THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN THE DEVELOPMENT OF ESSENTIAL HYPERTENSION

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TITLE: The Role of Endoplasmic Reticulum Stress in the Development of Essential Hypertension

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ABSTRACT

Essential hypertension is the leading contributor to premature death worldwide. Endoplasmic reticulum (ER) stress has recently been implicated in diseased blood vessels and hypertension. It is unclear whether ER stress is a cause or a consequence of hypertension. We hypothesized that ER stress inhibition would prevent the development of hypertension in the young spontaneously hypertensive rat (SHR) by improving vascular structure and function. The SHR was used as a genetic model of human essential hypertension, and the Wistar Kyoto (WKY) rat as its normotensive control. The first study conducted involved assessing the levels of ER stress in young SHRs, before they developed hypertension. The second study conducted involved treating rats with 1g/kg/day of the sodium salt of 4-phenylbutyric acid (4-PBA) orally for 8 weeks from 5 weeks of age. Blood pressure was measured weekly, noninvasively via radiotelemetry. Mesenteric arteries were collected at sacrifice. Finally, the third study conducted involved treating rats with 1g/kg/day 4-PBA orally for eight weeks from five weeks of age, and then withdrawing the drug for four weeks to determine if drug treatment created a sustained lowering of blood pressure. In the first study, ER stress markers were observed to be significantly increased in the young SHR when compared to the WKY. In the second study, blood pressure was observed to be significantly lower in the 4-PBA-treated SHR groups than in the untreated SHRs. In addition, mesenteric arteries from the 4-PBA treated SHRs had a significant decrease in media/lumen ratio, ER stress marker expressions, as well as improved vasodilatory response to carbachol and reduced contractile responses to phenylephrine. In the third study, 4-PBA was able to keep the blood pressure low for one week after withdrawal, however, blood pressure returned to similar levels as untreated SHRs by the end of three weeks. Overall, ER stress inhibition, via 4-PBA, blunted the development of hypertension in the SHR.

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LIST OF ABBREVIATIONS

4-PBA	4-phenylbutyric acid
ACE	angiotensin converting enzyme inhibitor
ARB	angiotensin II receptor blocker
ANOVA	analysis of variance
α-SMA	alpha smooth muscle actin
Ang II	angiotensin II
ATF6	activating transcription factor 6
BP	blood pressure
CCB	calcium channel blocker
CHOP	C/EBP homologous protein
CKD	chronic kidney disease
CO	cardiac output
DBP	diastolic blood pressure
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FDA	Food and Drug Administration
GRP78	glucose regulated protein, 78 kDa
GRP94	glucose regulated protein, 94 kDa
IRE1	inositol-requiring enzyme 1
kDa	kilodalton

mmHg	millimeter of mercury
NO	nitric oxide
PERK	protein kinase RNA-like endoplasmic reticulum kinase
RAAS	renin-angiotensin-aldosterone system
SBP	systolic blood pressure
SHR	spontaneously hypertensive rat
SPRINT	Systolic Blood Pressure Intervention Trial
TPR	total peripheral resistance
TUDCA	tauroursodeoxycholic acid
UPR	unfolded protein response
WKY	Wistar-Kyoto

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DECLARATION OF ACADEMIC ACHIEVEMENT

Telemetry implantation and vessel studies were performed with the assistance of Dr. Chao Lu.

1. INTRODUCTION

1.1 General Overview

Hypertension, high blood pressure, is a highly prevalent disease worldwide and in Canada. The current prevalence is greater than 22% within the Canadian population. Approximately 17% of people with hypertension are unaware of their condition. Thus, the exact prevalence of hypertension in Canada is most likely higher (Government of Canada, 2009). It is for this reason that hypertension is referred to many specialists as the 'silent killer' (CDC, 2017). According to the World Health Organization, hypertension is responsible for approximately 7.5 million deaths each year, which accounts for 12.8% of the total deaths worldwide (World Health Organization, 2017). Hypertension contributes to excess mortality primarily through inducing end organ damage in the heart, kidney, brain, and the vasculature (Government of Canada, 2009). The recent Systolic Blood Pressure Intervention Trial (SPRINT) found that recommending a target systolic blood pressure of lower than 120 millimeters of mercury (mmHg) was associated with lower cardiovascular disease and lower mortality rates when compared to current guidelines that recommend a systolic target of less than 140 mmHg (Cushman et al., 2016). Thus, new therapeutic interventions are required to make sure more challenging target such as this is achievable.

1.2 Hypertension

Hypertension is defined as an elevated blood pressure over 140 mmHg systole and over 90 mmHg diastole (Carretero & Oparil, 2000). This complex, multifactorial disease involves the interaction of multiple genes at various genetic loci and the influence of environmental factors, such as diet and lifestyle, to ultimately determine long-term arterial pressure. Hypertension increases the risk for cerebral, cardiac and renal events (Messerli, Williams, & Ritz, 2007). Cardiac and renal systems are essential for long-term blood pressure regulation, and thus damage to these systems can aggravate hypertension development. Approximately 95% of high blood pressure cases are primary hypertension, high blood pressure with no known cause, and the other 5% are secondary hypertension, high blood pressure with a known direct cause (Carretero & Oparil, 2000). Thus, despite its high prevalence, there is much controversy regarding the biological cause of primary hypertension. There are many antihypertensive agents that are given to hypertensive patients and that prevent the rise of blood pressure. Once a patient is diagnosed with hypertension, initial therapy consists of monotherapy or single pill combinations. Monotherapies include thiazide/thiazide-like diuretics, beta-blockers, angiotensin converting enzyme inhibitor (ACEI), angiotensin receptor blocker (ARB) or a long-acting calcium channel blocker (CCB). When target systolic and diastolic blood pressures are not achieved, single-dose monotherapy is changed to a combination therapy consisting of 2 or more first-line agents excluding the combination of ACEI and ARB. However, the effect of these drugs on long-term blood pressure, once treatment is stopped, depends on the type of treatment combinations (Hypertension Canada, 2017). There have been concerns over drug interactions between antihypertensive drugs' during combination therapy. There have also been concerns over many of the first-line hypertensive drugs metabolic effects (Karnes & Cooper-DeHoff, 2009).

1.3 Blood Pressure

Blood pressure (BP) is defined as the pressure of blood on the walls of blood vessels. It is expressed as a measurement of two numbers: systolic blood pressure over diastolic blood pressure. Systolic blood pressure is measured when the heart beats as it pushes blood to the rest of the body, placing pressure on the walls of the arteries. Diastolic blood pressure is measured when the heart is between beats, collecting blood and relaxing. Blood pressure is measured in millimeters of mercury (mmHg). The well-established normal blood pressure is 120/80 mmHg (Mohrman & Heller, 2006).

Two factors affect blood pressure: cardiac output (CO) and total peripheral resistance (TPR). CO is defined as the total volume of blood ejected by the heart per minute. TPR is a product of several factors: the size of the lumen of the blood vessels, blood viscosity, and total blood vessel length. The most significant contributor to TPR has been shown to be the size of the blood vessel lumen (Choi, Lim, Byeon, & Lee, 2016). Since BP is a product of CO and TPR, an increase in either elevates BP.

$$\mathbf{BP = (CO) * (TPR)} \qquad \textbf{- Equation 1}$$

There are three physiological processes that regulate blood pressure. Short-term regulation of blood pressure is achieved through direct innervations of the vascular smooth muscle cells by the sympathetic nervous system. The baroreceptor reflex operates on slightly longer time scale and is useful in preventing orthostatic hypotension (Kouchaki, Butlin, Lovett, & Avolio, 2015; Takahashi & Naruse, 2016). Long-term regulation of blood pressure is achieved through the kidney including the action of the renin-angiotensin-aldosterone system (RAAS) (Takahashi & Naruse, 2016). Additionally, the myogenic response, an autoregulatory

mechanism, maintains a constant blood flow by allowing arteries and arterioles to constrict or dilate. These systems regulate blood pressure by inducing a change in the relative size of the blood vessel lumen or by altering blood volume thereby influencing cardiac output (Mohrman & Heller, 2006). Overall, the ability of resistance blood vessels to respond to these stimuli becomes altered during the development of hypertension.

1.4 Endothelial Dysfunction and Hypertension

Blood vessel structure is made up of three layers: 1) the intima, the most inner layer, is comprised of endothelial cells, 2) the media, the middle layer, is comprised of smooth muscle cells, and 3) the adventitia, the most superficial layer, is comprised of fibroblasts, collagen and elastic fibers with embedded sympathetic nerve varicosities (Mohrman & Heller, 2006). One of the major contributors to hypertension is endothelial dysfunction, in which the ability of blood vessels to relax is compromised (Carlisle et al., 2016; Touyz, 2004). Changes in the structure and function of resistance blood vessels have been shown to precede hypertension development in the spontaneously hypertensive rat (Dickhout & Lee, 1997; Schiffrin, 2004). One important characteristic of vascular dysfunction is a decrease in nitric oxide (NO) bioavailability, which reduces endothelial vasodilatory response. This has been found in both categories of human essential hypertension (Giles, Sander, Nossaman, & Kadowitz, 2012; J. B. Park & Schiffrin, 2001) and hypertensive animal models (Rajapakse & Mattson, 2013). Endothelial nitric oxide synthase (eNOS) knockout mice, which have endothelial nitric oxide synthase deficiency, develop hypertension (Shesely et al., 1996). Studies have shown that endothelial dysfunction in human prehypertensives is one of the best predictors of hypertension development (J. B. Park &

Schiffrin, 2001; Quyyumi & Patel, 2010). Thus, therapeutic interventions that lower blood pressure and improve endothelial dysfunction usually result in the lowest rate of cardiovascular events (Ho, Karimi Galougahi, Liu, Bhindi, & Figtree, 2013; Kitta et al., 2009). Several studies have demonstrated that it is the lack of NO bioavailability, not a deficiency in smooth muscle to respond to NO donors, that result in hypertension (Giles et al., 2012). Free radicals such as superoxide anions have been shown to readily react with NO and could be the cause of the decrease in NO bioavailability (Dickhout et al., 2005; Touyz, 2004). Overall, correction of blood vessel structure and/or function is one systematic approach to lower blood pressure and prevent the end organ damage associated with essential hypertension.

1.5 Endoplasmic Reticulum Stress and the Unfolded Protein Response

One important factor that is potentially involved in the development of essential hypertension is endoplasmic reticulum stress. The endoplasmic reticulum (ER) is a network of tubules and flattened sacs that has a wide range of functions. The ER manufactures secretory and membrane proteins (Chiang et al., 2011). It is the site of synthesis of lipids and carbohydrates, and plays a role in calcium storage (Minamino, Komuro, & Kitakaze, 2010). It also provides the optimal biochemical environment for protein folding and assembly, producing functional and mature proteins (Kitamura, 2008). Certain protein mutations prevent proper protein folding which consequently causes an accumulation of unfolded proteins in the lumen of the ER (Wei, Rahman, Ayaub, Dickhout, & Ask, 2013). This excess of misfolded proteins and other disruptions in ER homeostasis cause ER stress and can be visualized by dilations in the rough ER (Dickhout, Carlisle, & Austin, 2011). Such accumulation of proteins activates an intracellular

signaling pathway called the unfolded protein response (UPR). The UPR pathway has two major roles: 1) to increase the cells protein folding capacity and/or 2) to signal the cell towards apoptosis if this cannot be done properly (Dickhout et al., 2011; Dickhout & Krepinsky, 2009). Initially, the UPR pathway deactivates protein translation and activates protein folding chaperones to bring the cell back to homeostasis. However, if the cell is continuously disrupted and the chaperones are unable to fold all the misfolded proteins, then UPR signals cell apoptosis (Malhotra & Kaufman, 2007). The UPR consists of three different pathways mediated by the protein kinase-ER kinase (PERK), inositol requiring kinase (IRE1), and activating transcription factors (ATF6) (Dickhout & Krepinsky, 2009). Glucose-related protein 78 (GRP78), a molecular chaperone, is bound to these three UPR activators, and its dissociation results in UPR activation. In the IRE1 pathway, GRP78 dissociation results in IRE1 (Inositol requiring enzyme 1) autophosphorylation and dimerization. The activation of the cytosolic domain of IRE1 catalyzes the splicing of the 253bp intron and the activation of the X-box binding protein (XPB1) transcription factor. This facilitates the expression of UPR genes which increases both ER expansion and the level of ER chaperones, ultimately enabling proper protein folding and degradation. Moreover, in the protein kinase-ER kinase (PERK) pathway, GRP78 dissociation results in PERK autophosphorylation and dimerization. The activation of the cytosolic domain of PERK phosphorylates the eukaryotic translation initiation factor 2alpha (eif2alpha). This phosphorylation results in global inhibition of protein translation and is required for ATF4 expression (Dickhout et al., 2011). ATF4 is a transcription factor leading to CCAAT enhancer binding protein (C/EBP) homologous protein (CHOP) expression. CHOP contributes to the dephosphorylation of eIF2alpha, and re-establishing translation. CHOP also has pro-apoptotic properties. CHOP is a common target for not only the PERK pathway, but also the ATF6

pathway. However, the exact mechanism by which CHOP induces apoptosis is unclear. Lastly, in the ATF6 pathway, GRP78 dissociation leads to the translocation of ATF6 from the ER to the Golgi body where site 1 and site 2 proteases cleave ATF6 to produce a catalytic fragment to produce a 50 kilodalton (kDa) fragment. This fragment is able to find and induce expression of UPR target genes by binding to their ER response element (ERSE) (Minamino et al., 2010) and facilitate the expression of ER chaperones and genes involved in endoplasmic reticulum associated degradation (ERAD) (Inagi, 2009).

1.6 Endoplasmic Reticulum Stress and Hypertension

There is increasing evidence demonstrating the involvement of ER stress and the UPR pathway in a variety of diseases including hypertension. Relevant to this project, the presence of ER stress in resistant blood vessels (Dickhout et al., 2005; Dickhout, Colgan, Lhoták, & Austin, 2007) may contribute to the development of hypertension (Kassan et al., 2012). It is important to note that ER-associated protein degradation to dispose of misfolded proteins occurs through disulfide bond disintegration which generate reactive oxygen species, specifically superoxide anions (Dickhout et al., 2011; Malhotra & Kaufman, 2007; Walter & Ron, 2011). Superoxide anions are known to readily react with NO to form peroxynitrite. Peroxynitrite is not a free radical; however, it is a powerful oxidant that can damage DNA and proteins. Peroxynitrite is an ER stress inducer (Dickhout et al., 2005). This is a plausible mechanism linking ER stress and hypertension and a visual representation is illustrated in **Figure 1**. In addition, recent studies have demonstrated a significant increase in the expression levels of many ER stress markers (GRP78, PERK, and CHOP) in the subfornical region of the brain in mice with angiotensin II-

induced hypertension. Co-treating with an ER stress inhibitor, tauroursodeoxycholic acid (TUDCA) prevented an increase in mean arterial pressure in these mice. Angiotensin II-induced hypertension has also been shown to result in cardiac hypertrophy and fibrosis in C57BL/6 (C57 black 6) mice. These effects were prevented by ER stress inhibition (Kassan et al., 2012). Another study demonstrated that ER stress inhibition, via TUDCA and 4-PBA, decreased systolic blood pressure and normalized aortic contractions to acetylcholine (Spitler, Matsumoto, & Webb, 2013). Yum et al. demonstrated that ER stress inhibition decreases salt-induced hypertension in Dahl salt-sensitive hypertensive rats (Yum et al., 2017). Carlisle et al. demonstrated that ER stress causes endothelial dysfunction, and that ER stress inhibition with 4-PBA lowers blood pressure in adult spontaneously hypertensive rats (SHR) (Carlisle et al., 2016). According to the work of Palao et al., there may be a genetic cause of ER stress in blood vessels of hypertensive animals. Different gene expression levels between spontaneously hypertensive rats and their control, the Wistar-Kyoto rats, were observed in their mesenteric arteries. The most upregulated gene in the SHR was thrombospondin 4, which encodes a protein that is involved in the ER stress response, specifically activating ATF6 levels and its downstream targets (Palao et al., 2015). These studies collectively suggest a link between ER stress and hypertension.

1.7 Endoplasmic Reticulum Stress Inhibitor, the Sodium Salt of 4-phenylbutyric Acid

4-phenylbutyric acid (4-PBA), a sodium salt, is a low molecular weight chaperone. 4-PBA is a drug approved by the food and drug administration (FDA) for clinical use to treat elevated blood ammonia in urea cycle disorders (Kolb et al., 2015; Teckman, 2004). 4-PBA has

three main mechanisms of action: it is an ammonia scavenger, a weak histone deacetylase inhibitor, and an ER stress inhibitor (Kolb et al., 2015). As an ammonia scavenger, 4-PBA decreases free ammonia levels through the clearance of glutamine. Glutamine is a substrate for ammoniagenesis, which can lead to hyperammoniaemia. 4-PBA can prevent hyperammoniaemia by causing the excretion of glutamine. In short, 4-PBA is converted to phenylacetate, which then covalently binds with circulating glutamine. This forms phenylacetylglutamine, which is excreted by the kidneys. Histone acetylation regulates gene expression by modulating chromatin structure. Histone deacetylase inhibitors prevent the removal of acetyl groups from histones, resulting in histone hyperacetylation, and altered gene expression. This can result in antifibrogenic effects in organs such as the liver, skin, lungs, and kidneys. However, the mechanisms behind these actions are unknown. Interestingly, histone deacetylase inhibitors acetylate spliced XBP1, increasing stability and transcriptional activity (F.-M. Wang, Chen, & Ouyang, 2011). Spliced XBP1 is a key transcriptional inducer of the IRE1 arm of the UPR and may protect against apoptosis by upregulating the expression of protein folding chaperones, including GRP78. 4-PBA dampens UPR marker expression by utilizing its chaperone-like property. It is expected to compensate for the unfolded proteins that were accumulating in the ER lumen as a result of ER stress (Kolb et al., 2015). This will allow the GRP78 protein to stay bound to the UPR activators and thus stalls and inhibits the induction of the UPR response (Ayala et al., 2012).

4-PBA has been used in a variety of disease models including cystic fibrosis where it reduced the number of unfolded proteins in the ER due to protein mutation and reduced lung injury (Zeitlin et al., 2002). 4-PBA has also been shown to attenuate cardiac hypertrophy in transverse aortic constriction (TAC)-operated mouse (C. S. Park, Cha, Kwon, Sreenivasaiah, &

others, 2012), attenuate apoptosis in bladder outlet obstruction in rats (Sawada et al., 2008) and have neuroprotective properties in cerebral ischemia injured rats (Qi, Hosoi, Okuma, Kaneko, & Nomura, 2004). Of relevance to this project is the use of 4-PBA to reduce high blood pressure and prevent the development of hypertension in spontaneously hypertensive rats. Kassan et al. demonstrated that 4-PBA was able to reduce BP in a mouse model of chronic angiotensin II-induced hypertension (Kassan et al., 2012). 4-PBA was also able to augment endothelium-dependent vasodilatory responses, elicited by acetylcholine, in mesenteric resistance arteries of angiotensin II-infused mice (Kassan et al., 2012). In our laboratory at St. Joseph's Healthcare Hamilton, our research group conducted a study looking at the effects of 4-PBA in adult spontaneously hypertensive rats (Carlisle et al., 2016) and Dahl salt-sensitive hypertensive rats (Yum et al., 2017). Our research group demonstrated that 4-PBA was able to significantly lower blood pressure in adult SHRs and Dahl SS rats treated with 4-PBA versus untreated rats. In addition, it was demonstrated that 4-PBA treated vessels had significantly lower media-to-lumen ratio, lower ER stress markers expression and reduced contractility (Carlisle et al., 2016; Yum et al., 2017). Thus, the use of 4-PBA in a variety of studies appears promising for its use in the context of reducing high blood pressure and in preventing the progression of end organ damage.

1.8 Animal Model

Several models of essential hypertension exist. The hypertensive animal model that was used for the purpose of this Master's project is the spontaneously hypertensive rat (SHR). The normotensive control is the Wistar-Kyoto (WKY) rat. In the 1960s, Okamoto and colleagues created the SHR strain by breeding Wistar-Kyoto rats with high blood pressure (Pfeffer, Pfeffer, Weiss, & Frohlich, 1977). In the absence of external factors, the SHR develops high BP

spontaneously; some reports have observed the development of significant BP difference between the SHR and WKY by 3 weeks of age (Lais, Rios, Boutelle, DiBona, & Brody, 1977; R. M. Lee, 1985; Smeda, Lee, & Forrest, 1988). However, other reports have found no significant difference between strains at 4 weeks of age (Adams, Bobik, & Korner, 1989; Dickhout & Lee, 1997; Tsuji, Su, & Lee, 1989). The SHR develops little to no renal injury. However, the kidney does play an important role in the development of hypertension in the SHR. Studies have shown that transplanting an SHR kidney to a WKY rat resulted in the WKY developing hypertension. Contrary, when a WKY kidney was transplanted into an SHR, the SHR did not develop hypertension (Kawabe, Watanabe, Shiono, & Sokabe, 1978; Rettig, 1993). Earlier studies have looked at the therapeutic effect of first-line antihypertensive drugs in lowering hypertension in the SHR. Beta-blockers and calcium antagonist given throughout the development of the SHR, have shown to prevent the rise of blood pressure, however, when treatment is stopped in adult life, high levels of blood pressure reoccur, to a similar extent as untreated SHR (Christensen, Jespersen, & Mulvany, 1989; Harrap, Merwe, Griffin, Macpherson, & Lever, 1990). In contrast, treatment with ACE inhibitors such as perindopril and ramipril have been shown to exert persistent antihypertensive effects, even after stopping of treatment (Harrap et al., 1990; Linz, Jessen, Becker, Schölkens, & Wiemer, 1997; H. Wang, Delaney, Kwiecien, Smeda, & Lee, 1997). Treatment with ACE inhibitors have been shown to significantly reduce contractility, enhance endothelial-dependent relaxation in the SHR aorta and normalize morphological alterations in SHR blood vessels compared to control (Bennett, Hillier, & Thurston, 1996; Clozel, Kuhn, & Hefti, 1990). Many studies have looked at therapeutic effects of various anti-hypertensive drugs, used clinically to treat hypertension disease, in lowering high blood pressure or preventing high blood pressure in SHR. Many of these studies are summarized in **Table 1**.

1.9 Question and Hypothesis

Based on our findings and the work of others, it is clear that ER stress plays a critical role in hypertension. An underlying question we sought to answer was if ER stress is a cause of essential hypertension or merely a consequence. Integrating Koch's postulates of causality to this project, for a change to be considered causative, it must precede the development of hypertension and a treatment that affects the change should lower blood pressure. Overall, we hypothesized that inhibiting ER stress in the blood vessels would prevent the development of essential hypertension in the SHR.

This project consisted of three aims to address our main hypothesis. In the first aim, we were interested in determining if ER stress is present in the SHR before the development of hypertension, and if there were differences in the levels of ER stress markers and vasculature dysfunction between WKY and SHR at 5 weeks of age. We hypothesized that in this study we would observe the presence of ER stress in the blood vessels before the onset of hypertension in the SHR. In the second aim, we were interested in determining if inhibition of ER stress with the small molecular chaperone 4-PBA is effective in preventing the development of high blood pressure in the SHR. We hypothesized that inhibiting ER stress with 4-PBA would restore proteostasis in the blood vessels and prevent the development of essential hypertension in the SHR. In the third aim, we were interested in determining if 4-PBA permanently lowers blood pressure after treatment withdrawal. We hypothesized that 4-PBA would permanently alter blood vessel structure and function, and thus permanently sustain low blood pressure after drug withdrawal.

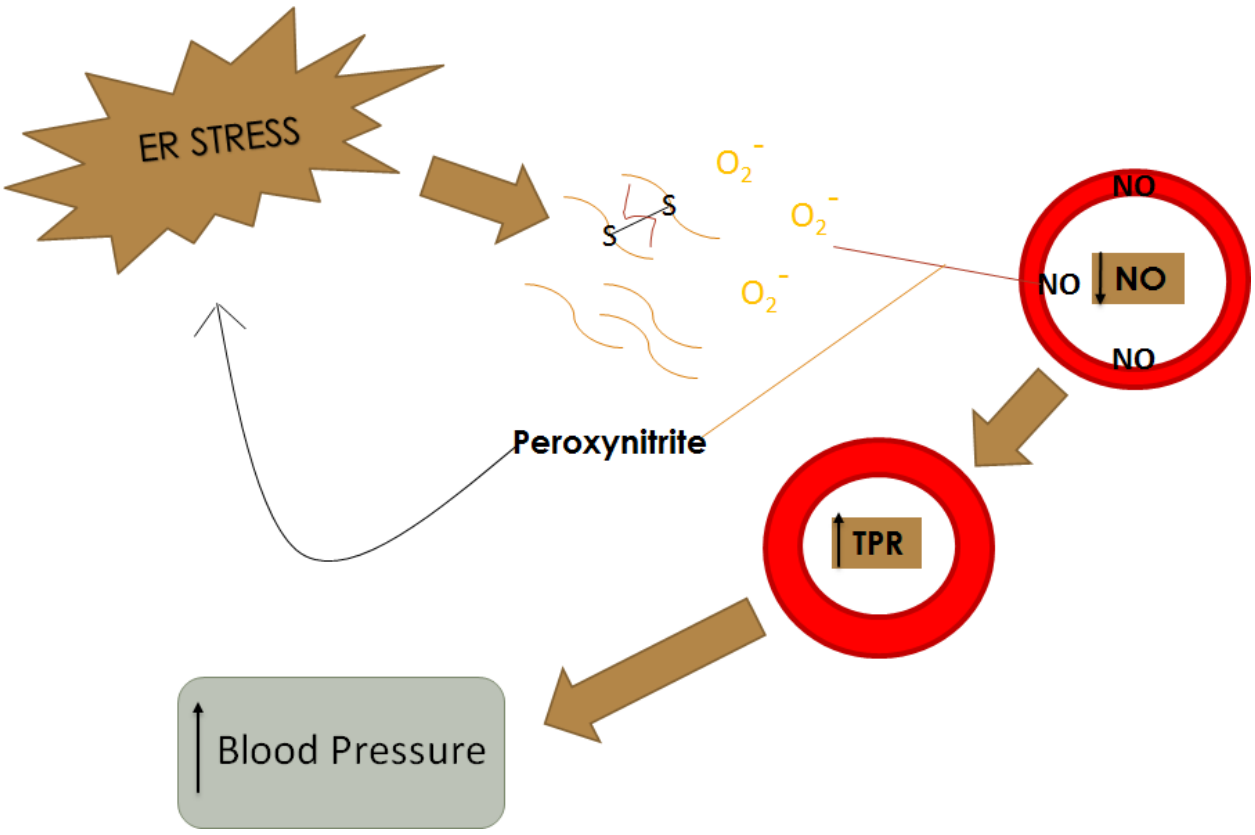


Figure 1. Proposed mechanism linking endoplasmic reticulum stress and hypertension. The degradation of misfolded or unfolded proteins, through disulfide bond disintegration, during excessive ER stress, produces superoxide anions (O_2^-). O_2^-, a free radical, is known to readily bind with nitric oxide (NO) in the blood vessels, resulting in a decrease of NO bioavailability in these blood vessels. NO is a common vasodilator found in blood vessels and thus a decrease in NO affects the vasodilatory responses of the blood vessels, resulting in a decrease in the lumen size of vessels. A decrease in lumen size causes an increase in the total peripheral resistance. A major determent of BP is total peripheral resistance, thus an increase in total peripheral resistance causes an increase in BP. The interaction between O_2^- and NO is involved in a negative feedback loop producing peroxynitrite, an ER stress inducer.

DRUG TX	MECHANISM OF ACTION	INTERVENTION	MAJOR RESULTS	AUTHOR/ DATE
PERINDOPRIL	ACEI	Dose: 3mg/kg/day Age: 2-6, 6-10, or 2-10 weeks	SBP measured via tail-cuff until 25 weeks of age revealed perindopril significantly reduced BP during treatment, increased when treatment was withdrawn, but plateaued significantly lower than untreated SHR.	(Harrap et al., 1990)
NADOLOL	Beta Blocker	Dose: 320mg/L Age: gestation until 28 weeks	Nadolol treatment caused some lowering of BP, but did not prevent the development of hypertension. The lumen of large mesenteric arteries from control SHR was smaller than from WKY, and nadolol treatment increased the lumen size in the SHR. However, the changes produced by nadolol did not reach the levels of control treated WKY.	(R. M. K. W. Lee, Tsoporis, & Wang, 1992)
LOSARTAN (L) Vs. CATOPRIL (C)	L: ARBs C: ACEI	Dose L: 15 mg/kg/day Dose C: 100 mg/kg/day Age: 3 weeks – 30 weeks Duration of TX: 4 or 10 weeks	Both losartan and captopril given for 4 and 10 weeks prevented the development of hypertension during treatment and redevelopment of hypertension after treatment was stopped. Treatment for 10 weeks was more effective than for 4 weeks in lowering long-term pressure. Four weeks of treatment did not affect the mesenteric resistance artery media/lumen ratio. In contrast, both losartan and captopril given for 10 weeks resulted in large and significant reductions in media/lumen ratio.	(Morton, Beattie, & MacPherson, 1992)
ATENOLOL	Beta Blocker	Dose: 25 mg Kg-1 day-1 Age: 8 weeks – 22 weeks	Atenolol attenuated the increase in BP by 30 mmHg in SHR. The moderate antihypertensive effect of atenolol in SHR was accompanied by enhancement of β-adrenoceptor-mediated and normalization of endothelium-dependent arterial relaxation.	(Kähönen, Mäkynen, Arvola, & Pörsti, 1994)
PERINDOPRIL (P), QUINAPRIL (Q), HYDRALAZINE (H), AMLODIPINE (A)	P: long-acting ACEI Q: ACEI A: CCB H: Vasodilator	Dose P: 1mg/kg/day Dose Q: 3mg/kg/day Dose A: 10mg/kg/day Dose H: 50mg/kg/day Age: 3-21 weeks	All drugs prevented the rise in blood pressure found in untreated SHRs. Ach-induced relaxation was significantly impaired in the untreated SHRs compared with WKY rats. ACEIs prevented the development of this impaired response. Responses to SNP were not different between untreated SHRs and WKY rats and were not affected by drug treatment.	(Bennett et al., 1996)
INDAPAMID (I), PERINDOPRIL (P)	I: Thiazide-like diuretic P: long-acting ACEI	Dose I: 0.24 Dose P: 0.76 mg/kg per day Age: 10–12 weeks old for 8 weeks	Indapamide + perindopril combination caused a significant lowering of both SBP and DBP. Media/lumen ratios were all significantly reduced by I + P treatment. There was some improvement in endothelium- independent vasorelaxation of mesenteric vessels.	(Ibrahim, Schachter, Hughes, & Sever, 1999)
NIFEDIPINE	CCB	Dose: 50mg/kg/day Age: 4 weeks old, and 8 weeks old treated for 4 weeks	BP of nifedipine-treated SHRs remained at initial levels, in contrast to NT SHRs which kept increasing. Maximum response to noradrenaline was significantly attenuated in the 12-weeks-old nifidepine-treated SHRs.	(Zemancíková & Török, 2009)
LACIDIPINE (L), ENALAPRIL (E)	L: CCB E: ACEI	Dose L: 1.5 mg/kg, twice daily for 8 weeks Dose E: 5 mg/kg twice daily for 8 weeks Age: 12 weeks	As expected, untreated SHR showed elevated SBP, impaired vascular reactivity, increased LVW and prolonged QT when compared with WKY. After treatment, both agents markedly improved vascular reactivity and reduced SBP in SHR. Additionally, enalapril reduced left ventricular width in both hypertensive and normotensive rats and, consequently, corrected QT duration in SHR.	(Klimas, Vaja, Vercinska, Kyselovic, & Krenek, 2012)

TABLE 1. The effect of first-line anti-hypertensive drugs in the spontaneously hypertensive rat. All drugs described in the table are used clinically to treat hypertension and fall in the classes of first-line anti-hypertensive drugs that are commonly used either as monotherapy or in combination therapy to combat hypertension disease.

2.0 MATERIALS AND METHODS

2.1 Animal Studies

All animal work was approved by and performed according to the McMaster University Animal Research Ethics Board guidelines. Animals (SHR and WKY available from McMaster University Central Animal Facility) were implanted with radio-telemetry transmitters (HD-X11 transmitter, Data Sciences International) to acquire blood pressure development. In the first study, 5 week old SHR and WKY, that were fed a normal-sodium diet (0.4% NaCl, AIN-76A, Research Diets Inc., New Brunswick, New Jersey, USA), had their BP measured, via tail-cuff plethysmography and endpoint cannulation, and were used to examine the level of ER stress in their blood vessels before the onset of hypertension. In the second study, 5-week-old young male SHRs, and their 5-week-old normotensive controls, WKY, were used to examine the effect of 4-PBA on BP development. Animals were randomized into either 4-PBA or untreated groups. Treatment with 1g/kg per day of 4-PBA in the drinking water proceeded for 8 weeks. 4-PBA was adjusted in fresh drinking water every 3 days. All animals were fed a normal-sodium diet (0.4% NaCl, AIN-76A, Research Diets Inc., New Brunswick, New Jersey, USA). BP was measured 1 week before treatment (week 0) and after each treatment using radio-telemetry, a method that accurately determines BP in conscious and free moving rats. For the duration of the 8 week treatment, animals had their blood pressure measurements collected at 0, 1, 2, 3, 4, 5, 6, 7 and 8 weeks of age. Animals were then anesthetized with isoflourane and sacrificed after 8 weeks. Organs were harvested and mesenteric resistance vessels were collected for functional and structural analysis. Vessels were perfused with Hank's buffered salt solution (HBSS) containing sodium nitroprusside (SNP) (10^{-4} mol/l) to place the resistance arteries in a maximally relaxed

state for perfusion fixation. Another group of SHRs had 4-PBA withdrawn for four weeks after 8 weeks of 4-PBA treatment to assess the ability of 4-PBA to permanently lower BP.

2.2 Catheter Implantation Procedure

Implantation of radio-telemetry catheter to abdominal artery was performed using a rodent anesthesia machine. 4% isofluorane was administrated to induce anesthesia and 2-2.5% isofluorane was given as maintenance with oxygen provided at 1.5 L/min. Depth of anesthesia was monitored by foot pinch. Larcrillube was applied to the eyes. Tissue oxygen was monitored by mucous membrane color, respiration and heart rate. The abdominal area was shaved and cleaned with a 3-part scrub (proviodine, ethanol, proviodine). Using surgical scissors, a 4-5 cm midline incision through the abdominal wall was made. Care was taken to not damage internal organs. The abdominal artery was carefully isolated from the surrounding tissue. Vessel dilators were used to separate the aorta from the vena cava just caudal to the left renal vein. One piece of 4-0 non-absorbable suture was positioned underneath the isolated artery section. Using vessel dilators, the aorta was carefully separated from the vena cava just cranial to the iliac bifurcation. One piece of 4-0 suture was carefully passed between the vena cava and aorta so that the suture lied underneath the aorta. This suture was used to temporarily occlude blood flow to allow introduction of the catheter into the vessel. The artery was pierced 1-2 mm cranial to the iliac bifurcation by using the 22-gauge needle as a catheter introducer and the catheter was inserted upstream toward the heart. Once the catheter was inserted into the vessel, the catheter introducer was withdrawn. The catheter was secured and artery clamp released. The peritoneal cavity was irrigated with warm, sterile saline. The intestines were gently massaged back into place. The transmitter was placed on top of the intestines, positioning the transmitter parallel to the long

axis of the body with the catheter directed caudally and the suture rib directed ventrally. The abdominal wall was closed using 4-0 non-absorbable suture with a simple interrupted pattern. The suture rib was incorporated on the transmitter into the closure. The skin incision was closed using a skin stapler. The animal was taken off anesthesia and put into a clean heated recovery cage to regain consciousness. After 5 days of recovery period, blood pressure was monitored by the radio-telemetry system.

2.3 Tail Cuff Plethysmography

To acquire BP noninvasively, the tail cuff plethysmography methods was used to accurately determine tail blood volume with a volume pressure recording (VPR) sensor and an occlusion tail-cuff (CODA system, Kent Scientific). BP parameters were measured by placing a tail cuff on the tail of the rat to occlude blood flow and a tail cuff with the VPR sensory intact was placed distal to occlusion cuff. The occlusion cuff was slowly deflated, allowing the VPR sensor to measure the physiological characteristics of the returning blood systolic blood flow resulting in values for systolic, diastolic and mean BP, as well as, heart pulse rate, tail blood volume and tail blood flow (Feng et al., 2008).

2.4 Carotid Artery Cannulation

The catheter was placed in the left carotid artery and positioned so that the sensing region of the catheter was in the aortic arch. The transmitter portion of the device was ideally positioned along the lateral flank between the forelimb and hind limb. A subcutaneous pocket was formed by blunt dissection from the neck incision down along the animal's flank. The animal was

positioned on the surgery table with the head closest to the surgeon. Using small surgical scissors, a 1.5 cm midline incision was made through the skin on the neck. The carotid artery was located along the left side of the trachea using sterile cotton tip. Using fine tipped, curved forceps, the vessel was carefully isolated from the surrounding tissue, making sure not to disturb the vagus nerve. Three pieces of 5-0 or 6-0 non-absorbable suture was passed underneath the isolated artery section. The ligation suture was positioned just proximal to the bifurcation of the interior and exterior carotid arteries. A secure knot was tied around the artery to ligate the vessel and the suture tails were taped to the surgery table. Tension was gently applied to the occlusion suture closest to the clavicle using a hemostat. This helped elevate the artery and occlude blood flow. Using the 25-gauge needle as an introducer, the artery was pierced just proximal to the ligation suture and the catheter was inserted upstream toward the aorta. Once the catheter was inserted into the vessel, catheter introducer was withdrawn. The middle suture was positioned around the artery and catheter. The catheter was secured by pulling the loose suture tail to tighten the knot. The catheter was further advanced so that at least 2 mm of the sensing region of the catheter was positioned in the aortic arch. The occlusion suture and the midline suture were tightened around the artery and catheter to seal the artery wall around the catheter stem. The tension on the ligation suture was released and tied the loose ends around the catheter stem to help anchor it in place. Small surgical scissors into the incision was inserted and a subcutaneous pocket was formed by using blunt dissection. Once the pocket was formed, the pocket was irrigated with a 3 cc syringe filled with warm, sterile saline. The transmitter was inserted against the body. The terminal end of the positive lead (red tubing) was grasped with a small hemostat and tunneled subcutaneously from the neck incision to the left caudal rib region. The terminal end of the negative lead (clear tubing) was grasped with a small hemostat and tunneled

subcutaneously from the neck incision to the right pectoral muscle. The lead was released and the hemostat was withdrawn leaving the lead in place under the skin. Both leads were placed flat against the muscle for the whole length of the lead. Both leads were secured near the neck incision by placing a stay suture through the chest muscle and around the leads using 5-0 or 6-0 non-absorbable suture. The skin incision was closed.

2.5 Vascular Contractility Studies

Dose-dependent vasoconstriction studies to phenylephrine (PHE - α1 adrenergic receptor agonist that stimulates vasoconstriction; 10^{-8} M to 10^{-5} M) were performed in mesenteric resistance arteries of SHR and WKY rats. Arteries were harvested within 30 minutes of animal sacrifice, and put into HBSS solution on wet ice. Vessels were then mounted on a small vessel wire style heated myograph (M4 series Myograph System; Radnoti LLC, Monrovia, CA) maintained at 37°C, and bubbled with 100% O_2. Mesenteric resistance arteries were mounted with 0.3g of tension. Vessels were washed with HBSS and allowed to sit and balanced for 60 minutes. To induce smooth muscle mediated contraction directly, vessels were treated with 60 mM KCl. After a plateau of the contraction was observed, vessels were then washed with HBSS to restore membrane potential. These methods were performed prior to all contractility protocols. To determine the effect of 4-PBA treatment on the contractility of SHR and WKY mesenteric resistance vessels, the vessels were induced to contract with phenylephrine (PHE) (10^{-6}M). To access endothelial mediated dilation, vessels pre-constricted to 50% maximum PHE constriction were then treated in a cumulative manner from 10^{-8} to 10^{-5} M with carbachol (CCh; a cholinergic agonist that stimulates NO release from the endothelium) to determine the effect of 4-PBA treatment on the response to NO mediated vasodilators on endothelium-derived relaxation. After

washing, vessels were again constricted with PHE. To assess smooth muscle response to NO, 10^{-7} to 10^{-5}M sodium nitroprusside (SNP; a nitric oxide donor) was added to the baths to determine the effect of 4-PBA on nitric oxide-mediated relaxation. Data from isolated vessel studies were recorded using WINDAQ data acquisition software through a DI-720-USB Series analog to digital converter (DATAQ Instruments, Inc. Akron, OH, USA). Responses induced by PHE were expressed as a percentage of the initial 60 mM KCl response. Responses induced by CCh were expressed as a percentage relaxation of PHE-pre-constricted vessels.

2.6 Structural Analysis of Mesenteric Arteries

At sacrifice, blood was removed from the mesenteric bed by perfusing with HBSS, placing the vessels in a maximally relaxed state. Second branch arteries were removed, dissected and fixed in 4% paraformaldehyde. Vessels were subsequently embedded in paraffin and then cut into sections (4μm thick) using a microtome. Sections were then air-dried, and de-paraffinzed through a series of xylene and graded ethanol baths. Slides were then stained with Masson's Trichrome for assessment of mesenteric artery's media-to-lumen ratio. Cross-sections of the mesenteric arteries were imaged using a light microscope at 40x magnification. A computer-aided tracing method (Metamorph image analysis software) was utilized to determine the area of the media and lumen. Using this data, the media-to-lumen ratio was determined for the vessels.

2.7 qRT-PCR Analysis

To determine the effect of 4-PBA treatment on ER stress marker expression in the blood vessels of SHR and WKY rats, quantitative reverse transcription polymerase chain reaction

(qRT-PCR) analysis was performed. In the case of blood vessel analysis, the mesentery was excised from the small intestine by blunt dissection and the superior mesenteric artery was cut away at the aortic branch point and perfused with RNAlater® Stabilization (Life Technologies, Ambion), an RNA preservation buffer. The excised mesenteric arcade was placed in ice-cold HBSS and dissection was immediately performed to remove the surrounding tissue. The cleaned second branches of mesenteric artery were stored at -80^{o}C for further RNA extraction. Total RNA was isolated from the frozen vessels using the RNeasy Mini Kit (Qiagen). Briefly, vessels were homogenized using a Sonic Dismembrator (Fisher Scientific) in RLT lysis buffer (Qiagen), RNA bound to spin columns, incubated in RNase-free DNase to eliminate DNA contamination, and eluted in RNase-free DNase-free water as per manufacturer's instructions. cDNA was synthesized from RNA using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Applied Biosystems) and reverse transcription performed on a Master cycler gradient (Eppendorf) thermocycler. Once cDNA was obtained, qRT-PCR was performed to determine relative expression levels of GRP78 and CHOP. Messenger RNAs were detected with Fast SYBR Green Master Mix (Life Technologies, Applied Biosystems) and qRT-PCR analysis was performed using 7500 Software (Life Technologies, Applied Biosystems).

Primers for GRP78 and CHOP were as follows:

GRP78 forward: 5-CTG GGT ACA TTT GAT CTG ACT GG-3
GRP78 reverse: 5-GCA TCC TGG TGG CTT TCC AGC CAT TC-3
CHOP forward, 5-AGC TGG AAG CCT GGT ATG AG-3
CHOP reverse, 5-GAC CAC TCT GTT TCC GTT TC-3

18S was used as an internal standard with forward and reverse primers as follows:

18S forward: 5-GTT GGT TTT CGG AAC TGA GGC-3
18S reverse, 5-GTC GGC ATC GTT TAT GGT CG-3

2.8 Statistical Analysis

Quantitative results are expressed as the mean ± standard error of the mean and were analyzed using the Student's t-test, one way analysis of variance (ANOVA) or two-way ANOVA with Bonferroni post-test correction for multiple comparisons. Significant differences were recognized at the 95% level.

3. RESULTS

3.1 ER stress markers, GRP78 and CHOP, are Upregulated in the SHR before the Onset of Hypertension

Indirect measurements, taken via tail cuff plethysmography, of SBP and DBP were similar between SHR (N=5 rats: 123-158 mmHg systole; 98-115 mmHg diastole) and WKY (N=6 rats: 123-140 mmHg systole; 82-108 mmHg diastole) rats at 5 weeks of age **(Figure. 2a)**. Direct measurements, taken via endpoint cannulation, of SBP and DBP were similar between SHR (N=5 rats: 119-123 mmHg systole; 76-98 mmHg diastole) and WKY (N=6 rats: 115-119 mmHg systole; 72-82 mmHg diastole) at 5 weeks of age **(Figure. 2b)**. To examine endoplasmic reticulum stress marker expression at 5 weeks of age, both WKY (N=15 rats) and SHR (N=18 rats) resistance vessels were processed for qRT-PCR analysis. RNA was extracted for qRT-PCR analysis of CHOP and GRP78. Both CHOP and GRP78 were significantly increased in the 5 week old SHR vessels compared with 5 week old WKY vessels **(Figure. 2c)**. To assess structural features of blood vessels at 5 weeks of age, mesenteric arteries stained with Masson's Trichrome were imaged using a light microscope (40X magnification). SHR mesenteric arteries (N= 12 rats) demonstrated a significantly higher media-to-lumen ratio in comparison with 5 weeks old WKY arteries (N=14 rats) **(Figure. 2d)**.

3.2 ER Stress Inhibitor, 4-PBA, Blunts the Development of Hypertension

Blood pressure measurements were similar between non-treated (N=6 rats, mean of 135.8 mmHg systole, mean of 102 mmHg diastole) and 4-PBA-treated (N=6 rats: mean of 137.2 mmHg systole; mean of 102 mmHg diastole) SHRs prior to 4-PBA treatment (week 0). Once 4-PBA treatment began, during week 1, differences between the groups were observed. SBP and DBP were significantly lower in the rats treated with 4-PBA at weeks 3, 4, 5, 6, 7 and 8. Final mean SBP was 180 mmHg in the untreated SHRs and 157 mmHg in the 4-PBA-treated SHRs. Final mean DBP was 136.8 mmHg in the untreated SHRs and 114.6 mmHg in the 4-PBA treated SHRs. Measurements of systolic BP and diastolic BP were similar between non-treated (N=3 rats) and 4-PBA-treated WKY rats (N=3 rats) prior to 4-PBA treatment. Throughout the 8 weeks of 4-PBA treatment, non-treated and 4-PBA-treated WKY rats displayed similar measures of SBP and DBP and no significant differences were observed **(Figure. 3a; Figure 3b)**.

3.3 ER Stress Marker Expression is increased in SHR Resistance Vessels

To examine endoplasmic reticulum stress marker expression, both untreated (N=4 rats) and 4-PBA-treated SHR (N=4 rats) and WKY second branch mesenteric arteries were collected at sacrifice and processed for qRT-PCR analysis. RNA was extracted for qRT-PCR analysis of CHOP and GRP78. Both CHOP and GRP78 were significantly increased in untreated SHR vessels compared with 4-PBA-treated SHR vessels. However, in WKY resistance vessels CHOP and GRP78 expression were not significantly changed between the non-treated and 4-PBA-treated groups **(Figure. 4a; Figure. 4b)**.

3.4 4-PBA Treatment Alters Resistance Blood Vessel Function

To determine if 4-PBA treatment had an effect on contractility, second branch mesenteric resistance arteries were collected at sacrifice and treated with cumulative doses of PHE (10^{-8} to 10^{-5} M). Resulting dose-response curves, analyzed by ANOVA, revealed a significantly attenuated contractile response in mesenteric arteries from 4-PBA-treated SHRs (N=4 rats) compared with those of the untreated animals (N=4 rats). Statistically significant differences between treatment groups were found at 10^{-6} M **(Figure. 5a)**. There was no significant difference in WKY mesenteric artery constriction with 4-PBA treatment **(Figure. 5b)**. Maximal PHE contracted vessels were treated with the stable acetylcholine mimetic, CCh (10^{-8} to 10^{-5} M), to study the effect of 4-PBA on endothelium-derived relaxation. Compared to untreated SHR mesenteric arteries, 4-PBA-treated SHR mesenteric arteries displayed a significantly higher relaxation to CCh **(Figure. 5c)**. There was no difference in endothelium-dependent relaxation of the mesenteric vessels from untreated (N=3 rats) or 4-PBA-treated WKY rats (N=3 rats) **(Figure. 5d)**. Mesenteric arteries were treated with 10^{-6} M PHE followed by the NO donor, SNP (10^{-7} to 10^{-5}M) to determine the effect of 4-PBA treatment on direct NO-mediated relaxation in these vessels. There was no difference in the NO-mediated relaxation of untreated and 4-PBA-treated SHR resistance vessels **(Figure. 5e)**.

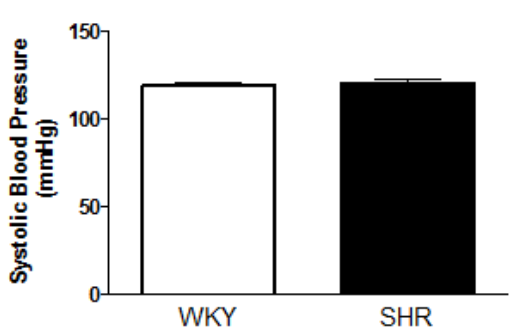
3.5 Effect of 4-PBA Treatment on Blood Vessel Structure

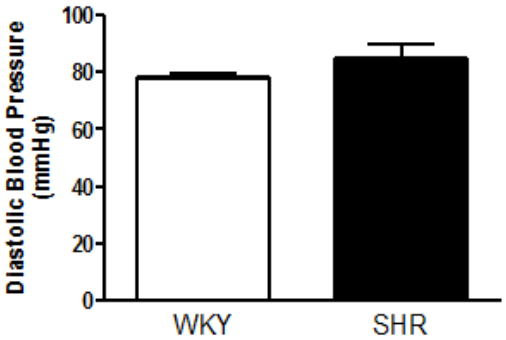
To assess structural features of blood vessels, second branch mesenteric arteries stained with Masson's Trichrome were imaged using a light microscope (40X magnification) **(Figure. 6a)**. Untreated SHR mesenteric arteries (N=5 rats) demonstrated a significantly higher media-to-lumen ratio in comparison with untreated WKY mesenteric arteries (N=3 rats). In addition, 4-PBA treated mesenteric arteries (N=5 rats) had a significantly reduced ratio when compared with untreated SHR arteries. No significant difference was found in the mesenteric artery media-to-lumen ratio between the treatment groups in WKY rats **(Figure. 6b)**.

3.6 4-PBA does not permanently lower BP after Treatment Withdrawal

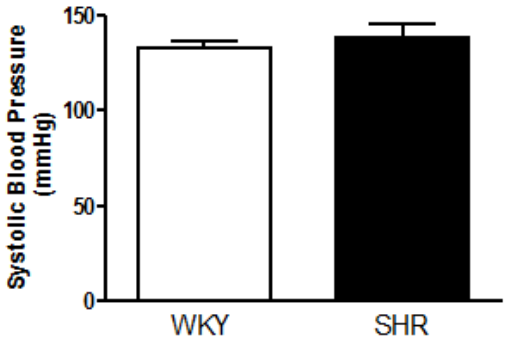
Direct radio-telemetry measurements of SBP and DBP were statistically different between untreated (N=4 rats: mean of 184 mmHg systole, mean of 136 mmHg diastole) and 4-PBA-treated SHRs (N=4 rats: mean of 156 mmHg systole; mean of 114 mmHg diastole) before 4-PBA withdrawal. After treatment withdrawal, similar levels of blood pressure were sustained between untreated (mean of 187 mmHg systole; mean of 138 mmHg diastole) and 4-PBA (mean of 168 mmHg systole; mean of 126 mmHg diastole) treated SHRs for one week. After 2 weeks of treatment withdrawal there were no significant difference between untreated SHRs and 4-PBA treated SHRs and by the fourth week of 4-PBA treatment withdrawal, SBP and DBP of 4-PBA-treated SHRs (mean of 181 mmHg systole; mean of 135 diastole) rose up to very similar levels as untreated SHRs (mean of 191 mmHg systole; mean of 141 mmHg diastole) **(Figure. 7a; Figure. 7b)**.

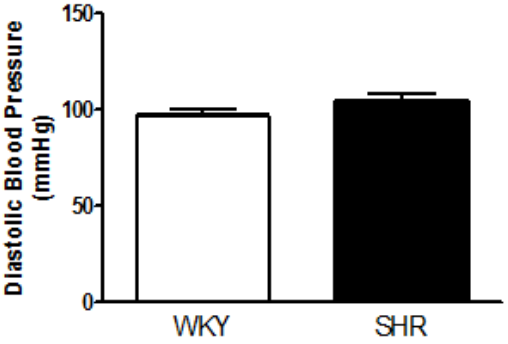
(a)


Systolic Blood Pressure (mmHg)
150
100
50
0
WKY
SHR


Diastolic Blood Pressure (mmHg)
100
80
60
40
20
0
WKY
SHR

(b)


Systolic Blood Pressure (mmHg)
150
100
50
0
WKY
SHR


Diastolic Blood Pressure (mmHg)
150
100
50
0
WKY
SHR

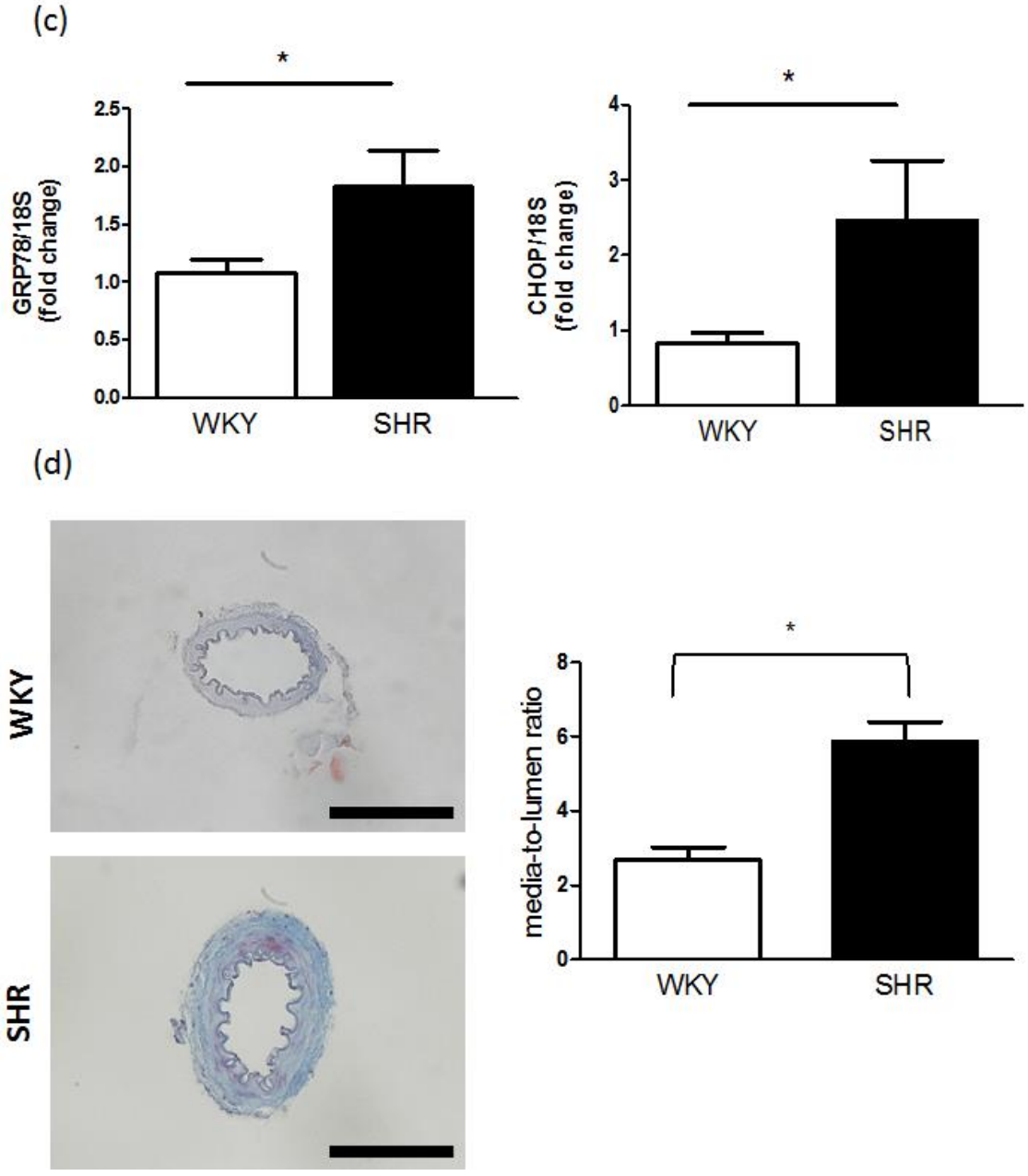
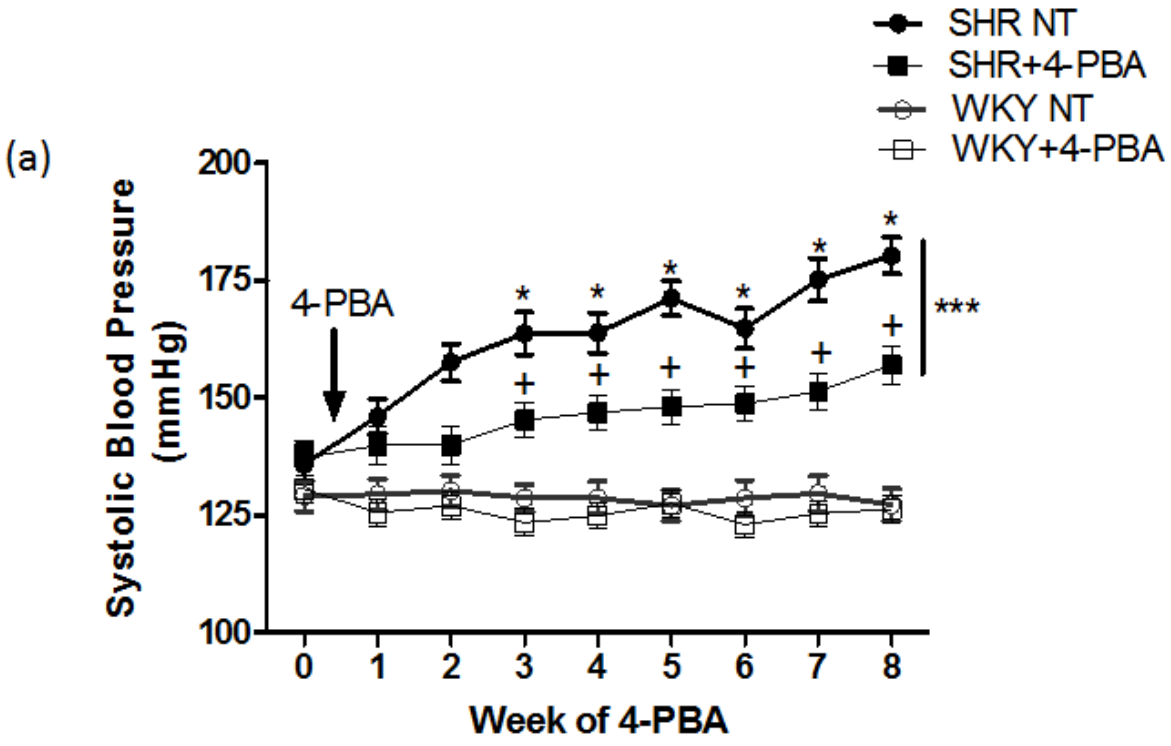

(c)
*
GRP78/18S (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
WKY
SHR
*
CHOP/18S (fold change)
4
3
2
1
0
WKY
SHR
(d)
WKY
SHR
*
media-to-lumen ratio
8
6
4
2
0
WKY
SHR

Figure 2. **ER stress markers, GRP78 and CHOP, and media-to-lumen ratio were elevated in the SHR before the development of essential hypertension.** a) Indirect blood pressure measured via tail cuff plethysmography demonstrated similar SBP and DBP between young SHR (N=5 rats) and WKY (N=6 rats) (b) Direct blood pressure measurements recorded through carotid artery cannulation demonstrated similar systolic and diastolic blood pressure between young SHR (N=4) and WKY (N=5) c) Reverse transcription-polymerase chain reaction analysis demonstrated an elevated expression in GRP78 and CHOP mRNA levels in SHR (N=18) versus WKY (N=15) d) Media-to-lumen ratio was significantly elevated in non-treated young spontaneously hypertensive rat mesenteric arteries (N=12) compared with Wistar-Kyoto vessels (N=14). *$P < 0.05$ vs. Wistar Kyoto. Scale: 150 μm.


(a)
SHR NT
SHR+4-PBA
WKY NT
WKY+4-PBA
Systolic Blood Pressure (mmHg)
200
175
150
125
100
4-PBA
*
+
***
0
1
2
3
4
5
6
7
8
Week of 4-PBA

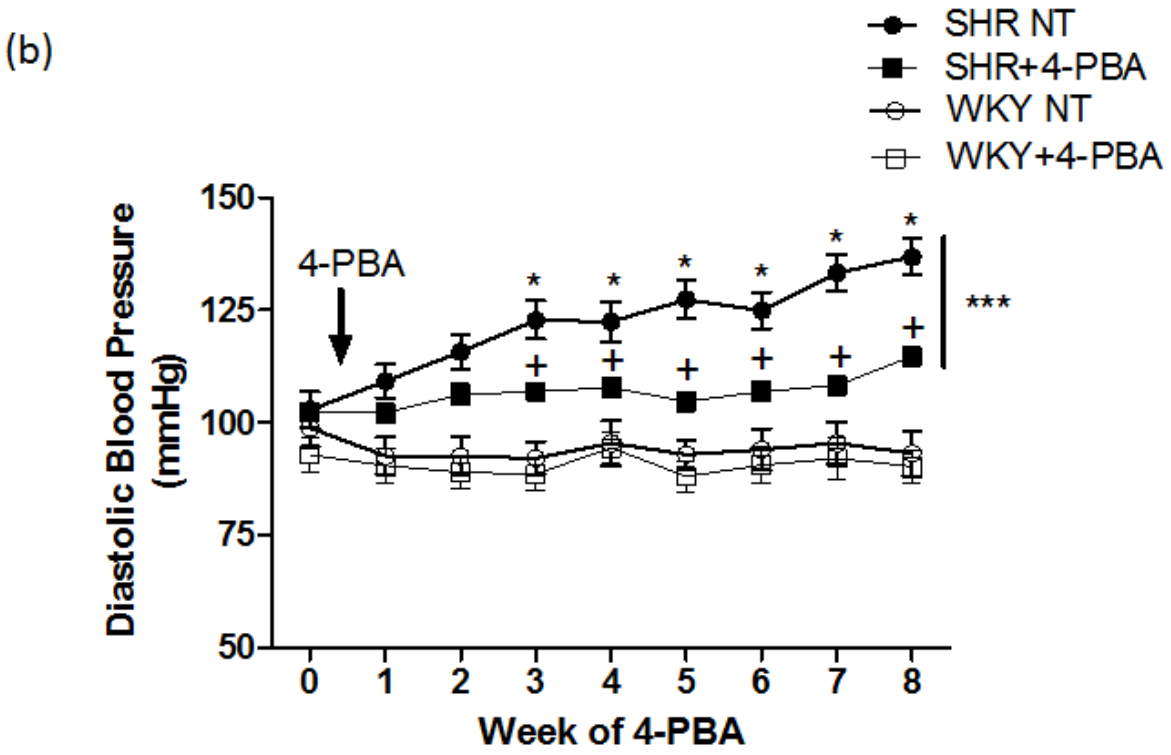
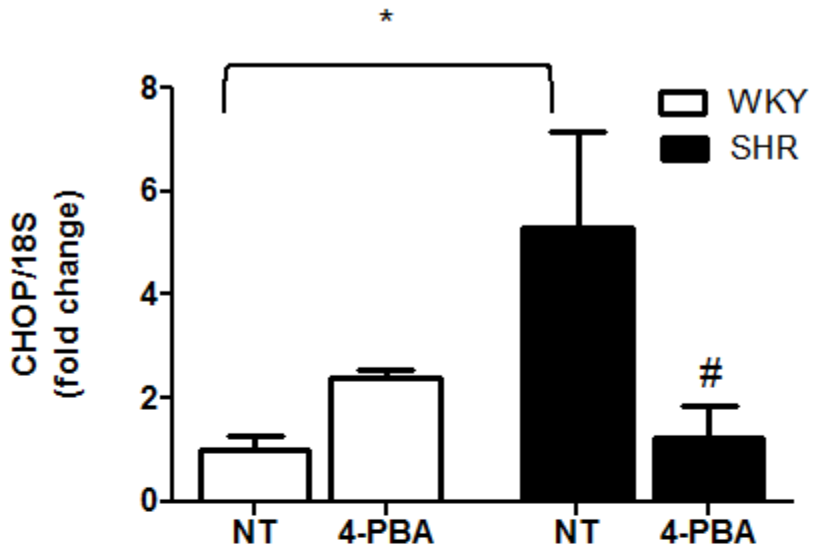

(b)
SHR NT
SHR+4-PBA
WKY NT
WKY+4-PBA
Diastolic Blood Pressure (mmHg)
150
125
100
75
50
4-PBA
*
+
***
0
1
2
3
4
5
6
7
8
Week of 4-PBA

***Figure 3*. 4-phenybutyric acid treatment blunts the development of SBP and DBP in young SHRs.** 5-week-old male SHRs and WKYs were implanted with radio-telemetry devices and randomized to receive normal drinking water (N=6 rats) or 4-PBA (1g/kg/day orally, N=6 rats) for 8 weeks. (a-b) Direct blood pressure measured via radio-telemetry demonstrated significantly lowered hypertensive rat SBP and DPB at 3, 4, 5, 6, 7, and 8 weeks of 4-phenybutyric acid treatment. There was no significant difference in systolic or diastolic blood pressure in untreated (N=3 rats) versus 4-PBA treated Wistar Kyoto rats (N=3 rats). ***P <0.01 vs untreated; *P <0.05 vs. untreated; $^{+}$P <0.05 vs. 4-PBA treated.

(a)


*
WKY
SHR
CHOP/18S
(fold change)
8
6
4
2
0
#
NT
4-PBA
NT
4-PBA

(b)

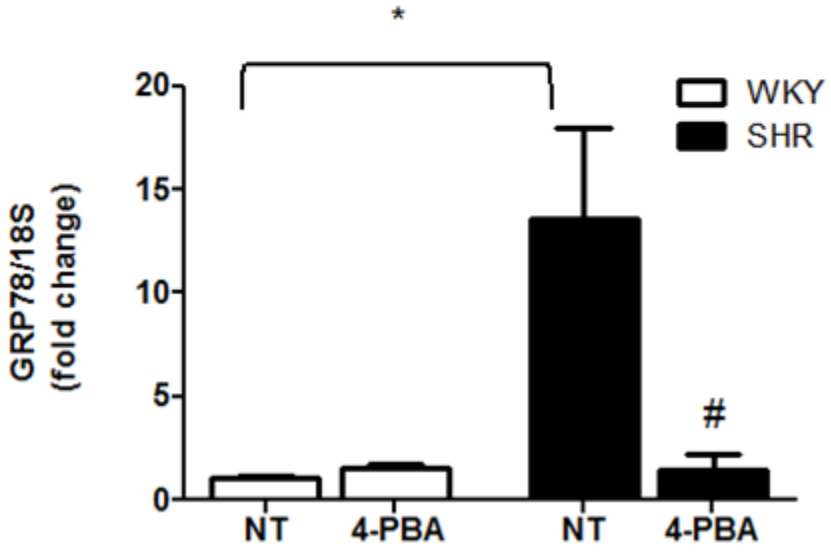

*
WKY
SHR
GRP78/18S
(fold change)
20
15
10
5
0
#
NT
4-PBA
NT
4-PBA

Figure 4. **4-phenylbutyric acid treatment decreases expression of endoplasmic reticulum stress markers.** Reverse transcription-polymerase chain reaction analysis demonstrates a significant decrease in (a) GRP78 and (b) CHOP mRNA levels with 4-phenylbutyric acid treatment in hypertensive rat resistance vessels. *P<0.05 vs. Wistar Kyoto untreated; $^{\#}P$<0.05 vs. hypertensive rat untreated. The following groups were studied: SHR untreated (N=4 rats), SHR-4PBA (N=4), WKY untreated (N=4), and WKY-4PBA (N= 4).

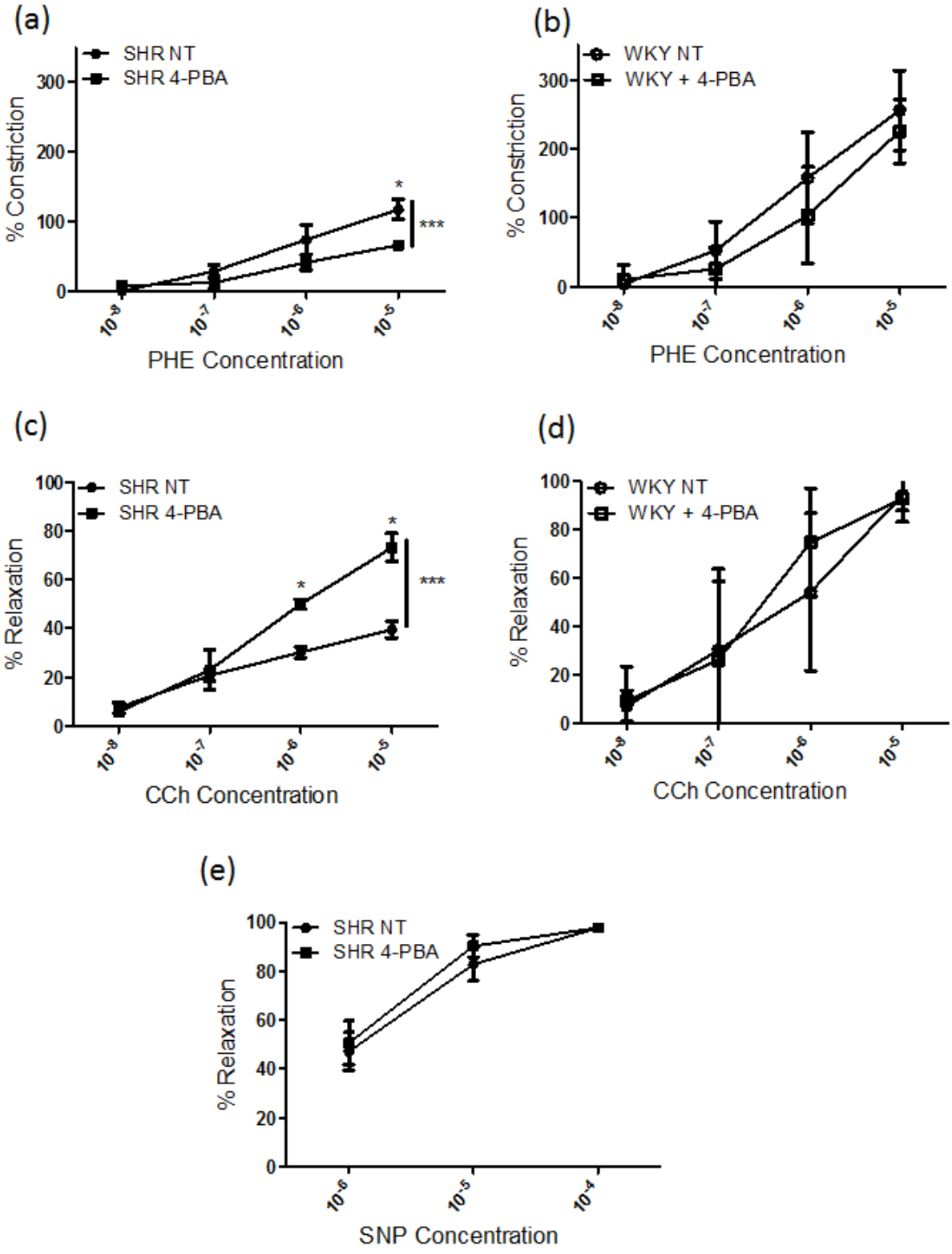
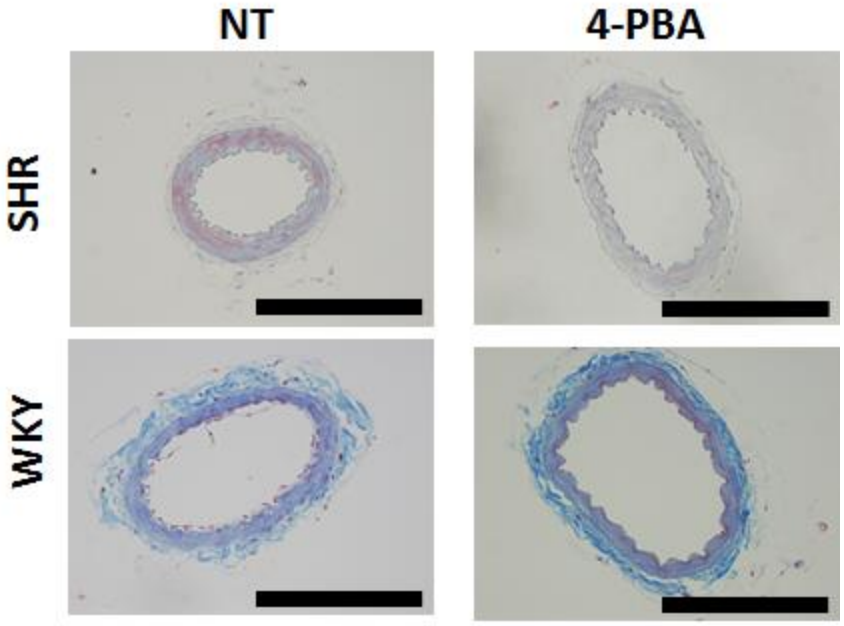

(a)
SHR NT
SHR 4-PBA
% Constriction
300
200
100
0
*
***
10^{-8}
10^{-7}
10^{-6}
10^{-5}
PHE Concentration
(b)
WKY NT
WKY + 4-PBA
% Constriction
300
200
100
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
PHE Concentration
(c)
SHR NT
SHR 4-PBA
% Relaxation
100
80
60
40
20
0
*
*
***
10^{-8}
10^{-7}
10^{-6}
10^{-5}
CCh Concentration
(d)
WKY NT
WKY + 4-PBA
% Relaxation
100
80
60
40
20
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
CCh Concentration
(e)
SHR NT
SHR 4-PBA
% Relaxation
100
80
60
40
20
0
10^{-6}
10^{-5}
10^{-4}
SNP Concentration

Figure 5. **4-phenylbutyric acid treatment reduces phenylephrine-mediated vasoconstriction and increases carbachol-mediated vasodilation in young hypertensive rat resistance vessels.** (a) Dose-response curves for phenylephrine-mediated vasoconstriction revealed a significantly attenuated contractile response in 4-phenylbutyric acid (4-PBA)-treated young hypertensive rat mesenteric arteries (N=4 rats). (b) 4-phenylbutyric acid had no effect on phenylephrine-mediated constriction in Wistar Kyoto mesenteric arteries (N=4). (c) 4-phenylbutyric acid treatment significantly increased carbachol-induced vasodilation in hypertensive rat mesenteric arteries (N=4). (d) 4-phenylbutyric acid had no effect on the carbachol-mediated dilatory response of Wistar Kyoto mesenteric arteries (N=4). (e) Sodium nitroprusside (SNP)-induced vasodilation revealed no difference between non-treated and 4-phenylbutyric acid-treated young hypertensive rat mesenteric arteries (N=4). *P<0.05 vs. non-treated; ***P<0.01 vs. untreated.

(a)


NT
4-PBA
SHR
WKY

(b)

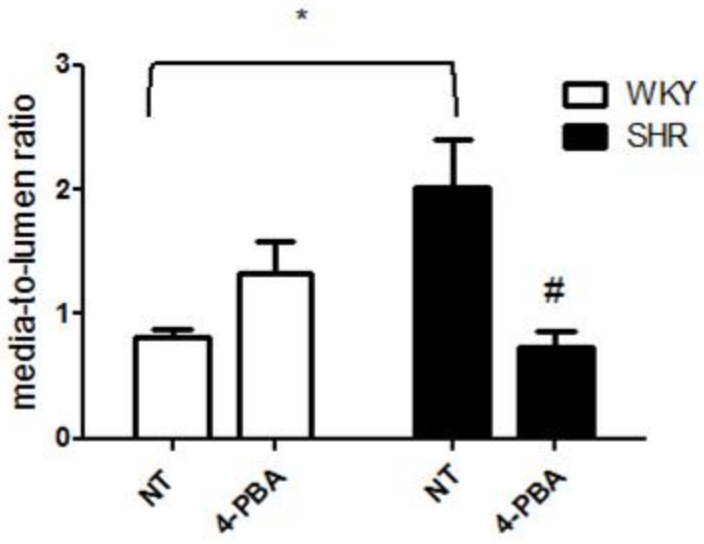
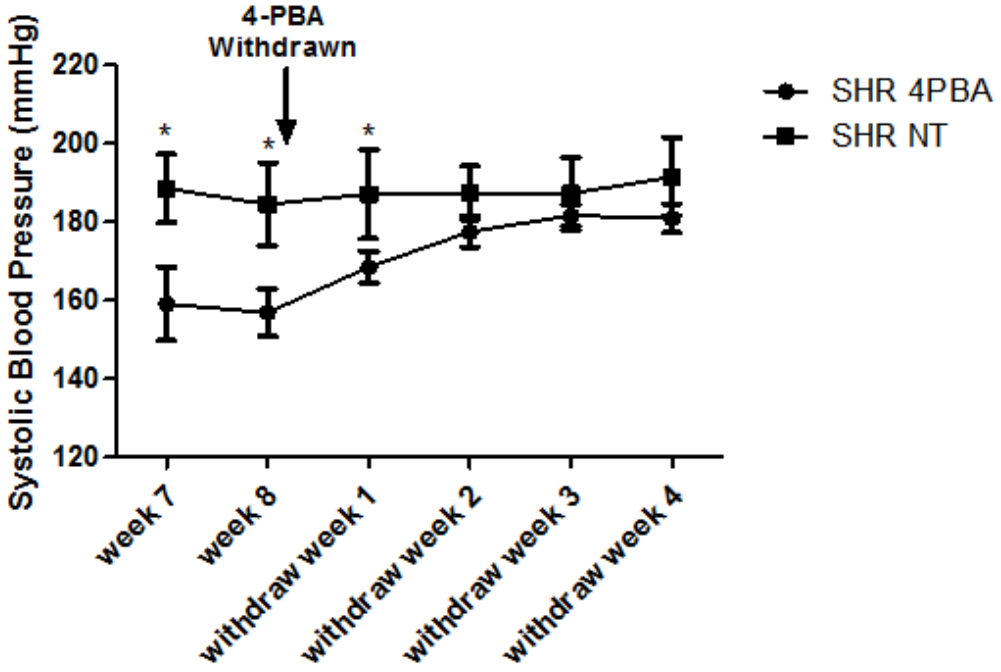

*
media-to-lumen ratio
3
2
1
0
WKY
SHR
#
NT
4-PBA
NT
4-PBA

Figure 6. **4-phenylbutyric acid treatment reduced the media-to-lumen ratio in young hypertensive rat arteries.** (a) Non-treated and 4-phenylbutyric acid-treated mesenteric arteries were stained with Masson's Trichrome and imaged. (b) Media-to-lumen ratio was significantly elevated in non-treated young hypertensive rat mesenteric arteries (N=6 rats) compared with Wistar Kyoto vessels (N=5). 4-phenylbutyric acid treatment significantly reduced media-to-lumen ratio in hypertensive rat vessels to a level similar to the Wistar-Kyoto. *P<0.05 vs. Wistar-Kyoto untreated; $^{\#}P$<0.05 vs. hypertensive rat untreated. Scale: 150 μm.

(a)


4-PBA
Withdrawn
Systolic Blood Pressure (mmHg)
220
200
180
160
140
120
*
*
*
week 7
week 8
withdraw week 1
withdraw week 2
withdraw week 3
withdraw week 4
SHR 4PBA
SHR NT

(b)

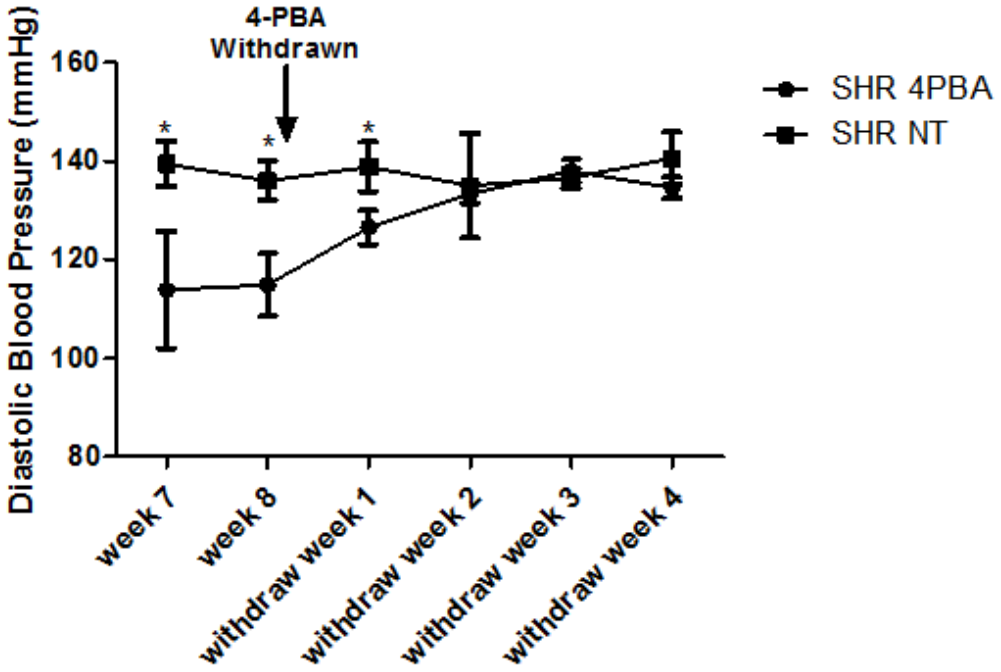

4-PBA
Withdrawn
Diastolic Blood Pressure (mmHg)
160
140
120
100
80
*
*
*
week 7
week 8
withdraw week 1
withdraw week 2
withdraw week 3
withdraw week 4
SHR 4PBA
SHR NT

Figure 7. **4-PBA does not sustain systolic or diastolic blood pressure lowering after treatment withdrawal.** Direct radio-telemetry measurements of systolic and diastolic blood pressure were statistically different between untreated (N=4 rats) and 4-PBA-treated SHRs (N=4 rats) before 4-PBA withdrawal (week 7 and 8 of 4-PBA treatment). After treatment withdrawal, 4-PBA was able to sustain similar levels of systolic and diastolic blood pressure. After 2 weeks of treatment withdrawal, systolic and diastolic blood pressures of 4-PBA-treated SHRs increased to same levels as untreated SHRs (Fig. 7a; Fig. 7b).

4. DISCUSSION

4.1 Differences between young SHR and WKY rats before the development of hypertension

4.1.1 Blood pressure levels before the onset of hypertension

The SHRs and their normotensive control, the WKY rats, come from the same breeding line. The SHR, however, develops high blood pressure during their lifetime, but the WKY does not. Thus, the SHR is considered a genetic model of essential hypertension. It has already been established that at a very young age, from birth to approximately 6 weeks of age, the SHR and WKY have similar systolic and diastolic blood pressure (Dickhout & Lee, 1997). In addition, earlier studies conducted on young SHRs have shown that for the first 4 to 6 weeks of life, SHRs are called pre-hypertensive with their systolic blood pressure measuring below 140 mmHg (Bennett et al., 1996; Dickhout & Lee, 1997; Spitler et al., 2013). In this study, we measured blood pressure indirectly through tailcuff, (Feng et al., 2008) as well as directly through carotid artery cannulation (Parasuraman & Raveendran, 2012) at 5 weeks of age. We confirmed the previously known findings that for the first 5 to 6 weeks of age, SHR and WKY have similar systolic and diastolic blood pressures.

4.1.2 Alterations in the blood vessels before the onset of hypertension

It is well established that the structure and function of small resistance arteries is altered in the young SHR when compared to young WKY (Bennett et al., 1996; Dickhout & Lee, 1997). Earlier studies conducted on young SHRs demonstrated that between 3 to 4 weeks of age, the

SHR develops structural changes in small resistant arteries compared to the normotensive WKY rats. These structural changes include increased medial volume and smooth muscle cell hypertrophy (Bennett et al., 1996; Dickhout & Lee, 1997; Spitler et al., 2013). These vascular changes in the resistant arteries may contribute to increased total peripheral resistance, resulting in the early development of hypertension in these animals. Other studies have also found the young SHR to have impaired vascular reactivity, increased left ventricular hypertrophy with and prolonged QT compared with the WKY (Klimas et al., 2012). These changes occur earlier on and before the development of hypertension and thus are considered primary changes of hypertension. In our study, we looked at media-to-lumen ratios of second branch mesenteric arteries of 5 week old SHR and WKY rats. Our study confirmed that the young SHRs had a significantly higher media-to-lumen ratio when compared to the WKY rats. This vascular dysfunction that occurs earlier in the SHR could contribute to the increase in total peripheral resistance seen in the SHR and the development of hypertension.

4.1.3 Levels of ER stress before the onset of hypertension

Recent studies have shown a relationship exists between endoplasmic reticulum stress and hypertension. As mentioned earlier, structural and functional changes are observed in the SHR resistance vessels at a young age (Dickhout & Lee, 1997). These are considered primary changes of hypertension. We were interested in taking this further and demonstrating that there is a causal relationship between hypertension and ER stress. According to Koch's postulates of causality, to prove causation between ER stress and hypertension, the ER stress in blood vessels must predate the onset of high blood pressure ("Koch's Postulates," n.d.). It is thus essential to compare the levels of ER stress in the blood vessels of the WKY and SHR before their blood

pressures diverge. In particular, we looked at gene expression of GRP78, a molecular chaperone and a common ER stress marker (Özcan et al., 2004). We also looked at the apoptotic gene CHOP, also shown to be expressed at high levels during ER stress (Zinszner et al., 1998). Our study with young SHRs and WKY demonstrated that both of these markers were significantly upregulated in the 5 week old SHR resistance vessels when compared to WKY. Up to this point, we have successfully shown that alterations in blood vessels as well as high levels of ER stress are found in the young SHR before they develop hypertension. In addition, a genetic study conducted by Palao et al. showed that there may be a genetic reason for the increased in protein misfolding in the vessels of the young SHR. At 6 weeks of age, SHR and WKY had differential gene expression levels. Specifically, they showed an increased activation of the unfolded protein response in the SHR (Palao et al., 2015).

4.2. Effect of ER stress inhibition in the development of essential hypertension

4.2.1 Reduction of blood pressure with 4-PBA

Recently, it was shown by Liang et al. that 4-PBA treatment in mice infused with tunicamycin or angiotensin II resulted in a reduction of SBP and DBP (Liang et al., 2013). Spitler et al. infused Sprague-Dawley rats with tunicaymcin, an ER stress inducer, and co-treated with 4-PBA and drug treatment also reduced systolic blood pressure (Spitler & Webb, 2014). Another study conducted by the same research group demonstrated a significant reduction in blood pressure in SHRs when they were treated with 4-PBA (Spitler et al., 2013). These results are consistent with our research groups earlier findings that demonstrate the blood pressure lowering effects of 4-PBA in adult SHR with already developed hypertension. In our study, we showed that 4-PBA significantly reduced SBP and DBP in the developing, young SHR. These studies collectively suggest that a component of the increase in blood pressure found in SHRs seems to be due to ER stress.

4.2.2 Effect of 4-PBA on the function, structure and levels of ER stress of the vasculature

Several studies have shown a role for endoplasmic reticulum stress in hypertension (Carlisle et al., 2016; Kassan et al., 2012; Mohammed-Ali et al., 2017; Spitler et al., 2013). However, our labs' earlier studies with adult SHR (Carlisle et al., 2016) were the first to demonstrate the effect of ER stress on restoration of vascular function in essential hypertension and normalization of blood vessel structure. In the adult SHR study conducted by Carlisle et al, ER stress inhibition with 4-PBA had a direct effect on resistance blood vessels, since an increase

in endothelial-dependent nitric oxide-mediated vasodilation, and a decrease in adrenergic agonist-mediated vasoconstriction were observed in resistance blood vessels isolated from the SHR undergoing long-term treatment with 4-PBA (Carlisle et al., 2016). In our study with young and developing SHRs, vessels dissected at sacrifice and the contractility studies that followed showed similar results observed in the adult SHR. We found that 4-PBA treatment improved vascular dilation and decreased vascular constriction. This may provide an understanding of how 4-PBA is able to reduce the severity of vascular dysfunction in this model of hypertension. These results are consistent with studies conducted with ACE inhibitors which showed significantly reduced contractility and enhanced endothelial-dependent relaxation in the SHR aorta compared to untreated SHR (Clozel et al., 1990; H. Wang et al., 1997). ER stress has also been associated with blood vessel dysfunction in other studies, particularly endothelial dependent relaxation (Kassan et al., 2012) and agonist mediated constriction (Spitler et al., 2013).

We were interested in observing how 4-PBA treatment may influence resistance blood vessel structure. Past studies have shown that treatment with ACE inhibitors normalized morphological alterations in SHR blood vessels and completely normalized the morphological alterations seen in the SHR blood vessels compared to WKY (Clozel et al., 1990; Harrap et al., 1990; Linz et al., 1997). In our study, 4-PBA treatment reduced media-to-lumen ratio in the mesenteric artery's. This effect of 4-PBA is consistent with the previously observed effects of ACEI to normalize SHR resistance blood vessel structure. Dysfunctional vascular function and alterations to the vascular structure found in the blood vessels of the SHRs may be linked to ER stress in the blood vessels, which in turn results in an increase in total peripheral resistance. 4-PBA treatment of a mouse model of chronic angiotensin II-induced hypertension resulted in reduced ER stress and oxidative stress (Kassan et al., 2012). The effects of 4-PBA treatment on

the expression of ER stress markers in the mesenteric arteries were assessed in our study of the developing SHR. The observations above were made concomitantly with 4-PBA treatment-induced reduction in resistance blood vessel ER stress marker expression of GRP78 and CHOP. The proposed mechanism of the role of ER stress in hypertension was illustrated in our laboratory's earlier studies with adult SHR, which examined the effect of ER stress on the systematic vasculature (Figure 1). Our previous studies have shown that ER associated protein degradation to dispose of misfolded proteins occurs through disulfide bond disintegration which generate reactive oxygen species, specifically superoxide anions. Superoxide anions are known to readily react with NO to form peroxynitrite. Peroxynitrite is an ER stress inducer and further exacerbates the pathologies observed in hypertension (Carlisle et al., 2016).

4.3. Effect of ER stress inhibition to permanently lower blood pressure

A variety of antihypertensive agents have been shown to prevent the rise of blood pressure in the SHR, however, the effect of these agents on long-term blood pressure measures, once treatment is stopped, depends on the type of treatment. Both long-term and short-term treatment with ACE inhibitors such as perindopril, ramipril and cilazapril have been shown to exert persistent antihypertensive effects, even after stopping of treatment (Harrap et al., 1990; Linz et al., 1997; H. Wang et al., 1997). We hypothesized that since 4-PBA was able to blunt the development of hypertension and correct the function and structure of the vasculature, similarly to ACE inhibitors (Clozel et al., 1990), that it will also have the same effects as ACE inhibitors when withdrawn. This study was the first to administer 4-PBA to an SHR at a young age and then withdraw it after 8 weeks of treatment. 4-PBA was withdrawn from SHRs drinking water and they were then given normal drinking water for four weeks until sacrifice. Before withdrawing 4-PBA, the SHR untreated group had a significantly higher systolic and diastolic blood pressure when compared to the 4-PBA treated group. A week after 4-PBA was withdrawn, 4-PBA was able to consistently keep similar levels of systolic and diastolic pressures in the 4-PBA pretreated SHRs. However, after one week, systolic and diastolic blood pressure started increasing, and eventually reached similar levels as untreated SHRs. In this withdrawal study, 4-PBA seems to be acting similarly to beta-blockers and calcium antagonists. When given beta-blockers and calcium antagonists through the development of an SHR and then stopped in adult life, high levels of blood pressure reoccur in the SHR to a similar extent as untreated SHR (Christensen et al., 1989; Harrap et al., 1990).

4.4 Future Directions

As observed in our studies with adult and young SHR, the ER stress inhibitor 4-PBA seems to play a role in blood vessel structure, function and hypertension. In future studies conducted by our laboratory, other ER stress inhibitors will be assessed to further investigate the relationship between ER stress and the development of hypertension. An example of an inhibitor that may be used is TUDCA, a chemical chaperone that is structurally distinct from 4-PBA, but shares the same property of inhibiting ER stress (Choi et al., 2016). TUDCA is an amphiphilic bile acid with chaperone properties (Choi et al., 2016; de Almeida et al., 2007)**.** TUDCA prevents apoptosis with its role in the Bax pathway. During ER stress, cytochrome C (cyC) is released and it is involved in initiating caspase enzymes that activate cellular mechanisms that cause apoptosis. Bax is a molecule that initiates the apoptosis pathway by translocating to the mitochondria and releasing cyC. TUDCA, however, prevents the translocation of Bax and thus inhibits apoptosis (Ramalho et al., 2004). Research on the significance of apoptosis in hypertension has recently been conducted by a few research groups. TUDCA treatment has been found to reduce blood pressure in a model of chronic Ang II infusion in mice (Kassan et al., 2012). The study by Hamet et al. demonstrated increased apoptosis in many of the organs of the SHR (Hamet et al., 1995). Spitler et al. demonstrated that there was increased apoptosis in aortic smooth cells of hypertensive rats (Spitler & Webb, 2014). In a study conducted on sixteen-week old SHRs and WKY rats that were treated with TUDCA (100mg/kg/day) for two weeks, TUDCA was able to lower SBP and ER stress markers. In addition, the pressure-induced myogenic tone and endothelium-dependent relaxation were normalized as result of TUDCA (Choi et al., 2016). Thus, TUDCA is anticipated to have similar effects as 4-PBA to blunt the development of hypertension in the young SHR.

4.5 Clinical Implications

Management of hypertension and vascular dysfunction remains limited to lifestyle modifications (diet, and/or exercise) and oral antihypertensive agents, including ACE inhibitors, ARBs, and CCBs. Nevertheless, the various clinical trials that evaluated the effectiveness of these therapeutic options had set a SBP target of less than 140 mmHg. However, the SPRINT trial, a recent landmark study, demonstrated that setting a SBP target of less than 120 mmHg results in fewer cardiovascular events, and mortality, compared to a SBP target of 140 mmHg in all tested age groups (Cushman et al., 2016). Thus, revisiting the current therapeutic options for the treatment of hypertension and vascular dysfunction is crucial for effective management and treatment of affected patients. This also warrants the development of novel antihypertensive drugs, as a quantity of patients respond sub-optimally to current front-line antihypertensive treatments. Thus, the development of more specific treatments that target the root cause of hypertension is warranted. 4-PBA, a currently FDA approved drug for urea cycle disorders, eliminating unnecessary ammonia from the blood through use of its metabolites (Kolb et al., 2015), is such an example. Research has demonstrated, from our group and others, that 4-PBA is a plausible therapeutic option for a few pathological diseases such as reversing the effects that contribute to chronic kidney disease (de Almeida et al., 2007; Mohammed-Ali et al., 2017), lowering hypertension in the adult SHR (Carlisle et al., 2016) and Dahl salt-sensitive rats (Yum et al., 2017), and in this study, restoring endothelium-dependent relaxation in the small arteries that regulate total peripheral resistance and blunting the development of essential hypertension in the young SHR. In addition, many hypertensive patients are prescribed combinations of standard anti-hypertensive drugs. 4-PBA may become a basis for new drug development in hypertension, targeting a novel biochemical pathway.

5. CONCLUSIONS

In this study, 4-PBA's molecular chaperone capability was used in reducing ER stress in the small arteries of young spontaneously hypertensive animals. The main effects of 4-PBA treatment on parameters that could influence the development of high blood pressure in the SHR were the reduction of resistant contractility and increased nitric-oxide mediated endothelial vasodilation. Overall, ER stress inhibition blunted the development of hypertension in the SHR model. This suggests that a component of the increase in blood pressure found in SHRs is due to ER stress, but not all, as a component of hypertension still remained despite ER stress inhibition. Thus, a component of hypertension may not be due to alterations of protein folding. Clinically, when used in conjunction with other drugs, 4-PBA has the potential to prevent the development of hypertension and thus reduce the morbidity and mortality seen in Canada and in the world that is associated with hypertension. The knowledge gained from this research study is intended to increase the awareness of ER stress as a process that plays an important role in hypertension development.

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