL/6 mice when eNOS has been deleted (195). In our study, we also observed increased protein excretion in EP1+/+, eNOS-/- mice as compared to EP1+/+, eNOS+/+ mice ($P = 0.009$). Deletion of the EP1 receptor on the background of eNOS-/- reduced urinary protein to a level similar to that observed in eNOS+/+ mice (EP1+/+, eNOS-/- vs. EP1-/-, eNOS-/- $P = 0.028$, Figure 5.4).

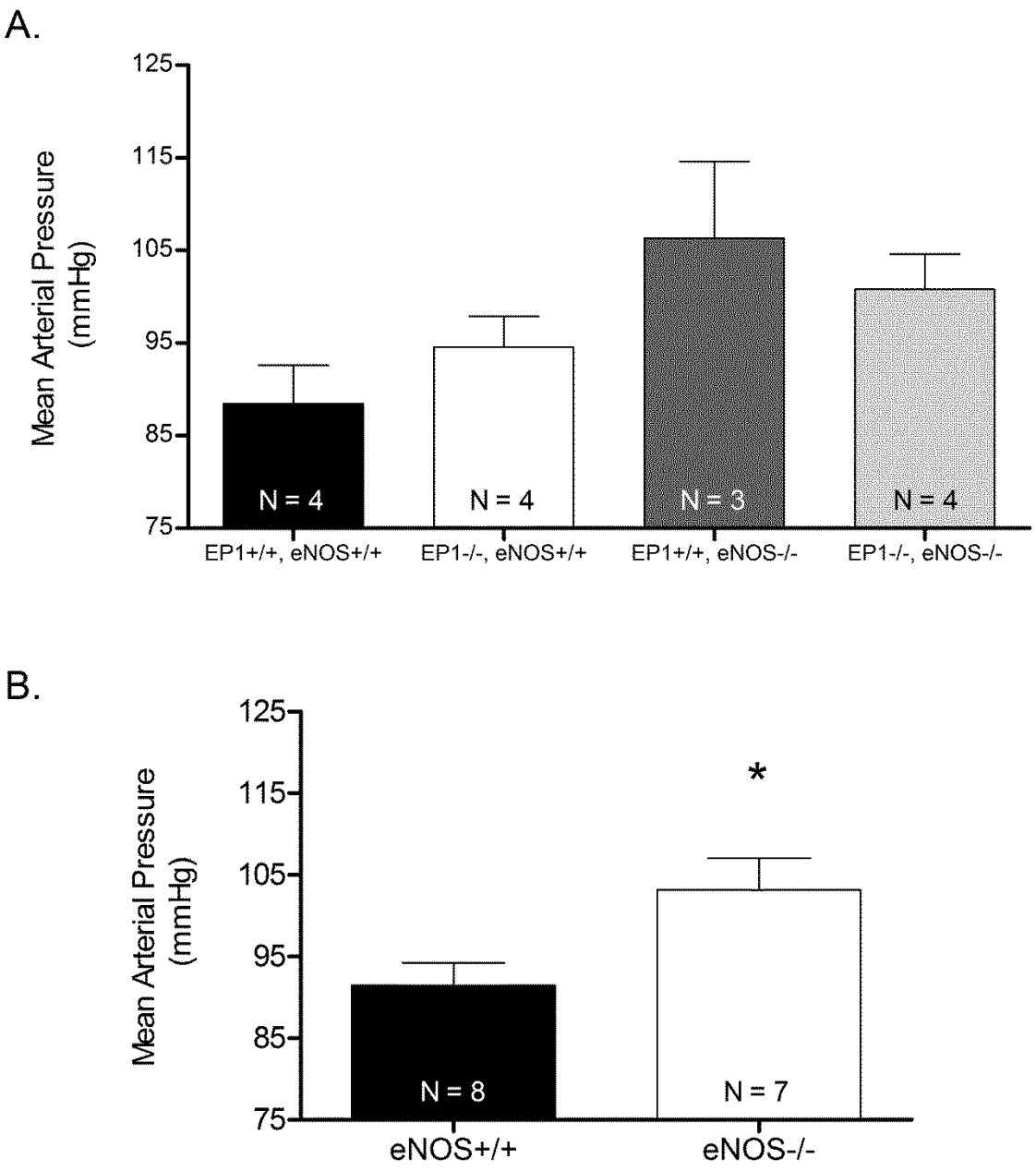



Figure 5.3 MAP was increased by genetic deletion of eNOS, but no change was observed due to EP1 gene disruption. A. Average MAP at 20 weeks diabetic. Anesthetized intracarotid blood pressure demonstrated no significant differences observed between any genotype (EP1+/+, eNOS+/+ vs. EP1-/-, eNOS+/+ P = 0.294; EP1+/+, eNOS-/- vs. EP1-/-, eNOS-/- P = 0.536; EP1+/+, eNOS+/+ vs. EP1+/+,eNOS-/- P = 0.090; EP1-/-, eNOS+/+ vs. EP1-/-, eNOS-/- P = 0.263 by t-test). B. MAP stratified based on eNOS genotype. MAP, regardless of EP1 gene status, was significantly increased with eNOS genetic ablation (P = 0.027).

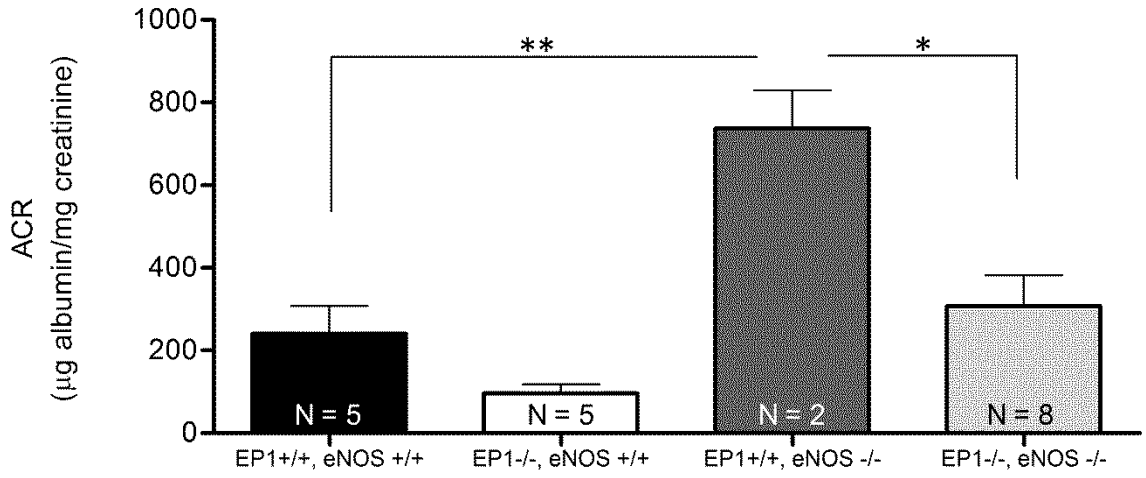



Figure 5.4 Albuminuria was significantly reduced by genetic disruption of EP1 in DN. Urinary albumin excretion was measured at 20 weeks post diabetes. Genetic deletion of eNOS significantly increased ACR on the background of EP1+/+ (P = 0.009), while no statistically significant increase was observed on the EP1-/- background (P = 0.054). Deletion of EP1reduced ACR on the eNOS-/- background (P = 0.028).

### *GFR was reduced by deletion of eNOS in the presence, but not absence of EP1*

Hyperfiltration is often observed in diabetes. In our study, we observed GFR of approximately 40 µL/min/gram body weight in EP1+/+, eNOS+/+ mice (Figure 5.5). Genetic deletion of eNOS significantly reduced GFR to approximately 15 µL/min/gram ($P = 0.0001$), indicating compromised renal function. Genetic deletion of EP1 on the eNOS-/- background had no statistically significant effect, but trended toward a slightly higher GFR (20 µL/min/gram, $P = 0.051$). However, genetic deletion of EP1 on the eNOS+/+ background significantly reduced GFR ($P = 0.011$). No significant reduction in GFR was observed between EP1-/-, eNOS+/+ and EP1-/-,eNOS-/- mice ($P = 0.301$).

## Discussion

In the present study we examined whether genetic disruption of EP1 protected eNOS-/- mice from STZ induced diabetic nephropathy. We demonstrated EP1-/-, eNOS-/- mice have a significant reduction in proteinuria as compared to EP1+/+, eNOS-/- control mice. No significant difference in blood pressure was conferred by disruption of EP1 in this model. Our data are consistent a previous report that showed treatment of STZ induced diabetes in Wistar rats with EP1 antagonist ONO-8713 reduced renal hypertrophy and suppressed proteinuria (88), suggesting the EP1 receptor contributes to renal decline in the setting of diabetes.

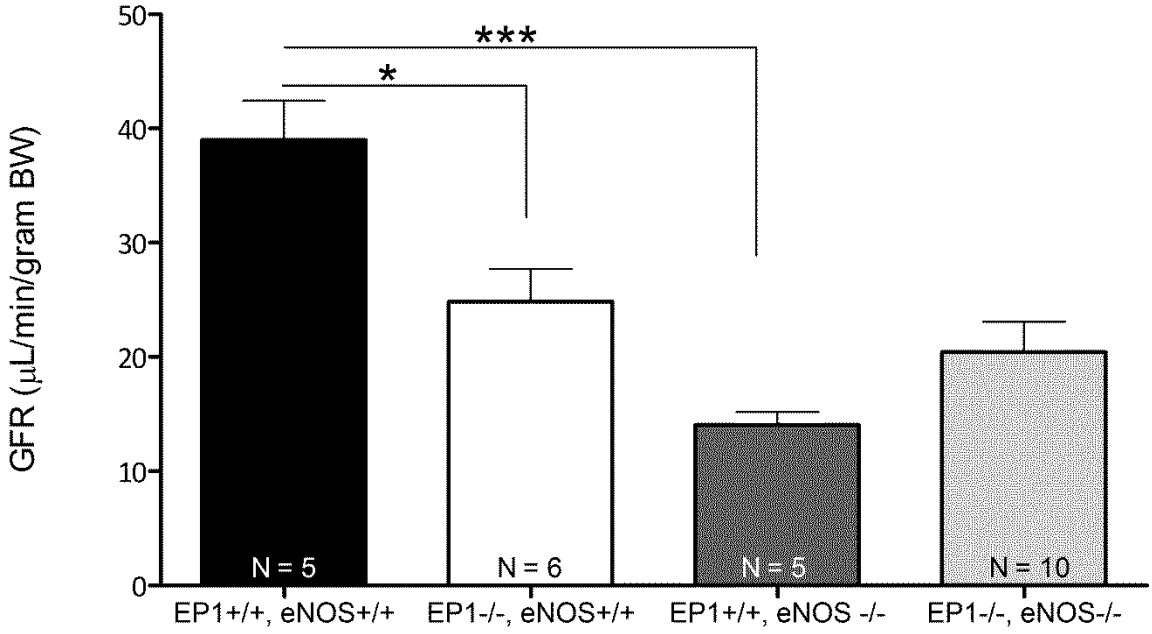



Figure 5.5 GFR was significantly reduced by deletion of eNOS in the presence, but not absence of EP1 receptor expression. GFR was measured at 20 weeks post diabetes. High GFR was observed in EP1+/+, eNOS+/+ mice, and was significantly reduced by deletion of eNOS (P = 0.0001) and deletion of EP1-/- (P = 0.011). Deletion of eNOS on the background of EP1-/- had no significant effect (P = 0.301). No statistically significant difference was observed between EP1+/+, eNOS-/- and EP1-/-, eNOS-/- mice (P = 0.051).

In our studies we were unable to detect a reduction in blood pressure due to disruption of EP1 suggesting suppression of proteinuria occurred by a mechanism other than blood pressure regulation. However, this may be a result of small sample size (3-4 mice per group). Deletion of eNOS conferred an average rise in BP of 10 mm Hg, and it may be expected that EP1 disruption reduce BP to an intermediate level. Such small changes would require larger sample sizes to measure with statistical significance. Rutkai et al demonstrated that BP and diabetes enhanced vasoconstriction could be reduced in the db/db diabetic model by treatment with AH6809 (86). The authors attribute the results to blockade of EP1 receptors although this particular antagonist is not very selective. In Xenopus oocytes expressing human EP1 receptors, AH6809 is able to inhibit calcium accumulation (21); in CHO cells stably expressing mouse prostanoid receptors AH6809 failed to compete $^{3}$H-PGE$_2$ for mouse EP1 (30 % displacement at 10 μM), while demonstrating a $K_i$ of 350 nM at mouse EP2 (34). It would be interesting whether their results could be reproduced with a more selective antagonist such as ONO-8713.

Another interesting finding uncovered in this study was lower GFR observed in EP1-/-, eNOS+/+ mice as compared to EP1+/+,eNOS+/+. C57BL/6J eNOS+/+ mice are resistant to development of diabetic nephropathy, while deletion of eNOS renders the strain susceptible (195,237). We predicted GFR would be elevated in both genotypes to a similar degree due to hyperfiltration. It may be possible that EP1-/- mice do not hyperfilter to the same extent as EP1+/+ mice. Alternatively it is possible that EP1 plays an important role in an age

dependent reduction in GFR. At the time GFR was measured, the mice were > 6 months old. This hypothesis is consistent with results shown in Chapter IV, Figure 4.5B. EP1-/- 129S6 mice, which were aged from 12 to 18 weeks without uninephrectomy or Ang II, displayed a significant reduction in GFR while no reduction was observed in EP1+/+ mice. It would be interesting to determine if this trend holds true in repeated experiments or is observed with prolonged treatment by pharmacological blockade in a healthy animal. Use of control animals, receiving no STZ, would be of importance in future experiment to eliminate hyperfiltration as a confounding variable.

In summary, our data demonstrate a detrimental role of EP1 in diabetes-induced proteinuria. This protection may be independent of blood pressure reduction. In addition, it would be of great interest to determine differences renal morphology as well. These results suggest the EP1 receptor may be a viable target for the treatment of diabetic renal damage.

# CHAPTER VI

# SUMMARY AND FUTURE DIRECTIONS

## Summary

Hypertension is a prevalent disease affecting one in three adults in the United States. It is estimated that 27.5 % of the adult population is either not receiving therapy for their hypertension or is unable to control their blood pressure with the current therapies, making treatment of hypertension an important public health goal.

$PGE_2$, a biologically active lipid-derived autacoid, contributes to the regulation of blood pressure and is able to exert vasopressor or vasodepressor effects depending upon the setting (17-19). EP1 does not appear to play a significant role in the blood pressure effects of systemically administered $PGE_2$, however, it does contribute to hypertension. EP1-/- mice have blunted pressor responses to both acute and chronic Ang II administration (85). In isolated vessel preparations, pre-treatment with the EP1/EP3 antagonist SC51322 reduced Ang II mediated vasoconstriction (85). Treatment of spontaneously hypertensive rats with SC51322 significantly reduces blood pressure (85), indicating blockade of the EP1/EP3 receptors may be a target for the treatment of hypertension.

EP1 blockade has been shown to positively affect renal function in stroke-prone spontaneously hypertensive rats (87), as well as cerebrovascular dysfunction induced by Ang II (161), implicating the EP1 receptor in hypertension and resultant end-organ damage. No data exists regarding the contribution of the EP3 receptor to hypertensive end-organ damage.

Three-quarters of diabetic patients have blood pressure ≥ 130 mm Hg, and diabetes is the leading cause of chronic kidney failure. In STZ and Akita diabetes models, renal EP1 and EP3 mRNA expression is increased, suggesting these receptors may play an important role in the pathogenesis of DN (236). Pharmacologic blockade of EP1 was able to ameliorate DN in a rat model of STZ induced diabetes (88). Treatment with the EP1 antagonist was able to reduce renal and glomerular hypertrophy, reduce mesangial expansion, and suppress proteinuria (88). However, little advance has been made on the role of EP1 in hypertensive and diabetic renal damage, as these studies were published about 10 years ago, nor have the effects been reproduced with a genetic approach or another antagonist. Therefore, we sought to determine the contribution of EP1 and/or EP3 receptors to hypertensive end-organ damage and DN using a genetic approach in mice.

Using the Nphx/DOCA-NaCl/Ang II model of hypertension, we have demonstrated that disruption of EP1 or EP3 can afford substantial protection from end-organ damage and reduce incidence of mortality (Chapter II and Chapter III). The beneficial effects of EP1 disruption, and likely EP3 disruption, appeared to be a result of reduction in MAP, since treatment with the

antihypertensive agent hydralazine was able to phenocopy the effect observed in EP1-/- mice. Mortality in the Nphx/DOCA-NaCl/Ang II model occurred primarily as a result of aortic aneurysm rupture, or after development of anasarca. We hypothesize that hypertension induced by DOCA-NaCl and Ang II results in volume loading and enhanced vasoconstriction, which places excessive stress on the vascular wall leading to enhanced permeability, resulting in edema and susceptibility to dissections and rupture. While we have shown that protection against end-organ damage is likely a result of reduced blood pressure, this does not eliminate the possibility that EP1 receptors might also provide protection directly at the target tissue, especially given the acute, severe nature of damage in this model.

Previous reports of the role of EP1 in renal injury are contradictory. In spontaneously hypertensive rats, treatment with an EP1 antagonist reduced proteinuria and tubulointerstitial damage (87), while in anti-GBM nephrotoxic serum nephritis genetic deletion of EP1-/- in mice resulted in enhanced mesangial expansion and tubular dilation and increased blood urea nitrogen and serum creatinine (173). In our studies with the Nphx/DOCA-NaCl/Ang II model, modest hypertensive renal damage was observed, although no significant differences in renal function were detected between genotypes. However, our interpretation was confounded by the differential mortalities in EP1+/+ and EP1-/- mice, potentially biasing our results. Examination of renal histopathology at time points prior to significant mortality failed to detect any severe renal damage or

differences between the genotypes, suggesting the role of EP1 in renal damage is highly context dependent.

Due to the high mortality and lack of severe renal damage observed in the Nphx/DOCA-NaCl/Ang II model, we pursued the use of another model involving uninephrectomy and Ang II on a 129S6 background (Chapter IV). This genetic background is more susceptible to development of renal damage, and it therefore requires less manipulation to achieve more renal damage. The uninephrectomy and Ang II model resulted in functional renal damage including a rise in BUN, elevated ACR and reduced GFR. Genetic ablation of EP1 reduced the rise in BUN and ACR while no change in hypertension was observed. This suggests the EP1 receptor plays an important role in hypertensive renal disease independent of blood pressure reduction. However, throughout these studies blood pressure was measured by tail cuff plethysmography. It is possible that blood pressure differences were not observed in our studies due to the lack of sensitivity of the tail cuff technique.

Lastly, we examined whether genetic disruption of EP1 protected eNOS-/- mice from STZ induced diabetic nephropathy (Chapter V). We demonstrated EP1-/-, eNOS-/- mice have a significant reduction in proteinuria as compared to EP1+/+, eNOS-/- control mice. No significant difference in blood pressure was conferred by disruption of EP1 in this model. Our data are consistent a previous report that showed treatment of STZ induced diabetes in Wistar rats with EP1 antagonist ONO-8713 reduced renal hypertrophy and suppressed proteinuria (88), suggesting the EP1 receptor contributes to renal decline in the setting of

diabetes. Again, we were unable to detect a reduction in blood pressure due to disruption of EP1 suggesting suppression of proteinuria occurred by a mechanism other than blood pressure regulation. Although blood pressure was measured using a direct intracarotid catheter, the lack of a difference may be a result of small sample size (3-4 mice per group).

Another interesting finding uncovered in these studies points to a potential role of the EP1 receptor in age-dependent renal decline. In the diabetic renal model (Chapter V), lower GFR was observed in EP1-/-, eNOS+/+ mice as compared to EP1+/+, eNOS+/+. At the time GFR was measured, the mice were > 6 months old. Furthermore, in the hypertensive renal model (Chapter IV), EP1-/- mice began the study with significantly higher BUN as compared to EP1+/+ mice. Similarly GFR declined with age in untreated EP1-/- mice and not EP1+/+ mice. This effect has not been observed previously, and it would be important to determine if this trend holds true in repeated experiments or is observed with prolonged treatment by pharmacological blockade in a healthy animal. This could indicate that treatment with EP1 antagonists would be better utilized for slowing the progression of renal damage and not advised for use in patients without renal damage.

In summary, our data demonstrate a detrimental role of EP1 in hypertensive and diabetic end-organ damage. Further characterization of EP1 in these diseases will be essential as the EP1 receptor may be a viable pharmaceutical target for the treatment of hypertension and subsequent organ damage.

## Future directions

The data presented in this thesis advances our knowledge of the role of EP1 and EP3 receptors in hypertension and subsequent sequalae. Several new questions have emerged as a result including: Does the EP1 receptor mediate the BP- independent actions of Ang II? What is the therapeutic relevance of EP1 blockade? What is the contribution of EP1 in vascular permeability? Which EP receptors contribute to aortic aneurysm formation? What is the mechanism of EP1/Angiotensin II hypertension? Future experiments should be designed to address these issues.

Drugs which block the renin-angiotensin-aldosterone pathway are considered superior to other anti-hypertensive treatments due to their beneficial actions directly on the kidney which occur independent of blood pressure reduction. Additionally, $PGE_2$ and the EP1 receptor have been demonstrated to mediate at least part of the actions of angiotensin II. It would be of great interest to determine whether genetic disruption of EP1also confers similar protection in the kidney. Preliminary studies presented in this thesis (Chapters IV and V) suggest this may be the case. Disruption of EP receptors suppressed increases in proteinuria and BUN or prevented reductions in GFR, while no significant differences in blood pressure were detected. However, throughout these studies blood pressure was measured by tail cuff plethysmography or contained small sample sizes of direct intracarotid measurements. It is possible that blood pressure differences were not observed in our studies due to the lack of

sensitivity or low power. To address this, future experiments should include more accurate blood pressure measurements using a direct arterial catherization, in either anesthetized mice or by telemetry. Additionally, the therapeutic relevance of EP1 blockade should be more thoroughly characterized. One major limitation associated with the use of genetic disruption verses pharmacologic blockade is the chronic disruption of receptor action. Lack of receptor action throughout development or in the absence of disease may result in compensatory changes which may not be observed with use of pharmacologic agents. Therefore, determining whether similar results can be obtained by treatment with an EP1 antagonist would be essential for verifying the therapeutic benefit of EP1 blockade. Furthermore, it would be of interest to determine whether there is additional benefit from dual EP1/EP3 blockade compared with EP1 or EP3 blockade only. The mechanism by which EP1 and EP3 mediate hypertension in unknown, however it is well understood that the EP1 and EP3 can mediate distinct signal transduction pathways. It is conceivable that blockade of EP1 and EP3 may have an additive effect and provide enhanced protection.

For the uninephrectomy/ DOCA-NaCl/Ang II model, we hypothesized that hypertension resulted in enhanced vascular permeability, causing edema and susceptibility to dissections and rupture (Chapter II). This hypothesis was based on eliminating the most common causes of edema. Furthermore both aneurysms and edema were reduced via treatment with hydralazine, supporting hypertension as the major mitigating factor for development of these pathologies.

Future experiments will be required to identify whether vascular permeability differences are observed between EP1+/+ and EP1-/- mice. In this model, vascular permeability could be examined using Evans Blue dye or dextran-rhodamine permeability. If differences were observed in vivo, the molecular mechanism could be identified using transwell vascular permeability assays, with cells isolated from EP1+/+ and EP1-/- mice or heterologously expressing EP1.

It has been previously shown that either selective inhibition of COX-2 or genetic deletion of COX-2 significantly reduced aortic aneurysm formation and macrophage infiltration (166,167). Furthermore, deletion of microsomal PGE synthase-1 has also been demonstrated to reduce aortic aneurysm formation and oxidative stress in LDLR-/- mice with an angiotensin II infusion (160), suggesting $PGE_2$ plays an important role in development of aneurysms and the EP receptors may be viable targets for treatment of aneurysm progression. It would be of interest to investigate the contribution of EP1 in vascular damage, utilizing models which are independent of increased blood pressure, such as wire injury.

Lastly, it is imperative that we identify the mechanism by which EP1 and EP3 mediate Ang II-induced hypertension. It has been demonstrated that Ang II-induced vasoconstriction can be blocked by treatment with EP1/EP3 antaogonist SC-51322. EP1 receptors in the sub-fornical organ of the brain also play a critical role in slow pressor Ang II hypertension (238). Ang II/EP1 signaling in the subfornical organ is dependent of COX activity, suggesting that Ang II results in local production of $PGE_2$, which then activates EP1 (238). It would be interesting

to determine whether a similar mechanism/interaction is observed in other target tissues such as the kidney. Furthermore, the mechanism of EP3 action in Ang II-mediated hypertension is still unknown but may have an important contribution in Ang II-induced vasoconstriction. Future experiment designed to address these remaining questions would increase our overall understanding of EP receptors in blood pressure regulation, and may also uncover new therapeutic targets for the treatment of hypertension and end-organ damage.

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