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Nan Zhang
Candidate

Biomedical Sciences

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Mary K Walker , Chairperson

THE ROLE OF ARYL HYDROCARBON RECEPTOR IN BLOOD PRESSURE REGULATION AND VASCULAR HOMEOSTASIS

BY

NAN ZHANG

B.S., Biological Sciences, Fudan University, 2003

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy

Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

July, 2009

DEDICATION

I would like to dedicate this work to my mother and father, Shumin Sun and Yongjiu Zhang, for instilling in me the integrity, persistence, a love of nature and the passion for life. I would not be able to achieve this accomplishment without their love, support and encouragement.

ACKNOWLEDGMENTS

I would like to acknowledge my graduate advisor, Mary Walker, who has always supported me in my graduate study, and who will always support me in my future endeavors. I cannot image to finish this work without her guidance and help, from experiment design to paper writing. One could not have asked for a better advisor.

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THE ROLE OF ARYL HYDROCARBON RECEPTOR IN BLOOD PRESSURE REGULATION AND VASCULAR HOMEOSTASIS

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ABSTRACT OF DISSERTATION

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The Role of Aryl Hydrocarbon Receptor in

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ABSTRACT

High blood pressure and endothelial dysfunction are two systemic abnormalities of the cardiovascular system that extensively contribute to the etiology of cardiovascular diseases, which represent the number one killer in the United States for decades and become more severe in developing countries. Previous studies have indicated that aryl hydrocarbon receptor (AHR), a transcription factor primarily mediating xenobiotic metabolism, plays an important role in cardiovascular development and physiology. AHR deficient mice have been demonstrated to develop hypotension at sea level and oxygen-dependent, endothelin-1 (ET-1) mediated hypertension at mild altitude. However, after generations at mild altitude, AHR deficient mice appear to acclimate to the lower atmospheric partial pressure of oxygen and exhibit normal levels of ET-1 and hypotension. Therefore, the goal of my research was to elucidate the mechanism by which endogenous AHR regulates blood pressure by studying blood pressure regulation

in the absence of the AHR. I found that AHR deficient mice exhibit significantly lower systemic arterial blood pressure, dysregulated circadian blood pressure rhythm, and significantly elevated vascular endothelial nitric oxide synthase (eNOS) expression and systemic production of nitric oxide (NO). I found that treating AHR deficient mice with the NOS inhibitor, N^{ω}-nitro-L-arginine (LNNA) failed to normalize blood pressure, suggesting that vascular eNOS over expression may not be the primary mediating factor of hypotension in AHR knockout mice. Further investigation on vascular activity *ex vivo* indicated increased sensitivity of vascular smooth muscle to NO which may simultaneously contribute to the hypotension in this scenario. *In vitro* studies using human aortic endothelial cells with AHR expression knockdown by siRNA suggested an intracellular role of endogenous AHR signaling in suppressing eNOS mRNA expression, which could be normalized by exogenous ligand activation of the residual AHR. My results suggest that AHR suppresses eNOS expression and acts to maintain basal vascular tone and normal blood pressure. Therefore, therapeutic approaches that antagonize AHR signaling could improve NO bioavailability and benefit patients with hypertension and vascular diseases commonly associated with endothelial dysfunction.

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CHAPTER I

INTRODUCTION

CARDIOVASCULAR DISEASES

Cardiovascular diseases are a group of disorders of the heart and blood vessels, including high blood pressure, coronary heart disease, stroke, and heart failure. Cardiovascular diseases are the number one killer in the United States, and about one third (80 million) of adults have one or more types of cardiovascular diseases nowadays (Lloyd-Jones, D. et al. 2009). The etiology of cardiovascular diseases is multifactorial and involves both genetic factors, such as family history, and environmental factors, such as high fat diet and tobacco use.

BLOOD PRESSURE ALTERATION IN CARDIOVASCULAR DISEASES

Blood pressure reflects the work intensity of cardiovascular system. It needs to be maintained at certain level to supply sufficient oxygen and nutrients and to remove metabolic by-products from each organ, especially the brain and heart. The effect of high blood pressure on the vascular wall dramatically promotes the development of atherosclerosis, and subsequently myocardial infarction and stroke, two types of cardiovascular diseases with the highest morbidity and mortality. Statistically, of the 80 million people who have at least one type of cardiovascular disease in the U.S., 73.6 million (92%) have hypertension, which is defined as systolic blood pressure $\geq$ 140 mm Hg and/or diastolic blood pressure $\geq$ 90 mm Hg (Lloyd-Jones, D. et al. 2009). Unfortunately, the etiology of 90% of the cases of hypertension is unknown.

In contrast to hypertension, hypotension, a systolic blood pressure $\leq$ 90 mm Hg and/or diastolic blood pressure $\leq$ 60 mm Hg (National Heart Lung and Blood Institute,

Diseases and Conditions Index, 2008), can deprive the brain and other vital organs of oxygen and nutrients, leading to a life-threatening condition called shock. Hypovolemia, resulting from severe hemorrhage or dehydration, is recognized as the most common cause of hypotension and shock. Hypotension also can be cardiogenic and caused by myocardial infarction and heart arrhythmias through decreasing cardiac output (Guyton, A.C. and Hall, J.E. 2006; Holmes, D.R., Jr. 2003). It also can be neurogenic, in which low blood pressure is caused by excessive vasodilation derived from abnormal autonomic nerve output after brain or spinal cord injury (Guyton, A.C. and Hall, J.E. 2006). In addition to loss of autonomic output, sepsis and severe systemic allergic reactions are two other conditions that induce marked vasodilation, thus hypotension and shock (Guyton, A.C. and Hall, J.E. 2006; Cauwels, A. 2007). These two types of shock are called septic shock and anaphylactic shock. Epidemiology studies indicate that the incidence of septic shock is growing by 9% per year (Annane, D. et al. 2005; Martin, G.S. et al. 2003) with an extremely high mortality rate of 50-70%. The frequency of anaphylaxis is also increasing due to the increased incidence of allergies and potential allergens to which people are exposed (Cauwels, A. 2007).

BLOOD PRESSURE REGULATION

Mean arterial blood pressure (MAP) is determined by cardiac output (CO), systemic vascular resistance (SVR) and central venous pressure (CVP) according to the following relationship:

MAP = CO × SVR + CVP

Because CVP is usually at or near 0 mm Hg, this relationship is often simplified to:

MAP = CO × SVR

Therefore, MAP could be affected by changes in either CO or SVR, which are normally independent variables. SVR can be calculated by SVR = MAP ÷ CO, but not determined by either MAP or CO. Cardiac output is determined by stroke volume (SV) and heart rate (HR), thus MAP can be expressed as:

MAP = SV × HR × SVR

Arterial blood pressure is continuously monitored by various sensors located within the body and regulated through adjustments in cardiac output and systemic vascular resistance when it varies from the normal value. In the short term, the arterial baroreceptor reflex is the primary regulatory mechanism. Arterial baroreceptors are predominantly located at the aortic arch and carotid arteries. The action potential of the receptors changes in response to the degree of stretch on vascular wall caused by blood pressure alteration, and travels to the medullary cardiovascular center for integration. Integrated feedback is sent through sympathetic and parasympathetic nerves to the heart and vasculature to regulate vascular tone, heart rate and stroke volume, and thus blood pressure (Figure 1). In the long term, baroreceptor firing rate adapts to prolonged changes in arterial pressure.

Chronic regulation of blood pressure is subjected to the glomerular filtration rate and renal fluid reabsorption, which is controlled by the renin-angiotensin system (RAS). Herein the affected parameters in determining blood pressure are CO (blood volume, precisely) and vascular resistance (by angiotensin II, Figure 2).

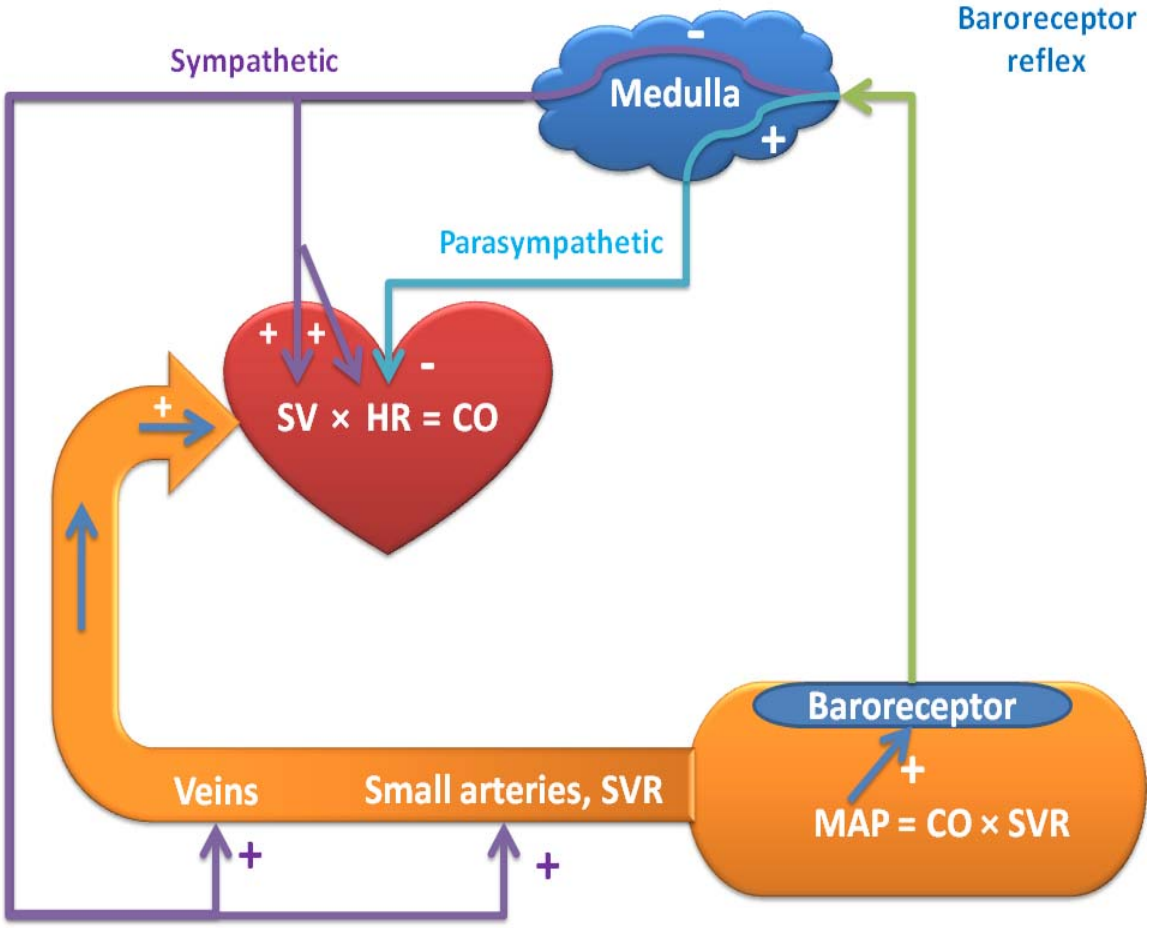

Figure 1. Arterial baroreceptor reflex pathway (Mohrman, D.E. and Heller, L.J. 2006). MAP, mean arterial blood pressure; CO, cardiac output; SVR, systemic vascular resistance; SV, stroke volume; HR, heart rate.

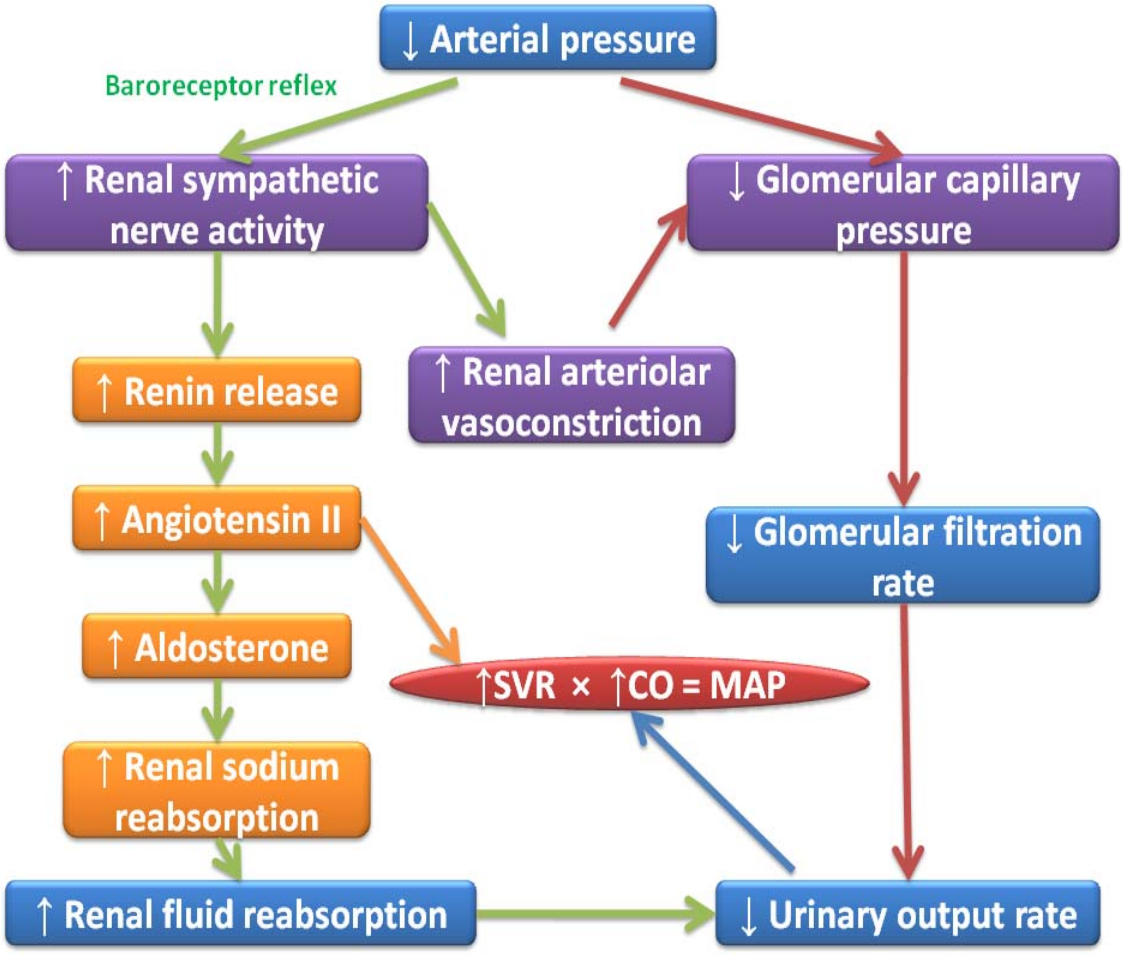

Figure 2. Mechanisms by which arterial pressure influences urinary output rate (revised from Mohrman, D.E. and Heller, L.J. 2006). MAP, mean arterial blood pressure; CO, cardiac output; SVR, systemic vascular resistance.

Vascular resistance. It is also well established that regulation of vascular resistance plays an important role in blood pressure control under both normal and pathological conditions.

Small muscular arteries and arterioles are the primary vessels that generate systemic vascular resistance (50-70%, Klabunde, R.E. 2004). The resistance (R) of a segment of vessel is determined by Poiseuille's Equation:

$$R \propto \frac{\eta \cdot L}{r^4}$$

η = viscosity of the blood, L = length of vessel and r = radius of vessel

From this equation, we can see that a 2 fold decrease in vessel radius would cause a 16 fold increase in vascular resistance. Since length and viscosity of the blood is usually consistent, the vessel radius becomes the primary driving force in systemic vascular resistance.

All small muscular arteries and arterioles basally exhibit a certain degree of smooth muscle contraction that determines the diameter, and hence the resistance. This smooth muscle contraction, also known as vascular tone, is determined by the balance of a number of competing vasoconstrictors and vasodilators. Those vasoconstrictors and vasodilators fall into two categories (Klabunde, R.E. 2004), extrinsic factors:

- Neurohormones released from autonomic nerves, such as norepinephrine (NE, vasoconstrictor) and acetylcholine (ACh, vasodilator) from sympathetic and parasympathetic innervations, respectively.
- Angiotensin II generated in RAS (vasoconstrictor).
- Atrial natriuretic peptide (vasodilator).

and intrinsic factors:

- Myogenic mechanisms (originating from vascular smooth muscle), which increase tone.
- Endothelial-derived vasodilatory factors, such as nitric oxide (NO), prostacyclin (PGI_2), endothelium-derived hyperpolarizing factor (EDHF), and vasoconstrictors, such as endothelin.
- Local hormones and chemical substances, such as arachidonic acid metabolites, histamine and bradykinin.
- Hypoxia.

Among all the tissues and organs involved in regulating vascular tone, the endothelium attracts particular attention not only due to its role in endothelium-dependent vasodilation, but also in immune and inflammatory reactions, both of which have significant impact on cardiovascular diseases, such as hypertension and atherosclerosis.

ENDOTHELIUM

Endothelial cells form a single layer across the entire vascular system. An adult has approximately 10^{13} endothelial cells (Galley, H.F. et al. 2004), with a comparable

weight to the liver. In addition to the function of maintaining vascular wall and molecular transportation as a semi-permeable barrier, endothelial cells are dynamic and have both metabolic and synthetic functions (Galley, H.F. et al. 2004). They synthesize and secrete a number of factors that modulate vascular tone, angiogenesis and inflammatory responses (Feletou, M. et al. 2006).

Endothelium in vascular tone regulation. Endothelium produces a pool of vasoactive factors, together with other neurohormonal stimuli, to act on smooth muscle cells to regulate vascular tone. NO, PGI_2 and EDHF are the most important factors produced by endothelium in relaxing the vasculature. In conduit vessels, endothelium-derived NO and PGI_2 are the predominant factors in causing vasodilation, while in small resistant vessels, EDHF tends to play a greater role in regulating vascular tone (Harrison, D.G. et al. 2003; Scotland, R.S. et al. 2005).

Endothelial cells also produce endothelin-1 (ET-1), particularly in pathological circumstances, to cause potent vasoconstriction. A number of receptors for paracrine and endocrine agonists are also expressed on the surface of endothelial cells, and mediate the release of endothelial-derived vasodilators. For instance, ET-1 can bind to its ET_B receptor on the endothelial cell surface to stimulate the release of NO (Hirata, Y. et al. 1993). In addition, angiotensin converting enzyme (ACE) is expressed on the surface of endothelial cells and catalyzes production of angiotensin II, a strong vasoconstrictor.

NITRIC OXIDE

NO is a highly lipophilic and diffusible gas and contains a single oxygen atom bonded to a nitrogen atom. It mediates diverse signaling pathways in multiple organ

systems (Dudzinski, D.M. et al. 2006) and is crucial in maintaining endothelial function to protect the cardiovascular system from pathologic damage.

NO as an endothelium-derived relaxing factor. In 1980, an endothelium-derived relaxing factor (EDRF) was discovered by Furchgott and Zawadzki. They demonstrated that ACh-induced vasodilation requires anatomical integrity of the endothelium (Furchgott, R.F. et al. 1980). Seven years later, this EDRF was identified as NO (Ignarro, L.J. et al. 1987). Nowadays, NO is one of the most well characterized vascular relaxing factors generated primarily by endothelial nitric oxide synthase (eNOS) in vascular endothelium. After being produced by endothelial cells, NO is transported into adjacent vascular smooth muscle cells (VSMC) by aquaporin (Herrera, M. et al. 2006), and causes VSMC relaxation through a variety of mechanisms, including decreasing intracellular calcium, desensitizing the contractile apparatus to calcium, and hyperpolarizing the cell membrane either directly or indirectly through cyclic guanosine monophosphate (cGMP) as a second messenger (Figure 3). NO is also involved in other important vascular functions, including leukocyte activation, platelet aggregation, interactions between the endothelium and circulating cells, and VSMC proliferation (Huang, P.L. 2000).

NO synthesis. NO is synthesized from L-arginine by three isoforms of nitric oxide synthases (NOS) in mammals: neuronal NOS, or nNOS (NOS I); inducible NOS, or iNOS (NOS II); and endothelial NOS, or eNOS (NOS III). nNOS and eNOS are predominantly expressed in nervous system and endothelium respectively, and iNOS is induced under inflammatory conditions. Three NOS isoforms are products of distinct genes but share 50-60% sequence identity and basic chemical properties (Dudzinski, D.M. et al. 2006). Generally they catalyze the reaction to produce NO:

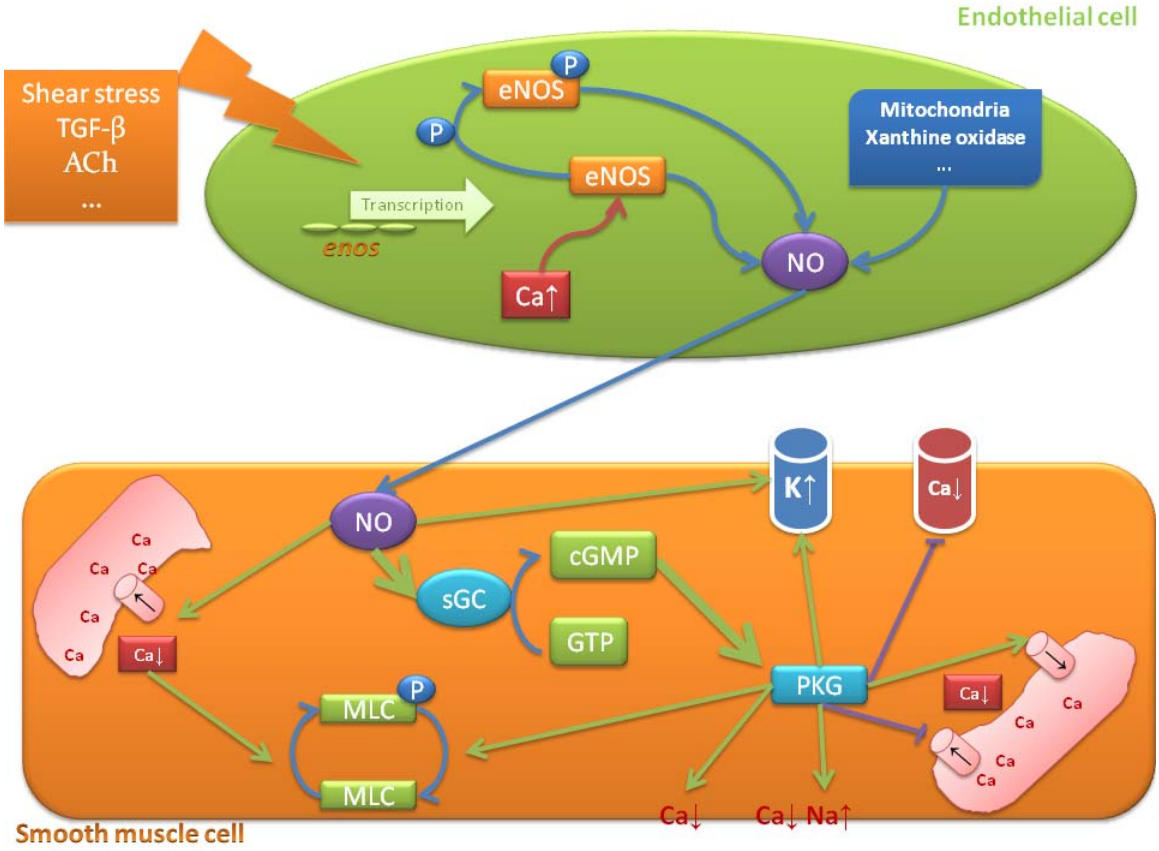

Figure 3. Vascular nitric oxide production and function on smooth muscle cells. Vascular NO is generated from eNOS and other sources, such as mitochondria and xanthine oxidase in endothelial cells. eNOS can be regulated at the transcriptional level, through protein phosphorylation and by factors that affect intracellular calcium concentration. NO causes relaxation of adjacent smooth muscle cells through variety of pathways. TGF-β, transforming growth factor-β; ACh, acetylcholine; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; sGC, soluble guanylyl cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; PKG, cGMP-dependent protein kinase; MLC, myosin light chain.

$$L-arginine+\frac{3}{2}NADPH+\frac{3}{2}H^{+}+2O_2 \xrightarrow{NOS} NO+L-citrulline+2H_2O+\frac{3}{2}NADP^{+}$$

NADPH: nicotinamide adenine dinucleotide phosphate

This reaction requires a number of cofactors, including flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in the reductase domain for accepting and transferring electrons from NADPH to heme iron, tetrahydrobiopterin (BH_4) in the oxygenase domain, and calmodulin/Ca^{2+} in the reductase domain (Alderton, W.K. et al. 2001). When either BH_4 or L-arginine substrate is limiting, NOS generates more superoxide rather than NO (Dudzinski, D.M. et al. 2006). This condition is designated as NOS uncoupling.

eNOS activity control. Nitric oxide has a short half-life of less than 5 seconds in vivo (Dudzinski, D.M. et al. 2006). Thus vascular NO bioavailability rigorously depends on eNOS activity, which is regulated by multiple interconnected factors at different levels. Vasoactive agonists, such as ACh and bradykinin, can increase intracellular calcium levels to stabilize the binding of eNOS to calmodulin, which is required for maximal NO synthesis. In addition, fluid shear stress, estrogen, insulin and inflammatory signals may stimulate the catalytic activity of eNOS through PI3K/Akt-dependent phosphorylation. eNOS can be phosphorylated at multiple sites, including two stimulatory sites: Ser1177 and Ser635, and two inhibitory sites: Thr495 and Ser116 (Dudzinski, D.M. et al. 2006). The expression of eNOS enzyme is also regulated at transcriptional and posttranscriptional levels (mRNA stability) by numerous factors, such as transforming growth factor-β1 (TGF-β1) and oxidized low-density lipoprotein (LDL) (Searles, C.D. 2006). Major factors that regulate eNOS expression and/or enzyme activity are listed in table I.

Table I. Regulatory factors of eNOS expression and activity.

Regulatory factor	Regulatory mechanism	References
Acetylcholine ↑	Intracellular Ca^{2+}	Dudzinski, D.M. et al. 2006
Bradykinin ↑	Intracellular Ca^{2+} Phosphorylation	Harris, M.B. et al. 2001
Estrogen ↑	Transcription Intracellular Ca^{2+} Phosphorylation	Caulin-Glaser, T. et al. 1997 Searles, C.D. 2006 Chambliss, K.L. et al. 2002
Hydrogen peroxide ↑	Phosphorylation	Thomas, S.R. et al. 2002
Insulin ↑	Phosphorylation	Montagnani, M. et al. 2001
Laminar shear stress ↑	Transcription Phosphorylation	Ziegler, T. et al. 1998 Fisslthaler, B. et al. 2000
Lysophosphatidylcholine ↑	Transcription	Zembowicz, A. et al. 1995
Statins (3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) ↑	mRNA stability	Searles, C.D. 2006
TGF-β1 ↑	Transcription	Inoue, N. et al. 1995
VEGF ↑	mRNA stability Phosphorylation	Papapetropoulos, A. et al. 1997 Tai, S.C. et al. 2004 Bouloumie, A. et al. 1999
Hypoxia ↓↑	Transcription mRNA stability Protein Phosphorylation Ca^{2+}	Dudzinski, D.M. et al. 2006
LPS↓	mRNA stability	Lu, J.L. et al. 1996
Oxidized LDL ↓	Transcription mRNA stability	Hernandez-Perera, O. et al. 1998 Tai, S.C. et al. 2004 Liao, J.K. et al. 1995
Thrombin ↓	Protein phosphorylation	Ming, X.F. et al. 2002
TNF-α ↓	Transcription mRNA stability	Yoshizumi, M. et al. 1993 Anderson, H.D. et al. 2004

↑: increases eNOS expression or activity; ↓: decreases eNOS expression or activity

NO and endothelial dysfunction. Endothelial dysfunction, manifested as impaired endothelium-derived nitric oxide (EDNO) bioactivity, refers to a loss of normal homeostatic functions of the endothelium, such as vasodilatation, inhibition of platelet aggregation and leukocyte adhesion. Reactive oxygen species (ROS) are very reactive and widely accepted as the major causal factor of endothelial dysfunction and cardiovascular diseases, including hypertension and atherosclerosis. They are naturally generated by normal metabolism of oxygen as a byproduct and serve as signaling molecules. Extra ROS are cleared by antioxidant enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, to protect cells from their harmful effects:

$$2O_2^- + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2 \xrightarrow{Catalase} H_2O + \frac{3}{2}O_2$$

$$2GSH + H_2O_2 \xrightarrow{Glutathione\ peroxidase} GS - SG + 2H_2O$$

Under pathological stimuli, excessive ROS are produced by various enzymatic systems, such as xanthine oxidase, cytochrome p450 and NADPH oxidase, which overwhelm the self-defense capacity of the antioxidant system. There is also an increase of eNOS expression in diseased vascular endothelium, which produces superoxide instead of NO. Under this condition, uncoupled eNOS transfers an electron from NADPH to oxygen, rather than the substrate, L-arginine, resulting in the production of superoxide instead of NO (Verhaar, M.C. et al. 2004). This eNOS uncoupling is primarily caused by reduced bioavailability of its cofactor, BH_4, through a mechanism of which BH_4 is oxidized by peroxynitrite generated from NO and ROS (Thomas, S.R. et al. 2008, Figure 4). Impaired EDNO production plus other factors can further contribute to the loss of endothelial function and development of cardiovascular diseases.

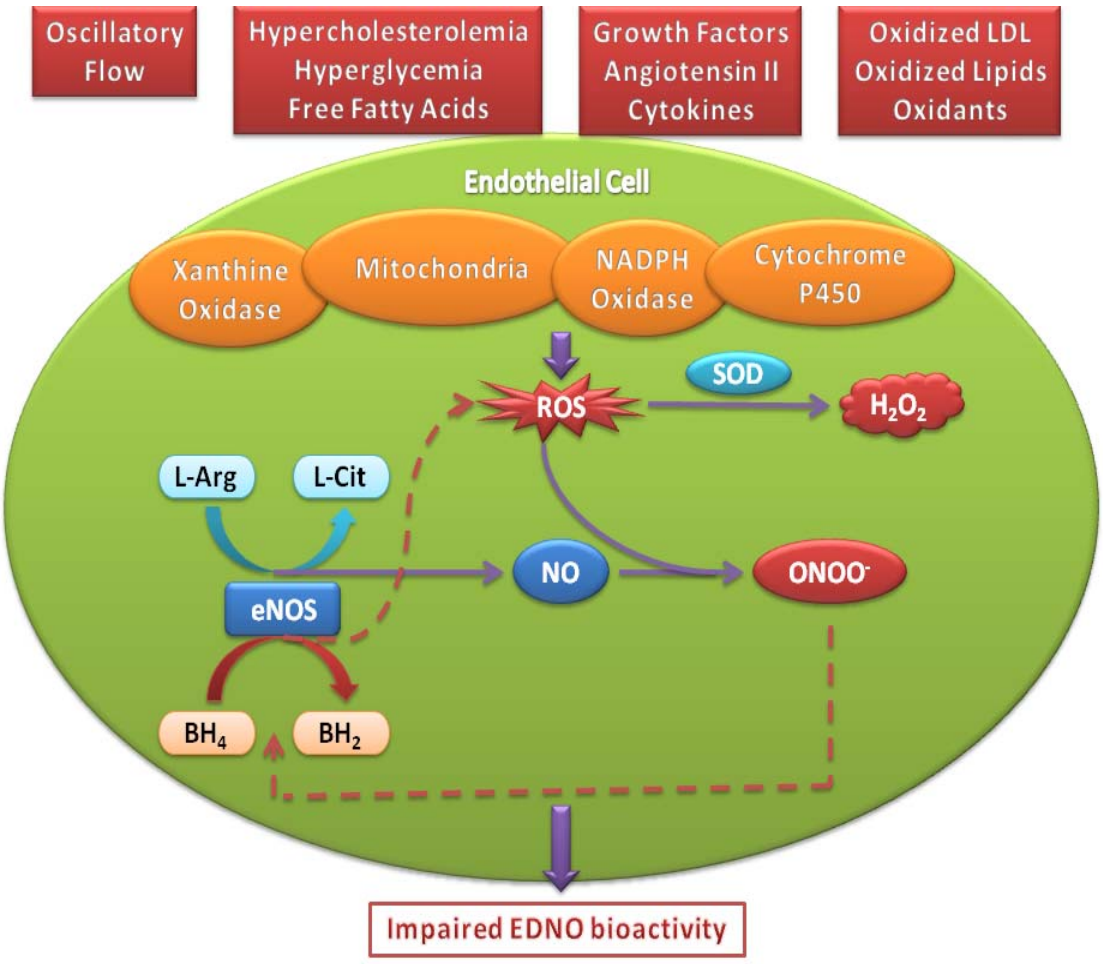

Figure 4. Effect of reactive oxygen species on endothelium-derived nitric oxide (revised from Thomas, S.R. et al. 2008). Under pathological stimuli, various enzymatic systems produce tremendous amount of superoxide, which reacts with NO to generate peroxynitrite. Accumulated peroxynitrite oxidizes BH_4 to uncouple eNOS, therefore produces ROS rather than NO and finally impairs the EDNO bioavailability to cause endothelial dysfunction. eNOS, endothelial nitric oxide synthase; NO, nitric oxide; $ONOO^-$, peroxynitrite; BH_4, tetrahydrobiopterin; BH_2, dihydrobiopterin.

NITRIC OXIDE AND CARDIOVASCULAR HOMEOSTASIS

NO has been generally accepted as a crucial factor in maintaining cardiovascular homeostasis including both blood pressure and cardiovascular organ function. Insufficient NO contributes to the development of hypertension and atherosclerosis (Huang, P.L. 2000), while excessive NO is the major cause of life-threatening hypotension in sepsis and anaphylactic shock (Cauwels, A. 2007).

NO and blood pressure regulation. Under physiological circumstances, vascular NO can either basally relax vasculature or be produced to counteract elevated blood pressure to maintain normotension, both of which are basically mediated by shear stress (Smiesko, V. et al. 1985). However dysregulation of NO can profoundly affect blood pressure to induce either hypertension or hypotension pathologically.

The role of NO in maintaining normal blood pressure has been demonstrated in rat and mouse models which develop different degrees of hypertension with NOS blockade (Arnal, J.F. et al. 1992; Duling, L.C. et al. 2006; Gardiner, S.M. et al. 1990; Gardiner, S.M. et al. 1992; Linder, A.E. et al. 2005). The significance of eNOS in NO mediated blood pressure regulation is also highlighted by *enos* gene knockout mice that exhibit high blood pressure (Huang, P.L. 2000). In contrast, over expression of eNOS induces hypotension in mice (Ohashi, Y. et al. 1998) and attenuates the high blood pressure in both stroke-prone spontaneously hypertensive rats and two-kidney one-clip hypertensive mice (Gava, A.L. et al. 2008; Miller, W.H. et al. 2005), suggesting a potential role of eNOS as a target in hypertension treatment.

NO-dependent hypotension also exists in pathological conditions, including sepsis, a serious medical condition characterized by a whole-body inflammatory state (Levy,

M.M. et al. 2003), and anaphylaxis, a sudden and severe systemic allergic reaction in the absence of allergy history. Both conditions have extremely high mortality. The fundamental role of NO in the development of hypotension in inflammation-associated shock has been demonstrated in a number of animal models, in which NOS inhibitors prevent hypotension induced by lipopolysaccharide (LPS), tumor necrosis factor (TNF), interleukin-1, 2 and sepsis (Kilbourn, R.G. et al. 1990a; Kilbourn, R.G. et al. 1990b; Kilbourn, R.G. et al. 1992; Kilbourn, R.G. et al. 1994; Petros, A. et al. 1991; Thiemermann, C. et al. 1990). In septic hypotension, NO is from eNOS and iNOS, as well as other sources, such as plasma nitrite (Cauwels, A. 2007). eNOS-derived NO seems to play both a pro-inflammatory role and an organ protective role, while iNOS-derived NO primarily contributes to excessive vasodilation (Cauwels, A. 2007; Connelly, L. et al. 2005). Therefore, iNOS selective inhibitors showed a better effect on septic shock than nonselective NOS inhibitors (Cauwels, A. 2007). In anaphylactic shock, eNOS-derived NO is the primary vasodilator, which is activated via the PI3K/Akt pathway (Cauwels, A. et al. 2006).

NO and atherosclerosis. In addition to vascular tone and blood pressure regulation, NO inhibits leukocyte-endothelial adhesion, vascular smooth muscle proliferation and migration, and platelet aggregation, all of which are critical steps in atherosclerosis development (De Caterina, R. et al. 1995; Garg, U.C. et al. 1989a; b; Sarkar, R. et al. 1996). Atherosclerosis has been demonstrated to be either accelerated by NO deprivation and *enos* gene knockout or attenuated by NO substrate supplementation and *enos* gene over expression in a number of animal models (Candipan, R.C. et al. 1996;

Chen, J. et al. 2001; Kauser, K. et al. 2000; Kuhlencordt, P.J. et al. 2001; van Haperen, R. et al. 2002).

ARYL HYDROCARBON RECEPTOR

The aryl hydrocarbon receptor (AHR) is a transcription factor that belongs to the basic helix-loop-helix/PER-ARNT-SIM family of DNA-binding proteins. AHR mRNA is expressed in multiple human tissues, with the highest expression in placenta, relatively high expression in lung, heart, pancreas and liver, and lowest expression in the kidney, brain and skeletal muscle (Dolwick, K.M. et al. 1993). AHR is well characterized for its role in mediating organismal responses to xenobiotics by inducing xenobiotic metabolizing enzymes. Halogenated aromatic hydrocarbons (HAH), such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons (PAH), such as benzo(a)pyrene are two major categories of environmental compounds that activate AHR signaling. The induction of metabolic enzymes is considered an adaptive response of the organism to its environment, which could decrease the potential toxicity of foreign chemicals. Through evolution of multicellular organisms, the function of AHR in environmental adaption has also been put to use in important physiological processes, which will be emphasized in the following discussion.

AHR alleles and molecular structure. Four murine AHR alleles, AHR^{b-1}, AHR^{b-2}, AHR^{b-3} and AHR^{d}, have been found and cloned from different inbred and wild mouse strains (Poland, A. et al. 1990; Poland, A. et al. 1987; Poland, A. et al. 1994; Schmidt, J.V. et al. 1996a). The AHR^{d} receptor has a lower ligand binding affinity compared to the AHR^{b-1} and AHR^{b-2} alleles (Poland, A. et al. 1994). The AHR^{b-1} allele encodes a protein of 805 amino acids, the AHR^{b-2} and AHR^{d} alleles encode proteins of 848 amino

acids, and the AHR^{b-3} allele encodes a protein of 883 amino acids. All proteins of four alleles contain a basic helix-loop-helix motif (bHLH), PER-ARNT-SIM (PAS) domains and a transactivation domain (TAD), and their varied amino acids exist at the carboxyl end (Schmidt, J.V. et al. 1996a, Figure 5). An $Ala_{375} \rightarrow Val_{375}$ polymorphism is responsible for the reduced ligand-binding affinity of AHR^{d} receptor compared with AHR^{b2} receptor in both rodents and humans (Ema, M. et al. 1994; Poland, A. et al. 1994).

AHR signaling pathway. Unliganded AHR forms a complex including two copies of 90-kDa heat shock protein (HSP90), one X-associated protein (XAP), and one p23 molecular chaperone protein in the cytoplasm (Carver, L.A. et al. 1998; Kazlauskas, A. et al. 1999; Ma, Q. et al. 1997; Meyer, B.K. et al. 1998). After being activated by its ligands, cytoplasmic AHR translocates into the nucleus, disassociates from the chaperone complex, dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and transactivates target genes through binding to dioxin response elements (DRE) in promoter regions (Figure 6). AHR target genes include Phase I and Phase II metabolic enzymes, such as cytochrome P450 1A1 (CYP1A1), cytochrome P450 1B1 (CYP1B1), NAD(P)H:quinone oxidoreductase I (NQO1) and aldehyde dehydrogenase 3 (ALHD3A1). In addition to the induction of these enzymes, AHR mediates the toxic endpoints of HAH exposure, including epithelial hyperplasia, porphyria, liver damage, thymic involution, cancer, teratogenicity, a severe wasting syndrome, and death (Gu, Y.Z. et al. 2000).

Studies using AHR deficient cells and animals indicate the role of AHR in normal physiological processes without the involvement of any known exogenous ligands,

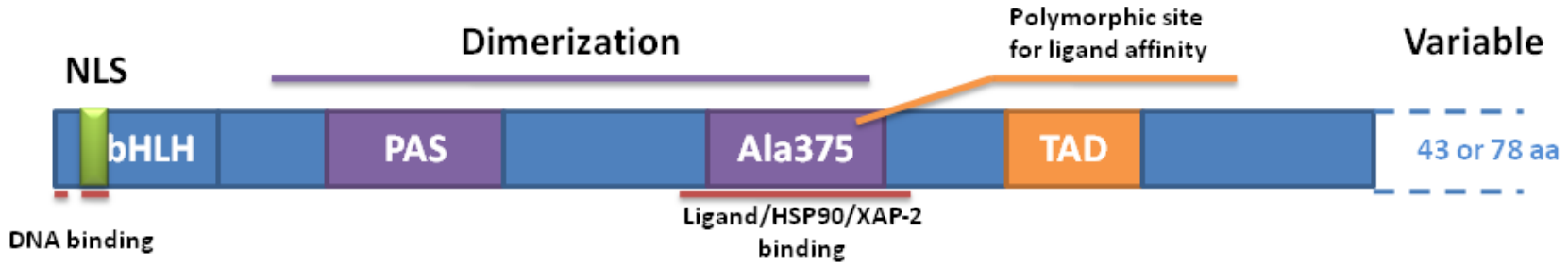

Figure 5. The molecular structure of aryl hydrocarbon receptor (revised from Schmidt, J.V. et al. 1996a). NLS, nuclear translocation signal; bHLH, basic helix-loop-helix; PAS, PER-ARNT-SIM; TAD, transactivation domain.

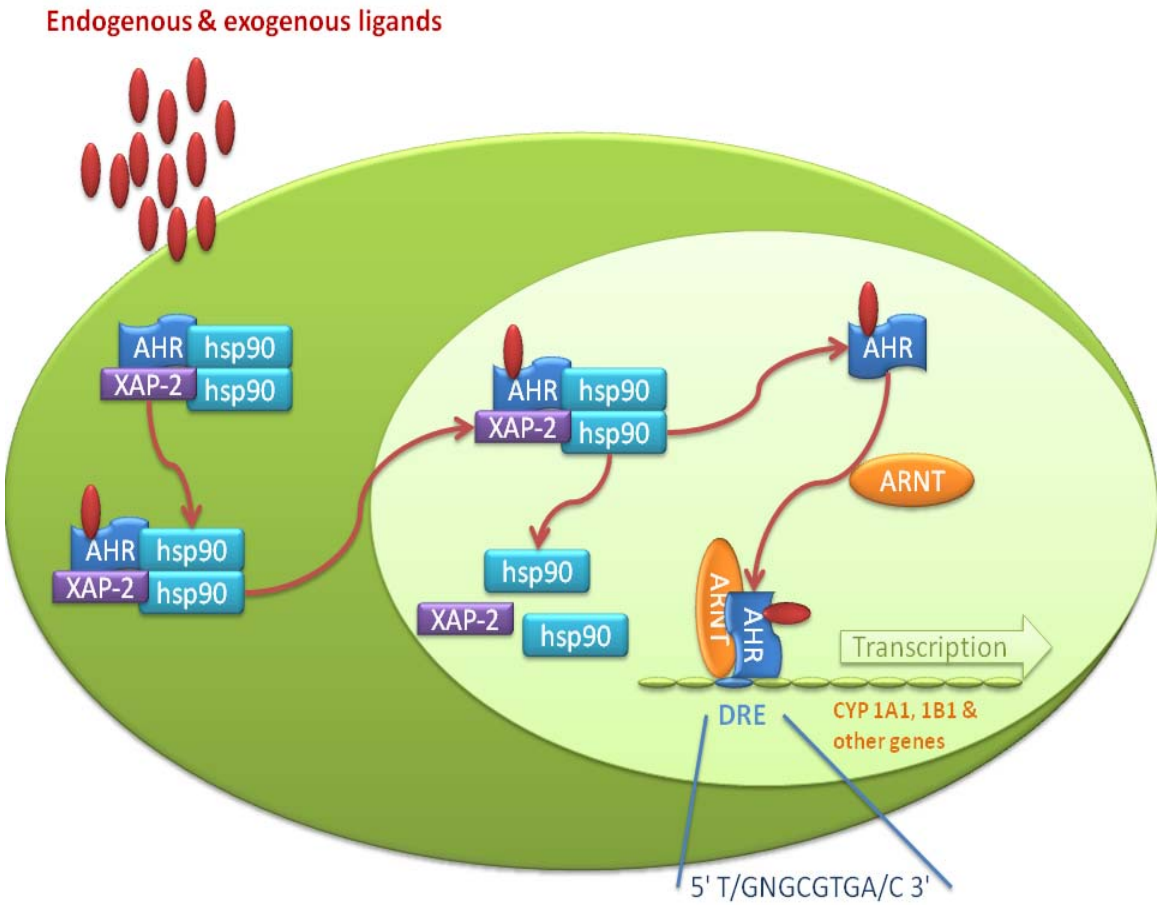

Figure 6. Aryl hydrocarbon receptor signaling pathway. Upon binding of ligand, AHR complex in the cytoplasm translocates into the nucleus and releases AHR/ligand to dimerize with ARNT. This heterodimer subsequently binds to the DRE in target genes to activate transcription. AHR, aryl hydrocarbon receptor; hsp90, heat shock protein 90; XAP, X associated protein; ARNT, aryl hydrocarbon receptor nuclear translocator; DRE, dioxin response element: 5'-T/GNGCGTGA/C-3', N stands for any nucleotide.

including cell cycle regulation, apoptosis, matrix metabolism, vascular development and cardiovascular physiology (Kung, T. et al. 2009; Lahvis, G.P. et al. 2005; Puga, A. et al. 2009). The intrinsic activity of AHR signaling has been suggested to be subject to the regulation by either endogenous ligands or nonligand activators. Variety of candidate endogenous ligands have been isolated from mammalian tissues, including indigo, indirubin, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester, arachidonic acid metabolites, such as lipoxin 4A and prostaglandin G_2, and heme metabolites, such as bilirubin (Adachi, J. et al. 2001; Henry, E.C. et al. 2006; Schaldach, C.M. et al. 1999; Seidel, S.D. et al. 2001; Sinal, C.J. et al. 1997). Moreover, ligand independent activators, such as shear stress and cAMP can also regulate endogeneous AHR activity and the expression of AHR target genes. The role of shear stress in AHR activation was initially demonstrated by the observation that shear stress can induce CYP1A1 expression in an AHR dependent manner (Eskin, S.G. et al. 2004; Mufti, N.A. et al. 1995a; Mufti, N.A. et al. 1995b). Afterwards, modified low-density lipoprotein (LDL) was identified as a mediator involved in the activion of AHR by shear stress (McMillan, B.J. et al. 2007). The second messenger cAMP also modulates AHR signaling in a ligand independent manner, in which AHR translocates into nucleus and binds to DRE without dimerizing with ARNT (Oesch-Bartlomowicz, B. et al. 2005). Stimulation with N^6-$O^{2'}$-dibutyryl-cAMP, a membrane-permeating derivative of cAMP or forskolin, a potent activator of adenylate cyclase, inhibits AHR-mediated response to TCDD in mouse hepatoma cells (Oesch-Bartlomowicz, B. et al. 2005). Those studies provide ample evidence of xenobiotic independent AHR signaling modulation that may perform more fundamental roles in developmental and physiological events.

Phenotype of AHR knockout mice. *ahr* gene knockout mice have become a powerful tool in investigating the role of endogenous AHR in physiological and developmental processes. In 1990s, AHR deficient mice were developed independently in three labs, by either deleting exon 1 (Fernandez-Salguero, P. et al. 1995; Mimura, J. et al. 1997) or exon 2 (Schmidt, J.V. et al. 1996b) of the gene. All three AHR null mice had a mixed C57BL/6 ×129 background and displayed a slower growth rate within the first few weeks after birth, TCDD resistance, failure of xenobiotic CYP1A1 and CYP1A2 induction, maintained but decreased fertility and liver pathology. One line had ~50% neonatal mortality rate (Fernandez-Salguero, P. et al. 1995; Mimura, J. et al. 1997), but this may have been dependent on the manner of gene deletion. The AHR knockout mice exhibit a spectrum of hepatic defects, including portal fibrosis and a smaller liver. The mechanism underlying the liver defect seems due to fetal hepatic necrosis caused by compromised perfusion (Harstad, E.B. et al. 2006; Walisser, J.A. et al. 2004a), and partially resulted from a patent ductus venosus in adulthood, which is mediated by loss of AHR in endothelial cells specifically (Lahvis, G.P. et al. 2000; Walisser, J.A. et al. 2004a; Walisser, J.A. et al. 2005). Furthermore, abnormal vascular structures have been reported in liver, kidney and hyaloid of AHR knockout mice (Lahvis, G.P. et al. 2000). It was originally reported that AHR knockout mice had an impaired immune system, which was demonstrated by decreased accumulation of lymphocytes in the spleen and lymph nodes (Fernandez-Salguero, P. et al. 1995). However, the role of endogenous AHR in immune function is not well-understood and needs to be further elucidated. Aged AHR knockout mice also develop multiple organ lesions including cardiac hypertrophy and fibrosis,

portal and uterine vascular remodeling, gastric hyperplasia and epidermal pathology (Fernandez-Salguero, P.M. et al. 1997).

ARYL HYDROCARBON RECEPTOR AND CARDIOVASCULAR PHYSIOLOGY

In addition to mediating PAH induced cardiovascular toxicity, AHR also plays a fundamental role in cardiovascular development and physiology, which has been demonstrated using AHR deficient mouse model.

AHR in cardiac hypertrophy. AHR is required in vascular development and in cardiovascular physiology. AHR deficient mice develop cardiac hypertrophy and fibrosis in adulthood (Fernandez-Salguero, P.M. et al. 1997; Thackaberry, E.A. et al. 2002; Vasquez, A. et al. 2003). The mechanism involved in the development of cardiac hypertrophy in AHR null mice may have many factors. Early characterization of the enlarged heart in AHR null mice suggested that enhanced VEGF expression may contribute to the hypertrophy phenotype (Thackaberry, E.A. et al. 2002). In 2003, Vasquez et al reported increased size of cardiomyocytes and an anatomic remodeling without typical features of molecular remodeling, which was not consistent with hypertrophic growth secondary to pressure or volume overload (Vasquez, A. et al. 2003). This suggested an intrinsic role of AHR in cardiomyocyte size control. In the same year, Lund et al. indicated that cardiac hypertrophy in AHR null mice was associated with high systemic arterial blood pressure as well as increased circulating angiotensin II (Ang II) and plasma ET-1 level (Lund, A.K. et al. 2003). This cardiac hypertrophic phenotype was primarily mediated by elevated circulating ET-1, thus treatment with BQ-123, an ET_A receptor antagonist, significantly attenuated the phenotype as well as the mRNA

expression of cardiac hypertrophy makers, atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC) (Lund, A.K. et al. 2006). All the research data above suggest that endogenous AHR contributes to the development of cardiac hypertrophy.

AHR in blood pressure regulation. The role of the AHR agonist, TCDD, in inducing high blood pressure has been demonstrated in both epidemiology studies and research using mouse models, in which AHR mediated cytochrome P450 induction may be involved (Dalton, T.P. et al. 2001; Kang, H.K. et al. 2006; Kim, J.S. et al. 2003; Kopf, P.G. et al. 2008; Pesatori, A.C. et al. 1998).

Besides ligand activated AHR, endogenous AHR also contributes to blood pressure regulation in addition to cardiovascular development mentioned above. Anesthetized AHR null mice were first found hypotensive in the absence of a heart rate difference at 8 months old (Vasquez, A. et al. 2003). They also reported a decreased cardiac output caused by diminished stroke volume in 4 month old AHR knockout mice (Vasquez, A. et al. 2003). This finding suggested a role of AHR in causing hypotension by decreasing cardiac function. Later in the same year, Lund reported high blood pressure in conscious AHR null mice, associated with elevated circulating Ang II and ET-1 levels (Lund, A.K. et al. 2003). In this study, angiotensin converting enzyme blockade by captopril attenuated, but did not normalize elevated arterial blood pressure. Afterwards, ET-1 was identified as the primary factor causing high arterial blood pressure in those AHR null mice (Lund, A.K. et al. 2006). Treatment with BQ-123, an ET_A receptor blocker, dramatically attenuated mean arterial blood pressure as well as plasma Ang II levels in AHR null mice, suggesting increased Ang II as a secondary effect of ET-1 elevation. Another group in Mexico City also reported elevated arterial blood

pressure in AHR null mice, which was normalized by captopril treatment (Villalobos-Molina, R. et al. 2008). Their model also suggested an increase of vascular α-1D adrenoceptor expression that was involved in the hypertensive phenotype. Interestingly, both groups reported hypertension in AHR null mice located at high altitude (Albuquerque NM, 1620m; Mexico City, 2240m). A further investigation of blood pressure in AHR null mice indicated that loss of endogenous AHR in mice led to hypotension at sea level and hypertension at mild high altitude, which was caused by different atmospheric oxygen levels (Lund, A.K. et al. 2008). These data suggested that a more complicated mechanism is involved in the role of endogenous AHR in regulating blood pressure, and needs to be further elucidated.

AHR in endothelial dysfunction. Previous research demonstrated that the AHR plays a very important role in mediating the toxic effect of its agonists to produce endothelial dysfunction and atherogenesis, which involves production of toxic metabolites and ROS in endothelial cells, abnormal cell adhesion and invasion and imbalance of cholesterol metabolism (Granberg, A.L. et al. 2000; Iwano, S. et al. 2005a; Iwano, S. et al. 2005b; Knaapen, A.M. et al. 2007; Korashy, H.M. et al. 2006; Meng, D. et al. 2009; Oesterling, E. et al. 2008; Toborek, M. et al. 1995).

Endogenous AHR has been found to play an indispensible role in vascular development in multiple organs (Lahvis, G.P. et al. 2000). The altered systemic blood pressure in AHR null mice was also reported to be associated with a change of circulating ET-1 levels, which was thought to be generated by endothelial cells primarily (Lund, A.K. et al. 2003). Those observations suggested that endogenous AHR may be involved in

cardiovascular development as well as vascular physiology in the absence of exogenous ligands.

AHR in circadian rhythms. The expression of AHR exhibits circadian oscillation and may be involved in mediating xenobiotic-induced disruption of circadian rhythms, which are observed in most physiological functions, such as metabolism, cell growth and immune responses (Shimba, S. et al. 2009). The daily cycle of relative AHR and ARNT protein expressions exhibit similar oscillation patterns in multiple organs of female Sprague-Dawley rats, respectively (Richardson, V.M. et al. 1998). In mice, the mRNA expression of AHR and its target gene, CYP1A1, also exhibit circadian changes in the suprachiasmatic nucleus and liver (Mukai, M. et al. 2008). The circadian rhythms of behavior and of the immune system can be disrupted by activation of AHR with TCDD (Garrett, R.W. et al. 2006; Mukai, M. et al. 2008). These observations suggest a possible cross-talk between AHR and circadian rhythm pathways.

RATIONALE FOR RESEARCH

Cardiovascular diseases have been the number one killer in the United States for decades. Hypertension and atherosclerosis are two types of cardiovascular diseases that systemically affect the whole cardiovascular system and dramatically contribute to the etiology of other types of cardiovascular defects, such as coronary heart disease and stroke. Extensive research has been conducted to investigate the role of AHR in mediating PAH/HAH induced cardiovascular diseases. Furthermore, AHR itself has been found to be involved in vascular development and cardiovascular homeostasis. These findings indicate that AHR has great potential impact in the etiology of cardiovascular diseases from both environmental and genetic perspectives.

AHR knockout mice have served as a very useful tool in studying the intrinsic developmental and physiological functions of AHR. AHR deficient mice develop vascular defects and cardiac hypertrophy (Fernandez-Salguero, P.M. et al. 1997; Lahvis, G.P. et al. 2000; Thackaberry, E.A. et al. 2002; Vasquez, A. et al. 2003). AHR null mice are hypotensive at sea level, but hypertensive at a mild high altitude (Lund, A.K. et al. 2008; Lund, A.K. et al. 2003; Vasquez, A. et al. 2003; Villalobos-Molina, R. et al. 2008). The hypertensive phenotype at high altitude seems to be caused by the lower oxygen concentration at high altitude, and through the mechanism of increased circulating endothelin-1, angiotensin II and vascular adrenoceptor levels (Lund, A.K. et al. 2008; Villalobos-Molina, R. et al. 2008). This may suggest that endogenous AHR contributes to blood pressure stability and leads to high blood pressure, thus loss of AHR causes lower blood pressure at sea level, but high blood pressure at mild high altitude, which may be due to an impaired blood pressure regulating mechanism.

Interestingly, after living for a few years at high altitude, our AHR null mice now have a hypotensive phenotype, which mimics the blood pressure phenotype observed at sea level. Additionally, the former proposed mediators of high blood pressure, including high circulating Ang II and ET-1 levels, no longer occur in these animals. This suggests that the AHR null mice in Albuquerque have physiologically adapted to the altitude and exhibit a blood pressure phenotype consistent with sea level animals. Although the mechanism underlying this hypotensive phenotype may differ from AHR null mice that are never raised at high altitude, there is no doubt that AHR plays a central role in this hypotensive phenotype. It is also very possible that these two types of hypotension caused by AHR deficiency share the same mechanism regardless of the altitude.

The long-term goal of this research is to elucidate the mechanism by which endogenous AHR regulates blood pressure, and loss of AHR contributes to blood pressure control. However, potential mechanisms that may mediate hypotension in AHR null mice have not been explored in great detail. Nonetheless, a few papers indicated that TGF-beta and VEGF are increased in AHR null mice (Chang, X. et al. 2007; Guo, J. et al. 2004; Ichihara, S. et al. 2007; Thackaberry, E.A. et al. 2002), both of which could regulate the expression or activity of eNOS, thus vascular NO, a potent vasodilator. Therefore, we hypothesize that AHR suppresses vascular eNOS in maintaining normal arterial blood pressure, and thus loss of AHR decreases arterial blood pressure due to an elevated vascular eNOS and increased NO bioavailability.

To test our hypothesis, we proposed the following specific aims:

I. Determine the degree of NO-mediated vasodilation and vascular eNOS expression in AHR knockout mice *in vivo*. The working hypothesis for this aim is that AHR suppresses eNOS expression, thus AHR knockout mice exhibit an increased eNOS expression and NO-mediated vasodilation.

II. Determine the degree by which eNOS over expression contributes to the hypotension of AHR knockout mice *in vivo*. The working hypothesis for this aim is that over expressed eNOS mediates the hypotension in AHR knockout, thus eNOS inhibition can normalize the blood pressure of AHR knockout mice to control level.

III. Determine the degree by which AHR signaling knockdown contributes to eNOS expression in human endothelial cells *in vitro*. The working hypothesis for this

aim is that endogenous AHR signaling suppresses eNOS expression, thus AHR signaling knock down can increase eNOS expression in human endothelial cells.

CHAPTER II

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Prazosin hydrochloride, hexamethonium chloride, N^{ω}-nitro-L-arginine (LNNA), phenylephrine hydrochloride (PE), acetylcholine chloride (ACh), S-Nitroso-N-acetyl-DL-penicillamine (SNAP), o-phenanthroline, p-hydroxymercuribenzoic acid, pepstatin A and bisbenzimide H 33258 were purchased from Sigma Aldrich (St. Louis, MO). RIPA (radio immuno precipitation assay) lysis buffer was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Endothelial cell basal medium-2 (EBM-2), endothelial cell growth medium-2 (EGM-2) and fetal bovine serum were purchased from Lonza (Switzerland). Lipofectamine 2000 and Opti-MEM media were purchased from Invitrogen (Carlsbad, CA). Precision Plus Protein All Blue Standard was purchased from Bio-Rad laboratories (Hercules, CA). Diaminofluorescein-2 Diacetate (DAF-2DA) was purchased from Cell Technology (Mountain View, CA). TCDD was a generous gift from Dr. Richard E. Peterson (University of Wisconsin-Madison).

ANIMALS

AHR knockout mice were originally obtained from Dr. Frank Gonzales (National Cancer Institute) and have been maintained by the Animal Resource Facility at University of New Mexico since 2000 (Albuquerque NM, altitude 1620 m). Mice were housed under conditions of constant temperature (22 °C), humidity (20%), a 12-h light/dark cycle, and fully provided with mouse chow and water. All animals have been backcrossed to C57B16N for more than 10 generations. Heterozygous sibling mice were used for breeding in order to generate wildtype, AHR heterozygous and AHR knockout mice. Age matched wildtype and AHR knockout mice were used in all experiments. All animal

protocols were approved by the University of New Mexico Animal Care and Use Committee and were in accordance with NIH guidelines.

CELLS

Primary human aortic endothelial cells (HAECs) were purchased from Lonza and cultured in EGM-2 media at 37 °C, 5% CO_2 according to the endothelial cell system instruction of use (Lonza). Usage of cells was restricted to passages 1 to 4.

BLOOD PRESSURE, HEART RATE AND ACTIVITY ASSESSMENT

PA-C20 pressure telemeters (Data Sciences International, St. Paul, MN) were used to assess arterial blood pressure, heart rate and activity of mice. Mice were subcutaneously injected with 0.1 mg/kg buprenorphine 30 min prior to surgery, and anesthetized with inhaled isoflurane (5% induction followed by 2-2.5% maintenance). The telemeter catheter was inserted into the left carotid artery to the aortic arch and the transmitter was placed subcutaneously in the abdomen. Mice were allowed to recover for at least 7 days prior to any data collection. Basal blood pressure including systolic, diastolic, mean and pulse arterial blood pressure, heart rate and activity were collected for 1 week before any treatments. All parameters were collected for 10 s every 15 min except when specifically noted. Mice were monitored for body weight twice per week, implanted telemeter signal strength and pinched or occluded catheter (reduced pulse-wave), and any data collected after appearance of those conditions were excluded from analyses.

DETERMINATION OF PLASMA ANGIOTENSIN II (ANG II)

Plasma sample preparation. Four month old wildtype and AHR knockout mice were anesthetized with ketamine (80 mg/kg)/xylazine (4 mg/kg), and euthanized by exsanguination. Blood samples were collected from the right ventricle with syringe containing 15 uL EDTA (0.5 M, pH8). Blood samples were mixed with a protease inhibitor cocktail immediately in collecting tubes. The inhibitor cocktail was a mixture of solution A (1.25 M EDTA, 50×, 20 uL for 1 mL of blood) and solution B (132 mM phenanthroline in DMSO, 36 mM pepstatine A in DMSO and 300 mM p-hydroxy-mercuribenzoic acid in 0.1 M potassium phosphate pH7.4 containing 1% 10 N NaOH, mixed at 1:1:1 ratio, 100×, 10 uL for 1 mL of blood). Within 30 min, plasma was separated from whole blood by centrifuging at 3,000 x g for 15 min at 4 °C. Plasma samples were stored at -80 °C before measurement and were thawed only at the time of analysis.

Ang II measurement. Ang II was extracted from 0.4 mL plasma using Strata phenyl cartridges (Phenomenex, Torrance, CA). The extracted sample was reconstituted using buffer provided in the enzyme immunoassay kit (Cayman, Ann Arbor, MI) and Ang II levels were measured in duplicate. Ang II concentration was determined from a standard curve.

DETERMINATION OF PLASMA ENDOTHELIN-1 (ET-1)

Plasma sample preparation. Four month old wildtype and AHR knockout mice were anesthetized with ketamine (80 mg/kg)/xylazine (4 mg/kg), and euthanized by exsanguination. Blood samples were collected from the right ventricle with syringe containing 15 uL EDTA (0.5 M, pH8). Within 30 min, plasma was separated from whole

blood by centrifuging at 3,000 x g for 15 min at 4 °C. Plasma samples were stored at -80 °C and thawed only at the time of analysis.

ET-1 measurement. ET-1 was extracted from 0.3 mL plasma by Strata C18-E cartridges (Phenomenex). Extracted samples were reconstituted in the buffer provided in the enzyme-linked immunosorbent assay (ELISA) kit (human ET-1QuantiGlo chemiluminescent ELISA kit, R&D, Minneapolis, MN) and subjected to measurement in duplicate. ET-1 concentration was calculated from a standard curve.

DETERMINATION OF URINARY NOREPINEPHRINE (NE)

24 hour urine collection. Wildtype and AHR knockout mice were placed into metabolic mouse cages, one animal per cage, to collect urine for 72 hours. Mice housed in cage had full access to food and water. The first 24 hours were used as an acclimation period, and urine generated in this period was discarded. Then, 24-hour urine samples were collected twice in the subsequent 48 hours separately. Cages were cleaned and dried between two collections, while urine samples were kept on ice. The 24-hour urine volumes were assessed and recorded as an average of two collections. Urine samples were stored at -80 °C.

Urine NE measurement. Urine samples were diluted 10 times in assay buffer and NE concentration was measured using NE EIA kit (Alpco Diagnostics, Salem, NH). NE concentration in each plate well was calculated from standard curve.

PRAZOSIN TREATMENT

Three month old wildtype and AHR knockout mice were implanted with PA-C20 pressure telemeters (Data Sciences International) for blood pressure and heart rate

assessment. After one week recovery and one week baseline data collection, prazosin (1 ug/g, 0.1 ug/uL in sterile water) was injected intraperitoneally into conscious animals. Telemetry parameters, including blood pressure and heart rate, were recorded for 10 s every min for 5 hours after injection.

HEXAMETHONIUM TREATMENT

Three month old wildtype and AHR knockout mice were implanted with PA-C20 pressure telemeters (Data Sciences International) for blood pressure and heart rate assessment. After one week recovery, one week base line data collection, prazosin administration and one week drug washout period, hexamethonium (30 ug/g, 3 ug/uL in sterile water) was injected intraperitoneally into conscious animals. Telemetry parameters, including blood pressure and heart rate, were recorded for 10 s every min for 5 hours after injection.

VASCULAR REACTIVITY ASSESSMENT

Physiologic saline solution preparation. Physiologic saline solution was prepared as mixed dry powder (130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 14.9 mM $NaHCO_3$, 5.5 mM glucose). Before aorta isolation, the proper amount of dry powder was dissolved in 2 L water and supplemented with 20 mL of $CaNa_2$ EDTA (2.6 mM) and 20 mL of $CaCl_2$ (180 mM) to achieve final concentrations of 26 uM $CaNa_2$ EDTA and 1.8 mM $CaCl_2$. Physiologic saline solution was bubbled for 30 min with 6% CO_2, 21% O_2 balanced N_2 gas, adjusted to pH 7.4 and kept on ice.

Aorta isolation and preparation. Four month old wildtype and AHR knockout mice were anesthetized with ketamine (80 mg/kg)/xylazine (4 mg/kg), and euthanized by

exsanguination. Aortas were carefully dissected and placed in ice cold physiologic saline solution. Aortas were carefully trimmed of connective tissue and cut into 2-3 mm segments under a microscope while in ice cold physiologic saline solution. To disrupt the endothelial cell layer, a piece of metal wire was inserted into the lumen of the aortic ring, and the wire and vessel rolled back and forth two times. The aortic ring was hung on two metal hangers that were carefully placed through the lumen, and rings were quickly placed in the tissue bath (Radnoti Glass Technology Inc., Monrovia, CA) with the lower hanger attached to a fixed stage and the upper hanger attached to a calibrated force transducer (Grass Technologies, West Warwick, RI). The tissue bath was filled with 37°C physiologic saline solution and constantly bubbled with 6% CO_2, 21% O_2 balanced N_2 gas. The vascular tension of aorta rings was brought up to 250 mg for 10 min, subsequently adjusted to 500 mg for 10 min, 1,000 mg for 10 min and 1,500 mg for 30 min before any experiments.

Aortic reactivity to KCl and PE ± LNNA preincubation. Aortic rings were contracted with 10^{-5} M PE and then 40 mM KCl for 10 min followed with 30 min washout after each treatment. Washout was performed every 10 min during the 30 min period. First, a KCl dose response (2.5 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM) was performed followed by a 30 min washout. The dose of KCl was increased every 5 min. After the KCl dose response, a PE dose response (10^{-9} M, $10^{-8.5}$ M, 10^{-8} M, $10^{-7.5}$ M, 10^{-7} M, $10^{-6.5}$ M, 10^{-6} M, $10^{-5.5}$ M, 10^{-5} M) was performed followed by a 30 min washout. The dose of PE was increased every 5 min. Finally, aortic rings were preincubated with 10^{-4} M LNNA for 30 min immediately followed by a

PE dose response (10^{-9} M, $10^{-8.5}$ M, 10^{-8} M, $10^{-7.5}$ M, 10^{-7} M, $10^{-6.5}$ M, 10^{-6} M, $10^{-5.5}$ M, 10^{-5} M) with the dose of PE increased every 5 min.

Aortic reactivity to ACh and SNAP. The aortic response to ACh was assessed on intact aortic rings, while the aortic response to SNAP was assessed on endothelium-disrupted aortic rings cut from the same aorta. A PE dose response (10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M) was performed on both intact and endothelium-disrupted aortic rings followed by a 30 min washout with the dose of PE increased every 5 min. The disruption of the endothelium was confirmed by a much greater contraction to PE, compared to endothelium-intact aortic rings. Then aortic rings were contracted with 80 mM KCl for 10 min followed by a 30 min washout. Intact smooth muscle was evaluated by an equal contraction of endothelium-disrupted aortic rings and intact aorta rings. Finally, aortic rings were contracted with 10^{-5} M PE for 10 min and relaxed with10^{-5} M ACh for 10 min. ACh was only added to intact rings but not endothelium-disrupted rings. After a 40 min washout on all aortic rings, both intact and endothelium-disrupted aortic rings were contracted with 10^{-6} M PE for 10 min. An ACh dose response (10^{-9} M, $10^{-8.5}$ M, 10^{-8} M, $10^{-7.5}$ M, 10^{-7} M, $10^{-6.5}$ M, 10^{-6} M, $10^{-5.5}$ M, 10^{-5} M) on intact aortic rings and a SNAP dose response (10^{-9} M, $10^{-8.5}$ M, 10^{-8} M, $10^{-7.5}$ M, 10^{-7} M, $10^{-6.5}$ M, 10^{-6} M, $10^{-5.5}$ M, 10^{-5} M) on endothelium-disrupted aortic rings were performed. Doses were increased every 5 min in both experiments.

DETERMINATION OF eNOS PROTEIN IN MOUSE AORTAS

Sample preparation. Four month old wildtype and AHR knockout mice were anesthetized with ketamine (80 mg/kg)/xylazine (4 mg/kg), and euthanized by exsanguination. Aortas were carefully dissected and placed in ice cold physiologic saline

solution. Aortas were carefully trimmed of connective tissues and cut into 2-3 mm segments under the microscope in ice cold physiologic saline solution. Aortas were frozen at -80 °C for later homogenization if necessary. Aortas were homogenized in ~ 40 uL of RIPA lysis buffer (Santa Cruz Biotechnology) using PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA) for 2 min on ice. Then homogenized aortas were frozen at -80 °C for ~15 min to freeze followed thawing on ice. Aortas were then sonicated for 20 s, frozen at -80 °C, thawed on ice and centrifuged at 15,000 x g 4°C for 10 min. Supernatant was saved at -80 °C as whole cell lysate. Protein concentration of aortic lysates was measured using Bio-Rad protein assay (Bio-Rad Laboratories).

Western blot. A 30 ug protein aliquot was mixed with 2 × loading buffer (100 mM pH6.8 Tris-HCl, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, 200 mM dithiothreitol added before using) and denatured at 95 °C for 5 min. Denatured samples were subjected to electrophoresis on 7.5% Tris-HCL polyacrylamide gel (Bio-Rad Laboratories) at 150 V for ~1 hour in running buffer (25 mM Tris-base, 150 mM glycine, 0.1% sodium dodecyl sulfate). Separated protein was transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories) at 350 mA for ~1 hour in cold transfer buffer (20 mM Tris-base, 150 mM glycine, 20% methanol). The PVDF membrane was then briefly washed with Tris-buffered saline with tween (TBST, 0.8% NaCl, 0.02% KCl, 0.3% Tris-base, 0.05% Tween-20, pH7.4) and blocked for 1 hour in 5% dry milk made with TBST on a rocker. The blocked membrane was cut into two pieces based on target protein size, thoroughly washed with TBST and then incubated with rabbit anti phospho-eNOS (Ser1177) antibody (1:1000, Catalogue No. 9571, Cell Signaling Technology, Danvers, MA) or goat anti-actin antibody (1:1000,

Santa Cruz Biotechnology) at 4 °C on shaker overnight. On the second day, the membrane was washed with TBST and incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL) or HRP conjugated donkey anti-goat IgG (Southern Biotech) for 1 hour at room temperature on a rocker. Then the membrane was washed with TBST and developed for 10 min by adding ~1 mL chemiluminescence reagent (Perkin-Elmer, Waltham, MA) under KODAK Image Station 4000MM digital imaging system (Perkin-Elmer).

The membrane was stripped with stripping buffer (7 M guanidine hydrochloride, 0.75% KCl, 0.38% glycine, 50 uM EDTA, 0.14% 2-mercaptoethanol, pH10) for 10 min on shaker and washed thoroughly with TBST. Then the membrane was blocked for 1 hour in 5% dry milk and incubated with mouse anti-eNOS antibody (1:2000, Catalogue No. 610296, BD Transduction Laboratories, San Jose, CA) at 4 °C on shaker overnight. On the second day, the membrane was washed with TBST and incubated with HRP-conjugated goat anti-mouse IgG (Southern Biotech) for 1 hour at room temperature on shaker. Then the membrane was washed with TBST and developed for 10 min by adding ~1 mL chemiluminescence reagent (Perkin-Elmer) under KODAK Image Station 4000MM digital imaging system (Perkin-Elmer). Precision Plus Protein All Blue Standard was used in estimating molecule size on all Western blots.

LNNA TREATMENT

Three month old wildtype and AHR knockout mice were implanted with PA-C20 pressure telemeters (Data Sciences International) for blood pressure and heart rate assessment. After one week recovery and one week base line data collection, 250 mg/L LNNA was administrated through the drinking water for two weeks and then followed

with 500 mg/L LNNA in drinking water for one additional week. Mice were returned to regular water for at least one week before sacrifice. During this entire treatment period, mouse body weight, water consumption, and blood pressure were carefully monitored. Telemetry parameters, including blood pressure and heart rate, were recorded for 10 s every 15 min. The drinking water containing LNNA was prepared fresh and changed every 3 days.

DETERMINATION OF URINARY NOx

24 hours urine collection. Wildtype and AHR knockout mice were placed into metabolic mouse cages, one animal per cage, to perform a urine collection for 72 hours as described above. Urine collections were performed either before starting LNNA treatment or while the mice were being dosed with 500 mg/L LNNA in the drinking water.

Urine nitrate/nitrite assessment. Urine samples were diluted 10 times in assay buffer and total nitrate/nitrite concentration was measured by Griess assay using Nitrate/Nitrite Colorimetric Assay Kit (Cayman). Total nitrate/nitrite concentration was predicted from standard curve.

siRNA TRANSFECTION

HAECs were grown in regular EGM-2 media at 37 °C, 5% CO_2 for cell expansion. Twenty-four hours before siRNA transfection, HAECs were plated at ~70-80% confluence in antibiotic-free EGM-2 media. The day 2, 50 nM ON-TARGETplus SMARTpool siRNA targeting AHR (Table II, Dharmacon, Chicago, IL) or 50 nM siGENOME RISC-Free Control siRNA (Dharmacon) were reconstituted in Opti-MEM

Table II. ON-TARGETplus SMARTpool AHR siRNA.

Duplex ID	Sense sequences	Antisense sequence
J-004990-05	GCAAGUUAAUGGCAUGUUUUU	PAAACAUGCCAUUAACUUGCUU
J-004990-06	GAACUCAAGCUGUAUGGUAUU	PUACCAUACAGCUUGAGUUCUU
J-004990-07	GCACGAGAGGCUCAGGUUAUU	PUAACCUGAGCCUCUCGUGCUU
J-004990-08	GCAACAAGAUGAGUCUAUUUU	PAAUAGACUCAUCUUGUUGCUU

media and used to transfect HAECs with Lipofactamine 2000 as a delivery system according to the manufacturer's protocol (Invitrogen). Twenty-four hours later, antibiotic-free EGM-2 media containing both lipofactamine 2000 and siRNA were removed, and fresh EGM-2 media were added. Forty-eight hours after transfection, total RNA was isolated from cells with RNeasy Mini Kit (Qiagen, Gmbh, Germany).

mRNA EXPRESSION ASSESSMENT

RNA concentration measurement. RNA concentration was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) using 1 uL of samples.

cDNA synthesis. cDNA was synthesized using iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories) with supplied random primers and 250 ng total RNA.

Primer efficiency assessment. PCR primers for human AHR, human eNOS and mammalian 18s (Table III) were purchased from Sigma Aldrich. Primers were thoroughly reconstituted with 100 uL DNase/RNase free water (Sigma Aldrich). Concentrations of primers were measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The optimal primer concentration was assessed by using 100 uM, 250 uM and 500 uM of sense and antisense primers in a 25 uL PCR reaction containing 12.5 uL iQ SYBR Green Supermix (Bio-Rad Laboratories) and 0.5 uL cDNA. The PCR reactions were started with a complete denaturation at 95 °C for 3 min; followed by 40 amplification cycles containing a 30 s denaturation at 95 °C and a 1 min annealing and extension at 60 °C. A melt curve was obtained for each sample at the end of the last amplification cycle, using the following parameters: 84 cycles starting at 54 °C and increasing 0.5 °C every 5 s. The primer concentration that generated the best melt

curve was chosen as the optimal concentration and used in the subsequent primer efficiency assessment.

A PCR amplification was performed using the same protocol as described above with 25 uL system including 12.5 uL iQ SYBR Green Supermix, 2 uL of the chosen concentration of primers and a series amount of cDNA: 0.125 uL, 0.25 uL, 0.5 uL, 1 uL, 2 uL. The C_T values for the target gene determined from the PCR reaction were plotted against the volume of cDNA and used to calculate primer efficiency, which was used in the target gene assessment.

Target gene expression analyses. Expression level of AHR mRNA, eNOS mRNA and 18s rRNA were quantitated by real time PCR amplification. PCR amplifications were performed on a 25 uL mixture of 12.5 uL iQ SYBR Green Supermix, 0.5 uL of synthesized sample cDNA and 500 uM primers for AHR and eNOS or 250 uM primers for 18s using iCycler PCR machine. The PCR reactions were started with a thorough denaturation at 95 °C for 3 min; followed by 40 amplification cycles containing a 30 s denaturation at 95 °C and a 1 min annealing and extension at 60 °C. A melt curve was also obtained as described above. Samples were run in triplicate and C_T (threshold cycle) values of each run were averaged. Relative expression was calculated by subtracting C_T value of 18s rRNA from C_T value of target gene as previously described (Muller, P.Y. et al. 2002).

eNOS PROTEIN ASSESSMENT IN HAECS

HAECs were grown at 37 °C, 5% CO_2 in EGM-2 media (Lonza). Cells were transfected with either 50 nM ON-TARGETplus SMARTpool siRNA targeting AHR (Dharmacon) or 50 nM siGENOME RISC-Free Control siRNA (Dharmacon). Forty

eight hours after transfection, cells were trypsinized using 0.25% trypsin-EDTA (Invitrogen) and centrifuged at 500 rpm for 5 min. Cell pellets were resuspended in proper amount of RIPA lysis buffer (Santa Cruz Biotechnology) and homogenized for 30 s on ice using hand-held motorized pestles (Sigma) in 1.5 mL centrifuge tubes. Cells were subjected to two freeze-thaw cycles at -80 °C and were centrifuged at 15,000 x g 4°C for 10 min. The supernatant was saved at -80 °C as whole cell lysate. Protein concentration of cell lysates was measured using Bio-Rad protein assay (Bio-Rad Laboratories). Total eNOS protein was assessed by western blot as described above. Protein aliquots (50 μg) were used in western blot for each sample.

NO SYNTHESIS ASSESSMENT IN HAECS

siRNA transfection. HAECs were transfected with 50 nM ON-TARGETplus SMARTpool siRNA targeting AHR (Dharmacon) or 50 nM siGENOME RISC-Free Control siRNA (Dharmacon) as described above.

NO production assessment. After 48 hours, cell culture media was removed, and cells were washed twice with 37 °C warm sterile Kreb Ringer (KR) buffer (20 mM HEPES, 10 mM glucose, 127 mM NaCl, 5.5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, pH7.4). DAF-2DA (Cell Technology) was diluted 1:500 in KR buffer, and 400 uL of diluted DAF-2DA was added to each well of the 24-well plate. KR buffer was added to control siRNA transfected cells as a background control. HAECs were incubated at 37 °C, 5% CO_2 in dark for 45 min to 1 hour. Then cells were washed thoroughly with warm KR buffer for 3 times to get rid of extra DAF-2DA. Then, 0.5 mL of KR buffer containing $10^{-6.5}$ M ACh was added to each well. Cells were incubated at 37 °C, 5% CO_2 in dark for 10 min. Fluorescence was measured with an excitation wavelength of 488 nm

Table III. Real time PCR primer sequences.

Gene	Sense primer	Antisense primer
Mammalian 18s	CGGAGGTTCGAAGACGATCAGATA	TTGGTTTCCCGGAAGCTGCC
Human AHR	GGCCGTGTCGATGTATCAGTG	GTACTGGATTGTACTGCATCTGAC
Human eNOS	TCTCCGCCTCGCTCATG	AGCCATACAGGATTGTCGCC
Human CYP1A1	CACATGCTGACCCTGGGAAAG	GGTGTGGAGCCAATTCGGATC

and an emission wavelength of 515 nm at an optimal gain using a GENios plate reader (Tecan, Mannedorf, Switzerland).

Normalization to DNA content. After DAF-2DA fluorescence measurement, cells in plate were frozen at -80 °C overnight. On the second day, the plate was thawed at room temperature for at least two hours. Two volumes of high salt TNE buffer (10 mM Tris-base, 2 M NaCl, 1 mM Na_2-EDTA, pH7.4) containing 18 nM bisbenzimide H 33258 was added to each well and incubated at room temperature in the dark over night. Fluorescence was measured with an excitation wavelength of 360 nm and emission wavelength of 465 nm at a gain of 96 using a GENios plate reader (Tecan). All DAF-2DA fluorescence values were normalized to DNA content. Plate wells without cells were loaded with all reagents and used as a background control.

ASSESSMENT OF SECRETED FACTOR IN MEDIATING eNOS INDUCTION IN HAECS

HAECs were grown at 37 °C, 5% CO_2 in EGM-2 media (Lonza) to expand cell population, and then cultured in EBM-2 media (Lonza) supplemented with only antibiotics and 5% fetal bovine serum for experiment. HAECs were transfected with 50 nM ON-TARGETplus SMARTpool siRNA targeting AHR (Dharmacon) or 50 nM siGENOME RISC-Free Control siRNA (Dharmacon) in EBM-2 media with only 5% serum on day 1. On day 2, media was changed back to EBM-2 suplemented with both antibiotics and 5% serum for transfected cells. On the same day, another set of HAEC was transfected with the same manner as the first set of cells. On day 3, the media collected from the first set of cells transfected with control siRNA or AHR siRNA were transferred into the second set of cells transfected with AHR siRNA or control siRNA,

respectively. RNA was isolated from the first set of cells on day 3, and RNA from the second set of cells was isolated on day 4. eNOS and AHR mRNA expressions were analyzed by real time PCR as described previously.

DETERMINE EFFECT OF AHR SIGNALING ON eNOS INDUCTION IN HAECS

HAECs were grown at 37 °C, 5% CO_2 in EGM-2 media (Lonza) for experiment. Cells were tranfected with either 50 nM ON-TARGETplus SMARTpool siRNA targeting AHR (Dharmacon) or 50 nM siGENOME RISC-Free Control siRNA (Dharmacon) on day 1, and then treated with either 0.1% DMSO or 1 nM TCDD after 24 hours in each siRNA treatment group respectively. 48 hours after transfection, RNA was isolated, and eNOS, AHR and CYP1A1 mRNA expressions were analyzed by real time PCR as described previously.

STATISTICAL ANALYSES

The data were expressed as mean ± SEM. Statistical analyses were performed with Student's t test, two-way analysis of variance (ANOVA), and two-way repeated measures ANOVA as noted. A $p < 0.05$ was considered significant in all cases.

CHAPTER III

HYPOTENSION AND ELEVATED ENDOTHELIAL NITRIC OXIDE SYNTHASE IN ARYL HYDROCARBON RECEPTOR KNOCKOUT MICE

SYSTEMIC BLOOD PRESSURE, HEART RATE AND LOCOMOTOR ACTIVITY OF ARYL HYDROCARBON RECEPTOR KNOCKOUT MICE

Systemic arterial blood pressure. Systemic blood pressure was measured by radiotelemetry 24 hours/day for 4 days before any treatments in conscious wildtype and AHR knockout mice. AHR knockout mice had significantly lower mean arterial blood pressure compared with wildtype animals ($AHR^{+/+}$: 105.9 ± 0.8 mm Hg; $AHR^{-/-}$: 89.3 ± 2.4 mm Hg, Figure 7A). Similarly, AHR knockout mice showed significantly lower systolic ($AHR^{+/+}$: 120.2 ± 0.9 mm Hg; $AHR^{-/-}$: 101.3 ± 2.9 mm Hg), diastolic ($AHR^{+/+}$: 91.4 ± 1.4 mm Hg; $AHR^{-/-}$: 76.2 ± 1.8 mm Hg) and pulse ($AHR^{+/+}$: 29.8 ± 1.4 mm Hg; $AHR^{-/-}$: 26.1 ± 1.1 mm Hg) pressures than those of wildtype animals (Figure 7B).

Heart rate. Heart rate was measured by radiotelemetry 24 hours/day for 4 days before any treatments in conscious wildtype and AHR knockout mice. There was no significant difference in heart rate of wildtype and AHR knockout mice ($AHR^{+/+}$: 528 ± 12 bpm; $AHR^{-/-}$: 548 ± 10 bpm, Figure 8).

Locomotor activity. Locomotor activity of animals was recorded by radiotelemetry 24 hours/day for 4 days before any treatments in conscious wildtype and AHR knockout mice. The 24 hour average locomotor activity of AHR knockout mice was significantly greater than wildtype mice ($AHR^{+/+}$: 4.6 ± 0.3 counts/min; $AHR^{-/-}$: 7.7 ± 0.8 counts/min, Figure 9).

Circadian systemic blood pressure, heart rate and locomotor activity rhythms. Systemic arterial blood pressures including mean, systolic, diastolic and pulse pressure, heart rate and locomotor activity were averaged for light/dark period or hourly over a 24

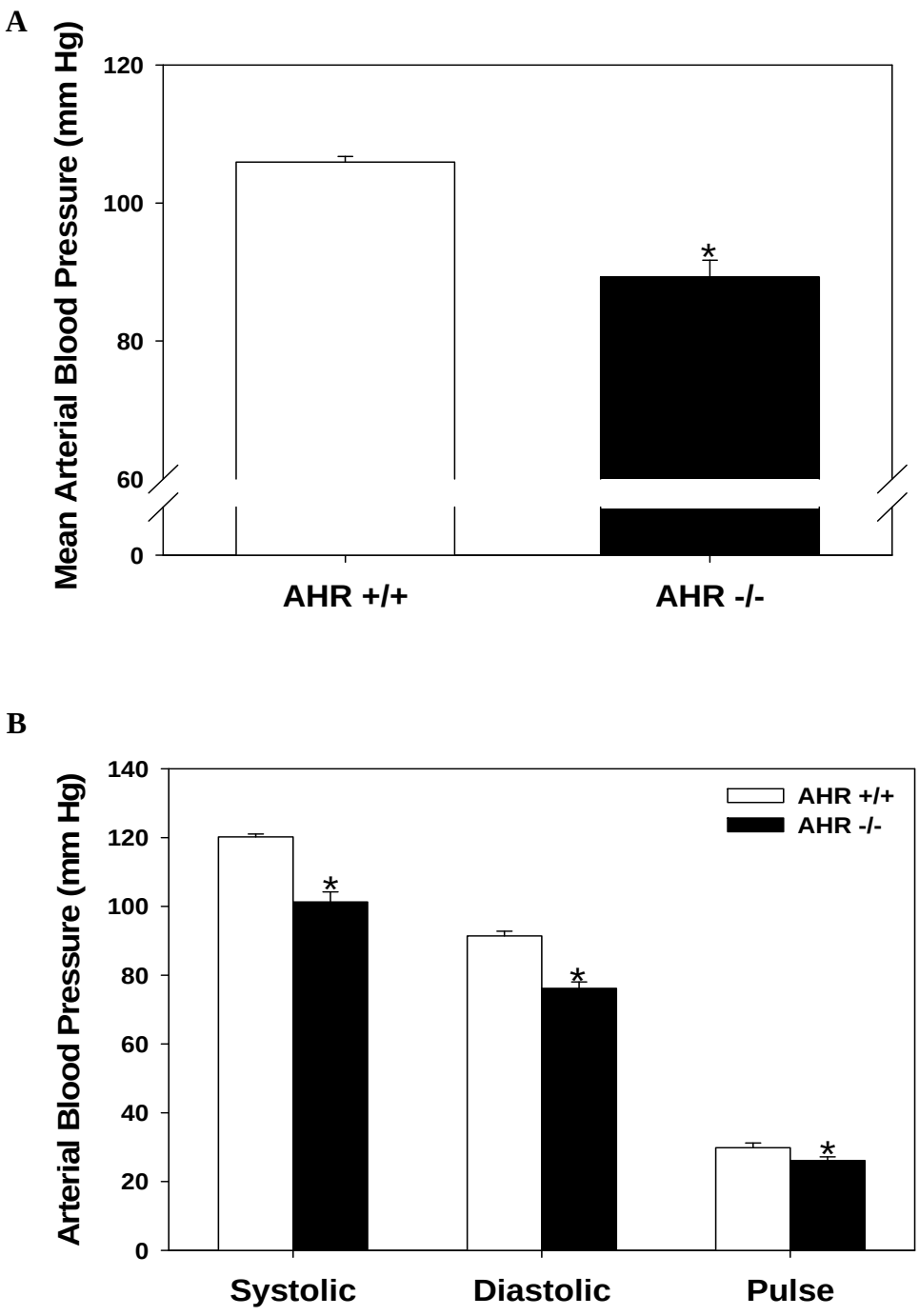

Figure 7. Systemic mean arterial blood pressure (A), systolic, diastolic and pulse arterial blood pressure (B) of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$: n=11; $AHR^{-/-}$: n=12. The data represent the mean ± SEM and were analyzed by Student's t-test; *$p < 0.05$, compared to $AHR^{+/+}$.

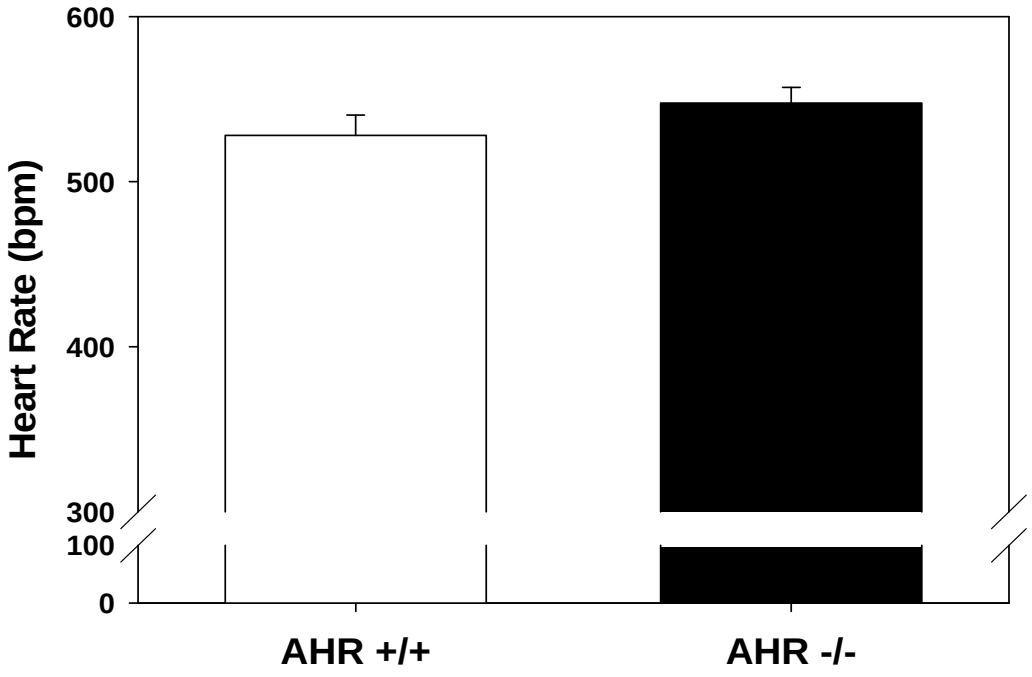

Figure 8. Heart rate of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=11; $AHR^{-/-}$, n=12. The data represent the mean ± SEM and were analyzed by Student's t-test.

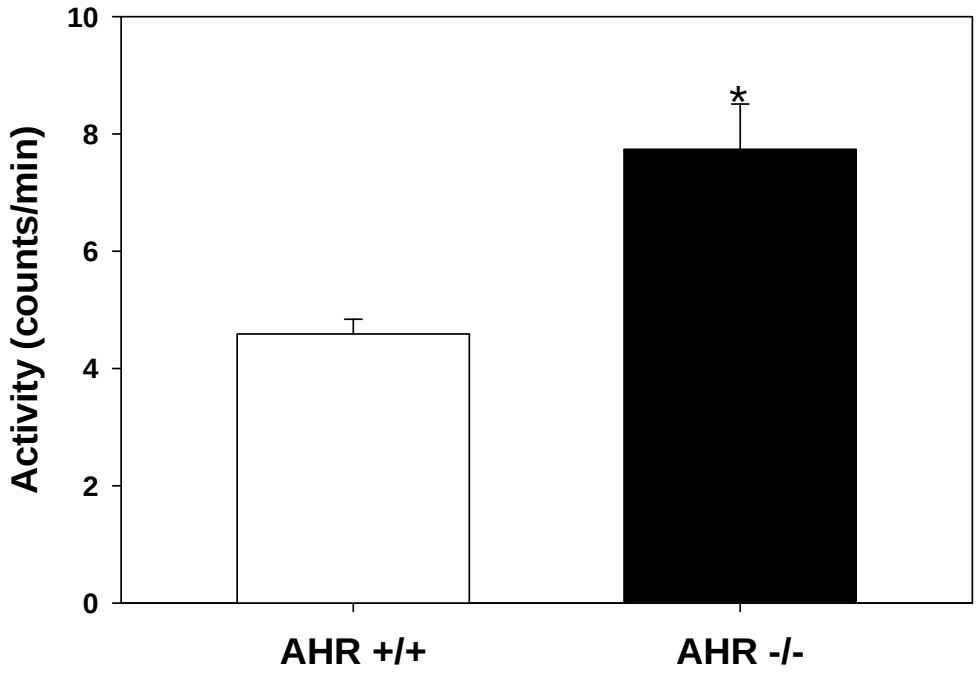

Figure 9. Locomotor activity of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=11; $AHR^{-/-}$, n=12. The data represent the mean ± SEM and were analyzed by Student's t-test; *$p < 0.05$, compared to $AHR^{+/+}$.

hour period for 4 days. All animals showed light/dark rhythms in systemic blood pressure, heart rate and locomotor activity. The ratio of dark (1800-0500) and light (0600-1700) values were compared between wildtype and AHR knockout mice. There was a significant difference only in the ratio of dark/light locomotor activity between wildtype and AHR knockout mice, but not in the ratio of mean, systolic, diastolic and pulse blood pressure and heart rate (Table IV). The dark/light locomotor activity ratio was significantly greater in AHR knockout mice. However, the 24 hour circadian pattern of systemic blood pressure, heart rate and activity were not identical between the two genotypes. The mean, systolic and diastolic blood pressure of wildtype animals exhibited the typical bimodal waveform with peak values evident early and late in the dark period (Li, P. et al. 1998, Figure 10 A, B and C, open circle, peaks indicated with arrows). In contrast, the blood pressure waveform of AHR knockout mice showed a single peak in the dark period (Figure 10 A, B and C, solid circle, peak indicated with arrow), and instead of increasing, the nocturnal high blood pressure fell precipitously at the end of dark period. Pulse pressure did not exhibit an obvious circadian change during 24 hours in both genotypes of mice, and was generally lower in AHR knockout mice than wildtype mice at most time points (Figure 11A). Heart rate increased at nighttime in both groups, and was significantly greater in AHR knockout mice than wildtype mice from 2300 to 0100 (Figure 11B). The locomotor activity of AHR knockout mice dramatically increased and was significantly higher than wildtype mice during most of the time at night (1800-0200 except 2100), which accounted for the difference of 24-hour locomotor activity between two genotypes (Figure 11C).

Table IV. Circadian analyses of systemic blood pressure, heart rate and locomotor activity of $AHR^{+/+}$ and $AHR^{-/-}$ mice.

		Light	Dark	Dark / Light	p-value
Mean arterial pressure (mm Hg)	+/+	102.6 ± 0.7	109.2 ± 1.3	1.06 ± 0.01	0.161
	-/-	85.1 ± 1.9	93.4 ± 3.0	1.10 ± 0.02	
Systolic arterial pressure (mm Hg)	+/+	116.7 ± 0.9	123.8 ± 1.1	1.06 ± 0.01	0.166
	-/-	97.5 ± 2.4	106.2 ± 3.5	1.09 ± 0.02	
Diastolic arterial pressure (mm Hg)	+/+	88.3 ± 1.5	94.6 ± 1.3	1.07 ± 0.01	0.156
	-/-	72.8 ± 1.4	80.7 ± 2.3	1.11 ± 0.02	
Heart rate (bpm)	+/+	504 ± 11	552 ± 14	1.10 ± 0.01	0.101
	-/-	514 ± 7	580 ± 13	1.13 ± 0.02	
Locomotor activity (counts/min)	+/+	3.2 ± 0.2	6.0 ± 0.4	1.93 ± 0.16	0.003*
	-/-	3.8 ± 0.4	11.7 ± 1.3	3.22 ± 0.35	

The data represent the mean ± SEM and were analyzed by Student's t-test; *$p < 0.05$, compared to $AHR^{+/+}$.

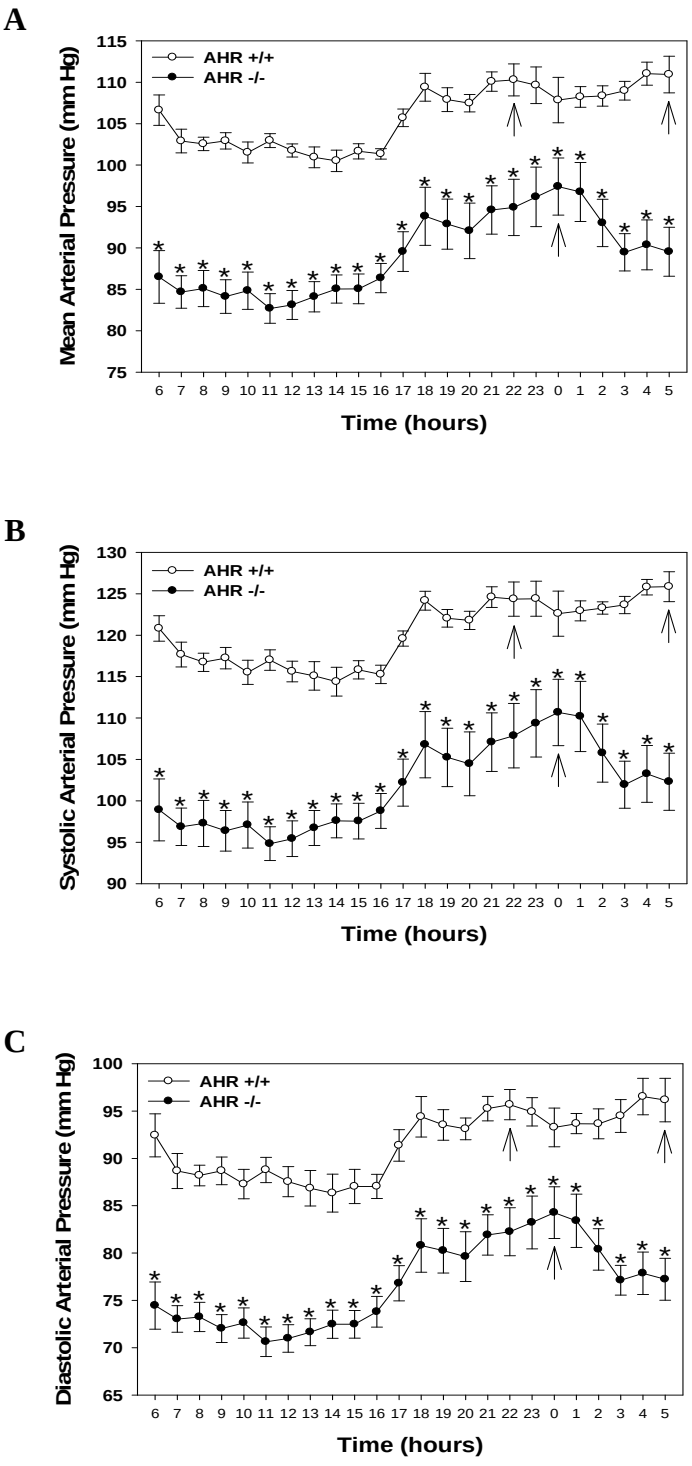

Figure 10. Circadian patterns of mean (A), systolic (B) and diastolic (C) arterial blood pressure of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=11; $AHR^{-/-}$, n=12. The data represent the mean ± SEM and were analyzed by two-way repeated measures ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to $AHR^{+/+}$ at the given time point. Blood pressure peaks are indicated with arrows.

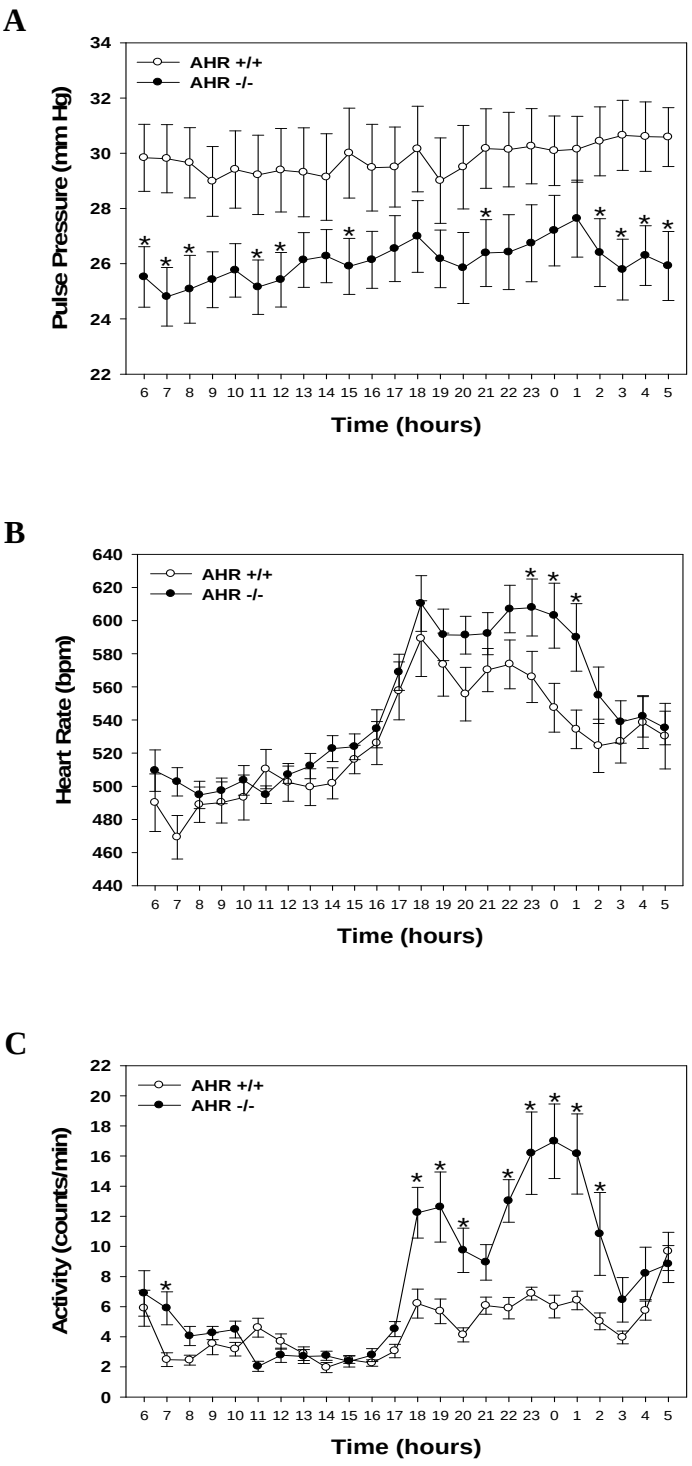

Figure 11. Circadian patterns of arterial pulse pressure (A), heart rate (B) and locomotor activity (C) of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=11; $AHR^{-/-}$, n=12. The data represent the mean ± SEM and were analyzed by two-way repeated measures ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to $AHR^{+/+}$ at the given time point.

PLASMA ANGIOTENSIN II OF ARYL HYDROCARBON RECEPTOR KNOCKOUT MICE

In order to determine the contribution of renin-angiotensin system to the hypotension in AHR knockout mice, circulating Ang II levels were measured using plasma collected from 4 month old wildtype and AHR knockout mice. There was no significant difference in plasma Ang II levels between the two genotypes (Figure 12).

PLASMA ENDOTHELIN-1 OF ARYL HYDROCARBON RECEPTOR KNOCKOUT MICE

To determine the role of ET-1 in the hypotensive phenotype of AHR knockout mice, plasma ET-1 were measured using plasma collected from 4 month old wildtype and AHR knockout mice. There was no significant difference in plasma ET-1 levels between the two genotypes (Figure 13).

AUTONOMIC NERVOUS SYSTEM CONTRIBUTION TO HYPOTENSION IN ARYL HYDROCARBON RECEPTOR KNOCKOUT MICE

The autonomic nervous system, including sympathetic and parasympathetic innervations, can be altered to cause blood pressure misregulation. Thus, to determine the possible contribution of this system to the low blood pressure in AHR knockout mice, we first indirectly assessed the sympathetic nerve activity by measuring excretion of urinary norepinephrine, which is the primary postganglionic neurotransmitter of sympathetic nerves. We also evaluated the role of the sympathetic α-adrenoceptor axis in systemic blood pressure regulation by blocking the α-adrenoceptor with prazosin in both genotypes of animals. Finally, we examined the effect of total autonomic nervous system blockade on blood pressure control using a ganglionic blocker, hexamethonium.

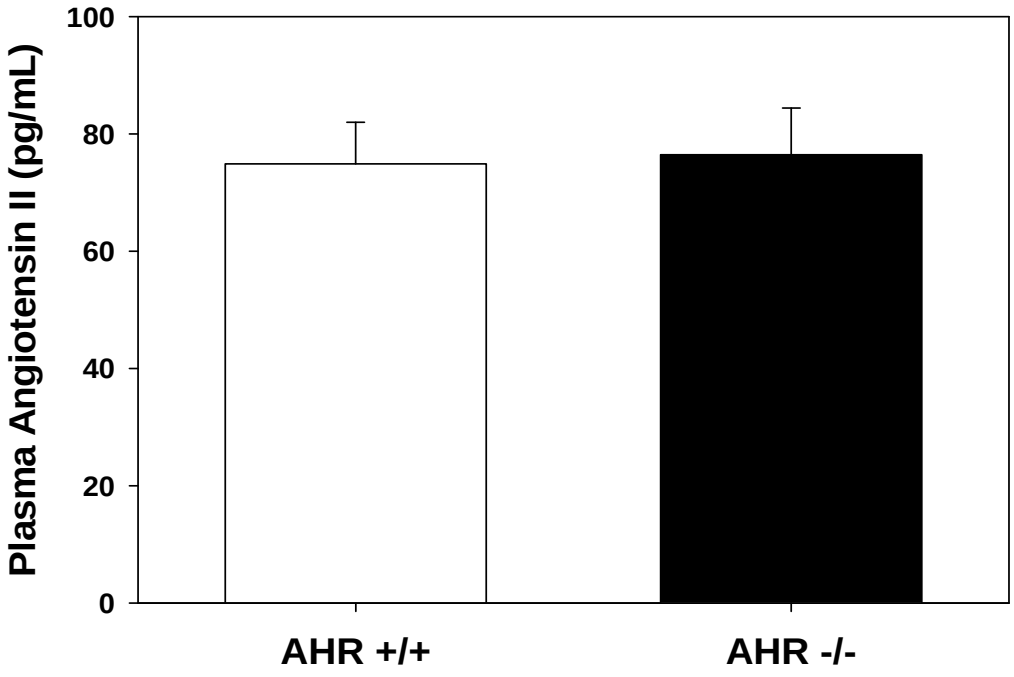

Figure 12. Plasma angiotensin II of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=6; $AHR^{-/-}$, n=7. The data represent the mean ± SEM and were analyzed by Student's t-test.

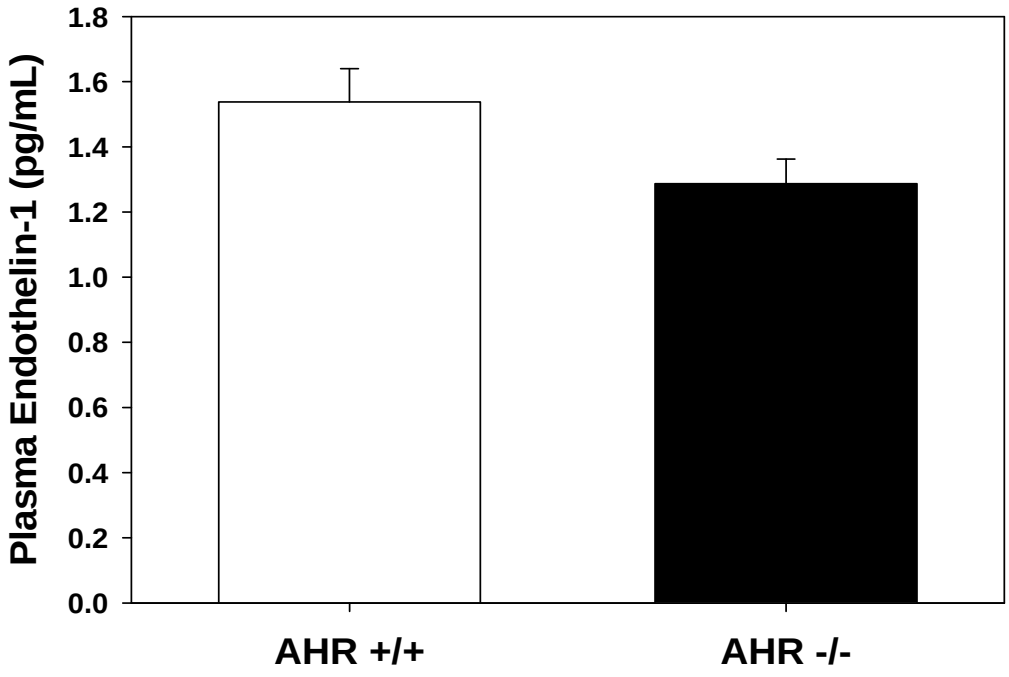

Figure 13. Plasma endothelin-1 of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=6; $AHR^{-/-}$, n=7. The data represent the mean ± SEM and were analyzed by Student's t-test.

Urinary norepinephrine. AHR knockout mice did not exhibit any differences in urinary norepinephrine concentration (Figure 14A) or 24-hour urine production (Figure 14B) compared to wildtype animals.

α-adrenoceptor blockade. We examined the contribution of systemic α-adrenoceptor expression in this blood pressure alteration by blocking α-adrenoceptor with prazosin in our animals. AHR knockout mice did not show any difference in the change of mean arterial blood pressure following prazosin injection, compared to wildtype mice (Figure 15).

Ganglionic blockade. In order to identify the contribution of autonomic nervous system, including both sympathetic and parasympathetic never, to the low blood pressure of AHR knockout mice, we treated our animals with a ganglionic blocker, hexamethonium. Blood pressure was expressed as a change from baseline pressure while heart rate was expressed as intrinsic heart rate with ganglion blockade. AHR knockout mice did not show any difference in the change of mean arterial blood pressure compared with wildtype mice (Figure 16A). In this study, AHR knockout mice showed a significantly greater basal heart rate than wild type (Figure 16B), which was different from what we determined previously (Figure 8). In addition, AHR knockout mice showed an elevated intrinsic heart rate following hexamethonium injection, compared to wildtype mice.

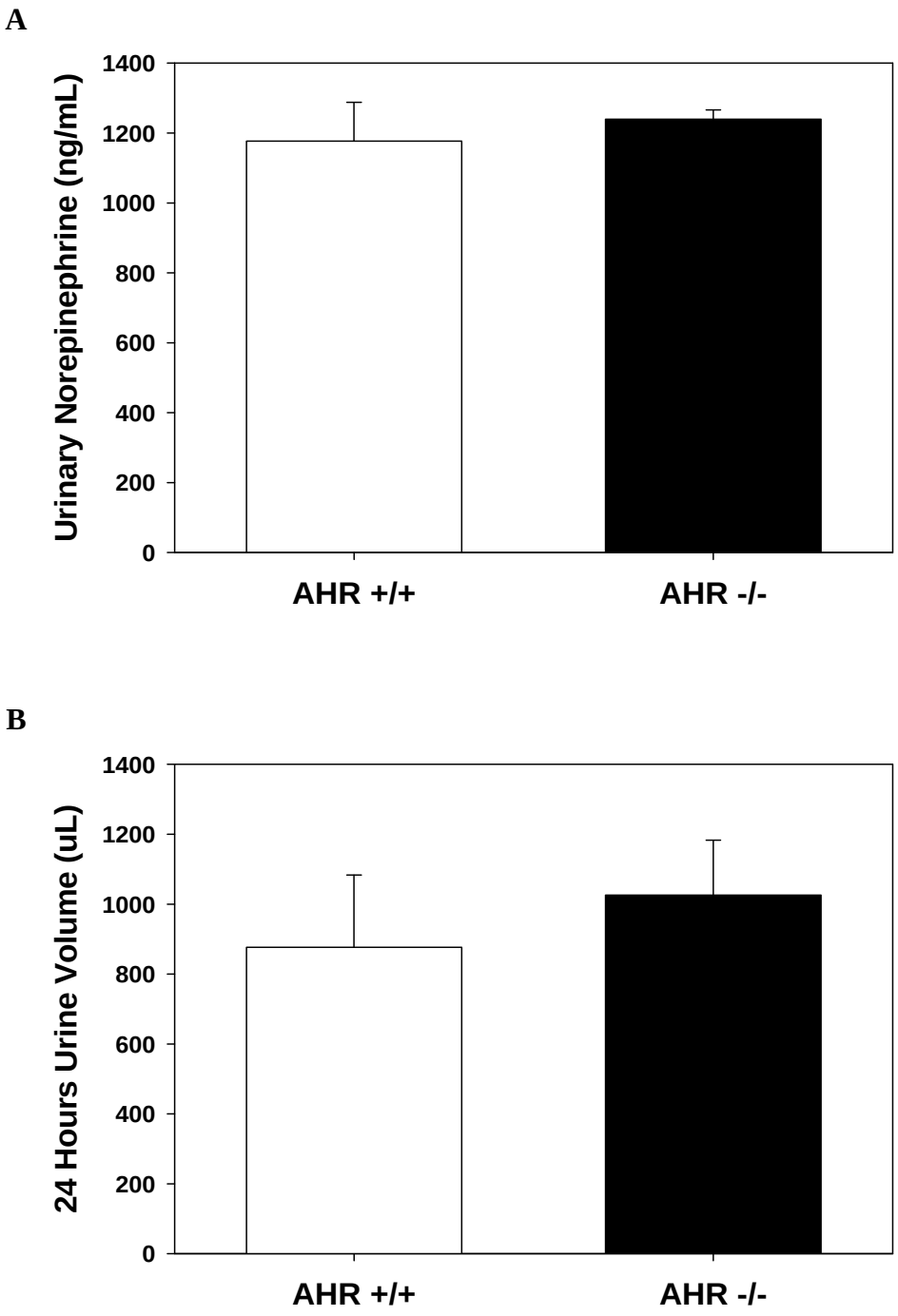

Figure 14. Urinary norepinephrine concentration (A) and 24 hour urine volume (B) of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=5; $AHR^{-/-}$, n=7. The data represent the mean ± SEM and were analyzed by Student's t-test.

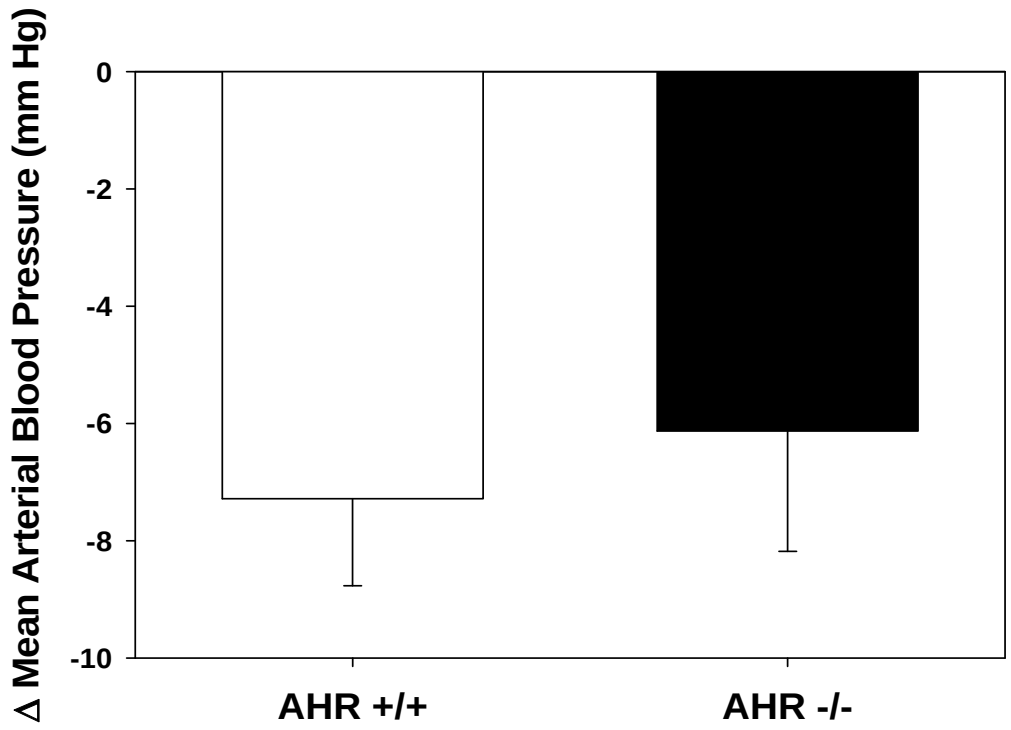

Figure 15. Effect of prazosin on mean arterial blood pressure change of $AHR^{+/+}$ and $AHR^{-/-}$ mice. Blood pressure was calculated by averaging readings for half hour 5 min after injection, and expressed as a change from 2 hours average pressure collected before injection. $AHR^{+/+}$, n=6; $AHR^{-/-}$, n=6. The data represent the mean ± SEM and were analyzed by Student's t-test.

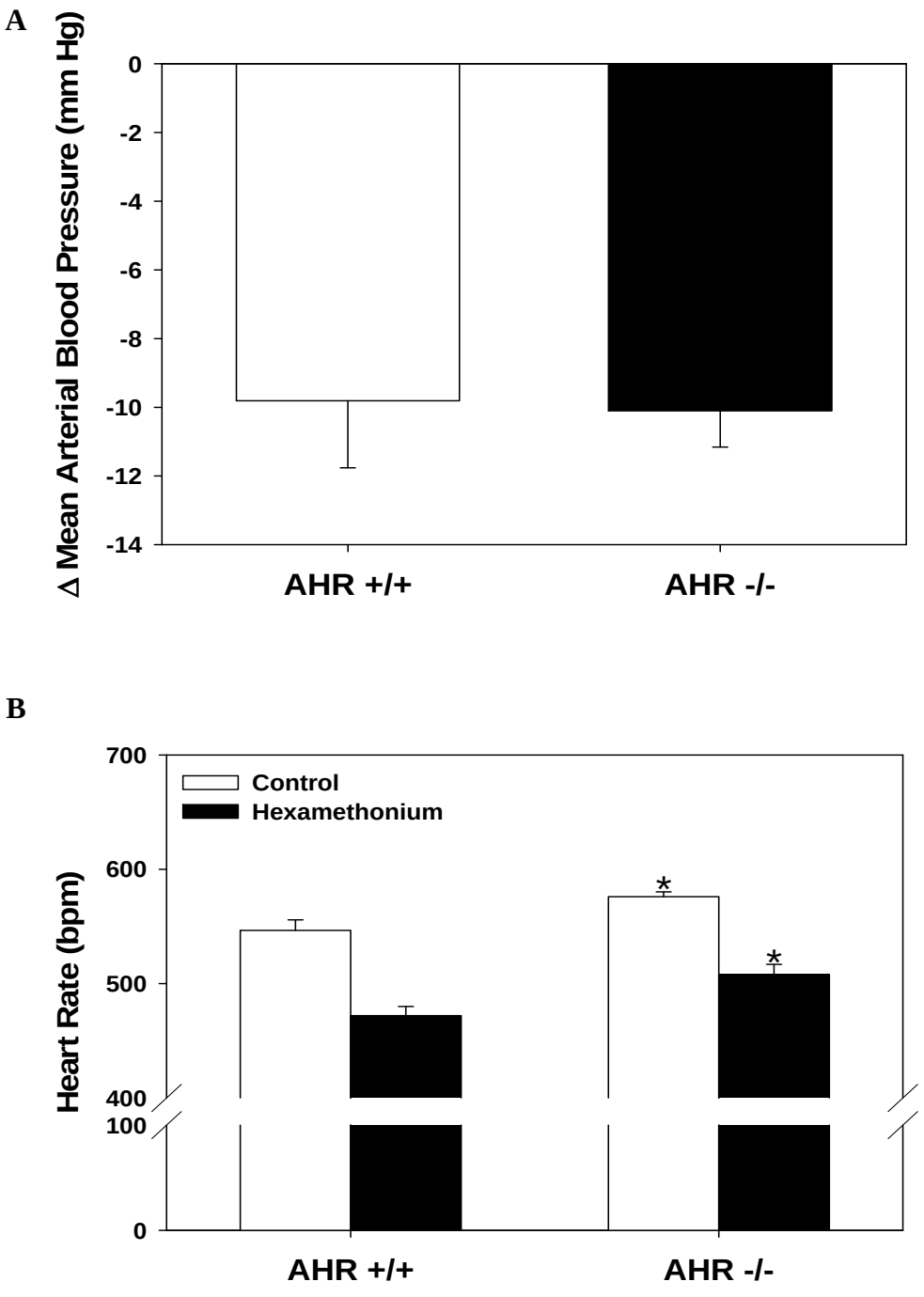

Figure 16. Effect of hexamethonium on mean arterial blood pressure (A) and heart rate (B) of $AHR^{+/+}$ and $AHR^{-/-}$ mice. Blood pressure and heart rate were calculated by averaging readings for half hour 5 min after injection, and expressed as a change from 2 hours average pressure collected before injection (A) or 4 days baseline heart rate (B), respectively. $AHR^{+/+}$, n=6; $AHR^{-/-}$, n=5. The data represent the mean ± SEM and were analyzed by Student's t-test (A) and two-way ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to $AHR^{+/+}$ (B).

VASCULAR REACTIVITY OF ARYL HYDROCARBON RECEPTOR KNOCKOUT MICE

Systemic vascular resistance (SVR) is one of the most important factors that directly determine systemic arterial blood pressure. SVR is regulated by multiple factors, including autonomic innervations, Ang II, ET-1 from endothelium and other local factors, such as nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factors. After investigating the possible role of autonomic nervous system, as well as Ang II and ET-1 in contributing to the hypotension in our mouse model, we further examined the vascular reactivity in respond to KCl, phenylephrine (PE) and exogenous NO donor, as well as the basal and ACh-induced NO-mediated vasorelaxation.

Vascular response to KCl. Intact aortic rings from wildtype and AHR knockout mice were stimulated with KCl in a dose dependent manner. We observed a significantly greater response with high doses KCl in aortic rings from AHR knockout mice compared to those from wildtype mice (Figure 17).

Vascular response to PE. To determine the vascular reactivity in response to neural hormone stimuli *in vitro*, we stimulated intact aortic rings isolated from both genotypes with PE and expressed the data as percentage of the maximal response to KCl. AHR knockout mice exhibited a diminished, but not significant, response to PE (Figure 18A).

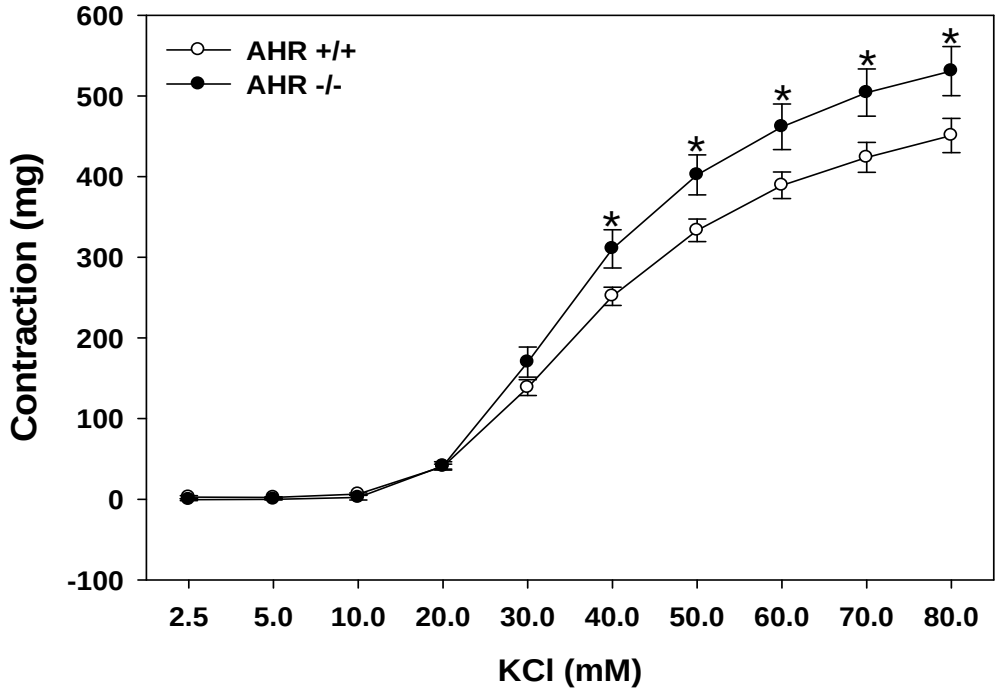

Figure 17. KCl induced vasocontraction of aortic rings from $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=6; $AHR^{-/-}$, n=6. The data represent the mean ± SEM and were analyzed by two-way repeated measures ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to $AHR^{+/+}$ at the given concentration.

Constitutive NO derived basal vasodilation. In order to determine the basal vasodilatory tone derived from constitutive NO expression, we preincubated intact aortic rings with LNNA, a NOS inhibitor, and then conducted a PE dose response. Contraction was expressed as a percentage of the maximal response to KCl. We observed a significantly greater vasocontraction in response to high concentrations of PE in AHR knockout mice in the presence of LNNA (Figure 18B).

We subtracted the contraction values to PE without LNNA preincubation from those with LNNA preincubation at each PE concentration. The result was expressed as a percentage of the maximal response to KCl. This result represents the degree of vasocontraction that is constitutively blocked by endogenous NO. There was a significantly greater degree of vasocontraction offset by constitutive NO in the aortas of AHR knockout mice than in wildtype mice (Figure 18C), suggesting that AHR knockout mice have significantly higher production of aortic NO than wildtype mice.

Vasorelaxation to ACh. As a neurotransmitter released from parasympathetic nerve, ACh can bind to muscarinic receptors on endothelial cells to activate NO production by eNOS through an intracellular calcium-mediated mechanism. To determine the level of ACh-induced vasorelaxation in aortas from both genotypes of mice, we precontracted the aortic rings with PE, and then stimulated them with a dose-dependent increase in ACh. We found a significantly greater relaxation at high dose of ACh (Figure 19A) and a significantly diminished vasocontraction to PE (Figure 19B) in the aortic rings from AHR knockout mice, compared to wildtype mice. The vasorelaxation is consistent with what we observed on basal NO-dependent vasodilation, again suggesting a higher level of NO production in the aorta of AHR knockout mice.

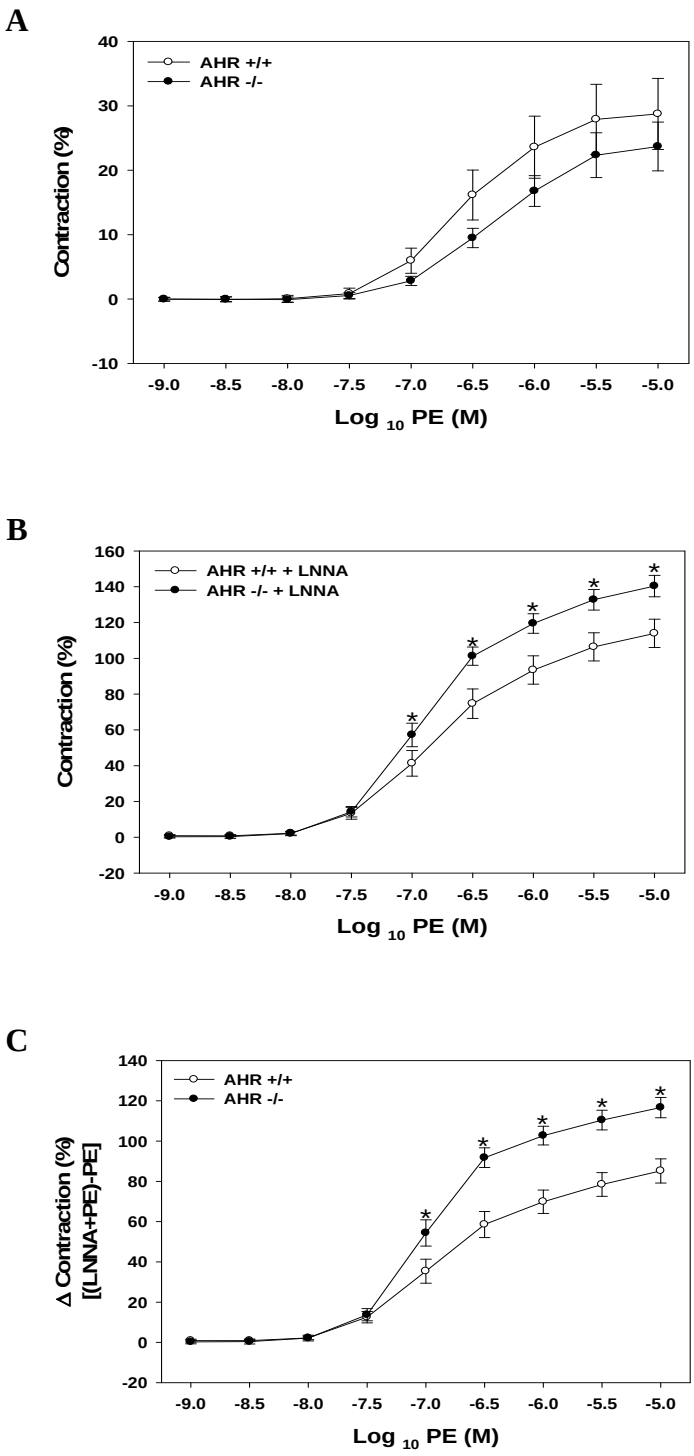

Figure 18. Vasocontraction to PE (A), LNNA + PE (B) and [(LNNA + PE) – PE] (C) of aortic rings from $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=6; $AHR^{-/-}$, n=6. The contraction was expressed as a percentage of the maximal response to KCl. The data represent the mean ± SEM and were analyzed by two-way repeated measures ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to $AHR^{+/+}$ at the given concentration.

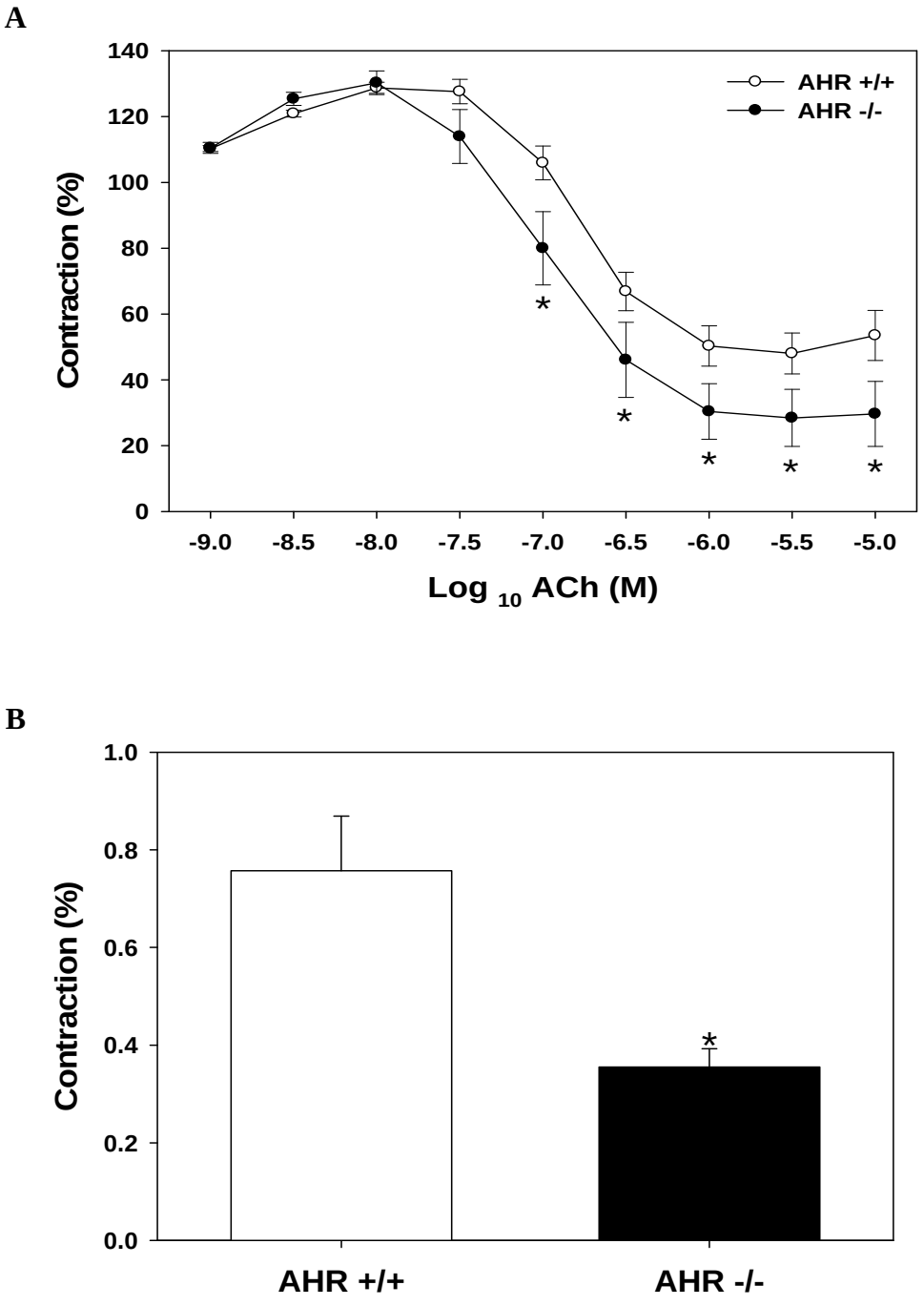

Figure 19. ACh-induced vasorelaxation (A) and PE-induced vasocontraction (B) of aortic rings from $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=7; $AHR^{-/-}$, n=6. The contraction was expressed as a percentage of response to PE (A) or 80 mM KCl (B). The data represent the mean ± SEM and were analyzed by two-way repeated measures ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to $AHR^{+/+}$ at the given concentration (A) and Student's t-test; *$p < 0.05$, compared to $AHR^{+/+}$ (B).

Vasorelaxation to exogenous NO. Nitric oxide produced by eNOS in endothelial cells diffuses into vascular smooth muscle cells to induce vasorelaxation through multiple mechanisms, such as sGC activation. Therefore, the sensitivity of the downstream pathways to endothelium derived NO in smooth muscle cells may also affect vasorelaxation. To determine the sensitivity of aortic vascular smooth muscle cells to NO, we conducted a dose response to the exogenous NO donor, S-Nitroso-N-acetyl-DL-penicillamine (SNAP), on PE precontracted, endothelium-disrupted aortic rings from both groups of animals. We detected a significantly greater sensitivity to exogenous NO in aortic rings from AHR knockout mice, compared to those from wildtype mice (Figure 20A). The vasocontraction of aortic rings to PE was the same between two genotypes of mice (Figure 20B).

ENDOTHELIAL NITRIC OXIDE SYNTHASE EXPRESSION IN AORTAS OF ARYL HYDROCARBON RECEPTOR KNOCKOUT MICE

To determine the expression of aortic eNOS, we measured total eNOS protein in isolated aortas from wildtype and AHR knockout mice. Since phosphorylation of serine ^{1177} in eNOS is a major mechanism of post-translational activation of this enzyme, we also assessed serine ^{1177} phosphorylated eNOS expression. Both total eNOS and phosphorylated eNOS protein expressions were dramatically increased (6 fold) in the aorta of AHR knockout mice, compared to wildtype mice (Figure 21). However, the ratio of phosphorylated eNOS to total eNOS in aortas from two groups of animals was not different.

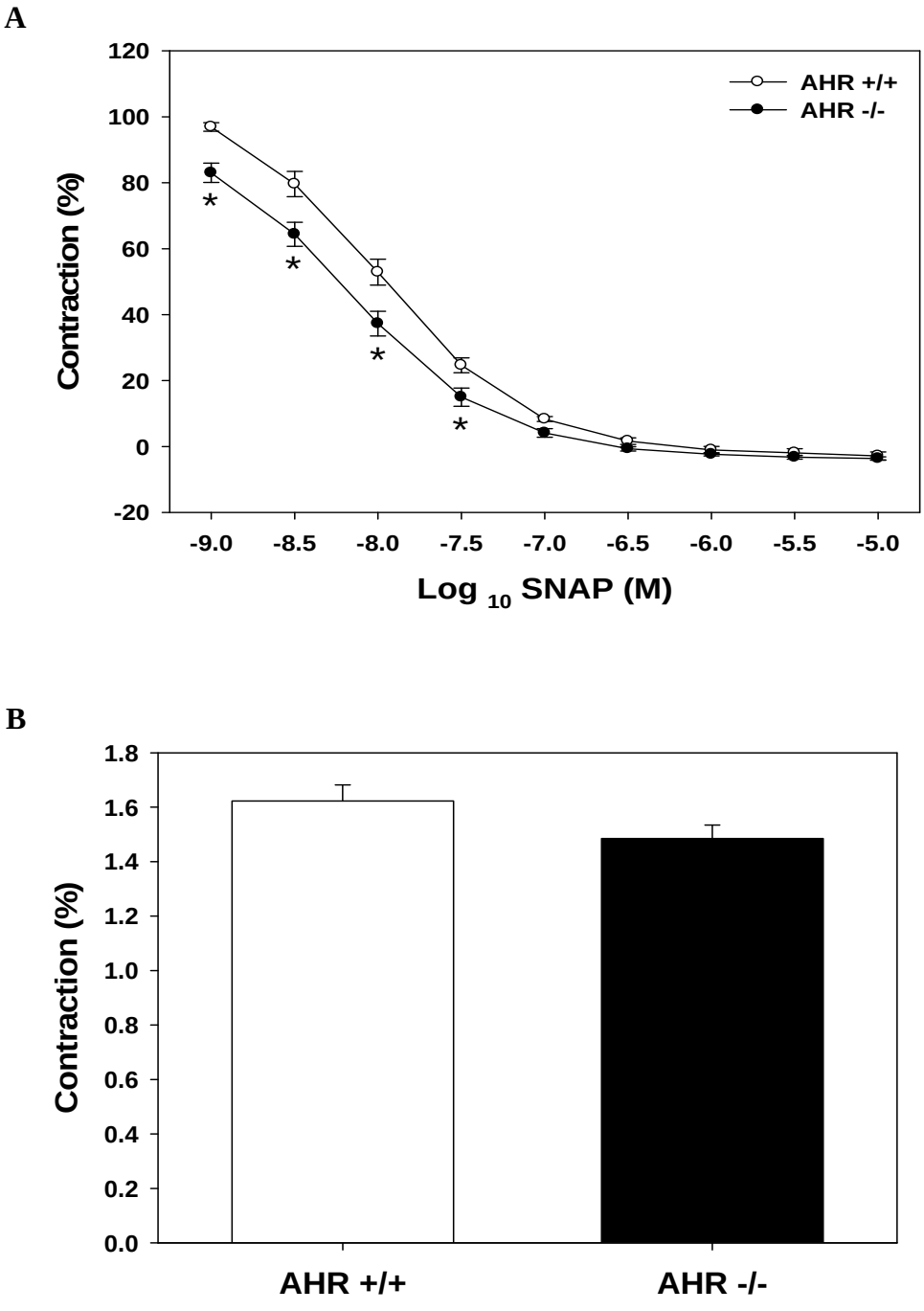

Figure 20. SNAP-induced vasorelaxation (A) and PE-induced vasocontraction (B) of endothelium-disrupted aortic rings from $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=6; $AHR^{-/-}$, n=6. The contraction was expressed as a percentage of response to PE (A) or 80 mM KCl (B). The data represent the mean ± SEM and were analyzed by two-way repeated measures ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to $AHR^{+/+}$ at the given concentration (A) and Student's t-test (B).

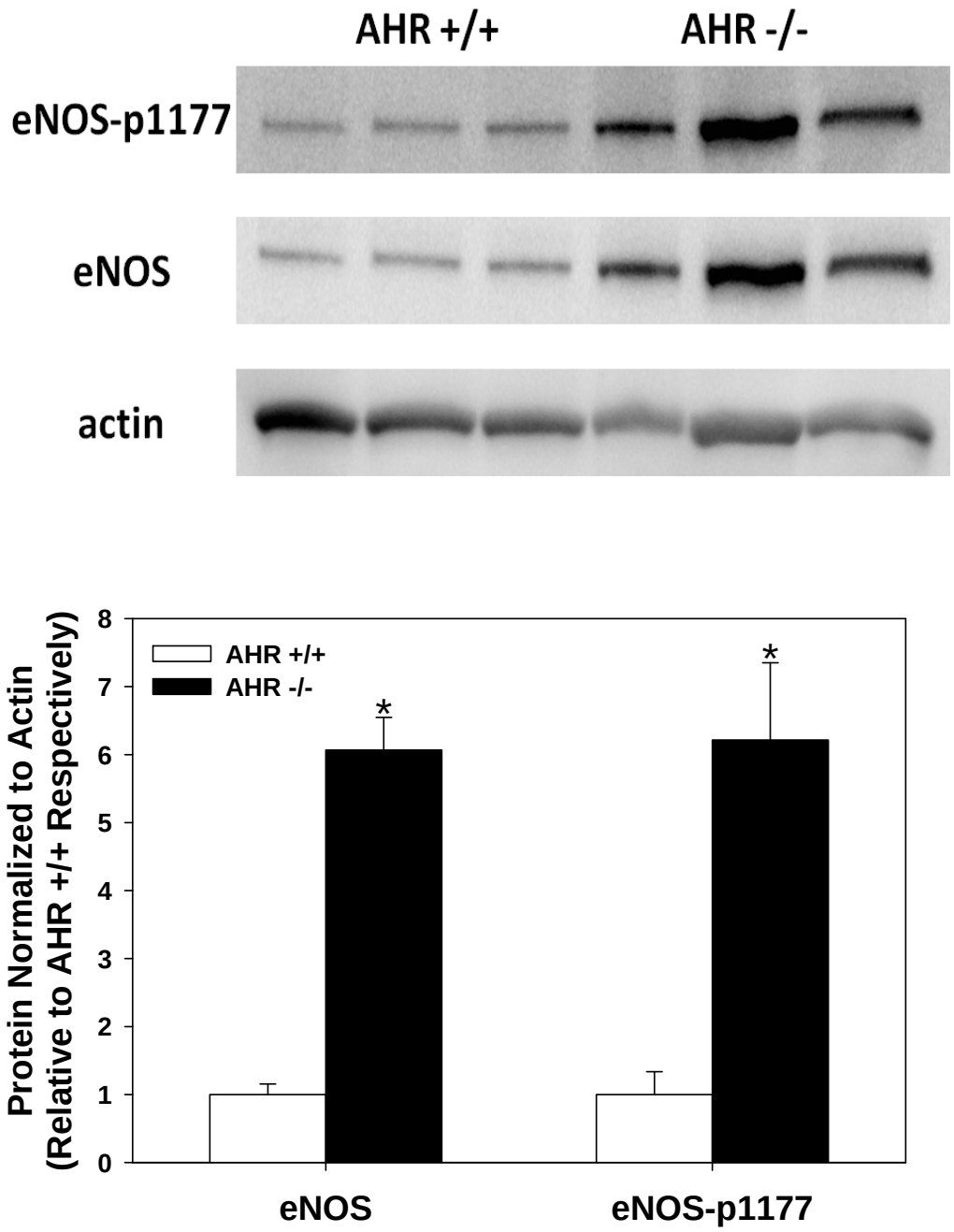

Figure 21. Aortic total eNOS and phosphorylated eNOS protein expression of $AHR^{+/+}$ and $AHR^{-/-}$ mice. n=3. The data represent the mean ± SEM and were analyzed by Student's t-test; *$p < 0.05$, compared to $AHR^{+/+}$.

EFFECT OF NITRIC OXIDE SYNTHASE BLOCKADE ON HYPOTENSION IN ARYL HYDROCARBON RECEPTOR KNOCKOUT MICE

Due to the pivotal role of NO in regulating systemic vascular resistance, thus systemic arterial blood pressure, we tested the hypothesis that hypotension is mediated by elevated NO production in AHR knockout mice. We treated wildtype and AHR knockout mice with NOS inhibitor, LNNA, in their drinking water, measured arterial blood pressure and heart rate by radiotelemetry, and measured urinary NOx before and during LNNA treatment.

Mean arterial pressure with LNNA treatment. Mean arterial blood pressure of wildtype mice significantly increased with 250 mg/L LNNA treatment (12.7 ± 1.4 mm Hg) and with 500 mg/L LNNA treatment (17.6 ± 3.8 mm Hg. Figure 22A). However, 500 mg/L LNNA did not further significantly increase blood pressure of wildtype mice compared to 250 mg/L LNNA treatment. Blood pressure dropped to baseline levels after LNNA treatment was stopped (Figure 22B). In contrast, in AHR knockout mice, mean arterial blood pressure only significantly increased with 500 mg/L LNNA treatment (9.6 ± 5.2 mm Hg. Figure 22A). The blood pressure peaked on day 2 of 250 mg/L LNNA treatment, but this increase was not sustained and blood pressure fell back to baseline levels by day 5 (Figure 22B). As with wildtype mice, blood pressure returned to baseline levels after LNNA treatment was stopped.

Heart rate with LNNA treatment. Heart rate was not altered by LNNA treatment in either AHR wildtype or knockout mice (Figure 23).

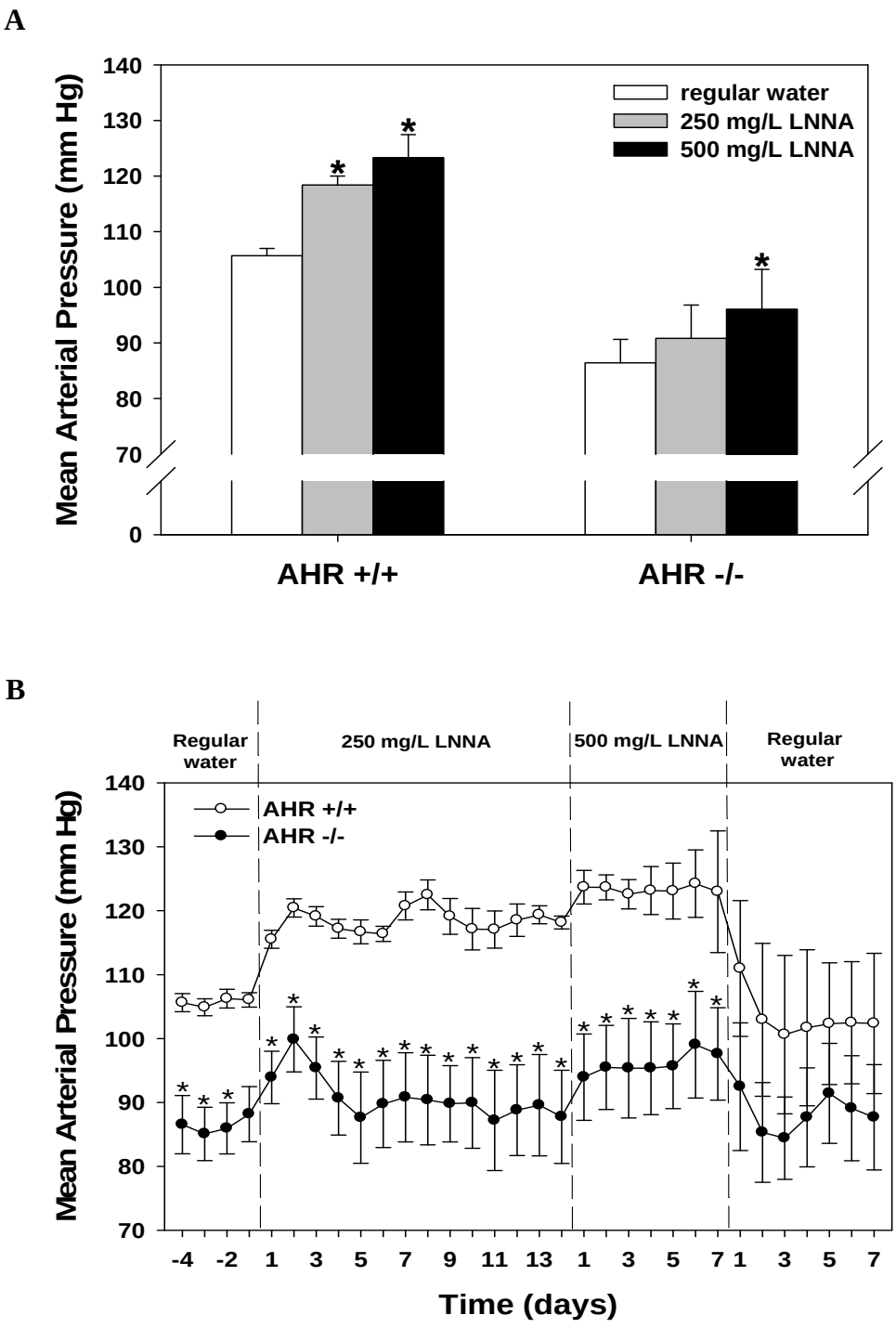

Figure 22. Effect of LNNA on mean arterial blood pressure of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=5; $AHR^{-/-}$, n=6. The data represent the mean ± SEM and were analyzed by two-way repeated measures ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to regular water in each genotype respectively (A), or compared to $AHR^{+/+}$ at the given time point (B).

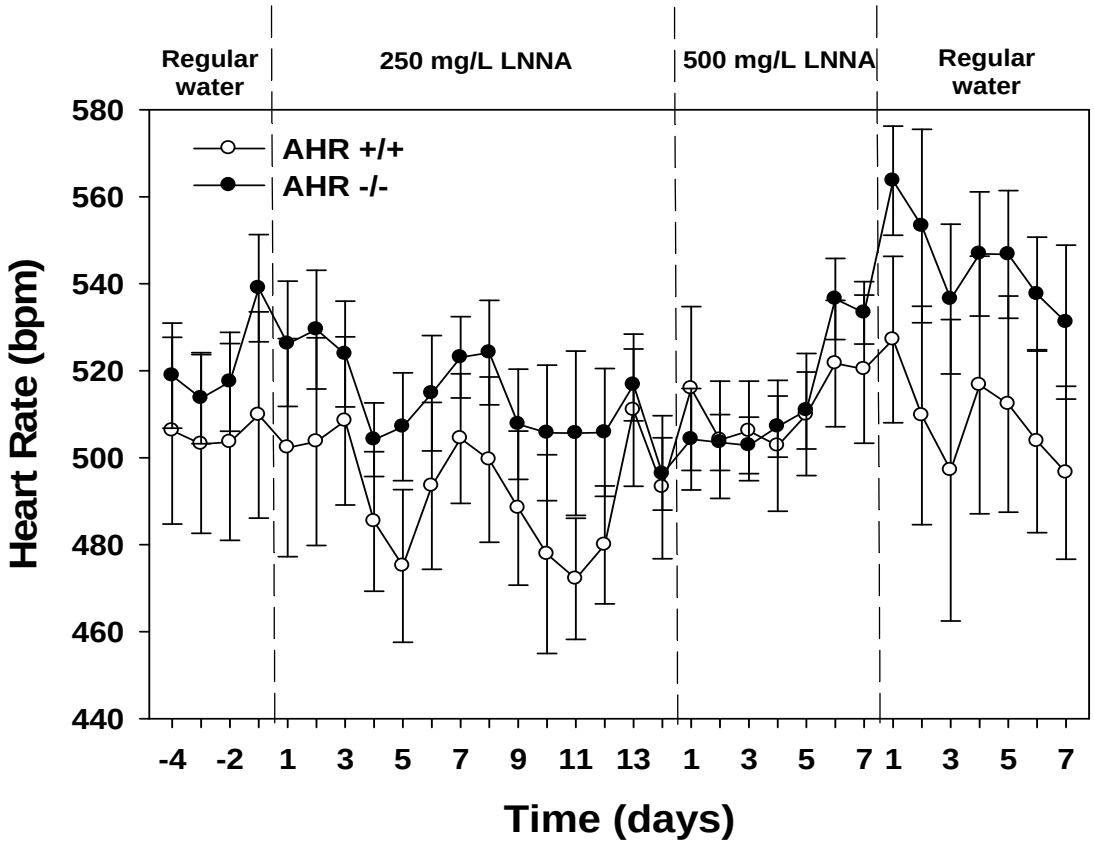

Figure 23. Effect of LNNA on heart rate of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=5; $AHR^{-/-}$, n=6. The data represent the mean ± SEM and were analyzed by two-way repeated measures ANOVA.

***Urinary NOx*.** Urinary NOx levels, an indirect indicator of systemic NO production, was significantly increased in AHR knockout mice about 2 fold, compared to wildtype mice (Figure 24). The elevated urinary NOx in AHR knockout mice was significantly attenuated and normalized to the same level as wildtype mice when being treated with 500 mg/L LNNA (Figure 24). AHR knockout mice did not show a difference in 24-hour urine production compared to wildtype animals (Figure 24B).

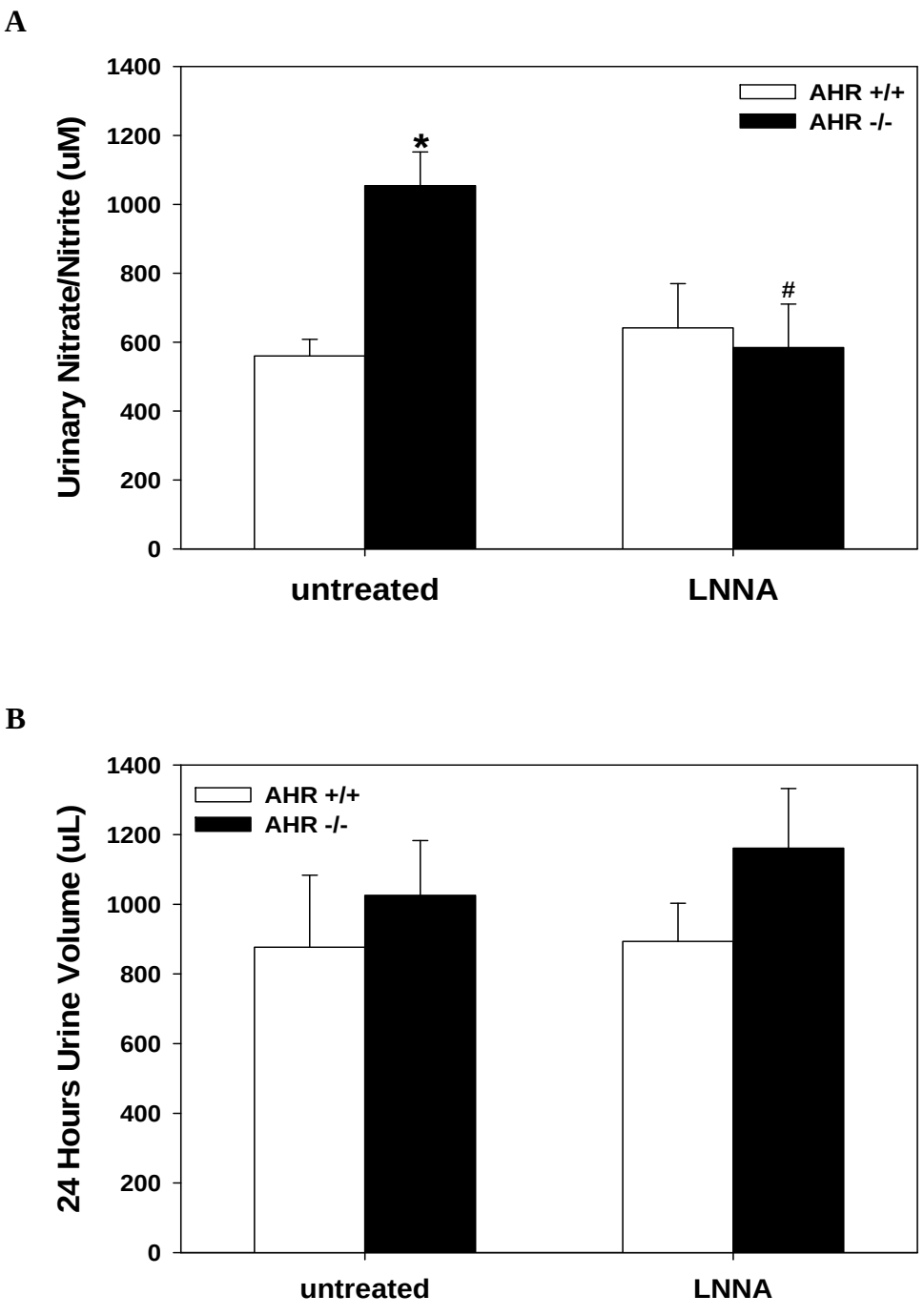

Figure 24. Effect of LNNA on urinary NOx production of $AHR^{+/+}$ and $AHR^{-/-}$ mice. $AHR^{+/+}$, n=5; $AHR^{-/-}$, n=7. The data represent the mean ± SEM and were analyzed by two-way ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to untreated $AHR^{+/+}$; #$p < 0.05$, compared to untreated $AHR^{-/-}$.

CHAPTER IV

ELEVATED ENDOTHELIAL NITRIC OXIDE SYNTHASE EXPRESSION IN HUMAN AORTIC ENDOTHEIAL CELLS WITH ARYL HYDROCARBON RECEPTOR KNOCKDOWN

AHR SIGNALING INHIBITION WITH AHR siRNA

To determine the function of AHR siRNA in inhibiting AHR signaling, we transfected HAECs with AHR siRNA, and then treated cells with TCDD. We assessed the mRNA expression of AHR and CYP1A1, which is a downstream target gene of AHR. AHR mRNA was significantly knocked down to ~15% of control by AHR siRNA, and a 75 fold induction of CYP1A1 mRNA by TCDD was significantly attenuated to 3-fold by simultaneous AHR siRNA treatment, suggesting an effective AHR signaling blockade by AHR siRNA (Figure 25).

eNOS mRNA EXPRESSION WITH AHR KNOCKDOWN

To determine the effect of AHR signaling blockade on eNOS mRNA expression *in vitro*, we inhibited AHR signaling with siRNA and then analyzed eNOS mRNA in HAECs. eNOS mRNA was significantly increased ~50% with AHR signaling knockdown in HAECs (Figure 26).

eNOS PROTEIN EXPRESSION WITH AHR KNOCKDOWN

To determine the effect of AHR signaling blockade on eNOS protein expression *in vitro*, we inhibited AHR signaling with siRNA and then analyzed eNOS protein by western blot in HAECs. We did not detect any changes in eNOS protein with AHR signaling knockdown in HAECs (Figure 27).

NO PRODUCTION WITH AHR KNOCKDOWN

In order to determine the activity of eNOS in producing NO with AHR signaling blockade, we knocked down AHR signaling with siRNA, and then assessed NO

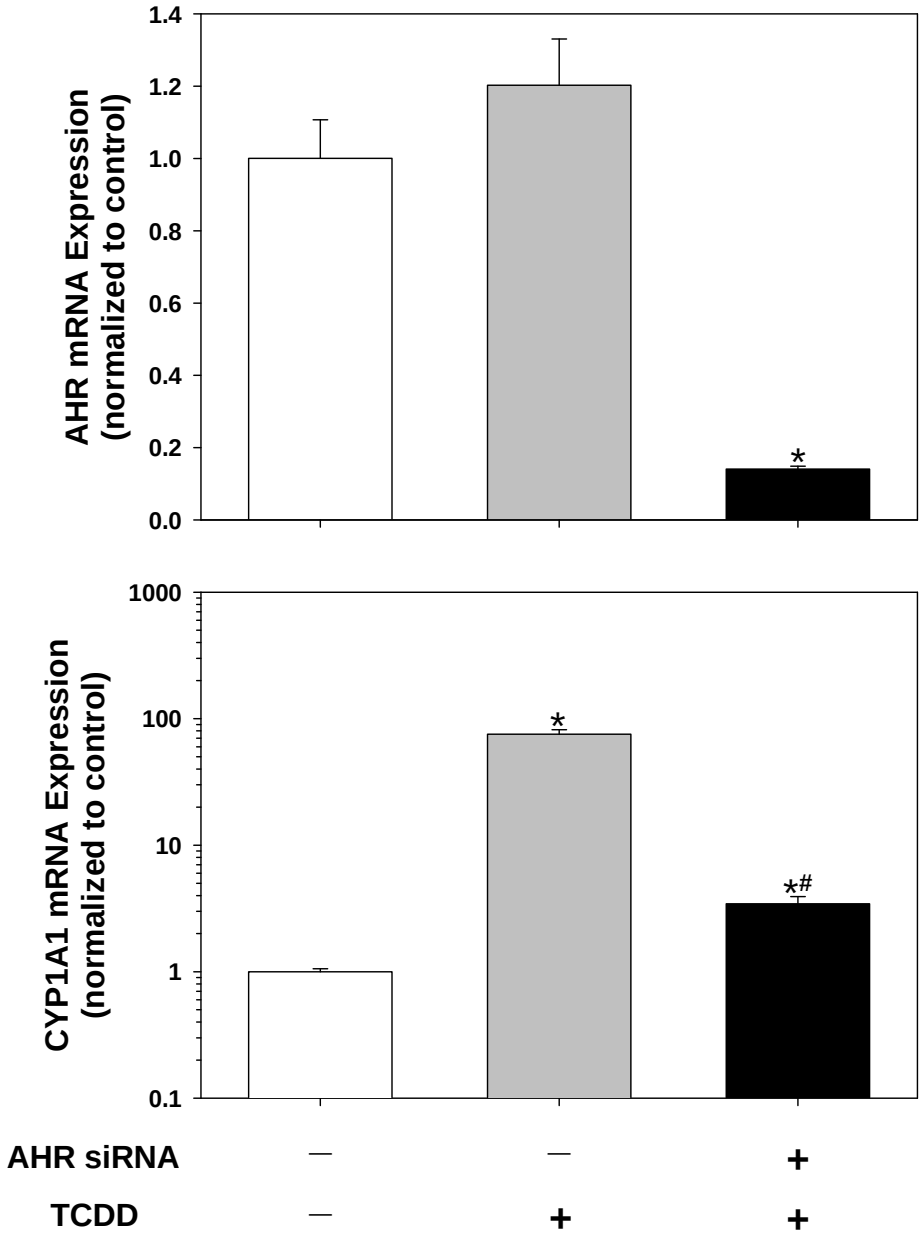

Figure 25. Effect of AHR siRNA on AHR signaling. mRNA expression was analyzed by real time PCR, and 18s rRNA was used as an internal control. n=3. The data represent the mean ± SEM and were analyzed by Student's t-test; *$p < 0.05$, compared to control siRNA treatment; #$p < 0.05$, compared to TCDD treatment.

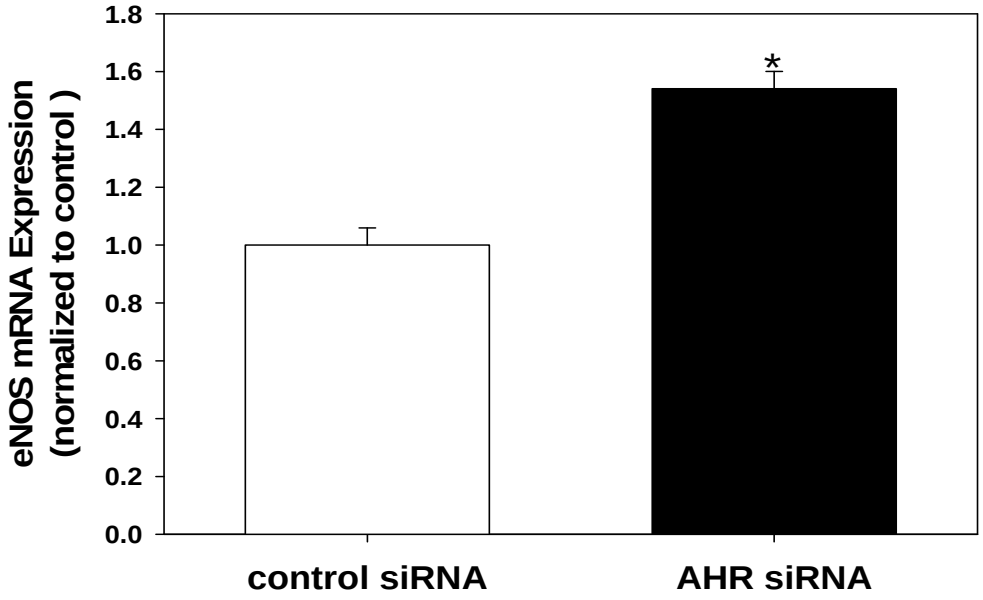

Figure 26. Effect of AHR mRNA knockdown on eNOS mRNA expression. mRNA expression was analyzed by real time PCR, and 18s rRNA was used as an internal control. n=3. The data represent the mean ± SEM and were analyzed by Student's t-test; *p < 0.05, compared to control siRNA treatment.

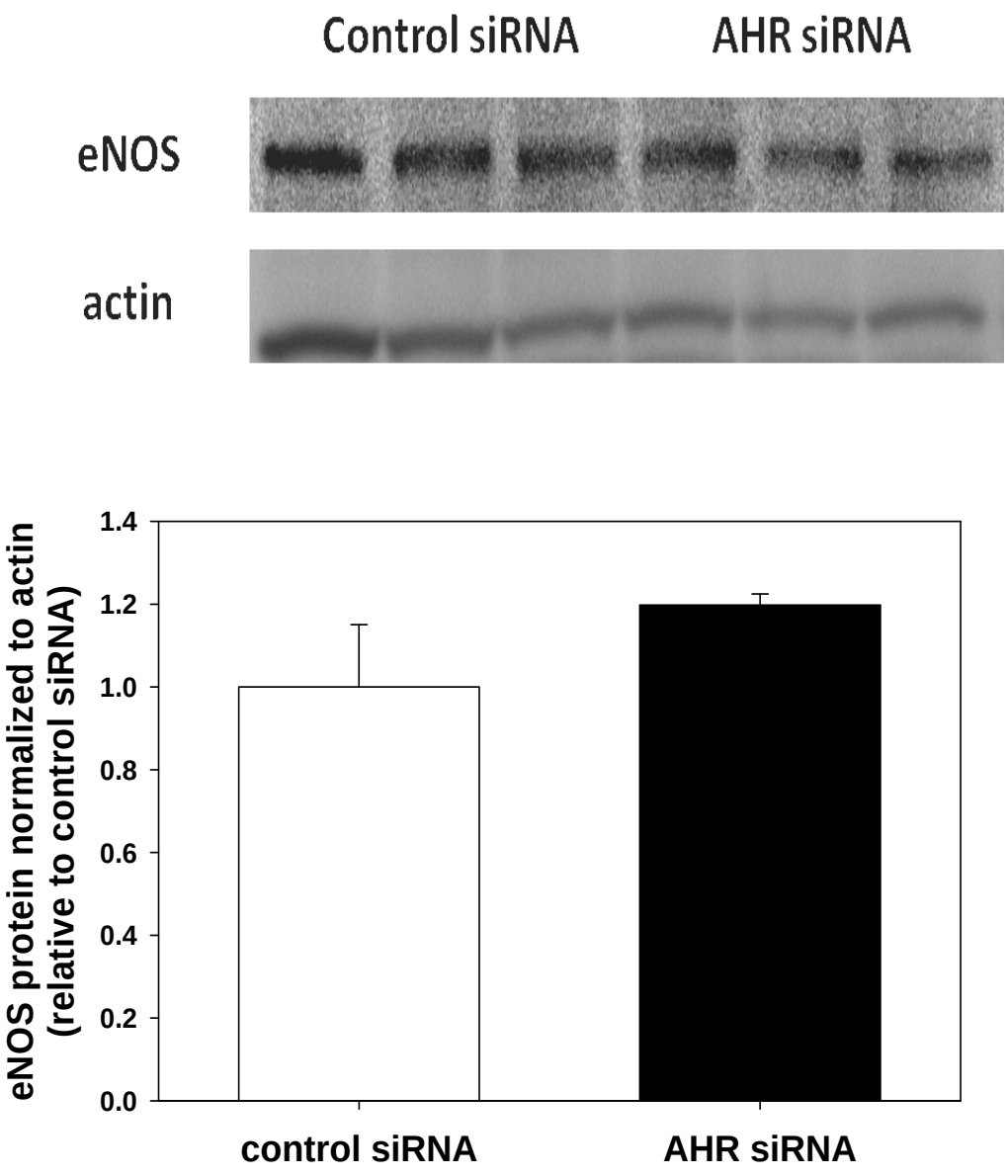

Figure 27. Effect of AHR mRNA knockdown on eNOS protein expression. n=3. The data represent the mean ± SEM and were analyzed by Student's t-test.

production with DAF-2DA fluorescent probe in presence and absence of ACh stimulation in cultured HAECs. eNOS catalytic activity was stimulated with ACh in cultured HAECs treated with control and AHR siRNA, and a significant higher level of NO production was observed in AHR siRNA treated cells, compared to control siRNA treatment (Figure 28). This suggests that ACh-stimulated eNOS activity is significantly increased when AHR signaling is inhibited in HAECs *in vitro*.

EFFECT OF AHR KNOCKDOWN ON SECRETED FACTORS THAT CAN MODULATE eNOS mRNA EXPRESSION

VEGF and TGF-β are two growth factors over expressed in AHR deficient mice (Chang, X. et al. 2007; Guo, J. et al. 2004; Ichihara, S. et al. 2007; Thackaberry, E.A. et al. 2002), and which can induce eNOS mRNA expression in cultured endothelial cells (Bouloumie, A. et al. 1999; Inoue, N. et al. 1995). To test the hypothesis that AHR signaling knockdown induces eNOS mRNA expression through increased secretion of VEGF and/or TGF-β1 in endothelial cells, we performed a media exchange assay using HAECs. Cell culture media collected from HAECs following treatment with AHR siRNA failed to alter eNOS mRNA expression in cells treated with control siRNA (Figure 29). Similarly, media from cells treated with control siRNA did not affect eNOS mRNA expression induced by AHR knockdown (Figure 29). These data suggest that secreted factors do not mediate the increase in eNOS mRNA expression observed following AHR knockdown.

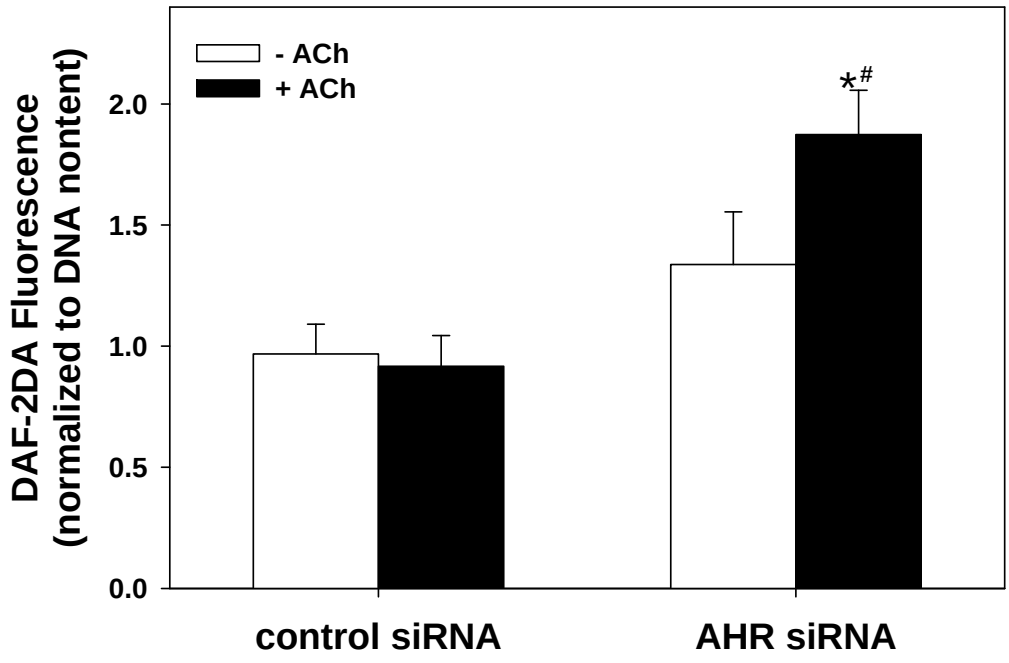

Figure 28. Effect of AHR mRNA knockdown on ACh-stimulated NO production. n=4. The data represent the mean ± SEM and were analyzed by two-way ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to control siRNA treatment with ACh stimulation; #$p < 0.05$, compared to AHR siRNA treatment without ACh stimulation.

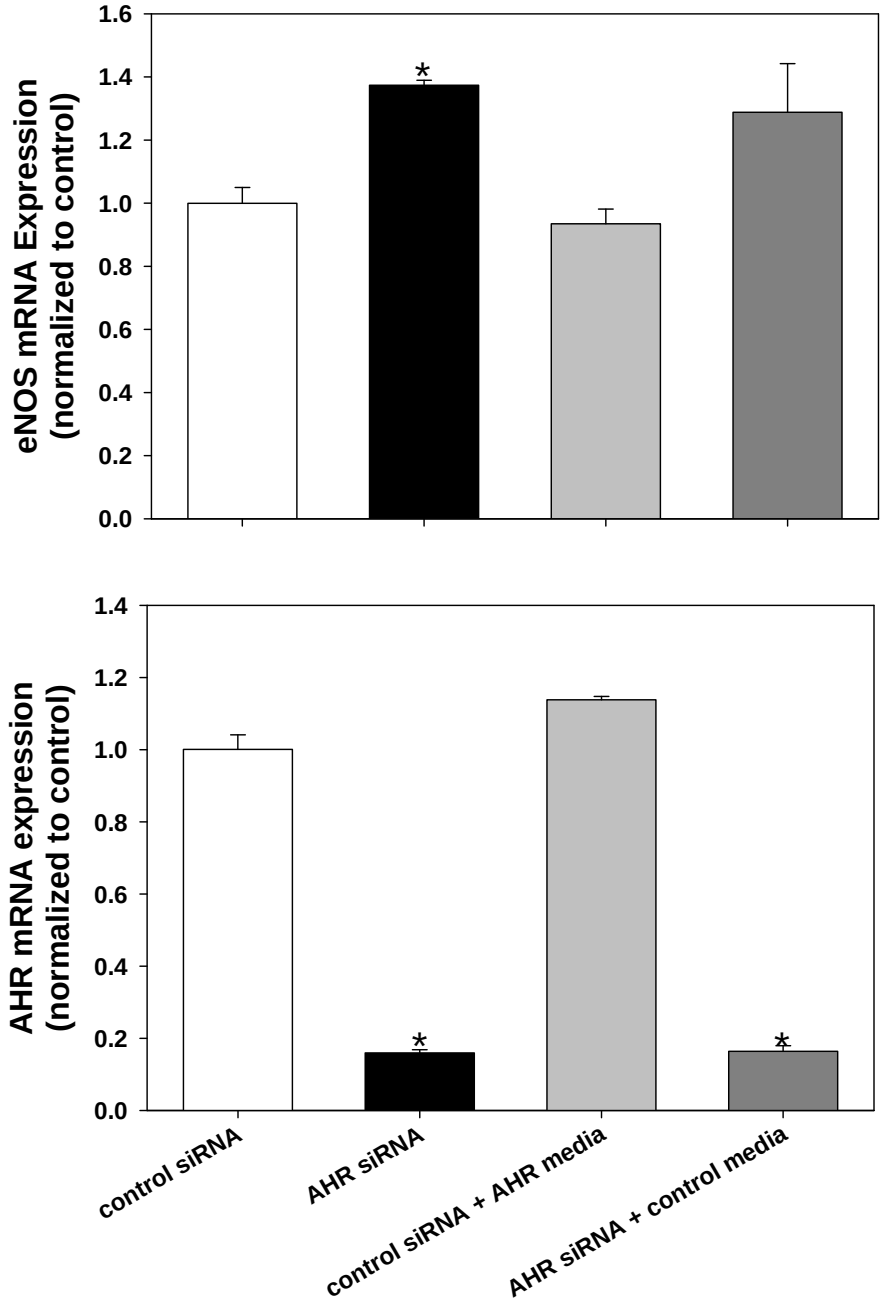

Figure 29. Effect of media collected from HAECs treated with AHR siRNA or control siRNA on eNOS mRNA expression. mRNA expression was analyzed by real time PCR, and 18s rRNA was used as an internal control. n=3. The data represent the mean ± SEM and were analyzed by Student's t-test; *$p < 0.05$, compared to control siRNA treatment.

REQUIREMENT FOR AHR-MEDIATED SIGNALING IN eNOS mRNA EXPRESSION

AHR is a transcription factor that primarily mediates transcription of xenobiotic metabolizing enzymes upon activation by exogenous ligands, such as TCDD. To determine the effect of activating AHR signaling on eNOS mRNA expression, we assessed eNOS and CYP1A1, an AHR target gene, mRNA expression following treatment of HAECs with TCDD, AHR siRNA, or both. TCDD dramatically activated AHR signaling as reflected by a ~75 fold induction of CYP1A1 mRNA, but did not alter eNOS mRNA expression (Figure 30). As observed previously, eNOS mRNA was significantly increased following AHR knockdown with siRNA. Interestingly, however, TCDD treatment of cells in which AHR was knocked down by siRNA resulted in a 3 fold increase in CYP1A1 mRNA expression and a normalization of eNOS mRNA expression back to control level (Figure 30). The ability of TCDD to induce a small increase in CYP1A1 mRNA indicates that a small amount of functional AHR is still present in HAECs when treated with AHR siRNA. Further, these data suggest that a potent stimulus of AHR signaling (i.e. TCDD) is sufficient to suppress eNOS mRNA expression, suggesting that AHR activation is mediating the suppression of eNOS mRNA.

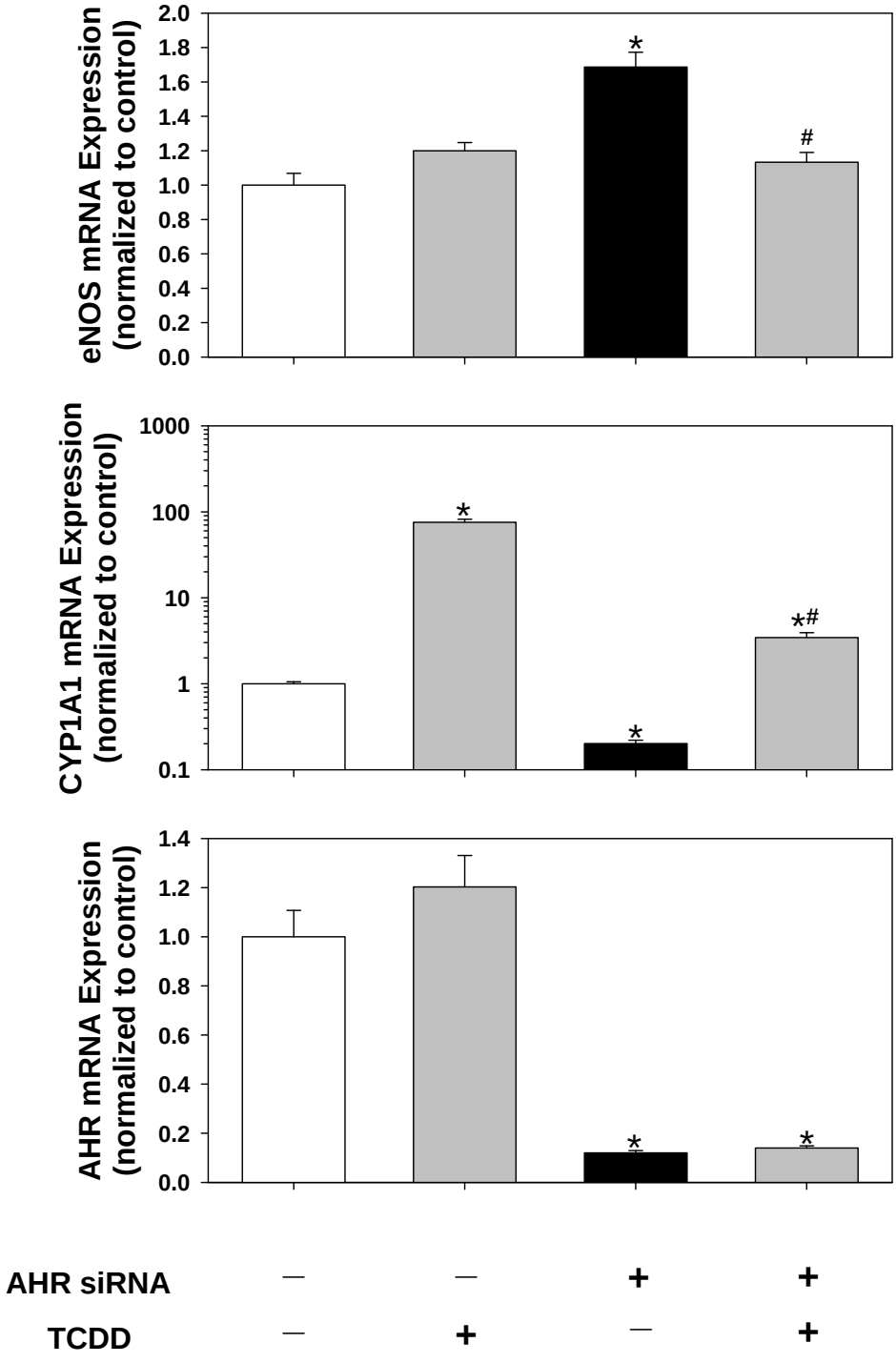

Figure 30. Effects of AHR activation and knockdown on eNOS mRNA expression. n=3. The data represent the mean ± SEM and were analyzed by Student's t-test; *$p < 0.05$, compared to control siRNA treatment; #$p < 0.05$, compared to AHR siRNA without TCDD treatment.

CHAPTER V

DISCUSSION

SYSTEMIC ARTERIAL BLOOD PRESSURE OF AHR KNOCKOUT MICE

Our research demonstrates the presence of a hypotensive phenotype in congenic AHR knockout mice at mild altitude, and an increased vascular NO bioavailability caused by eNOS over expression, which does not appear to account for the hypotension in these animals.

Previously, AHR knockout mice have been reported to be hypotensive at sea level and hypertensive at mild altitude (Lund, A.K. et al. 2008; Lund, A.K. et al. 2003; Vasquez, A. et al. 2003; Villalobos-Molina, R. et al. 2008). Vasquez et al. found that 4 month old AHR knockout mice developed significantly diminished stroke volume and anesthetized 8 month old AHR knockout mice had significantly lower blood pressure (Vasquez, A. et al. 2003). The AHR knockout mice used in this study were not congenic with the control animals, thus the segregation of blood pressure quantitative trait loci could influence the observations reported from these mice. After this study was published, our laboratory reported a hypertensive phenotype in C57Bl/6N congenic AHR knockout mice at mild altitude (Albuquerque NM, 1620 m) and hypotension in the same animals when maintained at sea level (Lund, A.K. et al. 2008; Lund, A.K. et al. 2003). Notably, the hypertension of AHR knockout mice at mild altitude was normalized within 2 weeks by exposure to the partial pressure of oxygen at sea level, revealing that blood pressure of AHR knockout mice is sensitive to small changes in atmosphere oxygen.

After more than 5 years maintaining these AHR knockout mice at mild altitude (Albuquerque NM, 1620 m), they appear to have acclimated to the levels of atmospheric oxygen and are no longer hypertensive. Thus, we began new studies to assess systemic arterial blood pressure using radiotelemetry on wildtype and AHR knockout mice. We

found that AHR knockout mice now exhibit a significantly lower mean arterial blood pressure, compared to wildtype animals ($AHR^{+/+}$: 105.9 ± 0.8 mm Hg; $AHR^{-/-}$: 89.3 ± 2.4 mm Hg), which is consistent with the blood pressure of these mice at sea level (Lund, A.K. et al. 2008). In addition, previously reported high plasma Ang II and ET-1 levels were now found to be normal. These data suggest that our AHR knockout mice have adapted to mild altitude and exhibit a blood pressure phenotype consistent with sea level animals. In order to determine the driving mechanism of hypotension in our AHR knockout mice, we began by assessing the autonomic nervous system and vascular resistance.

Autonomic nervous system. Blood pressure is continuously regulated by autonomic nervous system, including sympathetic and parasympathetic innervation of the heart and peripheral vasculature. The etiologies of neurogenic hypotension have been extensively documented clinically (Guyton, A.C. and Hall, J.E. 2006; Bradley, J.G. et al. 2003). Thus, we first tested the hypothesis that decreased sympathetic stimulation of blood pressure is responsible for the hypotension in AHR knockout mice. We examined the activity of sympathetic nerve activity indirectly by measuring the excretion of its functional neurotransmitter, norepinephrine, in urine. We found that urinary norepinephrine was not altered, failing to support our hypothesis and suggesting that sympathetic nerve activity is normal in AHR knockout mice.

To further assess the contribution of the sympathetic nervous system to blood pressure regulation, we used pharmacological blockade of this pathway focusing first on the primary receptor mediating catecholamine-induced vasoconstriction, the α-adrenoceptor. Mice lacking α1D-adrenoceptor or all three α-adrenoceptors have been

reported to develop systemic hypotension (Sanbe, A. et al. 2009; Tanoue, A. et al. 2002). Furthermore, an alteration of vascular α1D-adrenoceptor expression has been reported in AHR knockout mice, suggesting that this receptor may play a role in blood pressure regulation in AHR knockout animals (Villalobos-Molina, R. et al. 2008). Thus, we injected AHR knockout mice with the selective α1-adrenoceptor antagonist, prazosin, and recorded blood pressure. We observed that blood pressure dropped an equivalent amount in wildtype and AHR knockout mice following prazosin treatment, suggesting that the regulation of vascular tone by sympathetic stimulation of α1-adrenoceptors is the same in AHR knockout mice compared to wildtype mice, again failing to support our hypothesis. Finally, we assessed the global contribution of the autonomic nervous system to blood pressure control by treating mice with a ganglionic blocker, hexamethonium, which will block pre-ganglionic sites of both the sympathetic and parasympathetic nervous system. Again, blood pressure dropped equally in the wildtype and AHR knockout mice as did heart rate (Δ heart rate: $AHR^{+/+}$: 75 ± 11 bpm; $AHR^{-/-}$: 68 ± 6 bpm).

Taken together, all these data disprove our hypothesis and suggest that the autonomic nervous system is not likely involved in the hypotension of AHR knockout mice.

Systemic vascular resistance. Systemic vascular resistance represents a critical determinant of systemic arterial blood pressure which is regulated by circulating hormones, such as Ang II, autonomic nervous innervation, and local factors, such as endothelial-derived NO and ET-1. Since we have excluded Ang II, ET-1, and autonomic nervous system in causing hypotension in AHR knockout mice, we next assessed vascular reactivity and the presence of local dilatory factors that regulate vascular tone.

In addition, TGF-beta and VEGF are increased in AHR null mice (Chang, X. et al. 2007; Guo, J. et al. 2004; Ichihara, S. et al. 2007; Thackaberry, E.A. et al. 2002), both of which could regulate the expression or activity of eNOS, the enzyme that catalyzes vascular NO synthesis. We tested the hypothesis that AHR suppresses vascular eNOS in maintaining normal arterial blood pressure, and thus loss of AHR decreases arterial blood pressure due to an elevated vascular eNOS and increased NO bioavailability.

NO is a vasodilatory molecule that is generated either by basal or stimulated eNOS to control vascular tone, and thus blood pressure. In order to determine the contribution of NO to the control of vascular tone, we assessed NO-dependent vasorelaxation, the vascular smooth muscle response to NO and eNOS expression in aortas from our animals. While we did not observe a significant increase in the vasoconstrictor response of aortic segments to the α-adrenoceptor agonist, PE, we did observe that PE-mediated vasocontraction significantly increased following blockade of eNOS activity with LNNA. Further, the degree of basal vasorelaxation mediated by local NO was revealed by subtracting the PE-mediated vasocontraction in the absence of LNNA from that in the presence of LNNA. This revealed that the contribution of local NO to offset PE-mediated vasocontraction is significantly greater in AHR knockout mice than controls. In addition to the increase in basal NO-mediated vasorelaxation, we found an increase in the ACh-stimulated vasorelaxation in aortas of AHR knockout mice. These vascular reactivity data suggest that simulated NO production is elevated in aortas of AHR knockout mice. Moreover, we observed an enhanced vasorelaxation of endothelium-disrupted aorta to exogenous NO donor, suggesting an amplified signaling transduction downstream of NO in vascular smooth muscle cells of AHR knockout mice.

To determine vascular eNOS expression and systemic NO production, eNOS protein was assessed in isolated aortas and NOx was measured in urine samples. We found a 6-fold elevation in total and phosphorylated (Ser^{1177}) eNOS protein in aortas of AHR knockout mice compared with wildtype mice. This is the first time that an increased eNOS expression has been discovered in AHR knockout mice. The over expressed eNOS was accompanied by an increased urinary NOx level in AHR knockout mice, which suggests elevated functional eNOS rather than uncoupled enzyme.

Based on these data, we then hypothesized that pharmacological blockade of eNOS would normalize blood pressure in AHR knockout mice to control levels. To test our hypothesis, we treated wildtype and AHR knockout mice with LNNA, a nonselective NOS blocker, in the drinking water. Interestingly, blood pressure in AHR wildtype and knockout mice significantly increased on the first two days of treatment. However, while the increase of blood pressure was sustained in the wildtype mice, the increase in blood pressure in AHR knockout mice was only transient, returning to pre-treatment levels on day 3. An increase in the dose of LNNA did cause a persistent increase in blood pressure in AHR knockout mice, but it still failed to increase their blood pressure to control levels. Further, measurement of urinary NOx confirmed that LNNA treatment effectively reduced systemic NO to control levels in AHR knockout mice.

The role of NO derived from eNOS in blood pressure regulation has been demonstrated using both pharmacological blockade and genetically modified mouse models. Previous studies have demonstrated that C57Bl/6 mice treated with nonselective NOS inhibitor, N^{ω}-nitro-L-arginin-methylester (L-NAME), develop hypertension (Mattson, D.L. et al. 2006; Van Vliet, B.N. et al. 2003). eNOS deficient mice also

develop hypertension, while, in contrast, transgenic mice over expressing eNOS exhibit low blood pressure and low systemic vascular resistance (Huang, P.L. 2000; Ohashi, Y. et al. 1998). Over expression of eNOS also attenuates the high blood pressure in both the stroke-prone spontaneously hypertensive rat and the two-kidney one-clip hypertensive mouse (Gava, A.L. et al. 2008; Miller, W.H. et al. 2005). These data suggest a role of eNOS-derived NO in attenuating blood pressure in both physiological and pathological conditions. In addition, pharmacological blockade of NOS in C57Bl/6 mice with L-NAME induces blood pressure to the same level as eNOS deficient mice, and L-NAME treatment does not further alter blood pressure in eNOS knockout mice (Kojda, G. et al. 1999; Lemmer, B. et al. 2008; Van Vliet, B.N. et al. 2003), suggesting a dominant role of eNOS-derived NO in blood pressure control.

Our AHR knockout mice exhibit a similar increase in vascular eNOS expression and a similar degree of hypotension as previously reported in eNOS transgenic mice (Ohashi, Y. et al. 1998; van Haperen, R. et al. 2002). However, AHR knockout mice exhibit quite different vasodilatory responses to ACh and the endothelium-independent NO donor, SNAP. Transgenic eNOS over expressing mice show blunted vascular responses to ACh and an exogenous NO donor, which are caused primarily by diminished sGC/PKG signaling manifested as a lower cGMP level upon NO stimulation (Adlam, D. et al. 2007; Ohashi, Y. et al. 1998; van Deel, E.D. et al. 2007; Yamashita, T. et al. 2000). In contrast, aortas from our AHR knockout mice exhibit significantly increased vasodilatory responses to ACh stimulation and significantly greater sensitivity to an endothelium-independent NO donor. Those data suggest that AHR knockout mice exhibit a similar degree of blood pressure drop and endothelial eNOS expression

compared with eNOS over expressing mice, but different NO signaling transduction in vascular smooth muscle cells.

In vascular reactivity assessment, we observed that aortas from AHR knockout mice exhibit a significantly increased vasocontraction response to PE following blockade of eNOS activity with LNNA, which may suggest an elevation of α-adrenoceptor expression in aortas. This is consistent with previous report of vascular α1D-adrenoceptor over expression in AHR knockout mice (Villalobos-Molina, R. et al. 2008). In their experiment, intact aorta isolated from AHR knockout mice showed significant greater response to PE. However this elevated responsiveness to PE in our AHR knockout mice was only observed after LNNA incubation, which blocks the basal vasodilatory effect of NO generated by basal eNOS. Obviously, the absence of greater responsiveness of intact AHR negative aorta to PE is due to eNOS over expression in aortic endothelium. However, the equivalent amount of blood pressure drop caused by prazosin treatment suggests that this difference in α-adrenoceptor expression may be only limited to aorta rather than the systemic vasculature. Moreover, we also observed a significantly greater contraction of aortas from AHR knockout mice to potassium stimulation. These data may suggest alterations in adrenoceptor expression/distribution and depolarization machinery in vasculature of AHR knockout mice that needs to be further elucidated.

Blockade of eNOS with 500 mg/L LNNA in our AHR knockout mice failed to normalize blood pressure to the same level of wildtype mice. Previous studies have shown that doses as low as ~ 100 mg/L L-NAME, a LNNA precursor, could completely block basal eNOS activity in blood pressure regulation in C57Bl/6 mice and bring their

blood pressure to the same level of eNOS knockout C57Bl/6 mice (Lemmer, B. et al. 2008). The mean arterial blood pressure of eNOS deficient C57Bl/6 mice has also been reported as ~120 mm Hg measured by radiotelemetry (Van Vliet, B.N. et al. 2003), which is consistent with what we observed in wildtype mice with LNNA treatment. Moreover, 1g/L L-NAME in drinking water can normalize blood pressure of eNOS over expression mice in two weeks (Ohashi, Y. et al. 1998). These data suggest that the dose of LNNA we used was sufficient for complete eNOS inhibition in our wildtype mice; however, we cannot exclude the possibility that 500 mg/L LNNA was not sufficient to block over expressed eNOS in our AHR knockout mice. In addition, we cannot accurately estimate the efficiency of 500 mg/L LNNA in blocking over expressed eNOS in AHR knockout mice by analyzing urinary NOx excretion either. We observed that urinary NOx failed to reflect a change in vascular NO production before and after LNNA treatment in wildtype mice, suggesting a high background level of systemic NOx. Although this result suggests that LNNA normalized urinary NOx in AHR knockout mice, there may be a significantly higher residual vascular NO that cannot be detected by this method. Therefore, a double knockout mouse lacking both AHR and eNOS would be one approach to define the degree to which eNOS over expression mediates hypotension of AHR knockout mice.

Another mechanism that may be involved in causing hypotension and accounting for the resistance of AHR knockout mice to LNNA is the increased sensitivity of vascular smooth muscle cells to NO. eNOS over expressing mice exhibit a blunted vascular response to NO, which is mediated by diminished sGC/PKG signaling in smooth muscle cells (Adlam, D. et al. 2007; Ohashi, Y. et al. 1998; van Deel, E.D. et al. 2007;

Yamashita, T. et al. 2000). Interestingly, we detected an increased vasodilatory response to an exogenous NO donor in aortas from AHR knockout mice. Although sGC is regarded as the principal intracellular NO receptor in mediating vasorelaxation in vascular smooth muscle cells, NO can also activate K^+ channels in an sGC-independently manner, leading to K^+ efflux and membrane hyperpolarization, or increase cytosolic Ca^{2+} uptake by sarcoplasmic reticulum through its oxidative product, peroxynitrite (Cauwels, A. 2007). No direct evidence has been reported to connect AHR and the NO downstream pathways, however, we did observe increased aortic contraction to extracellular K^+ stimulation, which may suggest an alteration of K^+ channel in the vasculature of AHR knockout mice. Taken together, these data suggest that NO downstream signaling pathways could also be altered and contribute to the hypotension and resistance to NOS blockade in AHR knockout mice.

Myogenic response is characterized as cell membrane depolarization and consequently constriction of vascular smooth muscle in response to increases in transmural pressure (Huang, A. et al. 2005). It is primarily dependent on vascular smooth muscle and contributes to the regulation of basal vascular tone and organ perfusion (Schubert, R. et al. 1999). NO has been found to profoundly inhibit myogenic response (Just, A. et al. 2005; Skarsgard, P.L. et al. 2000), which may be mediated by the level of 20- hydroxyeicosatetraenoic acid (20-HETE) (Huang, A. et al. 2005). 20-HETE is synthesized from arachidonic acid by CYP450/ω-hydroxylation (CYP4A) and can inhibit K^+ channel and membrane hyperpolarization in vascular smooth muscle cells. NO is a factor that can inhibit the expression and function of CYP4A (Harder, D.R. et al. 1995), and thus increased NO production can diminish 20-HETE production and activate K^+

channel in smooth muscle cells, resulting in hyperpolarization and attenuation of myogenic tone. Therefore, attenuated myogenic response by eNOS-derived NO may also be involved in the development of hypotension in AHR knockout mice.

Others factors in blood pressure regulation. Mean arterial blood pressure is determined by cardiac output and systemic vascular resistance. We have examined the roles of autonomic nervous system, circulating ET-1, Ang II and vascular NO in the blood pressure regulation of our animals, but failed to identify the primary driving factors of hypotension in AHR knockout mice. To systemically assess the factors that may be involved in hypotension of AHR knockout mice, we need to determine the cardiac output and total peripheral resistance of those mice. Previous research shows that AHR knockout mice exhibit diminished stroke volume (Vasquez, A. et al. 2003), cardiac hypertrophy (Fernandez-Salguero, P.M. et al. 1997; Lund, A.K. et al. 2003; Thackaberry, E.A. et al. 2002; Vasquez, A. et al. 2003) and kidney hypertrophy and fibrosis (Lund, A.K. et al. 2003). Consistently, we observed cardiac and renal hypertrophy in our AHR knockout mice. The abnormality of the heart in AHR knockout mice may contribute to the hypotension by decreasing cardiac contractility, and thus cardiac output, which needs to be further investigated. The possible functional alteration in the kidney of AHR knockout mice may also cause hypotension through a disruption of fluid balance, which is the long term regulatory mechanism of blood pressure. Although the hematocrit of AHR null mice was not different from that of wildtype mice (data not shown), suggesting the same blood volume between two genotypes of mice, a direct assessment of blood volume may also need to be conduct to determine the effect of blood volume on hypotension of AHR null mice in future studies.

Arterial pulse pressure. Arterial pulse pressure is the change of blood pressure during systole and is determined by stoke volume and aortic compliance (Klabunde, R.E. 2004). Vascular compliance is described as the ability of a vessel to distend and increase volume with increasing transmural pressure. We found a significantly lower pulse pressure in our AHR knockout mice ($AHR^{+/+}$: 29.8 ± 1.4; $AHR^{-/-}$: 26.1 ± 1.1). According to the factors that could affect pulse pressure, the AHR knockout mice may have a smaller stroke volume or an increased vascular compliance or both. Smaller stroke volume has been reported in non-congenic AHR knockout mice (Vasquez, A. et al. 2003). Previous studies also have demonstrated that pulse pressure is increased in eNOS deficient mice, eNOS over expression may mediate the beneficial effect of exercise training on aortic stiffness in rats, and the eNOS polymorphism influences aortic stiffness in human (Chen, W. et al. 2004; Maeda, S. et al. 2005; Van Vliet, B.N. et al. 2003). These data suggest that decrease in cardiac output and eNOS over expression may be involved in determining the low pulse pressure phenotype of AHR knockout mice.

eNOS EXPRESSION AND AHR SIGNALING IN ENDOTHELIAL CELLS

eNOS expression and activity are regulated by a number of factors at multiple levels, including transcription, mRNA stability, protein phosphorylation and enzyme cofactor availability. On the transcriptional level, eNOS can be transactivated by various factors, such as TGF-β, laminar shear stress, H_2O_2 and estrogen (Inoue, N. et al. 1995 Chambliss, K.L. et al. 2002; Ziegler, T. et al. 1998). The mRNA level of eNOS is also regulated at posttranscriptional level by factors such as VEGF, which stabilizes eNOS mRNA (Bouloumie, A. et al. 1999).

In our *in vivo* study, we observed an elevated vascular eNOS expression in the aortas of AHR deficient mice, suggesting a significant role of AHR in regulating eNOS expression in vasculature. In our *in vitro* study on human aortic endothelial cells, we detected elevated eNOS mRNA and ACh-induced NO production when AHR protein was depleted by siRNA. Previous studies have provided very limited evidence connecting eNOS expression with AHR signaling. Elevated TGF-β, which could activate eNOS mRNA transcription (Inoue, N. et al. 1995), has been found in liver, and in fibroblast and smooth muscle cells of AHR knockout mice (Chang, X. et al. 2007; Guo, J. et al. 2004; Zaher, H. et al. 1998). Additionally, VEGF is also increased in AHR deficient mice (Ichihara, S. et al. 2007; Thackaberry, E.A. et al. 2002). Therefore we tested the hypothesis that loss of AHR in HAECs increases eNOS mRNA by secreted factors in an autocrine manner. However, culture media from HAECs that contain secreted factors produced by AHR knockdown failed to induce eNOS mRNA expression, suggesting the absence of autocrine pathway in eNOS induction. In contrast, our experiments demonstrated that diminished endogenous AHR signaling is required for eNOS induction, which could be abolished by AHR signaling activation through TCDD treatment. However, AHR signaling activation by TCDD did not further suppress eNOS mRNA level. This is consistent with a previous research on the role of endogenous AHR in liver vascular development. AHR deficient mice develop patent ductus venosus after birth (Lahvis, G.P. et al. 2000). The mice carrying the hypomorphic AHR allele also develop patent ductus venosus, which could be rescued by TCDD treatment (Walisser, J.A. et al. 2004a). In addition, nuclear translocation and DNA binding abilities of AHR are both required in the closure of ductus venosus, suggesting a transactivation

mechanism in endogenous AHR function (Bunger, M.K. et al. 2008; Bunger, M.K. et al. 2003). Taken together, these models suggest that the endogenous and exogenous ligand-activated AHR signaling may share the same signal transduction mechanism, in which AHR translocation and DNA binding is required. Our model indicates a role for endogenous activation of AHR in suppressing eNOS mRNA expression in endothelial cells.

However, western blot failed to detect the difference in total eNOS protein expression in HAECs treated with control and AHR siRNA, which was not consistent with the 6 fold induction of eNOS protein in the aortas of AHR knockout mice. This inconsistency could result from a number of factors. First, the degree of eNOS suppression by AHR signaling may be greater in mice than in humans; therefore, AHR signaling inhibition can cause greater induction of eNOS protein in mice than in humans. *In vivo*, eNOS expression is maintained and regulated by blood flow shear stress, which was not applied to the culture cells. The sustained exposure of endothelial cells to shear stress amplifies both basal and inducible eNOS expression, and thus the increase of eNOS protein expression caused by AHR blockade may also be amplified and easierto detect in AHR knockout mice. Third, AHR signaling was completely blocked in AHR knockout mice, but was only attenuated in HAECs, which may also account for the different degree of eNOS protein induction between two models. Finally, factors generated by smooth muscle cells or in circulation of AHR knockout mice may also contribute to the eNOS protein over expression *in vivo*. In contrast, cultured cells were not exposed to those factors and may not be subjected to an extensive regulation by AHR signaling as *in vivo*.

SIGNIFICANCE OF eNOS OVER EXPRESSION

Beyond the role in blood pressure control, eNOS derived NO is also involved in other important functions, thus increased NO production by over expressed eNOS is beneficial in various pathological conditions.

NO has been demonstrated to inhibit leukocyte-endothelial adhesion, vascular smooth muscle proliferation and migration, and platelet aggregation, all of which are critical steps in atherosclerosis development (De Caterina, R. et al. 1995; Garg, U.C. et al. 1989a; b; Sarkar, R. et al. 1996). The atherosclerosis development has been found to be accelerated in apolipoprotein E/eNOS double-knockout mice, which is not dependent on the hypertension of those mice (Chen, J. et al. 2001; Kuhlencordt, P.J. et al. 2001). In contrast, atherogenesis process is dramatically attenuated by eNOS over expression in mice (van Haperen, R. et al. 2002). These data indicate a protective role of eNOS derived NO in atherosclerosis development.

eNOS derived NO is important in preserving cerebral blood flow under ischemia conditions, such as stroke, which has been demonstrated by the observation that eNOS knockout mice develop larger strokes after middle cerebral artery occlusion (Huang, Z. et al. 1996). Myocardial reperfusion injury is another pathological condition in which over expressed eNOS in cardiomyocyte may play protective role (Brunner, F. et al. 2003; Elrod, J.W. et al. 2006).

Based on the spectrum of beneficial effects of eNOS derived NO in cardiovascular deleterious conditions, elucidation of the role of AHR in suppressing

eNOS expression will offer a novel and promising target in cardiovascular disease treatment.

CIRCADIAN SYSTEMIC BLOOD PRESSURE, HEART RATE AND LOCOMOTOR ACTIVITY RHYTHMS

Circadian rhythms are observed in most physiological functions, including metabolism, cell growth and immune responses (Shimba, S. et al. 2009). The daily cycle of relative AHR and ARNT protein expressions exhibit similar oscillation patterns in multiple organs of female Sprague-Dawley rats, respectively (Richardson, V.M. et al. 1998). In mice, the mRNA expressions of AHR and its target gene, CYP1A1, also exhibit circadian changes in suprachiasmatic nucleus and liver (Mukai, M. et al. 2008). Circadian rhythms of behavior and immune system can also be disrupted by activation of AHR with TCDD (Garrett, R.W. et al. 2006; Mukai, M. et al. 2008). These observations suggest that endogenous AHR signaling may affect circadian rhythms of certain physiological functions.

A circadian oscillation of blood pressure occurs by both humans and rodents. In humans, blood pressure increases on awakening, decreases during sleep and peaks in early morning, which is highly associated with an excessive incidence of cardiovascular events (White, W.B. 2007). As a nocturnal species, mice exhibit higher blood pressure and heart rate at nighttime rather than in the light period. Furthermore, the waveform of their blood pressure is bimodal, with evident peak values early and late in the dark period (Li, P. et al. 1999). Their heart rate generally peaks at dark onset and reaches its lowest point in the middle of both the light and dark periods (Li, P. et al. 1999).

Our data demonstrate for the first time an alteration in the circadian haemodynamic and locomotor activity rhythms of AHR knockout mice. In AHR knockout mice, the light/dark differences of arterial blood pressure and heart rate were not significantly different from wildtype mice. However, there were differences in 24 hr blood pressure and heart rate waveforms between the two genotypes. The blood pressure of AHR knockout mice increases normally at the onset of the dark period; however, blood pressure in AHR knockout mice drops precipitously at the end of dark period. This blood pressure drop led to a shorter window of time during the dark cycle when blood pressure is elevated in AHR knockout mice. Patients who lack the normal fall in blood pressure at the onset of the sleep cycle (non-dippers) have been shown to have an excessive incidence of cardiovascular events, including stroke and atherogenesis (Routledge, F.S. et al. 2007; White, W.B. 2007). Therefore, a shorter non-dipping period might be beneficial to the cardiovascular system of AHR knockout mice in addition to their low blood pressure.

The heart rate of AHR knockout mice was significantly greater than wildtype mice from 2300 to 0100 hours, when the locomotor activity of AHR knockout mice also reached its maximal values. The higher heart rate in AHR knockout mice may be a result of the higher locomoter activity during that time period, since a higher metabolic level would be required in those animals and result in an increase in cardiac output.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

SUMMARY

Previously, AHR knockout mice have been reported to be hypotensive at sea level and hypertensive at mild altitude (Lund, A.K. et al. 2008; Lund, A.K. et al. 2003; Vasquez, A. et al. 2003; Villalobos-Molina, R. et al. 2008). After more than 5 years maintaining AHR knockout mice at mild altitude (Albuquerque NM, 1620 m), they appear to have acclimated to mild altitude and become hypotensive. Therefore, the research performed here is to understand the mechanism of AHR in blood pressure regulation, or specifically loss of AHR in causing hypotension.

Our research demonstrates no changes of plasma Ang II and ET-1 level in AHR knockout mice compared to wildtype mice, indicating a loss of prior reported driving factors of hypertension in those animals at mild altitude (Lund, A.K. et al. 2008; Lund, A.K. et al. 2003). In addition, we did not detect any alterations in the contribution of autonomic nervous system to hypotension in AHR knockout mice. First of all, we detected a normal sympathetic nerve activity in AHR knockout mice, which was reflected by urinary norepinephrine level. Prazosin, a selective α1-adrenoceptor blocker, caused the same degree of blood pressure drop in both genotypes, which excludes the possible role of different systemic α1-adrenoceptor expression in mediating lower vascular tone, thus hypotension in AHR knockout mice. Finally, ganglionic blocker, hexamethonium, induced the same change of blood pressure and heart rate between both genotypes, suggesting an equal contribution of autonomic nervous system to blood pressure.

The research also demonstrates a significantly elevated vascular eNOS expression in AHR knockout mice. We found an increased NO-dependent basal vasorelaxation in isolated aortas of AHR knockout mice, which was revealed by a significantly greater PE-

mediated vasocontraction offset. The ACh-dependent vasodilation of aortas was also increased in AHR knockout mice. Additionally, we demonstrated a greater sensitivity of vascular smooth muscle to exogenous NO-mediated vasorelaxation in AHR deficient aortas, indicating an amplified NO downstream signaling transduction in vascular smooth muscle cells, which may also contribute to the hypotension. eNOS over expression was confirmed by a 6 fold elevation of Ser^{1177} phosphorylated and total eNOS protein in AHR deficient mouse aortas, and a 2 fold increase of systemic urinary NOx production in AHR knockout mice.

Previous research has extensively demonstrated that NO derived by eNOS can cause hypotension or attenuate high blood pressure either in eNOS over expressing mice (Gava, A.L. et al. 2008; Miller, W.H. et al. 2005; Ohashi, Y. et al. 1998) or under pathological shock conditions (Cauwels, A. et al. 2006). Therefore we blocked systemic NO production with a nonselective NOS inhibitor, LNNA, in both wildtype and AHR knockout mice. However, this treatment did not normalize the blood pressure of AHR knockout mice to control level. The measurement of urinary NOx indicated a normalization of systemic NOx production in AHR knockout mice to control level in wildtype mice, but failed to prove a complete inhibition of eNOS in AHR knockout mice. Moreover, the amplified NO downstream signaling transduction in vascular smooth muscle cells could be another factor involved in this resistance to LNNA in AHR knockout mice.

In addition to low blood pressure, we revealed the first time an altered circadian rhythm in the locomotor activity and cardiac parameters of AHR knockout mice. The AHR knockout mice exhibit significantly higher locomotor activity than wildtype mice

during nighttime, which may contribute to a significantly greater midnight heart rate in those animals. They also lose the typical bimodal pattern of nighttime blood pressure and exhibit a precipitous blood pressure drop at the end of nighttime period, which differs from the 24 hour blood pressure pattern of wildtype mice. The data suggest an important role of endogenous AHR in regulating behavioral and cardiac circadian rhythmicity in mice.

Our research on human aortic endothelial cells *in vitro* suggests an inhibitory role of endogenous AHR signaling in eNOS mRNA expression. Endogenous AHR signaling blockade with siRNA increased eNOS mRNA and NO production level in HAECs. The increase of mRNA with AHR signaling blockade was abolished by exogenous AHR agonist, TCDD, treatment. However, AHR signaling activation by TCDD did not further attenuate eNOS mRNA expression, which implicates exogenous ligand-independent role of AHR in eNOS mRNA regulation.

In summary, we reveal the first time that endogenous AHR signaling plays a very important role in regulating circadian blood pressure and locomotor activity rhythms and vascular eNOS expression, a pivotal factor in vascular homeostasis. Although our initial research has not fully demonstrated the function of over expressed eNOS in hypotension of AHR deficient mice, it indicates a very promising future of the research on AHR in cardiovascular physiology and disease treatment.

CONCLUSION

Beyond mediation of adaptive and toxic responses to xenobiotics, the significant role of endogenous AHR in embryonic development and adult physiology starts to attract more attention and remains to be further investigated.

As an orphan receptor, no high-affinity endogenous ligand of AHR has been identified to date (Beischlag, T.V. et al. 2008). Although a few candidate endogenous AHR ligands have been isolated from mammalian tissues (Nguyen, L.P. et al. 2008), they are inclined to establish an intrinsic environment that sustains the endogenous signaling of AHR in an integrated manner, rather than activate AHR individually. This exogenous ligand-independent AHR signaling plays a relatively subtle but crucial role in variety of cellular, developmental and physiological processes. AHR deficient cells exhibit aberration in cell cycle progression and apoptosis (Chang, X. et al. 2007; Wu, R. et al. 2007). AHR knockout mice develop patent ductus venosus, persistent hyaloid arteries and abnormal kidney vasculature (Lahvis, G.P. et al. 2000). Loss of AHR in mice also causes blood pressure alteration and instability (Lund, A.K. et al. 2008; Lund, A.K. et al. 2003; Vasquez, A. et al. 2003; Villalobos-Molina, R. et al. 2008). Our results demonstrate hypotension, alteration of circadian blood pressure, heart rate and activity rhythms in AHR knockout mice, and elevated eNOS expression in both AHR knockout mice and cultured human endothelial cells with diminished endogenous AHR signaling. All the data suggest that the role of endogenous AHR signaling is significant in blood pressure regulation and vascular homeostasis and more complicated than its role in mediating xenobiotic responses.

Due to the absence of potent endogenous ligands, the knowledge related to the function of endogenous AHR signaling is very limited. The role of endogenous AHR in maintaining basal level CYP1A1 mRNA expression has been observed in human endothelial cells (Zhang, N. et al. 2007). AHR deficient mice develop patent ductus venosus after birth (Lahvis, G.P. et al. 2000). In this model, nuclear translocation and DNA binding abilities of AHR are both required in normal liver vascular development, and the mice carrying hypomorphic AHR or ARNT allele also develop patent ductus venosus, which could be rescued by TCDD treatment (Bunger, M.K. et al. 2008; Bunger, M.K. et al. 2003; Walisser, J.A. et al. 2004a; Walisser, J.A. et al. 2004b). The research on the function of endogenous AHR in vascular development suggests that endogenous AHR signaling may also function through a gene transactivation pattern similar to ligand-activated AHR signaling. Our observation of elevated eNOS expression in both AHR knockout mice and AHR signaling diminished endothelial cells is consistent with previous data on liver vasculature development, suggesting a resemblance of endogenous signaling with xenobiotic activation of AHR. However, eNOS is fully suppressed by constitutive AHR signaling, which is not further attenuated by TCDD treatment in endothelial cells. These data suggest an exogenous ligand-independent role of AHR signaling, which is not addressed in the patent ductus venosus model. Because TCDD does not affect eNOS expression in endothelial cells, our model cannot be interpreted by the theory of common factor competition either, in which the deprivation of shared factors could be facilitated by TCDD treatment.

In conclusion, endogenous AHR plays an important role in blood pressure regulation and vascular homeostasis. Our data indicate that AHR is involved in blood

pressure control, and suppresses eNOS expression thus vascular production of NO, the pivotal molecule in endothelial function. Therefore, therapeutic approaches that target the connection of AHR and eNOS could be beneficial to patients with hypertension and vascular diseases, such as atherosclerosis.

FUTURE DIRECTIONS

eNOS gene knockout. The result of LNNA treatment suggests that over expressed eNOS may not be the primary driving factor of hypotension in AHR knockout mice, however, we cannot completely exclude the possible role of over expressed eNOS in blood pressure regulation in those animals. Therefore, a genetic deletion of eNOS gene in AHR knockout mice is required to fully address the degree by which eNOS over expression contributes to the hypotension of AHR knockout mice. Due to the elevated sensitivity of vascular smooth muscle to NO in AHR knockout mice, we may hypothesize that *enos* gene knockout out can causes greater blood pressure increase in AHR knockout mice compared to wildtype mice, but cannot normalize the blood pressure to the same level of eNOS deficient mice. In this case, the difference in the degree of blood pressure elevation caused by eNOS deletion between wildtype and AHR knockout mice pressure should indicate the contribution of eNOS over expression to the hypotension in AHR knockout mice.

Signaling pathways downstream of NO. In contrast to the blunted vascular NO responsiveness caused by diminished sGC/PKG signaling in eNOS over expressed transgenic mice (van Deel, E.D. et al. 2007; Yamashita, T. et al. 2000), our vascular reactivity assessment reveals a significantly more sensitive vasodilatory response to exogenous NO in endothelium-disrupted aorta of AHR knockout mice, suggesting an

amplified NO downstream signaling transduction in smooth muscle cells. This downstream signal amplification may be caused by an increase of intracellular sGC activity, which can be tested using 1*H*-(1,2,4)oxadiazolo(4,3-α)quinoxalin-1-one (ODQ), a sGC inhibitor in AHR knockout mice aorta *ex vivo*. sGC inhibition by ODQ in endothelium-disrupted aortas could abolished the different responses to NO if high sGC activity is the mediator of vascular hypersensitivity to NO. Other mediators of NO downstream signaling, such as BK potassium channel, may also be involved in this hypersensitivity to NO in AHR deficient vasculature and can be further investigated using vascular reactivity assay *ex vivo*.

Other vasodilators. In conduit vessels, endothelium-derived NO and PGI_2 are the predominant factors in causing vasodilation, while in small resistant vessels, EDHF tends to play a greater role in regulating vascular tone (Scotland, R.S. et al. 2005). Previous research on eNOS knockout mice indicates a compensatory role of EDHF in vasodilatory response (Huang, A. et al. 2000; Waldron, G.J. et al. 1999). NO has been reported to attenuate EDHF release (Bauersachs, J. et al. 1996), and in contrast eNOS expression could be up-regulated by EDHF treatment in endothelial cells (Wang, H. et al. 2003). Those data suggest a cross talk between eNOS pathway and EDHF signaling. Thus EDHF production may also be altered in AHR knockout mice, which may contribute to hypotension and need further investigation. Additionally, AHR signaling may suppress the expression of bradykinin, another potent vasodilator (Nukaya, M. et al. 2001), thus loss of AHR may cause bradykinin mediated hypotension in mice. Finally, our data suggest alterations of vascular reactivity in response to KCl and PE in AHR deficient mice, which may disrupt the homeostasis of vascular tone control. Further investigation

on these pathways that are involved in blood pressure control in AHR knockout mice will help us to elucidate the mechanism of hypotension in those animals.

Cell culture studies. Our *in vitro* results suggest that eNOS expression is suppressed by endogenous AHR signaling, but is not further attenuated by ligand activated AHR. Endogenous AHR signaling inhibition causes eNOS over expression in HAECs, which could be abolished by activating the residual AHR with TCDD. The data implicate that endogenous AHR signaling suppresses eNOS mRNA expression either directly through a mechanism of transcriptional regulation or indirectly through interaction with other intracellular pathways.

A number of transcription factors involved in different pathways usually accumulate in promoter to regulate gene transcription, thus the transcription level of a target gene depends on the integrated effect of those factors. A typical transcription activator could also perform as a suppressor if its participation into the transcription machinery interferes with the efficiency of the entire transcriptional complex. AHR is well recognized as a transcription factor in mediating gene transcription through binding to dioxin response element (DRE) in promoter. Previous research suggests that inhibitory DRE (iDRE) which contains DRE core sequences (5'-GCGTG-3') for AHR binding may exhibit in the promoter of 17β-estradiol (E2) target genes and disrupt the E2-mediated transactivation of these genes (Duan, R. et al. 1999; Gillesby, B.E. et al. 1997; Krishnan, V. et al. 1995; Porter, W. et al. 2001; Wang, F. et al. 2001). There are two putative pentanucleotide core sequences of DRE (-168 to -164 and -739 to -735) in the promoter of human eNOS gene, another target gene regulated by estrogen on transcription level (Kleinert, H. et al. 1998; Zhang, R. et al. 1995). However, no research

has demonstrated any inhibitory effects of activated AHR on eNOS induction by E2. Our data suggest an inhibitory effect of endogenous AHR on basal eNOS mRNA expression, which may involve a recruitment of AHR to eNOS promoter to disrupt constitutive transcription activity of this gene. Therefore, we can hypothesize that endogenous AHR suppresses eNOS transcription by interrupting constitutive activity of transcription machinery, thus loss of endogenous AHR increases transcription of eNOS mRNA in endothelial cells.

To test this hypothesis, we need to determine: 1) whether any mediating factors are involved in eNOS mRNA suppression by AHR signaling; 2) whether the eNOS mRNA suppression by AHR is on transcription level; 3) whether AHR binding in promoter of eNOS is required for the transcription suppression.

To determine whether any mediating factors are involved in eNOS mRNA suppression by AHR signaling, we can block protein synthesis after AHR knockdown with cycloheximide. Inhibition of protein translation will not affect eNOS mRNA over expression by AHR knockdown, and eNOS mRNA normalization by TCDD simultaneous treatment if the hypothesis is correct.

To determine whether the eNOS mRNA suppression by AHR is on transcription level, we can perform nuclear run on assay on cells treated with control or AHR siRNA. Isolated nuclei will exhibit a higher transcription rate of eNOS gene with AHR siRNA knockdown if our hypothesis is true.

To determine whether AHR binding in promoter of eNOS is required for the transcription suppression, we will transfect cells with eNOS promoter-driving luciferase

reporter containing intact or mutated putative iDRE in promoter. The difference in luciferase activity of different reporters with and without AHR siRNA treatment will indicate the role of DNA binding in transcription suppression.

Pharmaceutical therapy. As a regulator of blood pressure and a suppressor of eNOS gene, AHR represents a potential target of pharmaceutical drugs in hypertension and vascular diseases treatment. Drugs that antagonize AHR may be able to reduce high blood pressure and increase vascular NO production in patients with hypertension and vascular diseases. Our research also demonstrated an enhanced vascular response to PE in AHR heterozygous mice, which exhibit normal blood pressure, suggesting a greater contribution of vasoconstrictor in vascular tone control and maintaining blood pressure with attenuated AHR signaling. The normal blood pressure of AHR heterozygous mice may be caused by a combined effect of eNOS over expression and increase of vasoconstrictor mediated vascular resistance. Since AHR heterozygous mice resemble the condition of AHR antagonist treatment, simultaneous administration of AHR antagonists and other drugs that target vasoconstrictor pathway may improve the treatment of hypertension by decreasing blood pressure more sufficiently.

Gene therapy. Our initial research on the role of endogenous AHR on eNOS expression also offers a potential target for gene therapy of high blood pressure and other vascular diseases. As an activator of xenobiotic metabolizing enzyme transcription, AHR may not represent an ideal target of genetic modification. If our hypothesis above is true, which means AHR suppresses eNOS expression through binding to its promoter, the binding site of AHR in eNOS promoter could be a perfect target for gene therapy. Low affinity sequence for AHR could be inserted into this region to substitute the iDRE and

eliminate the suppression effect of AHR. The expression level of eNOS could be controlled by delivering sequences with different AHR binding affinity.

CHAPTER VII

APPENDIX

ONE ALLELE OF AHR IS SUFFICIENT IN MAINTAINING NORMAL BLOOD PRESSURE AND VASCULAR eNOS EXPRESSION

Hypothesis. One allele of AHR is sufficient in maintaining normal blood pressure and vascular eNOS expression, thus AHR heterozygous mice exhibit normal blood pressure, vascular constitutive NO dependent vasodilation and eNOS expression.

Methods. Systemic arterial blood pressure and heart rate of $AHR^{+/-}$ mice were measured as previously described. Aortic reactivity to PE ± LNNA was assessed on $AHR^{+/-}$ aortas as previously described. Aortic eNOS protein expression was assessed by western blot as previously described.

Results. Systemic mean arterial blood pressure and heart rate were measured by radiotelemetry 24 hours/day for 4 days in conscious wildtype and AHR heterozygous mice. AHR heterozygous mice exhibited the same mean arterial blood pressure ($AHR^{+/+}$: 105.9 ± 0.8 mm Hg; $AHR^{+/-}$: 107.7 ± 1.7 mm Hg, Figure 31A) and significantly greater heart rate ($AHR^{+/+}$: 528 ± 12 bpm; $AHR^{+/-}$: 573 ± 9 bpm, Figure 31B) compared with wildtype animals.

AHR heterozygous mice exhibited a significantly greater vasocontraction in response to high concentrations of PE either with or without LNNA incubation compared with wildtype animals (Figure 32A and B). In addition, the LNNA incubated aortas from AHR heterozygous exhibited a significantly greater response to as low as $10^{-7.5}$ M PE (Figure 32B). We subtracted the contraction values to PE without LNNA preincubation from that with LNNA preincubation at each PE concentration. This result represents the degree of vasocontraction that is constitutively blocked by endogenous NO. There was a

significantly greater degree of vasocontraction offset by constitutive NO in the aortas of AHR heterozygous mice than in wildtype aortas only at medium PE concentrations (Figure 32C).

Total eNOS protein expression was not altered in the aortas of AHR heterozygous mice, compared to wildtype mice (Figure 33).

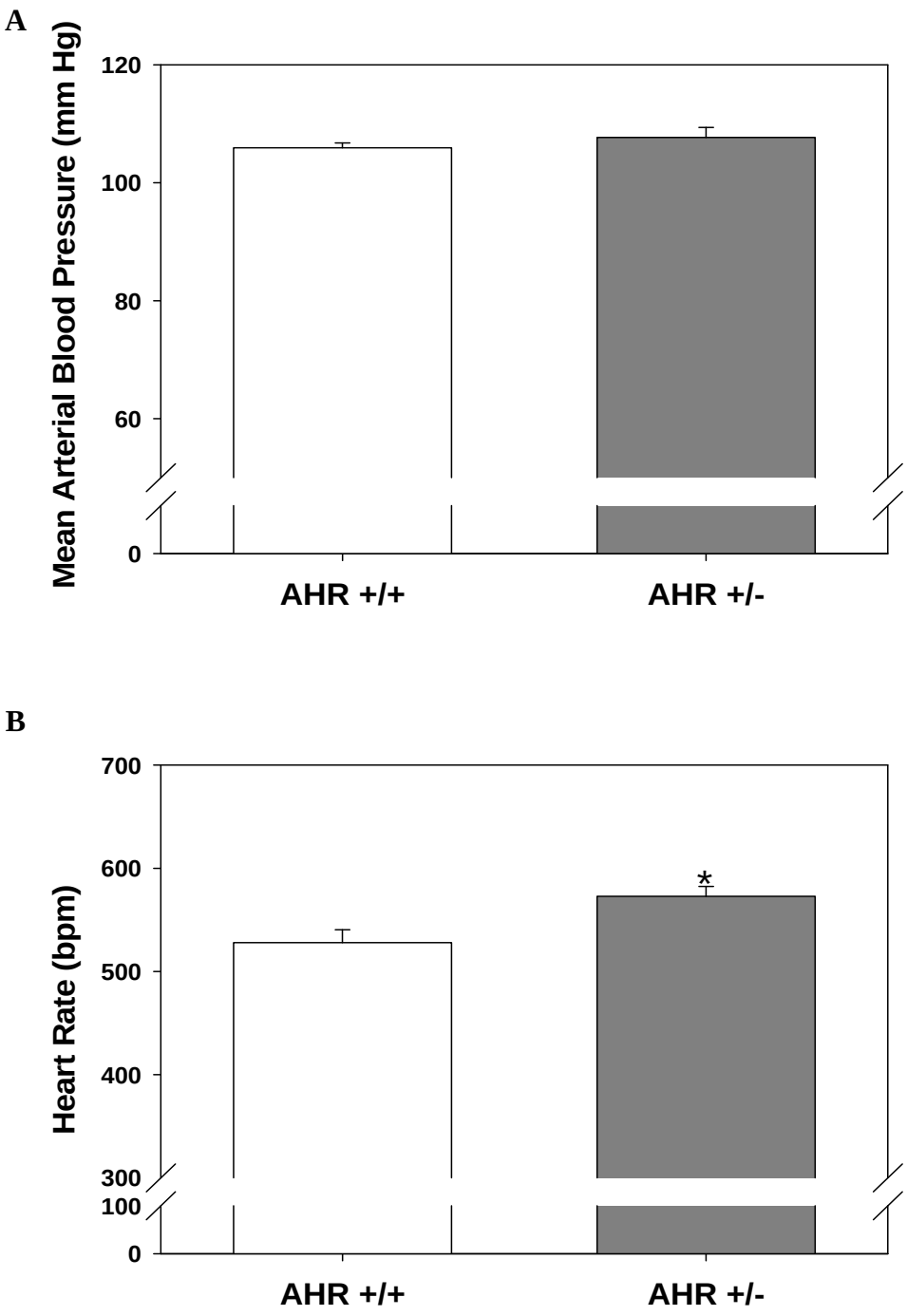

Figure 31. Systemic mean arterial blood pressure (A) and heart rate (B) of $AHR^{+/+}$ and $AHR^{+/-}$ mice. $AHR^{+/+}$: n=11; $AHR^{+/-}$: n=6. The data represent the mean ± SEM and were analyzed by Student's t-test; *$p < 0.05$, compared to $AHR^{+/+}$.

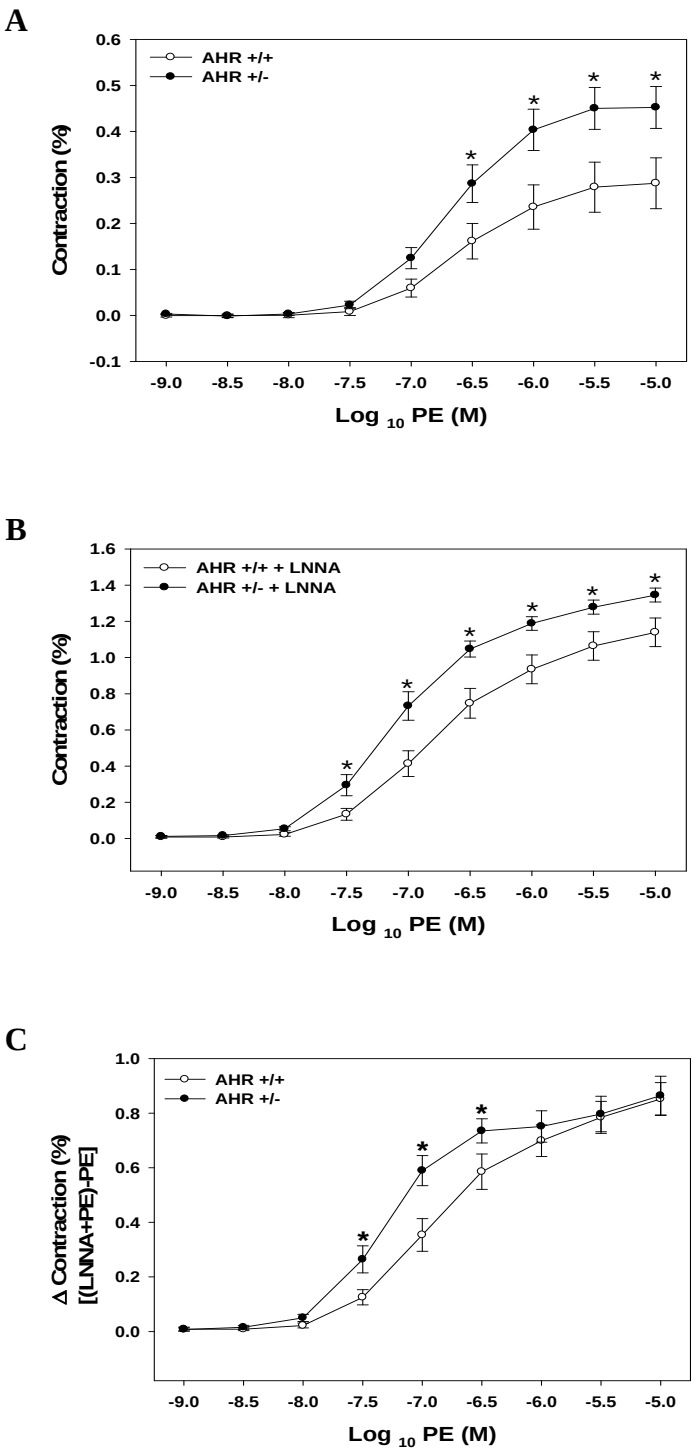

Figure 32. Vasocontraction to PE (A), LNNA + PE (B) and [(LNNA + PE) – PE] (C) of aortic rings from $AHR^{+/+}$ and $AHR^{+/-}$ mice. $AHR^{+/+}$, n=6; $AHR^{+/-}$, n=6. The data represent the mean ± SEM and were analyzed by two-way repeated measures ANOVA, using post hoc Holm-Sidak comparisons; *$p < 0.05$, compared to $AHR^{+/+}$ at the given concentration.

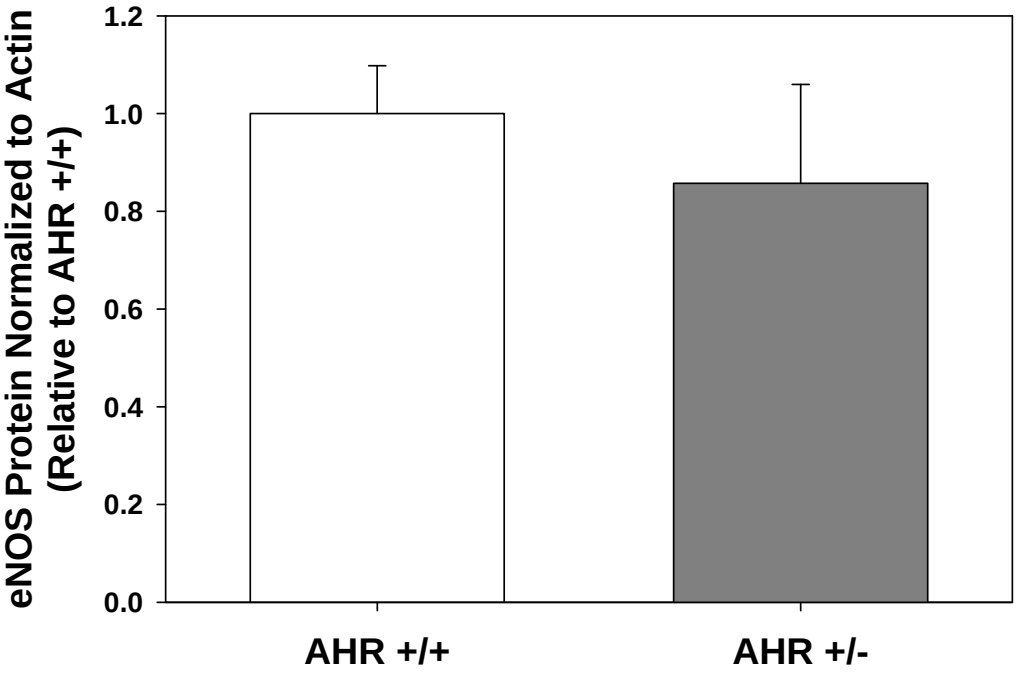

Figure 33. Aortic total eNOS protein expression of $AHR^{+/+}$ and $AHR^{+/-}$ mice. $AHR^{+/+}$, n=4; $AHR^{+/-}$, n=8. The data represent the mean ± SEM and were analyzed by Student's t-test.

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