Hydrogen Peroxide is Vasoactive in the Mesenteric Arteries of Spontaneously Hypertensive Rats

by

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ABSTRACT.

It is well established that hypertension decreases endothelium-dependent vasomotor function, partially by excessive generation and reduced scavenging of reactive oxygen species (ROS). Nevertheless, at appropriate levels, some ROS can act as signaling molecules in the vasculature and contribute to endothelium-dependent dilation. Recent evidence in healthy resistance arteries suggests that the ROS species hydrogen peroxide (H_2O_2) acts as an endogenous endothelium-dependent dilator through a non-nitric oxide, non-prostaglandin (3NP) pathway. The aim of this study was to investigate the role of endogenous H_2O_2 in 3NP-mediated endothelium-dependent dilation of rat mesenteric arteries, and the changes that occur in these vessels with essential hypertension. 18-20wk old male spontaneously hypertensive rats (SHR; n=24) had an elevated systolic blood pressure of 198±6mmHg compared to 93±4mmHg ($p<0.001$) in the age matched normotensive Wistar-Kyoto rat (WKY; n=22). Isolated mesenteric arteries were preconstricted with norepinephrine (NEPI), followed by exposure to increasing doses of the endothelium-dependent dilator acetylcholine (ACh), which revealed vasomotor dysfunction in the SHR (maximal dilation: WKY: 94.8±1.3% vs. SHR: 75.2±2.9%, $p<0.001$). Incubation of the vessels with the non-specific cyclooxygenase (COX) inhibitor indomethacin (INDO) restored the ACh response in the SHR to the level of the WKY control (area under the curve: WKY: 354.6±8.6 vs. SHR INDO: 350.2±12.2, $p>0.05$) indicating that the release of constrictory prostaglandins from COX contribute to endothelial vasomotor dysfunction. Co-incubation of vessels with INDO and the nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine (LN) inhibited dilation in SHR (46.2±4.8%, $p<0.001$) but not in WKY (98.3±1.5%, $p>0.05$), indicating an elevated 3NP

component in WKY over SHR. Further co-incubation with the H_2O_2 scavenger catalase (CAT), LN, and INDO inhibited the 3NP component to a greater extent in SHR (29.7±3.1%, p=0.062) than in WKY (91.6±2.5%, p<0.05). The responses of SHR and WKY mesenteric arteries to the endothelium-independent dilator sodium nitroprusside, the receptor-mediated constrictor NEPI, and the electrochemical constrictor KCl were no different between LN INDO and CAT LN INDO conditions. These data suggest that endogenous H_2O_2 has a greater role in mediating endothelium-dependent dilation in the mesenteric resistance arteries of SHR. Interestingly, in SHR, co-incubation with LN INDO improved dilation over LN alone (46.2±4.8% vs 23.3±3.2±, p=0.001), and CAT LN INDO decreased dilation from LN INDO to a similar extent, suggesting that COX-inhibition could be a source of H_2O_2 for endogenous vasodilation. Western blotting revealed a 54% increase in COX-1 protein expression in the SHR mesenteric arteries (WKY: 1.00±0.18 (n=9) vs. SHR: 1.54±0.17 (n=13), p<0.05), but no difference in the expression of the pro-oxidant enzyme p47phox, and the anti-oxidant enzymes CAT, SOD-1, and SOD-2. Administration of exogenous H_2O_2 to NEPI preconstricted mesenteric arteries revealed a dose-dependent dilation that was no different between SHR and WKY, and incubation of isolated WKY and SHR mesenteric arteries with CAT reduced the accumulation of H_2O_2 to a similar extent, as assessed by the H_2O_2-specific fluorescent dye Amplex Red. In conclusion, endogenous H_2O_2 is a vasodilator in the mesenteric arteries of SHR and WKY rats in the absence of nitric oxide and prostaglandins. In the SHR, COX-1 inhibition may allow endogenous H_2O_2 to become bioavailable for vasodilation. This study is the first to show a role for endogenous H_2O_2 in maintaining endothelium-dependent dilation in hypertensive rat resistance arteries, and provides evidence to support a role for COX-1-inhibition in the increased availability of H_2O_2 for dilation.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS.

α_1-AR	*α_1 adrenergic receptor*
3NP	*non-nitric oxide, non-prostaglandin*
5-LO	*5-liopoxygenase*
AA	*arachidonic acid*
ACE	*angiotensin converting enzyme*
ACh	*acetylcholine*
AII	*angiotensin-II*
AT	*aminotriazole*
ATP	*adenosine triphosphate*
AUC	*area under dose-response curve*
BKY	*bradykinin*
BSO	*L-butathione-(S,R)-sulfoxamine*
BW	*body weight*
CAT	*catalase*
cGMP	*guanosine 3',5'-(cyclic)phosphate*
CON	*control*
COX	*cyclooxygenase*
Cu,Zn-SOD	*copper zinc superoxide dismutase*
DBP	*diastolic blood pressure*
DCF	*2'7'-dichlorofluorescein*
DETCA	*diethyldithiocarbamate*
E-	*endothelium denuded*
E+	*endothelium intact*
EC_{50}	*50% effective concentration*
ecSOD	*extracellular superoxide dismutase*
EHDF	*endothelium derived hyperpolarizing factor*
eNOS	*endothelial nitric oxide synthase*
GPx	*glutathione peroxidase*
H_2O_2	*hydrogen peroxide*

HM	*heart mass*
HR	*heart rate*
INDO	*indomethacin*
K_{ATP}	*ATP-sensitive potassium channel*
K_{Ca}	*calcium-sensitive potassium channel*
KPSS	*potassium salt solution*
L_0	*normalized diameter*
LK	*left kidney*
LN	*N^{ω}-aginine-L-arginine*
L-NMMA	*N^{G}-monomethyl-L-argninine*
L-NOARG	*L-N^{G}-nitroarginine*
LV	*left ventricle*
MAP	*mean arterial pressure*
MC	*maximal constriction*
Mn-SOD	*manganese superoxide dismutase*
MR	*maximal relaxation*
mRNA	*messenger ribonucleic acid*
NAD(P)H oxidase	*nicotinamide adenine dinucleotide phosphate-oxidase*
NEPI	*norepinephrine*
NO	*nitric oxide*
NOS	*nitric oxide synthase*
O_2-	*superoxide anion*
OH^-	*hydroxyl radical*
ONOO	*peroxynitrite*
PC	*preconstriction*
PE	*phenylephrine*
PEG-SOD	*polyethylene glycated superoxide dismutase*
PG(s)	*prostaglandin(s)*
PGE_2	*prostaglandin E_2*
$PGF_{2\alpha}$	*prostaglandin $F_{2\alpha}$*
PGH_2	*prostaglandin H_2*

phospho-eNOS	*phosphorylated endothelial nitric oxide synthase*
PKG	*protein kinase G*
PRO	*propranolol*
PSS	*physiologic salt solution*
ROS	*reactive oxygen species*
RV	*right ventricle*
SBP	*systolic blood pressure*
SD	*Sprague-Dawley rat*
sGC	*soluble guanylate cyclase*
SHR	*spontaneously hypertensive rat*
SHRSP	*stroke-prone spontaneously hypertensive rat*
SNP	*sodium nitroprusside*
SOD	*superoxide dismutase*
SOD-1	*Cu,Zn – superoxide dismutase*
SOD-2	*Mn – superoxide dismutase*
TEMPOL	*4-hydroxy-tempol*
TP	*thromboxane-prostanoid receptor*
TXA_2	*thromboxane* A_2
VGCC	*voltage gated calcium channel*
VSM	*vascular smooth muscle*
WKY	*Wistar-Kyoto rat*

INTRODUCTION.

The study of vascular function has progressed immensely since Furchgott and Zawadski (1980) first noted the importance of the vascular endothelium in the maintenance and control of vascular tone. Since this seminal work, three major pathways responsible for endothelium-dependent dilation have been identified, as has a relationship between endothelial dysfunction and the development of cardiovascular disease (Feletou and Vanhoutte, 2006). Nevertheless, many questions, both basic and applied, remain unanswered due to the complex interaction of the multiple and redundant mechanisms controlling vascular function and blood flow (Laughlin and Korzick, 2001). Among these mechanisms, a role for reactive oxygen species (ROS) is apparent, where pathological levels of ROS may cause impaired cell function whereas physiological levels of ROS act as signaling molecules necessary for normal cell function (Stone and Yang, 2006). Recent evidence suggests that the ROS species hydrogen peroxide (H_2O_2) may partially mediate the component of endothelium-dependent dilation remaining when nitric oxide (NO) and prostaglandin (PG) production are inhibited (Fujiki *et al*, 2005; Lacza *et al*, 2002; Matoba *et al*, 2000; Matoba *et al*, 2002; Matoba *et al*, 2003; Yada *et al*, 2003). This is termed non-prostaglandin, non-nitric oxide (3NP)-mediated dilation. However, the mechanisms responsible for the action of H_2O_2 as a vascular signaling molecule, and its role in the pathogenesis of hypertension, have not been fully explained.

ROS IN THE VASCULATURE.

A) ROS Species

Several species of ROS are present in the vasculature, including the short-lived superoxide anion (O_2^-) and hydroxyl radical (OH^-), the more stable hydrogen peroxide (H_2O_2), and the reactive nitrogen species nitric oxide anion (NO) and peroxynitrite ($ONOO^-$) (Paravicini and Touyz, 2006; and Droge *et al*, 2002) (Figure 1). The major producer of endogenous ROS in the vasculature is the NAPD(P)H oxidase enzyme, which is comprised of both membrane bound and cytosolic subunits (Rush and Aultman, 2007), but ROS are also produced by the mitochondrial respiratory chain, xanthine oxidase, cyclooxygenase (COX), cytochrome P450, and uncoupled endothelial nitric oxide synthase (eNOS) (Fulton *et al*, 1997; Feletou and Vanhoutte, 2006; Paravicini and Touyz, 2006). O_2^- is the major ROS produced by these sources and can then be spontaneously or enzymatically dismutated to H_2O_2 or react with NO to produce $ONOO^-$ (Feletou and Vanhoutte, 2006). H_2O_2, in turn, can be further dismutated to water and molecular oxygen by catalase (CAT), and by peroxidases such as the thiolate-dependent glutathione peroxidase (GPx) (Stone and Yang, 2006). H_2O_2 can also be spontaneously converted to the highly reactive OH^- in the presence of metal cations via the Fenton and Haber-Weiss reactions (Feletou and Vanhoutte, 2006). Thus, the enzymatic conversion of H_2O_2 to H_2O provides an antioxidant defense mechanism eliminating the oxidative stress and cellular damage caused by the non-enzymatic conversion of H_2O_2 to OH^-. Interestingly, GPx appears to be a more effective antioxidant than CAT in endothelial cells since blockade of glutathione reductase, an enzyme necessary for the function of the glutathione redox cycle, greatly increased H_2O_2-induced cytotoxicity compared to no effect when CAT was inhibited (Suttorp *et al*, 1986). Nevertheless, addition of exogenous CAT

almost completely prevents endothelial cell damage that normally occurs in response to H_2O_2 exposure, suggesting that the additional CAT provides an extracellular "sink" for the H_2O_2 (Suttorp *et al*, 1986). In a complex interaction between many endogenous enzymes responsible for the production and scavenging of ROS, the level of cellular ROS is carefully regulated in vascular tissue, thereby controlling downstream signaling cascades in physiologic and pathologic scenarios.

B) ROS as Signaling Molecules

Although ROS are typically associated with damage, oxidative stress may also play an important role in vascular signaling. The most well known example of ROS signaling in the vasculature is the role of NO as an endothelium-derived relaxing factor causing dilation of the vascular smooth muscle (Palmer *et al*, 1987; Ignarro *et al*, 1987). Recent evidence supports the role of other ROS as signaling molecules. $ONOO^-$, is typically associated with nitrosylation and impairment of both vascular and renal tissue (Chu *et al*, 2003), but is also a dilator of cerebral vessels through an ATP-sensitive potassium channel (K_{ATP}) mechanism (Wei *et al*, 1996). Similarly, O_2^- produced by xanthine oxidase causes dilation of cerebral vessels that is partially dependent upon calcium-activated potassium channels (K_{Ca}) (Wei *et al*, 1996). H_2O_2 is required for the formation of PG by COX, since the presence of GPx causes a dose-dependent suppression of COX activity (Marshall *et al*, 1987). When compared to O_2^- and H_2O_2, the highly reactive OH^- is not likely to have a major role as a signaling molecule beyond its site of production (Paravicini and Touyz, 2006). In general, ROS species are involved in vascular signaling; however, the contribution of each species to overall vascular function remains undefined.

C) ROS and Gene Expression

ROS may also play an important role in the modulation of gene expression in vascular cell types in a manner that has consequences in blood vessel function. For instance, Drummond and colleagues (2000) elegantly demonstrated that H_2O_2 causes a dose-dependent increase in eNOS mRNA and protein expression leading to increased NO production in bovine aortic endothelial cells. Similarly, Lauer *et al* (2005) demonstrated a 1.5-fold increase in eNOS protein content in the aorta of endurance trained wild-type mice with no training-induced change noted in transgenic mice over-expressing human endothelial CAT. This suggests that H_2O_2 was necessary for the exercise-induced up-regulation of eNOS. Finally, transgenic mice over-expressing the p22phox subunit of the NAD(P)H oxidase enzyme in vascular smooth muscle cells have a greater extracellular O_2^- (Laude *et al*, 2005). As a compensation for greater O_2^-, these mice also over-expressed the extracellular isoform of SOD (ecSOD), leading to a greater dismutation of O_2^- to H_2O_2 that can readily diffuse into the endothelium (Laude *et al,* 2005). As a consequence of increased intracellular H_2O_2, eNOS expression is increased leading to greater NO production. Over-expression of CAT inhibits the up-regulation of eNOS, confirming the role of H_2O_2 as a stimulator of transcription for eNOS (Laude *et al,* 2005). Taken together, these studies imply that H_2O_2 may affect NO-mediated vasodilation through altered eNOS expression.

THE ROLE OF H_2O_2 AS A DILATOR IN THE VASCULATURE.

A) General actions of H_2O_2 as a dilator

Several studies have illustrated the complex role of exogenous H_2O_2 as a vasoactive chemical. Gil-Longo and Gonzalez-Vazquez (2005) uncovered four separate responses to exogenous H_2O_2 administered to WKY rat aortic rings. At low concentrations, H_2O_2 causes 1) an initial COX-dependent constriction followed by 2) an endothelium-dependent dilation related to K^+ channel activation and NO release, and 3) an endothelium-independent dilation related to K^+ channel activation at the smooth muscle layer. Furthermore, at concentrations of H_2O_2 greater than 100μmol/L there was 4) an irreversible dilation caused by vessel damage. Similar responses have been shown in the rat mesenteric vessels, where H_2O_2 dose-response curves result in a constriction at low concentrations and a phasic constriction followed by an endothelium-independent dilation at concentrations of H_2O_2 greater than 300μmol/L (Gao *et al*, 2003). This effect of exogenous H_2O_2 as an endothelium-independent dilator is not altered by mannitol, an inhibitor of OH^- destruction, or by stimulation of OH^- production through the Haber-Weiss and Fenton reactions, suggesting that OH^- is not responsible for the dilatory effects of H_2O_2 (Beny and von der Weid, 1991). Since exogenous H_2O_2 stimulates a complex interaction of mechanisms and may cause cellular damage, endogenous levels of H_2O_2 must be tightly regulated by robust antioxidant mechanisms to achieve the desired effect as a signaling molecule in the vasculature.

B) H_2O_2 as an Activator of the COX Pathway and Hyperpolarization

Few studies describe the precise mechanism by which H_2O_2 causes dilation in the mesenteric vasculature; however, a proposed mechanism is summarized in Figure 1. Hattori *et*

al (2003) administered exogenous H_2O_2 to denuded rabbit mesenteric vessels and measured changes in membrane potential. Through the extensive use of pathway inhibitors, it was shown that H_2O_2 activates COX to produce prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) leading to the opening of sarcolemmal K_{ATP}-channels causing vascular smooth muscle hyperpolarization. The authors also demonstrated the involvement of the 5-lipoxygenase (5-LO) pathway; however, they were unable to identify the downstream leukotrienes responsible for hyperpolarization. Unfortunately, the importance of this finding in terms of 3NP-mediated dilation is uncertain since the CAT inhibition of dilation occurs in the presence of the COX inhibitor INDO (Matoba *et al*, 2000), suggesting that PGs are not the main dilatory substances, although 5-LO derivatives may still be involved. Interestingly, H_2O_2 seems to have a differential effect on vascular function through a common K^+-channel pathway. In a study by Lucchesi *et al* (2005), low concentrations of H_2O_2 (1-50μmol/L) caused endothelium-dependent dilation in phenylephrine (PE)- and pressure-contracted mouse mesenteric arteries. However, >50μmol/L H_2O_2 caused endothelium-independent constriction in KCl pre-constricted vessels, and this response was eliminated by the p38 MAP-kinase inhibitor SB203850 (Lucchesi *et al*, 2005). In addition, both dilation and constriction to H_2O_2 were completely eliminated by the Ca^{2+}-channel inhibitor nimodipine, while the addition of the K_{Ca}-channel inhibitors iberiotoxin and apamin did not alter dilation or constriction to exogenous H_2O_2. Since KCl preconstriction and Ca^{2+}-channel inhibition eliminated dilation to exogenous H_2O_2, it suggests that H_2O_2 operates through hyperpolarizing the vascular smooth muscle, possibly by opening K^+-channels separate from K_{Ca}-channels. Thus, it can be speculated that through an unknown K^+-channel, H_2O_2 may cause the hyperpolarization of the cell membrane, closing voltage-gated Ca^{2+}-channels (VGCC) in a manner similar to EDHF

(McGuire *et al*, 2001). The closed VGCC inhibits the entry of extracellular Ca^{2+}, thereby reducing intracellular Ca^{2+} (McGuire *et al*, 2001) and inhibiting Ca^{2+}-calmodulin-dependent myosin light chain kinase (CaCM-MLCK), reducing cross-bridge formation and causing relaxation (Guyton and Hall, 2000). However, this theory has not been thoroughly tested. In the mesenteric vasculature, mechanisms involved in the dilatory vasomotor effects of H_2O_2 include metabolites of COX and 5-LO that cause hyperpolarization of the vascular smooth muscle (VSM) by K^+ channel activation.

C) H_2O_2 as a Dilator Through NO or Second Messenger Pathways

H_2O_2 may be an endothelium-dependent dilator operating through mechanisms common to the NO pathway, although this theory remains controversial (Figure 1). In a study by Itoh *et al* (2003), SOD improved endothelium-dependent dilation of rabbit mesenteric arteries to ACh, an effect that was eliminated by the further addition of CAT or L-N^G-nitroarginine (L-NOARG). Furthermore, the endothelium-independent dilation to an NO donor was improved with SOD and inhibited with CAT while exogenous H_2O_2 increased cGMP induced vasodilation (Itoh *et al*, 2003). These findings support the theory that H_2O_2 causes dilation through cellular mechanisms common to the NO pathway. Leung *et al* (2006) provided additional support for this theory as CAT caused a rightward shift in endothelium-dependent dilation of rat femoral arteries to ACh, an effect that was eliminated with N^{ω}-nitro-L-arginine (L-NAME, abbreviated LN) or the sGC inhibitor ODQ. Incubation with either CAT or LN eliminated ACh-induced H_2O_2 accumulation in the endothelium of rat femoral arteries, and CAT reduced dilation in the presence of the "EDHF blockers" charybdotoxin plus apamin (Leung *et al*, 2006). This study suggests that H_2O_2 may be released by eNOS and

act through sGC to cause dilation at the vascular smooth muscle. However, H_2O_2 administration has also been shown not to alter NO-induced dilation. The inhibition of endogenous CAT through the incubation of rabbit aortic rings with aminotraizole (AT) does not alter dilation to ACh, the calcium ionophore A23187, or the NO donor nitroprusside (Mügge *et al,* 1991). Thus, H_2O_2 may act as a dilator through stimulating the effectors of the NO pathway, although this theory remains controversial.

H_2O_2 may also cause the release of a separate substance from the endothelium to cause dilation at the smooth muscle layer. Miura *et al* (2003) used cannulated coronary arterioles from patients undergoing cardiopulmonary bypass surgery and demonstrated that CAT alone inhibited 2/3 of the flow-mediated dilation and, along with LN, completely abolished flow-induced H_2O_2 production in intact vessels. CAT did not alter endothelium-independent dilation to papaverine, and flow caused the accumulation of H_2O_2 only in the endothelium of intact vessels, and not in the smooth muscle layer. These findings suggest that H_2O_2 may activate a second messenger to cause dilation at the VSM. In a second human study, intestinal submucosal arterioles were excised from patients who did not have cardiovascular disease but were undergoing bowel operations (Hatoum *et al,* 2005). CAT completely inhibited the 3NP component of ACh-induced dilation; however, exogenous H_2O_2 caused dilation in endothelium-intact vessels, and constriction in denuded vessels (Hatoum *et al*, 2005). Since the exogenous H_2O_2 did not cause direct dilation of the smooth muscle, these results suggest that endogenous H_2O_2 produced in the endothelium stimulates the release of a substance distinct from H_2O_2 to cause dilation at the VSM.

ENDOGENOUS H_2O_2 IN NON-NO, NON-PG (3NP)-MEDIATED DILATION.

A) Evidence Supporting H_2O_2 Involvement in 3NP-mediated Dilation

Recent evidence suggests that H_2O_2 may be involved in 3NP-mediated dilation, the component of dilation that remains following NO and PG blockade. This reveals an important link between oxidative stress and vascular function (Figure 1). Careful work by Matoba *et al* (2000), where the effectiveness of the NO and PG blockade was ensured with additional downstream blockers, demonstrated that the addition of CAT eliminated stimulated endothelial H_2O_2 production, 60% of the endothelium-dependent dilation, and greatly reduced hyperpolarization to ACh in mouse mesenteric arteries. The inhibited endothelium-dependent dilation to CAT was reversed following incubation with AT, and hyperpolarization in response to an endothelium-independent agonist (levcromakalim) was not altered by CAT (Matoba *et al*, 2000). Interestingly, when the vessels of control mice were incubated with only CAT and INDO, dilation was not altered from control levels in wild-type mice, but was greatly inhibited in $eNOS^{-/-}$ mice (Matoba *et al*, 2000). This suggests that H_2O_2 acts as an endothelium-dependent dilator only in the absence of NO. In 2003, Matoba *et al* (2003) identified H_2O_2 as a 3NP-mediated dilator in pig coronary vessels through the inhibitory actions of CAT on BKY induced dilation, hyperpolarization, and H_2O_2 production identified by electron spin resonance detection. Similarly, Yada *et al* (2003) measured significant inhibition of ACh-induced dilation in the presence of N^G-monomethyl-L-argninine (L-NMMA), the non-selective COX inhibitor ibuprophen, and CAT in the small coronary arteries (>100μm) and arterioles (<100μm) of dogs. A further study by Morikawa *et al* (2003) provides possibly the most convincing evidence that H_2O_2 is involved in 3NP-mediated dilation by using the Cu,Zn-SOD knockout ($SOD\text{-}1^{-/-}$) mouse model. In the presence of CAT,

the 3NP component of ACh-induced dilation is reduced from 75% to 25% in the mesentery of control mice with a corresponding 50% reduction in hyperpolarization. Conversely, SOD-$1^{-/-}$ mice demonstrate no H_2O_2 release with ACh stimulation and no effect of CAT on ACh-induced dilation or hyperpolarization (Morikawa *et al*, 2003). This finding supports the involvement of H_2O_2 in 3NP-mediated dilation since the knockout mice would not produce H_2O_2 endogenously, thus it would not be available for dilation. In the SOD-$1^{-/-}$ mice, the SOD mimetic TEMPOL that scavenges O_2^- and produces H_2O_2 (Chen *et al*, 2007; Fulton *et al*, 1997), improved ACh-induced dilation, an effect which was reversed by the further addition of CAT (Morikawa *et al*, 2003). Conversely, the incubation of wild-type mesenteric vessels with TEMPOL did not alter ACh-induced dilation; however, co-incubation with TEMPOL and CAT caused a reduction in dilation, suggesting that the CAT effect on H_2O_2 was preserved. These findings suggest that when adequate endogenous O_2^- scavengers exist, the endogenous H_2O_2 is involved in 3NP-mediated dilation; however, when the level of SOD is reduced, TEMPOL improves dilation by providing additional H_2O_2 to act as a dilator. In this context, SOD-1 may act as a major producer of H_2O_2 for dilation.

B) Evidence Against H_2O_2 Involvement in 3NP-mediated Dilation

In light of the evidence suggesting that H_2O_2 is involved in 3NP-mediated dilation, Ellis *et al* (2003) undertook a thorough study of the role of this ROS in the mouse mesenteric arteries. In wild type or type-II diabetic mice (db/db), CAT did not alter endothelium-dependent dilation in the presence of INDO and L-NAME. Likewise, inhibition of SOD-1 with DETCA, augmentation of SOD with the cell permeable polyethylene-glycolated-SOD (PEG-SOD), and inhibition of endogenous CAT with AT did not alter the 3NP component of

mesenteric artery dilation, suggesting that endogenous H_2O_2 does not cause dilation. Even altering the CAT brand to mimic other studies where endogenous H_2O_2 acted as a dilator did not affect the ACh-induced dilation in this study. Although CAT inhibited dilation to exogenous H_2O_2, 1mmol/L H_2O_2 impaired the $PGF_{2\alpha}$-induced constriction following washout, suggesting that H_2O_2 causes damage to the vessels at high concentration that could be mistaken for dilation. Further studies in catalase-over-expressing (CAT^{++}) mice suggest that H_2O_2 may not be a simple endothelium-dependent dilator in the same manner as NO. Suvorava *et al* (2005) observed that CAT^{++} mice were hypotensive compared to their catalase-negative (CAT^{n}) counterparts, and that the addition of AT to CAT^{++} caused a normalization of blood pressure to the levels of CAT^{n}. Interestingly, aortic endothelium-dependent and –independent dilation was no different between CAT^{++} and CAT^{n}; and H_2O_2 caused endothelium-independent constrictions that were greater in the CAT^{n} strain. Thus, these findings suggest that H_2O_2 does not act as an endogenous dilator, but rather as an endothelium-independent constrictor leading to hypertension. Other studies in humans and other mammals suggest that H_2O_2 may not be involved in 3NP-mediated dilation. CAT does not inhibit the 3NP component of endothelium-dependent dilation in the radial arteries of coronary artery bypass patients (Hamilton *et al*, 2001). Similarly, co-incubation with CAT and SOD does not alter the 3NP component of dilation of coronary vessels to BKY in the Wistar rat perfused heart model (Fulton *et al*, 1997). Finally, incubation with CAT alone does not alter endothelium-dependent dilation or hyperpolarization of pig coronary arteries to BKY or Substance P (Bény and von der Weid, 1991). Thus, a wide body of evidence refutes the hypothesis that H_2O_2 is involved in 3NP-mediated dilation.

VASCULAR FUNCTION AND HYPERTENSION.

It is generally agreed that agonist-induced increases in intracellular calcium levels in endothelial cells lead to the activation of eNOS and COX, causing the release of NO and PGs respectively, that cause dilation (McGuire *et al*, 2001). Factors distinct from NO and PGs are also released from the endothelium to cause dilation (McGuire *et al,* 2001). All of these endothelium-derived relaxing factors are altered in hypertension leading to impaired vascular function.

A) NO-bioavailability and Hypertension

Elevated ROS in hypertension can impair NO-bioavailability leading to vascular dysfunction (Rathaus and Bernheim, 2002; Paravicini and Touyz, 2006; Ardanaz and Pagano, 2006). Renal hypertension is associated with a decrease in NO provision leading to impaired endothelium-dependent relaxation of blood vessels (Stankevicius *et al,* 2002) and complete blockade of NOS with chronic LN administration causes significant hypertension (Sainz *et al*, 2005). In the spontaneously hypertensive rat (SHR) and in aortic banding hypertension, O_2^- is elevated and endothelium-dependent dilation is impaired (Chu *et al*, 2003; Bouloumie *et al*, 1997). The increased O_2^- can interact with NO to create an elevated $ONOO^-$, thereby causing nitrosylation and damage of proteins as well as reduced NO available for dilation (Bouloumie *et al*, 1997). Thus impaired NO-bioavailability may be important in hypertension.

B) The PG Pathway and Hypertension

The PG pathway is also involved in hypertension. PGs are endothelium-derived constricting and relaxing factors formed by the COX enzymes from arachidonic acid

(McGuire *et al*, 2001) and are inhibited by INDO (Kodama *et al*, 1995; Ruiz *et al*, 1994). INDO improves endothelium-dependent dilation to ACh and reduces receptor-dependent constriction to norepinephrine (NEPI) in the mesentery of SHR rats without altering either response in WKY (Luscher *et al*, 1990). In addition, INDO does not alter endothelium-independent dilation to sodium nitroprusside (SNP) (Luscher *et al*, 1990) and has been shown not to alter LN-induced hypertension in SHR (Ruiz *et al*, 1994), suggesting that vasoconstricting PGs are produced in SHR rats thereby impairing vascular function without altering NO-bioavailability.

C) 3NP-mediated Dilation and Hypertension

Like NO and PGs, the remaining 3NP component of dilation may be altered in hypertension. SHR femoral arteries have reduced 3NP-mediated dilation at 15 and 25wks of age corresponding to the development of severe hypertension (Mori *et al*, 2006). In the mesenteric vessels, SHR have both impaired ACh-induced hyperpolarization and only ~10% dilation that is 3NP-mediated compared to ~55% in WKY (Fujii *et al*, 1992). Conversely, Sprague-Dawley (SD) rats fed high (8%) salt diets developed significant hypertension; however, the 3NP component of ACh-induced dilation is 81% in the high salt rats and only 44% in the low salt (0.4%) group (Sofola *et al*, 2002). Thus, hypertension may involve the impairment of endothelium-dependent dilation through reduced dilation via the NO and PG pathways as well as reduced 3NP-mediated dilation. The effects on specific pathways depend upon the model of hypertension.

H_2O_2 AS A COMPENSATORY DILATOR IN HYPERTENSION.

The phenomenon of endogenous H_2O_2 as a dilator may compensate for reduced NO-bioavailability. The injection of ecSOD into two-kidney one-clip ecSOD knockout ($ecSOD^{-/-}$) mice causes an improvement in endothelium-dependent dilation in the aorta which may be due to augmented H_2O_2 since the administration of CAT caused complete inhibition of dilation in all groups (Jung *et al,* 2003). TEMPOL reduces LN induced hypertension in Wistar rats possibly through greater H_2O_2 instead of reduced O_2^- since the increased activity of SOD-1, SOD-2, GPx, and glutathione reductase enzymes following LN administration are reduced to normal levels in the kidney with TEMPOL treatment (Sainz *et al,* 2005). These findings suggest that the reduction in the SOD enzymes may be due to the presence of TEMPOL; however, the reduction in the endogenous H_2O_2 scavenging enzymes may suggest that the accumulated H_2O_2 following TEMPOL treatment may contribute to the reduction in blood pressure. Hatoum *et al* (2005) demonstrated that ACh caused CAT-sensitive endothelium-dependent H_2O_2 production in human mucosal arteries only in the presence of LN and INDO and not with ACh alone, suggesting that H_2O_2 production may compensate for inhibited NO and PG production in the endothelium. The inability of CAT to inhibit dilation to ACh in the absence of LN and INDO is supported by several other studies (Matoba *et al,* 2000; Beny and von der Weid, 1991). Thus, endogenous H_2O_2 may compensate for a decrease in NO-bioavailability in hypertension.

Nevertheless, several studies suggest that endogenous H_2O_2 does not compensate for reduced NO. CAT does not alter ACh-induced dilation in the femoral arteries of 15wk old WKY in the presence of LN and INDO and who have a blood pressure of 125mmHg, suggesting that H_2O_2 does not contribute to 3NP mediated dilation in normotensive animals

(Mori *et al,* 2006). Similarly, Fujiki *et al* (2005) describe an increase in the CAT-sensitive 3NP component of dilation in normotensive (SBP=116mmHg) mouse mesenteric arteries following treatment with an ACE-inhibitor and suggest that this treatment rather than the increased blood pressure causes greater H_2O_2-mediated dilation. Finally, the injection of TEMPOL along with the SOD-inhibitor DETC causes an increase in blood pressure, likely through increased endogenous H_2O_2, which is normalized by the addition of CAT (Makino *et al*, 2003). Thus, several groups suggest that H_2O_2 does not compensate for reduced NO, and may instead cause hypertension.

PURPOSES.

The following study aims to investigate the role of endogenous H_2O_2 as a 3NP-mediated dilator in resistance arteries, and the extent of the contribution of endogenous H_2O_2 to dilation in hypertension. This study will investigate the effect of CAT on the function of rat mesenteric arteries and how the CAT effect changes with hypertension. The response of mesenteric arteries to exogenous H_2O_2 will also be assessed. The SOD-mimetic TEMPOL will be acutely administered to both hypertensive and normotensive arteries and changes in vasomotor function will be measured. Finally, the level of H_2O_2 released from the vessel under myography conditions will be measured using a fluorescent probe.

The contribution of endogenous H_2O_2 to vasodilation has been supported and refuted by numerous studies, and the contribution of this ROS in rat resistance arteries has not been established. In addition, hypertension may increase or decrease the 3NP component of dilation, but the role of H_2O_2 in 3NP dilation is unknown. The following study aims to address the controversial role of endogenous H_2O_2 as a 3NP-mediated dilator, the dependence

of the endothelium on this response, and how endogenous H_2O_2-mediated dilation is altered with hypertension and antioxidant treatment.

HYPOTHESES.

Hypothesis 1: SHR vessels will exhibit reduced endothelium-dependent dilation, a reduced 3NP component, with normal vascular smooth muscle dilation and exaggerated constrictory functions when compared to WKY.

Hypothesis 2: CAT will inhibit 3NP-mediated dilation in SHR rats but not in WKY and will not affect dilation or constriction in the absence of NO and PG blockade. ACh will cause an increase in vascular H_2O_2 accumulation that is sensitive to CAT.

Hypothesis 3: Exogenous H_2O_2 will cause both constriction and dilation in both SHR and WKY vessels, depending on the concentration of H_2O_2.

Hypothesis 4: TEMPOL will improve both the NO and the 3NP components of endothelium-dependent dilation and the improvement will be greater in SHR than WKY. The improvement in 3NP with TEMPOL will be eliminated with CAT.

Hypothesis 5: In isolated vessels, CAT will reduce H_2O_2 release while TEMPOL will increase H_2O_2 release in a CAT sensitive manner. Vessels stimulated with ACh in the presence of NO and PG blockade will release H_2O_2 that will be inhibited with CAT.

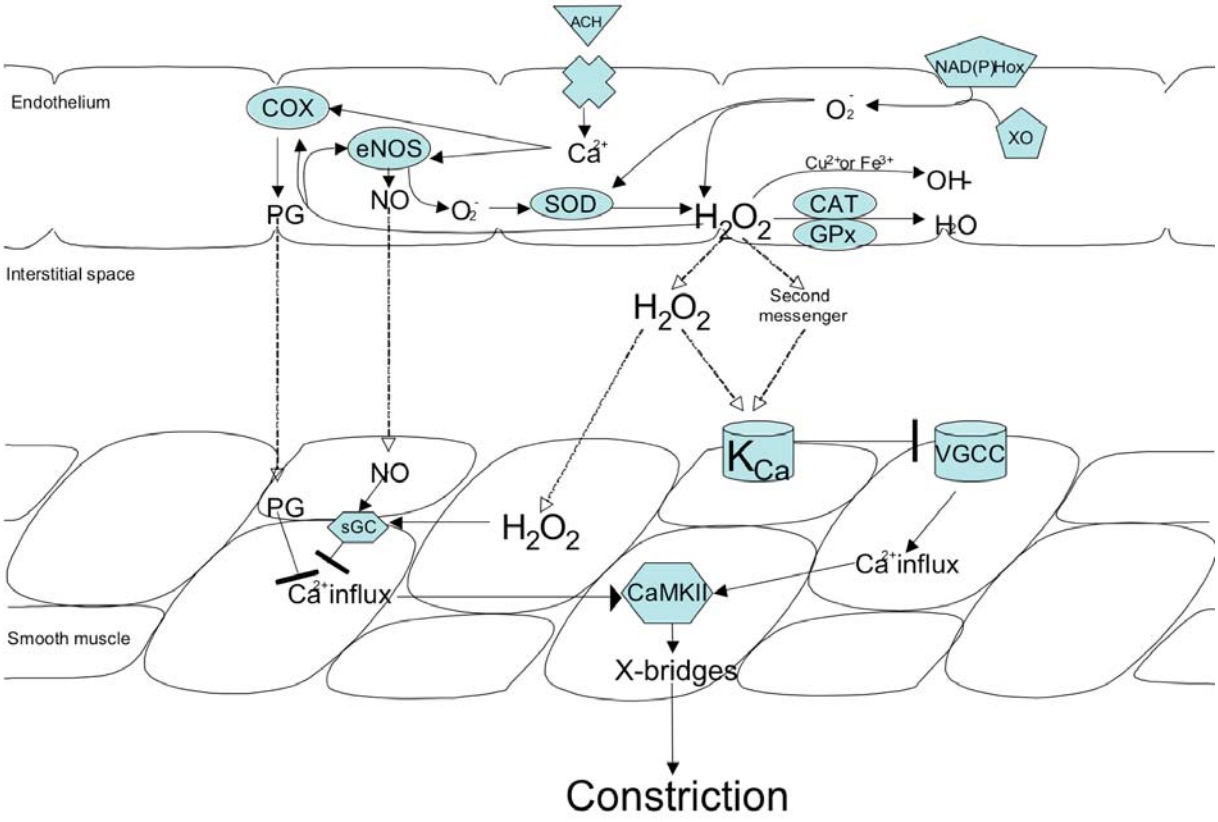

Figure 1: *H_2O_2 in 3NP-mediated dilation.* Arrows indicate activation of an enzyme or formation of ROS. T-bars indicate inhibition of a cellular pathway (i.e. inhibited Ca^{2+}-influx ultimately reduces constriction leading to dilation).

METHODS.

ANIMALS.

Male SHR (n=60) and male Wistar Kyoto rats (WKY, n=47) were obtained from Harlan (Indianapolis, IN) at ~12weeks of age. Male Sprague Dawley rats (SD, n=29) were obtained from the University of Waterloo breeding colony. Rats were group housed in a temperature and humidity controlled room and acclimatizing to the reverse light cycle. Rats were fed standard rat chow (Harlan) and tap water *ad libitum*. At 16-20weeks of age, rats were injected with sodium pentobarbital in saline (~85mg/kg i.p.; Bimeda-MTC, Cambridge, ON). For measurements of hemodynamics, the level of sedation was monitored through the withdrawal reflex from a toe pinch, and sodium pentobarbital was additionally titrated at to ensure withdrawal reflex was absent and breathing rate was regular during measurements. Following measurement of hemodynamics, the rats were sacrificed by exsanguination and the mesentery was removed. All drugs were obtained from Sigma Chemical (St. Louis, MO) except for $NaHCO_3$ (BioShop; Burlington, ON).

HEMODYNAMIC MEASURES.

Blood pressure was directly assessed through the left carotid artery of a subset of rats (WKY; n=22, SHR; n=24). Anaesthetized rats were placed supine on a heating pad at 37°C (Gaymar TP-500, Orchard Park, New York and Temp-Pad; Seabrook Medical Systems). Following an incision into the neck, the left carotid artery was exposed and a small cut was made in the artery. The calibrated catheter (size 2F, 140cm: Millar Instruments, Houston, TX) was inserted into the artery, oriented towards the heart, and secured with silk suture (4-0 silk,

Look, Reading, PA). The systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) outputs were registered on a Powerlab console (ADInstrumetnts, Colorado Springs, CO) using Chart 5 software (ADInstruments, Colorado Springs, CO, v5.5.4).

ASSESSMENT OF VASOMOTOR FUNCTION.

A) Surgery

A section of the small intestine spanning from the pylorus to the proximal large intestine was removed, and was immediately placed in oxygenated ($95\%O_2/5\%CO_2$) physiologic salt solution (PSS; concentration (mmol/L): 118.99NaCl, 4.69KCl, $1.17MgSO_4 \circ 7H_2O$, $1.18KH_2PO_4$, $2.50CaCl_2 \circ 2H_2O$, $25.00NaHCO_3$, 0.03EDTA, 5.50Glucose) at 4°C. The intestine was pinned about the perimeter of a surgery dish embedded with silicone elastomer (Sylgard 184, Dow Corning Inc, Midland, MI) with the mesenteric arcades splayed. The superior mesenteric artery was identified and the 3rd order artery branches were cleaned of venous branches under a microscope (Zeiss; VWR, Mississauga, ON). A single 3rd order artery was divided into two segments for mounting to the myograph, and a neighboring 3rd order artery was used as an alternate. All vessels were placed into oxygenated PSS at room temperature (22°C) and then mounted onto the calibrated single wire myograph (model 310A, DMT, Aarhus, Denmark) in a chamber filled with 10mL 37°C oxygenated PSS as outlined by Mulvany and Halpern (1977). Briefly, two adjacent horizontal wires were carefully threaded through the lumen of the artery and secured to adjacent steel jaws. One jaw was directly connected to a force transducer, while the other was attached to a micrometer, allowing the distance between the adjacent wires, and hence the vessel lumen diameter and wall tension, to

be adjusted. Output from the force transducer was linked to a personal computer through a Myo-Interface unit (model 310A, v2.03) attached to a Powerlab console.

B) Vessel Preparation for Myography Experiments

Each excised vessel was subjected to normalization and standard start protocols following by a defined experimental protocol.

I) Normalization protocol

Following mounting, the vessels were incubated for 30min in 37°C PSS with bubbled 95%O_2/5%CO_2 prior to normalization. The normalization procedure involved the stepwise increase in diameter increments of 10μm/min to a maximal tension of 10mN as outlined by Mulvany and Halpern (1977). The normalized diameter (L_o) was derived from the intersect of the 100mmHg isobar (the tension required to elicit a pressure of 100mmHg), and the vessel wall tension was set in accordance with the LaPlace relationship (pressure=wall tension/(internal circumference/2π)) for each vessel for the remainder of the protocol. $0.9L_o$ was used as the experimental diameter as it has been established as the diameter that elicits the greatest active tension in studies by Falloon et al (1995), as well as by our own pilot work.

II) Standard Start Protocol

Following incubation, the normalized vessel underwent a standard start protocol involving the exchange of bathing solutions containing 1) 10mL potassium physiologic salt solution (KPSS; concentration: substitutes 123.70mmol/L total KCl for KCl+NaCl in PSS) supplemented with NEPI (10^{-5}mol/L), 2) 10^{-5}mol/L NEPI in PSS only, 3) and KPSS only, for 3min respectively. Exposure to each solution was followed by 4 exchanges with PSS. After

the last wash, the endothelium function test was performed and both vessels were incubated with 3μmol/L (+/-)-Propranolol hydrochloride (propranolol, abbreviated PRO) to block the ß-adrenergic receptor mediated effects of NEPI (Yang *et al,* 2005; Mulvany *et al,* 1982). In addition to PRO, some vessels were incubated with the following drugs: 1) Catalase (CAT, 860U/mL; from bovine liver, 2) 4-Hydroxy-TEMPO (TEMPOL; 1mmol/L), 3) N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, abbreviated to LN; 300μmol/L), 4) Indomethacin (INDO; 5μmol/L).

The dose of LN (300μmol/L) was similar to the dose commonly used in our lab (100μmol/L). 300μmol/L LN has been shown to inhibit dilation in SD rat small mesenteric vessels, an effect that was not altered by the additional administration of other NOS inhibitiors or the soluble guanylate cyclase (sGC) inhibitor (Chauchan *et al*, 2003), suggesting that 300μmol/L LN is sufficient to inhibit NOS in this preparation. Similarly, our lab and others use 5μmol/L INDO to inhibit COX-mediated vasomotor function in the mesenteric arteries of SHR, WKY and SD rats (Chauhan *et al,* 2003; Le Marquer-Domagala and Finet, 1997). Finally, in studies demonstrating the role of H_2O_2 as an endogenous vasodilator, the concentration of NOS inhibitor was 100μmol/L and the concentration of COX-inhibitor was 1μmol/L (Matoba *et al*, 2000, 2003; Morikawa *et al*, 2003), and closely match the doses used in the present study.

Similarly, the dose of TEMPOL and CAT were taken from findings in the literature. 1mmol/L TEMPOL has previously been shown to cause a dilation in vessels that was reversed by CAT in rat mesenteric arteries (Chin *et al*, 2007). 860U/mL of CAT was chosen to approximate the 1000U/mL shown to eliminate H_2O_2-induced dilation and constriction to 10^{-3}mol/L H_2O_2 in WKY (Gao *et al*, 2003) and the constriction of mesenteric arteries to $5x10^{-}$

^{4}mol/L H_2O_2 in both WKY and SHR (Gao and Lee, 2001). Similarly, 1250U/ml CAT from bovine liver has been shown to greatly inhibit ACh-induced dilation in mouse small mesenteric arteries in the presence of LN and INDO (Matoba *et al*, 2000), suggesting that 860U/mL would be sufficient to reveal an effect of endogenous H_2O_2 on vasodilation in our preparation.

C) Myography Experimental Protocols

I) Denuding protocol and endothelial function test

Selected SD vessels (n=12) were denuded by rubbing the lumen for 1min with straight human hair, as described by Mulvany (2004). Following denudation, the vessel normalization, standard start, and experimental protocols proceeded as usual.

The endothelial function test was performed in all rat strains (SHR, WKY, SD) and followed the standard start protocol. This procedure consisted of a 10^{-5}mol/L NEPI pre-constriction to a steady plateau followed by administration of ACh (10^{-6} or 10^{-4}mol/L) to stimulate endothelium dependent dilation. Following the application of ACh, the bathing solution was exchanged 4x with PSS and PRO was re-administered.

II) KCl dose-response curve

The KCl dose-response curve consisted of the equimolar replacement of KCl for NaCl (0-100mmol/L KCl in 10mmol/L increments, see Appendix A for solution preparation) in the constant presence of PRO, and in the presence or absence of CAT, TEMPOL, LN, or INDO. The equimolar replacement of KCl for NaCl ensured that the effects were due to changes in K^+ but with a constant total ionic concentration. The solution bathing the vessel was removed

and replaced with each subsequent KCl solution. Following the final dose of the KCl dose-response curve, the bathing solution was exchanged 3-4x with PSS and the inhibitors were replaced.

III) NEPI and H_2O_2 Constriction Dose-Response Curves

The constriction curves were preceded by 15-30min incubation with selected inhibitors (PRO, LN, INDO, CAT, TEMPOL). The NEPI dose-response curve (10^{-11}-10^{-4}mol/L and half-concentrations) assessed receptor-mediated constriction, and the H_2O_2 dose-response curve (10^{-9}-10^{-2} mol/L and half-concentrations) revealed the effect of H_2O_2 in a quiescent vessel. Developed tension was calculated as the difference between peak constriction and baseline (in mN). Following the response from the final dose of either agonist, baths were replaced 3-4x.

IV) ACh, SNP, and H_2O_2 Dilation Dose-Response Curves

To assess the dilatory function of the mesenteric vessels, an ACh dose-response curve (10^{-11}-10^{-6} mol/L and half-concentrations) measured endothelium-dependent dilation and a SNP dose response curve (10^{-10}-10^{-4} mol/L and half-concentrations) measured endothelium-independent dilation. The H_2O_2 dose response curve (10^{-9}-10^{-2} mol/L and half-concentrations) was also conducted on selected vessels. All dose-response curves were preceded by 15-30min incubation with selected inhibitors (PRO, LN, INDO, CAT, TEMPOL). The vessels were pre-constricted with 10^{-5}mol/L NEPI and the dilation curve began once a plateau in the constriction was achieved. The peak dilation response was used to calculate the change in

tension from the NEPI pre-constriction plateau and was expressed as % dilation. Following the response from the final dose of agonist baths were replaced 3-4x.

V) Tempol and Catalase experiment

The TEMPOL and CAT experiment occurred following a 15min incubation in PSS containing PRO and simulated an experiment performed by Chen *et al* (2007) in rat mesenteric arteries. The vessels were pre-constricted with 10^{-5}mol/L NEPI and 1mmol/L TEMPOL was added causing dilation to a plateau over 20-30min. Once the plateau was achieved, 860U/mL CAT was added and a reconstriction was measured over 10-20min. 1mmol/L TEMPOL was present in the CAT solution in order to maintain the TEMPOL concentration and the plateau dilation and constriction response were calculated (in mN).

H_2O_2 ACCUMULATION

Mesenteric vessel arcades (1^{st} through 4^{th} order) were obtained as described above and kept in PSS at 4°C. Groups of mesenteric arcades with equal weight (1-3arcades/treatment) were incubated in 200μL volumes of 37°C PSS, or with PSS containing ACh (10^{-4}mol/L), LN (300μmol/L), INDO (5μmol/L), CAT (860U/mL), and TEMPOL (1mmol/L) alone or in combination. Each vessel group, and non-vessel blank solutions, were incubated with 200uL volumes of the fluorescent probe 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; 10 μmol/L,) and horseradish peroxidase (0.2U/mL) dissolved in PSS as a stock solution for 1hr at 37°C in the dark in brown tubes. The Amplex Red Assay reagents and H_2O_2 standards were purchased in a Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen Detection Technologies, Eugene, OR**)** and the reagent concentrations were chosen

to limit autofluorescence (Dikalov *et al*, 2007). Following incubation, the solutions were mixed and the supernatant was aliquoted in triplicates of 100μL into a 96well dark plate. Fluorescence of supernatant was measured on a fluorescent spectrometer (SpectraMax GeminiXS, Molecular Devices, Sunnyvale, CA), and H_2O_2 concentration was obtained from a standard curve and each plate also contained H_2O standards (10^{-9}-10^{-6}mol/L).

Following removal of the supernatant for H_2O_2 measurement, the vessels were washed, snap-frozen in liquid nitrogen, removed from tubes, and hand homogenized with a glass homogenizer (Kontes-Glass, Vineland, NJ) in 100uL of lysis buffer (20mM Hepes, 10mM NaCl, 1.5mM MgCl, 1mM DTT, 20% Glycol, 0.1% Triton X 100) with protease inhibitor (40μL/mL, Complete, Roche, Basel, Switzerland). Aliquots were then incubated at 37°C at 350rpm for 3hrs. Protein concentration was measured using a BCA assay (see Western protocol). H_2O_2 concentration was normalized to the protein content of tested vessel homogenates and expressed as pmol/mg protein.

WESTERN BLOTTING.

Frozen vessel segments (1-3segments of 3-5mg) were thawed and homogenized as described above and homogenates were combined for each rat. Protein concentration was then assessed using the BCA protein assay method where samples were combined with BCA working reagent (50parts bicinchoninic acid + 1 part Copper II sulphate) and compared to a bovine serum albumin protein standard. The protein concentration was read on a spectrometer (OD 527nm, SpectraMax plus 384, Molecular Devices, Sunnyvale, CA).

Each homogenate was aliquoted into individual samples for westerns to reduce the impact of freezing and thawing the stock homogenate for each western. Samples were

prepared with ¼ of the total volume of sample buffer (see Appendix A**)** and with protein diluted to 1μg/μL in lysis buffer to ensure equal loading.

A glass plate sandwich was loaded with 5-15% running gel and stacking gel. Samples were thawed, denatured at 95°C for 2-5min and loaded to randomly assigned lanes along with a rainbow (3-6μL GE Healthcare, Buckinghamshire, UK), a biotin ladder (3-8μL, Cell Signaling, Danvers, MA), and a protein standard (SHR mesenteric artery homogenate). The gel was electrophoresed for 30-60min at 175V using electrophoresis units (Bio-Rad, Mississauga, ON). For high molecular weight proteins (COX-1 and eNOS), non-methanol transfer buffer was used, and transfer buffer containing methanol was used for all other proteins (Appendix A). The gels were then transferred to the membrane at 25V for 45min. Following the transfer, the membrane could be dried or stained with Ponceau to expose protein loading.

For immunodetection, membranes were blocked for 1-3hrs in 10% milk or 5%BSA dissolved in TBS-T to block non-specific biding sites, and subsequently incubated for 1-12hrs with a primary antibody for Catalase (Chemicon, Danvers, MA), COX-1 (Cayman, Ann Arbor, MI), p47phox (Transduction Laboratories, BD, Mississauga, ON), SOD-1 (Stressgen Biotechnologies, Ann Arbor, MI) and SOD-2 (Stressgen Biotechnologies, Ann Arbor, MI). The membranes were then washed in TBST and exposed to the appropriate horseradish-peroxidase conjugated secondary antibody for 1hr (Santa Cruz Biotechnologies, Santa Cruz, CA). The membranes were washed again and exposed to the ECL detection solution (1:1, GE Healthcare, Buckinghamshire, UK) and developed in a Syngene Bio-imaging system (Cambridge, UK). Samples were run in duplicate and the average response for each rat was

averaged across the strain. Results were compared using a Student t-test with $p<0.05$ considered significant.

DATA FILTERING AND STATISTICAL PROCEDURES

To eliminate the high frequency but low amplitude force oscillations in dose-response curves, the data was filtered with a 0.1Hz low-pass filter with a 0.02Hz transition width. The responses of interest remained generally unchanged by the filtering, but this procedure eliminated the oscillations from contributing to changes in peak responses.

For constrictor curves (NEPI and H_2O_2), data was calculated as developed tension (peak constriction – resting tension). For dilation curves (ACh, SNP, H_2O_2), data was calculated as peak %dilation ((peak dilation/plateau in pre-constriction) x 100). For the dose-response curves, the curve parameters were calculated using a sigmoidal model with the bottom set to 0 (GraphPad Prism, v5.0a, San Diego, CA). Maximum Constriction (MC, in mN), Maximum Relaxation (MR, in %), sensitivity (EC_{50}, in mol/L or log mol/L), and total area-under-the-curve (AUC, in arbitrary units (AU)) were calculated for each artery.

The preconstrictions and parameters of the dose-response curves were compared with a 2-way ANOVA (rat strain vs drug condition, $\alpha = 0.05$) using SPSS statistical software (v16.0, SPSS Inc, Chicago, IL). The rat tissue weights, hemodynamic measures, intact vessel H_2O_2 production, and vessel characteristics were also compared using a 2-way ANOVA. In the event of a significant interaction, a one-way ANOVA was run for each strain (where $\alpha = 0.05/\#strains$) and a LSD post-hoc test was completed ($\alpha = 0.05$). A Student T-test for independent means compared differences between strains from the 2-way ANOVA ($\alpha = 0.05/\#drug\ conditions$). For Western blots, SD experiments, and wherever appropriate,

Student T-tests for independent means were used ($\alpha = 0.05$). Values are expressed mean±s.e.m.

RESULTS.

RAT AND ARTERY BASELINE PARAMETERS.

As anticipated, the SHR had an elevated blood pressure and heart rate compared to WKY (Table 1B). In addition, the SHR had greater body weight and an increased absolute heart and kidney weight; however, only the left kidney-to-body weight ratio was different between strains (Table 1A). For the vessel parameters, there was no difference in normalized vessel diameter between strains; but, as expected, there was a ~50-60% reduction in dilation to 10^{-4}mol/L ACh in the endothelial function test in SHR compared to WKY (Table 1C). In WKY, HR was significantly correlated to MAP ($r= 0.853$, $p<0.001$), SBP ($r=0.794$, $p<0.001$) and DBP ($r=0.868$, $p<0.001$) but no other correlations existed between LK/BM, endothelial function test (%), and BP parameters. In addition, no correlations existed between BP parameters, LK/BM, endothelial function test (%), or HR in SHR**.**

VASORELAXATIONS.

A) Endothelium-dependent vasomotor function – ACh dose-response curves

I) Preconstrictions

Differences in preconstrictions were noted prior to the ACh dose-response curves. When collapsed across strain, CAT incubation caused a decrease in preconstriction (7.5±1.5mN, n=12, p=0.044), and TEMPOL CAT LN INDO caused an increase in preconstriction (15.9±1.2mN, n=16, p=0.001) when compared to CON (11.0±0.7mN, n=72); however, no differences were noted between strains (Table 2A-D). In addition, preconstriction prior to

CAT LN INDO did not differ from CON (p=0.850), LN INDO did not differ from CON (p=0.907), and CAT LN INDO did not differ from LN INDO (p=0.936).

II) Maximal Responses, Sensitivity and Total Responses to ACh

Endothelium-dependent dilation was modulated both by condition and by the rat strain (Table 2A-D, Figures 2-4). For simplicity, the maximal responses and $\log EC_{50}$ will be described for differences between conditions and strains. Since changes in AUC were comparable to changes in maximal dilation, AUC will be mentioned only when these parameters do not follow the same pattern.

III) Comparison of control group responses and LN and INDO treatments

Both the maximum response and total response of SHR vessels was ~20% less than WKY under CON conditions ($p<0.001$, $p<0.001$) (Table 2B, Figure 2). LN inhibited both SHR and WKY responses (SHR: $p<0.001$, WKY: $p=0.012$), while INDO improved maximal dilation in SHR only (SHR: $p=0.023$, WKY: $p=0.287$) (Figure 2). However, AUC in WKY was increased by INDO compared to CON ($p=0.031$) (Table 2B). Although, maximal dilation was greater in WKY CON than SHR INDO ($p<0.001$), the AUC was no different ($p=0.448$). Co-incubation with LN and INDO inhibited ~38% of maximal dilation from CON ($p<0.001$) in SHR. In addition, in SHR, LN INDO had greater maximal dilation ($p=0.001$) than LN. Conversely, in WKY, LN INDO did not alter dilation from CON ($p=0.287$), but did improve dilation from LN ($p=0.003$).

IV) Comparison of responses with CAT treatment

Incubation with CAT did not alter dilation from CON in WKY (p=0.124) or SHR (p=0.985) (Table 2B-C, Figure 3). Co-incubation with CAT LN INDO caused further inhibition in maximal dilation from LN INDO; however, this response only tended towards significance in SHR (p=0.062 in ANOVA, p=0.041 in Student T-test), while achieving significance in WKY (p=0.013). However, AUC measures revealed no difference between CAT LN INDO and LN INDO in WKY (p=0.313). Interestingly, the maximal response with CAT LN INDO treatment was no different from LN in SHR (p=0.517) and WKY (p=0.457).

V) Comparison of responses with TEMPOL treatment

Incubation with TEMPOL did not alter dilation from CON in SHR (p=0.160) or WKY (p=0.928) (Table 2A and D, Figures 2 and 4), but TEMPOL did enhance the reconstriction phase in SHR. In both SHR and WKY, co-incubation with TEMPOL LN INDO did not alter dilation from LN INDO (SHR: p=0.129, WKY: p=0.377), from CON (SHR: p=0.081, WKY: p=0.920), or from TEMPOL CAT LN INDO (SHR: p=0.506, WKY: p=0.866). However, in contrast to the aforementioned maximal response measures, TEMPOL LN INDO in SHR yielded a lower AUC than SHR CON (p=0.017). Finally, TEMPOL CAT LN INDO had reduced dilation from CON in SHR (p=0.001) but not in WKY (p=0.751).

Following co-incubation with TEMPOL CAT LN INDO in WKY, both maximal dilation and AUC was no different from CAT LN INDO (p=0.353, p=0.366); however, in SHR, TEMPOL CAT LN INDO caused greater preconstriction, maximum dilation, and AUC than CAT LN INDO (p=0.009; p=0.009; p=0.008).

B) Endothelium-independent vasomotor function – SNP dose-response curves

I) Preconstrictions

Endothelium-independent responses were assessed by an SNP dose-response curve in NEPI preconstricted vessels (Table 3 A-D, Figures 5-7). Preconstriction was greater in SHR than WKY when collapsed across all drug conditions (WKY: 10.6±0.7mN (n=56) vs SHR: 13.3±0.7mN (n=67)); however, there was no main effect of drug condition on preconstriction; and NEPI preconstriction was not significantly different between SHR CON and WKY CON. The preconstrictions preceding the first and second dilatory dose-response curves, in the absence of inhibitors, were highly correlated in both WKY CON (PC1: 12.6+/-1.1mN vs PC2: 13.7+/-0.9mN (n=15), r=0.761, p=0.001) and SHR CON (PC1: 9.5+/-1.5mN vs PC2: 8.8+/-1.4mN (n=15), r=0.946, p<0.001).

II) Maximal Responses, Sensitivity and Total Responses to SNP

Maximal responses to the endothelium-independent dilator SNP were no different between SHR and WKY; however, there was a main effect of drug condition (Table 3A). The main effect of rat strain was also found for AUC, where SHR had ~6% greater total response than WKY when collapsed across drug conditions (423.0±5.2 vs 399.6±6.6). Similarly, there was a main effect of rat strain on $\log EC_{50}$ where SHR were slightly, but significantly, more sensitive to SNP than WKY when collapsed across drug conditions (-8.5±0.6 vs -8.3±0.1log mol/L). In addition to the main effects of strain, there was also a main effect of drug condition for maximal relaxation, $\log EC_{50}$, and AUC, which is described in the following sections.

III) Comparison of control group responses and L-NAME and INDO treatments

There were no interaction effects for maximal relaxation, $logEC_{50}$, and AUC, so only the main effects of drug condition have been described (Table 3A). Incubation with LN did not alter the maximal response, AUC, or $logEC_{50}$ from CON (p=0.925; p=0.340; p=0.436) (Table 3B, Figure 5). Similarly, incubation with INDO did not alter maximal response, AUC, or logEC50 from CON (p=0.206; p=0.088; p=0.240). However, co-incubation with LN and INDO caused a greater maximal response (p=0.037) and AUC (p=0.009) compared to CON with unchanged sensitivity (p=0.140).

IV) Comparison of responses with CAT treatment

There were no interaction effects for maximal relaxation, $logEC_{50}$, and AUC, so only the main effects of drug condition have been described (Table 3A). Incubation with CAT did not alter maximal response to SNP (p=0.255), AUC (p=0.953), or $logEC_{50}$ (p=0.896) from CON (p=0.255, p=0.953, p=0.896) (Table 3C, Figure 6). In addition, co-incubation with CAT LN INDO did not alter maximal response, AUC, or sensitivity from LN INDO (p=0.532, p=0.259, p=0.080). CAT LN INDO treatment had greater maximal response (p=0.049), AUC (p=0.001), $logEC_{50}$ (p=0.003) than LN, regardless of strain.

V) Comparison of responses with TEMPOL treatment

There were no interaction effects for maximal relaxation, $logEC_{50}$, and AUC, so only the main effects of drug condition have been described (Table 3A). Neither TEMPOL incubation nor TEMPOL LN INDO co-incubation altered maximal response, AUC, or sensitivity from CON (TEMPOL vs CON: p=0.068, p=0.718, p=0.613; TEMPOL LN INDO

vs CON: p=0.080, p=0.976, p=0.336) (Table 3D, Figure 7). Similarly, TEMPOL LN INDO was no different from LN INDO or from TEMPOL CAT LN INDO for maximal response, AUC, or sensitivity (TEMPOL LN INDO vs LN INDO: p=0.724, p=0.088, p=0.139; TEMPOL CAT LN INDO vs TEMPOL LN INDO: p=0.243, p=0.252, p=0.384).

Interestingly, TEMPOL CAT LN INDO had lower AUC than CAT LN INDO (p=0.039) and a decrease in sensitivity (p=0.006) but no difference in maximal relaxation (p=0.851). In addition, TEMPOL LN INDO had a decreased sensitivity and AUC to SNP over TEMPOL CAT LN INDO (p=0.001; p=0.004) without altering maximal relaxation (p=0.795).

VASOCONSTRICTIONS.

A) Receptor-mediated Constriction – NEPI dose-response curves

I) Maximal responses, Sensitivity and Total Responses to NEPI

Receptor-mediated constriction was assessed by the NEPI dose-response curve in the presence of the β-blocker PRO. Main effects for rat strain and drug condition were observed for the maximal constriction response, where the SHR had ~23% greater constriction than WKY when collapsed across drug conditions (13.4±0.6mN vs 10.9±0.6mN) (Table 4A, Figures 8 and 9). Similar to maximal constriction, AUC analysis showed a main effect of both rat strain and drug condition, where SHR had ~21% greater AUC than WKY (46.4±2.0 vs 38.5±2.2). For $\log EC_{50}$, there was an interaction effect of rat strain*drug condition but significant post-hoc differences were not found in either strain (WKY p=0.05, for SHR p=0.241**)**.

II) Comparison of control group responses L-NAME and INDO treatments

There were no interaction effects for maximal constriction, $logEC_{50}$, and AUC, so only the main effects of drug condition have been described (Table 4A). INDO did not alter maximal response or AUC from CON (p=0.883, p=0.606) or from LN INDO (p=0.472, p=0.362) (Table 4B, Figure 8-9). In addition, maximal constriction and AUC for LN INDO was no different from CON (p=0.406, p=0.477), irrespective of strain.

III) Comparison of responses with CAT and TEMPOL treatments

There were no interaction effects for maximal constriction, $logEC_{50}$, and AUC, so only the main effects of drug condition have been described (Table 4A). The maximal contraction and the AUC in the presence of TEMPOL were no different from CON (p=0.145, p=0.230) (Table 4C, Figure 8-9). In addition, the maximal contraction and the AUC in the presence of CAT LN INDO were no different from LN INDO (p=0.808, p=0.754). Interestingly, treatment with TEMPOL CAT LN INDO increased the maximal response and AUC above CON (p=0.001, p=0.001), LN INDO (p=0.037, p=0.023), and CAT LN INDO (p=0.049, p=0.035) regardless of strain.

B) Electromechanical coupling – KCl dose-response curve

I) Maximal responses, Sensitivity and Total Responses to KCl

Electromechanical coupling was assessed using a KCl dose-response curve with a equimolar exchange of KCl for NaCl. No significant main or interaction effects were

observed for maximal constriction; however, a main effect of drug condition was observed for both AUC and EC_{50} (Table 5A).

II) Comparison of control group responses and L-NAME and INDO treatments

There were no interaction effects for maximal relaxation, EC_{50}, and AUC, so only the main effects of drug condition have been described for AUC and EC_{50} (Table 5A). INDO did not alter AUC or sensitivity compared to CON ($p=0.845$, $p=0.846$); however, LN INDO increased both AUC and sensitivity over CON ($p=0.011$, $p<0.001$) (Table 5B, Figure 10). Although AUC was not significantly different between INDO and LN INDO ($p=0.063$), EC50 was improved in LN INDO compared to INDO ($p=0.003$).

III) Comparison of responses with CAT and TEMPOL treatments

There were no interaction effects for maximal relaxation, EC_{50}, and AUC, so only the main effects of drug condition have been described for AUC and EC_{50} (Table 5A). For the CAT LN INDO condition, the AUC and EC50 did not differ from LN INDO ($p=0.818$, $p=0.131$) (Table 5C, Figure 11). In addition, TEMPOL did not alter AUC or sensitivity from CON ($p=0.906$, $p=0.055$).

RESPONSES TO EXOGENOUS H_2O_2.

The response to exogenous H_2O_2 was assessed both in preconstricted and quiescent vessels in SHR and WKY (Table 6, Figures 12 and 20). To illustrate the relationship between H_2O_2 constrictory and dilatory responses, H_2O_2-induced constriction in each SHR and WKY artery was expressed relative to the NEPI preconstrictions achieved in the endothelial function

test, and these results are illustrated in Figure 12 with error bars omitted for clarity (Figure 20 is the same figure with the error bars). The maximal relaxation, logEC_{50}, and AUC did not differ between strains. However, the SHR preconstrictions were ~46% lower than the WKY vessels but did not achieve statistical significance. The maximal constriction, logEC_{50}, and AUC did not differ between SHR and WKY in the H_2O_2 constriction curve from the quiescent state, which is expressed as developed tension in Table 7. In addition, the NEPI reconstriction that followed either the H_2O_2 constriction or dilation dose-response curves did not differ between SHR and WKY, but was greatly blunted compared to the preconstrictions for the H_2O_2 dilation curve in both strains (p=0.001).

The ability of the endothelium to modulate the direct effects of H_2O_2 was assessed in both endothelium-intact (E+) and endothelium-denuded (E-) mesenteric arteries in SD rats. In addition, both E+ and E- vessels were pre-incubated with 860U/mL CAT to assess changes in the H_2O_2 dilatory response. Detailed descriptions of the results are included in Appendix B (Figure 21). Briefly, both E+ and E- vessels dilate with H_2O_2, but the AUC in E+ is ~40% greater than in E-. In addition, H_2O_2 to quiescent E+ and E- vessels causes a similar constriction. Finally, pre-incubation with CAT shifts both the E+ and E- curves to the right, and decreases the AUC by 55-75% without altering the maximal relaxation.

VASCULAR H_2O_2 ACCUMULATION.

A) Comparisons between rat strains

Levels of H_2O_2 accumulated from intact vessels were measured using the Amplex Red Assay (WKY n=5-6/group; SHR n=7-8/group). There was an interaction effect for H_2O_2 accumulation (strain*condition: p=0.050). For the SHR, no differences existed for PSS or

ACh standards between Basal and Stimulated groups (PSS-Basal vs PSS-Stimulated, p=0.219; ACH-Basal vs ACH-Stimulated, p= 0.407). Similarly, in isolated WKY mesenteric arteries, no differences existed for PSS or ACh standards between Basal and Stimulated groups (PSS-Basal vs PSS-Stimulated, p=0.178; ACH-Basal vs ACH-Stimulated, p= 0.055) Thus, for clarity, the PSS and ACh responses were collapsed across basal and stimulated experiments in the text and in Table 7A, and statistical tests reflected differences from the appropriate standard.

Generally, no differences existed between strains, although there were significant trends approaching the p=0.05 significance level (Table 7A-C). Several trends favored the increased release of H_2O_2 by SHR vessels over WKY in the ACh (p=0.055), TEMPOL (p=0.055), and ACh TEMPOL (p=0.062) conditions. Conversely, there were trends towards reduced H_2O_2 accumulation in SHR below WKY in the ACh CAT LN INDO (p=0.053) and CAT (p=0.057) conditions. Otherwise, there were no differences between strains (PSS p=0.174, LN INDO p=0.566, ACh LN INDO p=0.134, ACh -CAT p=0.425, CAT LN INDO p=0.166, ACh TEMPOL LN INDO p=0.983, TEMPOL CAT p=0.850, and ACh TEMPOL CAT LN INDO p=0.852).

B) Comparison of control group responses and L-NAME and INDO treatment

ACh treatment did not stimulate H_2O_2 release in SHR vessels (p=0.147) and co-incubation with ACh LN INDO did not alter H_2O_2 production from PSS (p=0.501) or from LN INDO (p=0.544),

In isolated WKY mesenteric arteries, ACh does not stimulate H_2O_2 release over PSS in either (p=0.055) (Table 7A). In addition, ACh LN INDO did not release additional H_2O_2

when compared to PSS (p=0.084) or LN INDO (p=0.946), nor did LN INDO release H_2O_2 when compared to PSS (p=0.098).

C) Comparison of responses with CAT treatment

In SHR vessels treated with CAT alone or in the presence of LN and INDO had reduced H_2O_2 production below PSS levels (CAT p<0.001, CAT LN INDO p<0.001, ACh-CAT p=0.031, ACh CAT LN INDO p=0.003) (Table 7B). Also, the additional presence of ACh did not alter H_2O_2 production when co-incubated with CAT LN INDO (p=0.854) or with CAT (p=0.448). Notably, ACh CAT LN INDO had lower H_2O_2 release compared to ACh LN INDO (p<0.001) in SHR.

In a similar pattern to SHR, WKY vessels treated with ACh and CAT either together or in combination LN and INDO reduced H_2O_2 release below PSS (ACh CAT p=0.043, CAT LN INDO p=0.032, ACh CAT LN INDO p=0.018) (Table 7B). In contrast to the SHR experiments, WKY vessels incubated in CAT alone did not have reduced H_2O_2 below the PSS condition (p=0.199). These results confirm that CAT scavenges H_2O_2 in isolated mesenteric arteries.

D) Comparison of responses with TEMPOL treatment

In SHR, TEMPOL alone did not increase H_2O_2 release over PSS (p=0.879) (Table 7C). ACh TEMPOL and ACh TEMPOL LN INDO increased H_2O_2 release over PSS; however, these effects failed to achieve statistical significance (p=0.085, p=0.064). Any solutions containing both TEMPOL and CAT had a level of H_2O_2 release below PSS (TEMPOL CAT p<0.001, ACh TEMPOL CAT LN INDO p=0.021)

In WKY, incubation with ACh TEMPOL caused an increase in H_2O_2 release from PSS, although this trend did not reach statistical significance (p=0.063) (Table 7C). However, ACh TEMPOL LN INDO did not increase H_2O_2 release from PSS (p=0.374) and ACh TEMPOL CAT LN INDO reduced H_2O_2 release below the levels of PSS (p=0.003). Finally, in contrast to SHR, TEMPOL CAT treatment was not able to lower H_2O_2 levels below those of PSS (p=0.072).

VASCULAR ENZYME EXPRESSION.

A) Anti-oxidant enzyme expression

Expression of the H_2O_2 scavenger Catalase was no different between SHR and WKY (WKY1.00±0.06 (n=8) vs SHR 0.93±0.08 (n=14); p=0.550) (Figure 13). Similarly, the antioxidant enzyme SOD-1 was no different between strains (WKY 1.00±0.10 (n=10) vs SHR 1.05±0.09 (n=14); p=0.714) (Figure 14). Furthermore, SOD-2 expression was no different between strains (WKY 1.00±0.07 (n=10) vs SHR 1.06±0.05 (n=14); p=0.479) (Figure 15).

B) Pro-oxidant enzyme expression

The expression of p47phox, one cytosolic subunit of the pro-oxidant enzyme NAD(P)H oxidase, was similar between both strains (WKY 1.00±0.27 (n=8) vs SHR 0.80±0.10 (n=14); p=0.592) (Figure 16). Conversely, preliminary work showed differences in the expression of gp91phox, one membrane bound subunit of NAD(P)H oxidase, where SHR expressed ~50% of WKY levels.

C) Vasoactive enzyme expression

eNOS expression was reduced ~44% in SHR compared to WKY (WKY 1.00±0.20 (n=10) vs SHR 0.56±0.07 (n=14); p=0.029) (Figure 17). Conversely, COX-1 was elevated by ~54% in SHR (WKY 1.00±0.18 (n=9) vs SHR 1.54±0.17 (n=13), p=0.045) (Figure 18). Preliminary work failed to detect COX-2 in the mesenteric vessels of both strains, however, this enzyme was highly expressed in the thoracic aorta of 30wk old male SHR.

Table 1. *Rat and Ring Parameters*

A

Parameter	WKY	n	SHR	n	p -value
BM (g)	314±3	37	347±2	43	<0.001
HM (mg)	1372±55	24	1560±47	23	0.013
LV (mg)	935±31	25	1045±48	24	0.061
RV (mg)	224±11	23	246±11	21	0.166
LK (mg)	974±20	20	1108±16	21	<0.001
HM/BM (mg/g)	4.35±0.17	24	4.50±0.13	23	0.489
LV/BM (mg/g)	2.97±0.09	25	3.01±0.14	24	0.819
RV/BM (mg/g)	0.71±0.03	23	0.71±0.03	21	0.967
LK/BM (mg/g)	3.07±0.04	20	3.18±0.03	21	0.030

B

Parameter	WKY	n	SHR	n	p -value
MAP (mmHg)	75±4	22	168±5	24	<0.001
SBP (mmHg)	93±4	22	198±6	24	<0.001
DBP (mmHg)	65±4	22	153±5	24	<0.001
HR (bpm)	312±9	22	418±6	24	<0.001

C

Parameter	WKY	n	SHR	n	p -value
Normalized diameter (μm)	235±7	74	221±6	92	0.112
PC in Endo fx (plateau, mN)	11.5±0.7	54	13.5±0.9	53	0.094
Endo fx -4ACh (peak, %)	79.5±2.2	54	35.0±2.3	53	<0.001

Values are mean±s.e.m. *A:* Rat tissue weights; *B*. Hemodynamics *C.* Vessel baseline characteristics. BM, body mass; HM, heart mass; LV, left ventricle mass; RV, right ventricle mass; LK, left kidney mass; HM/BM, heart mass-to-body mass ratio; LV/BM, left ventricle mass-to-body mass ratio RV/BM, right ventricle mass-to-body mass ratio; LK/BM, left kidney mass-to-body mass ratio; MAP, mean arterial pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; Endo fx; endothelial function test; PC, norepinephrine preconstriction; ACh, acetylcholine. p-value from a Student T-test for independent means

Table 2*. Endothelium-Dependent Dilation to ACh*

A

ACh	P values		
	Strain	Drug	Strain*Drug
PC (mN)	0.071	0.010	0.716
MR (%)	<0.001	<0.001	<0.001
$logEC_{50}$ (mol/L)	0.079	0.133	0.998
AUC (AU)	<0.001	<0.001	<0.001

B

ACh	WKY				SHR			
	CON	LN	INDO	LN INDO	CON	LN	INDO	LN INDO
PC (mN)	10.9±1.0	12.0±2.7	7.0±0.8	9.1±1.4	11.0±0.9	11.4±1.5	12.5±1.2	12.1±1.4
MR (%)	94.8±1.3	83.3±7.7	98.6±1.6	98.3±1.5	75.2±2.9	23.3±3.2	89.7±2.0	46.2±4.8
$logEC_{50}$ (mol/L)	-9.8±0.1	-9.3±0.2	-10.1±0.1	-9.6±0.1	-9.6±0.2	-9.2±0.2	-9.9±0.1	-9.3±0.1
AUC (AU)	354.6 ±8.6	279.8 ±31.4	396.5 ±12.1	350.4 ±14.3	283.6 ±10.4	76.1 ±12.9	350.2 ±12.2	154.6 ±18.8

Values are mean±s.e.m. *A:* p-values from ANOVA; *B.* Effects of L-NAME and INDO. ACh, acetylcholine; PC, norepinephrine preconstriction; MR, maximal relaxation; AUC, area under the dose-response curve; LN, L-NAME; INDO, indomethacin; Strain, main effect of rat strain; Drug, main effect of drug condition; Strain*Drug, interaction effect of rat strain and drug condition.

***Table 2**. Endothelium-Dependent Dilation to ACh*

C

ACh	WKY		SHR	
	CAT	CAT LN INDO	CAT	CAT LN INDO
PC (mN)	6.4±2.2	11.0±2.3	8.7±2.2	10.2±2.1
MR (%)	99.9±3.0	91.6±2.5	75.1±7.4	29.7±3.1
$logEC_{50}$ (mol/L)	-10.0±0.1	-9.7±0.1	-9.8±0.2	-9.4±0.2
AUC (AU)	382.8 ±13.7	332.1 ±14.2	275.6 ±25.9	101.6 ±12.1

D

ACh	WKY			SHR		
	TEMPOL	TEMPOL LN INDO	TEMPOL CAT LN INDO	TEMPOL	TEMPOL LN INDO	TEMPOL CAT LN INDO
PC (mN)	12.8±2.2	12.3±2.0	14.6±2.0	14.1±2.4	14.4±2.3	16.6±1.5
MR (%)	95.7±1.9	95.7±1.7	95.1±0.8	63.4±7.6	60.5±9.6	54.0±8.5
$logEC_{50}$ (mol/L)	-9.8±0.2	-9.5±0.1	-9.7±0.0	-9.3±1.0	-9.5±0.3	-9.6±0.1
AUC (AU)	360.2 ±24.1	330.2 ±8.6	350.9 ±3.2	270.5 ±29.7	210.2 ±32.6	191.8 ±26.9

Values are mean±s.e.m. *C.* Effect of CAT; *D.* Effect of TEMPOL. ACh, acetylcholine; PC, norepinephrine preconstriction; MR, maximal relaxation; AUC, area under the dose-response curve; LN, L-NAME; INDO, indomethacin; CAT, catalase; Strain, main effect of rat strain; Drug, main effect of drug condition; Strain*Drug, interaction effect of rat strain and drug condition.

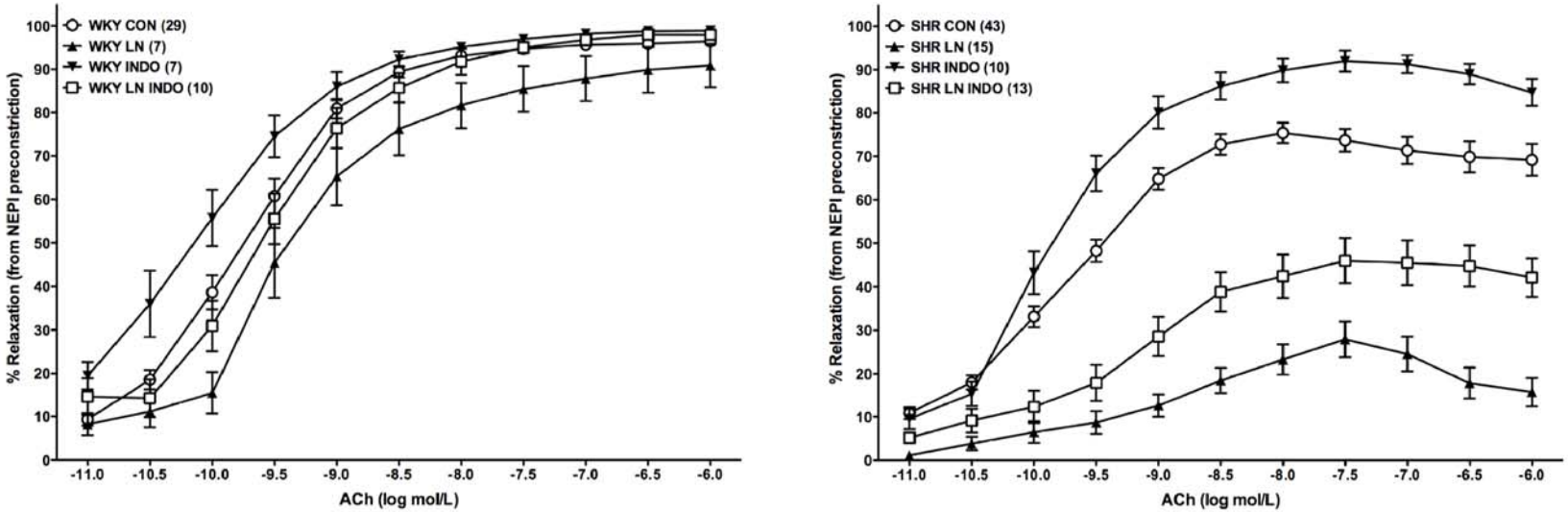

Figure 2. *Endothelium-dependent dilation to ACh in the presence of LN and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. ACh, acetylcholine; NEPI, norepinephrine; CON, untreated; LN, L-NAME; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details.

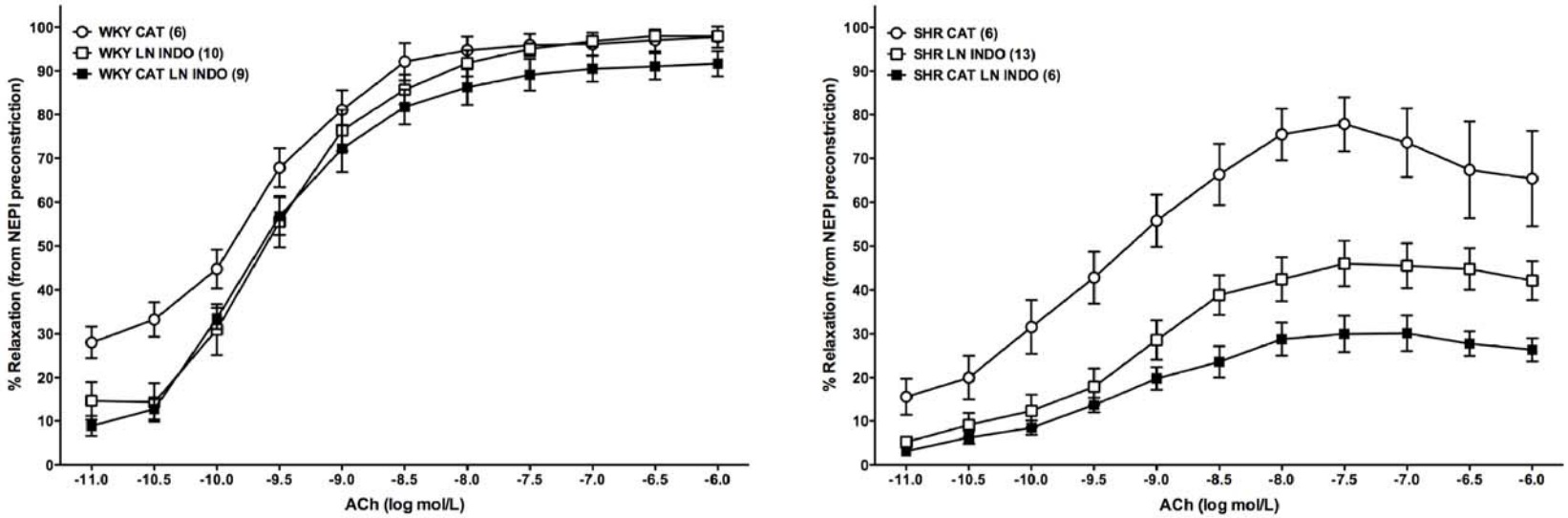

Figure 3. *Endothelium-dependent dilation to ACh in the presence of CAT, LN, and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. ACh, acetylcholine; NEPI, norepinephrine; CAT, catalase; LN, L-NAME; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details.

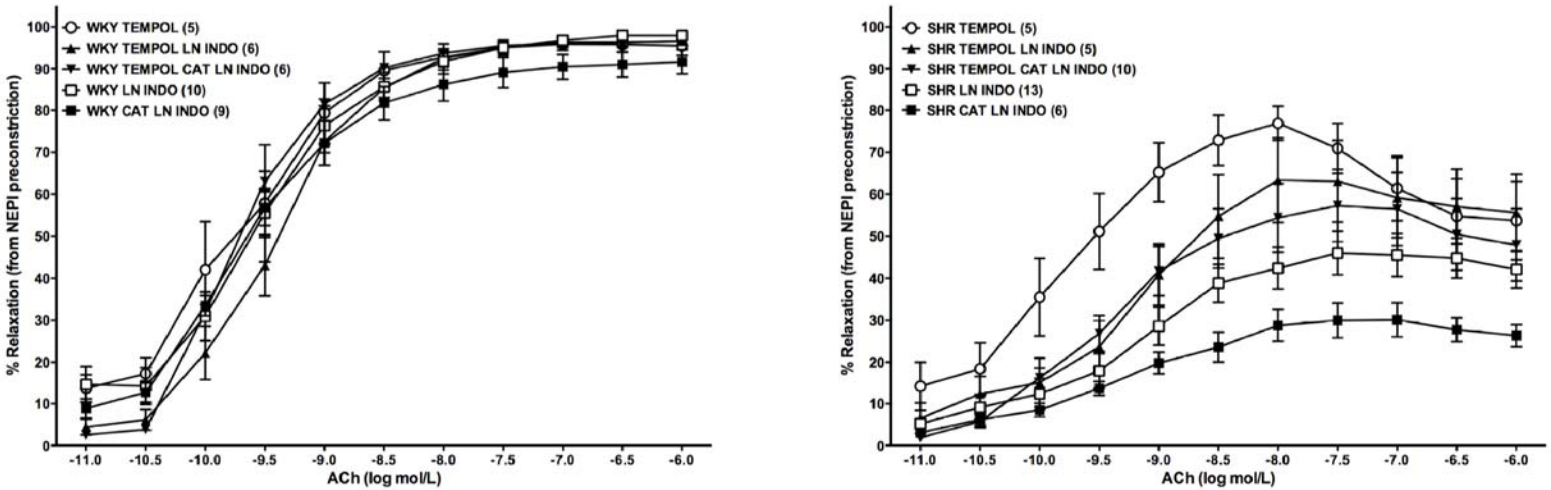

Figure 4. *Endothelium-dependent dilation to ACh in the presence of TEMPOL, CAT, LN, and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. ACh, acetylcholine; NEPI, norepinephrine; CAT, catalase; LN, L-NAME; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details.

Table 3*. Endothelium-Independent Dilation to SNP*

A

SNP	P values		
	Strain	Drug	Strain*Drug
PC (mN)	0.024	0.111	0.929
MR (%)	0.937	0.002	0.162
$logEC_{50}$ (mol/L)	0.008	0.004	0.340
AUC (AU)	0.009	<0.001	0.254

B

SNP	WKY				SHR			
	CON	LN	INDO	LN INDO	CON	LN	INDO	LN INDO
PC (mN)	10.7±2.0	10.9±2.7	9.1±1.2	10.6±1.0	9.0±1.3	14.6±1.8	13.9±2.4	15.3±1.6
MR (%)	93.1±2.7	89.6±2.8	94.1±1.7	96.6±0.7	92.5±1.6	94.1±1.0	97.0±1.5	96.4±0.6
$logEC_{50}$ (mol/L)	-8.2±0.2	-8.2±0.3	-8.4±0.2	-8.5±0.1	-8.3±0.2	-8.5±0.1	-8.6±0.3	-8.5±0.1
AUC (AU)	385.5 ±18.4	372.4 ±28.9	410.5 ±19.3	434.2 ±13.5	389.9 ±12.5	417.4 ±10.5	438.4 ±17.4	433.6 ±10.9

Values are mean±s.e.m. *A:* p-values from ANOVA; *B.* Effects of L-NAME and INDO. SNP, sodium nitroprusside; PC, norepinephrine preconstriction; MR, maximal relaxation; AUC, area under the dose-response curve; LN, L-NAME; INDO, indomethacin. Strain, main effect of rat strain; Drug, main effect of drug condition; Strain*Drug, interaction effect of rat strain and drug condition.

Table 3*. Endothelium-Independent Dilation to SNP*

C

SNP	WKY		SHR	
	CAT	CAT LN INDO	CAT	CAT LN INDO
PC (mN)	7.8±2.0	9.2±2.3	9.6±2.1	10.3±2.1
MR (%)	96.3±1.7	95.1±2.1	92.8±2.0	95.8±0.9
$logEC_{50}$ (mol/L)	-7.8±0.2	-8.7±0.2	-8.6±0.2	-9.0±0.2
AUC (AU)	357.0 ±16.8	437.0 ±13.7	412.1 ±14.0	472.7 ±14.2

D

SNP	WKY			SHR		
	TEMPOL	TEMPOL LN INDO	TEMPOL CAT LN INDO	TEMPOL	TEMPOL LN INDO	TEMPOL CAT LN INDO
PC (mN)	12.4±1.8	10.9±2.1	12.8±1.5	13.6±2.7	14.0±2.3	15.9±1.5
MR (%)	97.8±0.6	95.4±1.6	99.0±1.4	94.4±1.1	96.4±0.7	96.5±0.7
$logEC_{50}$ (mol/L)	-8.2±0.3	-8.3±0.1	-8.1±0.1	-8.1±0.1	-8.3±0.1	-8.6±0.2
AUC (AU)	407.5 ±23.1	401.9 ±10.2	400.1 ±8.8	381.4 ±8.6	408.5 ±10.5	440.1 ±14.0

Values are mean±s.e.m. *C.* Effect of CAT; *D.* Effect of TEMPOL. SNP, sodium nitroprusside; PC, norepinephrine preconstriction; MR, maximal relaxation; AUC, area under the dose-response curve; LN, L-NAME; INDO, indomethacin; CAT, catalase; Strain, main effect of rat strain; Drug, main effect of drug condition; Strain*Drug, interaction effect of rat strain and drug condition.

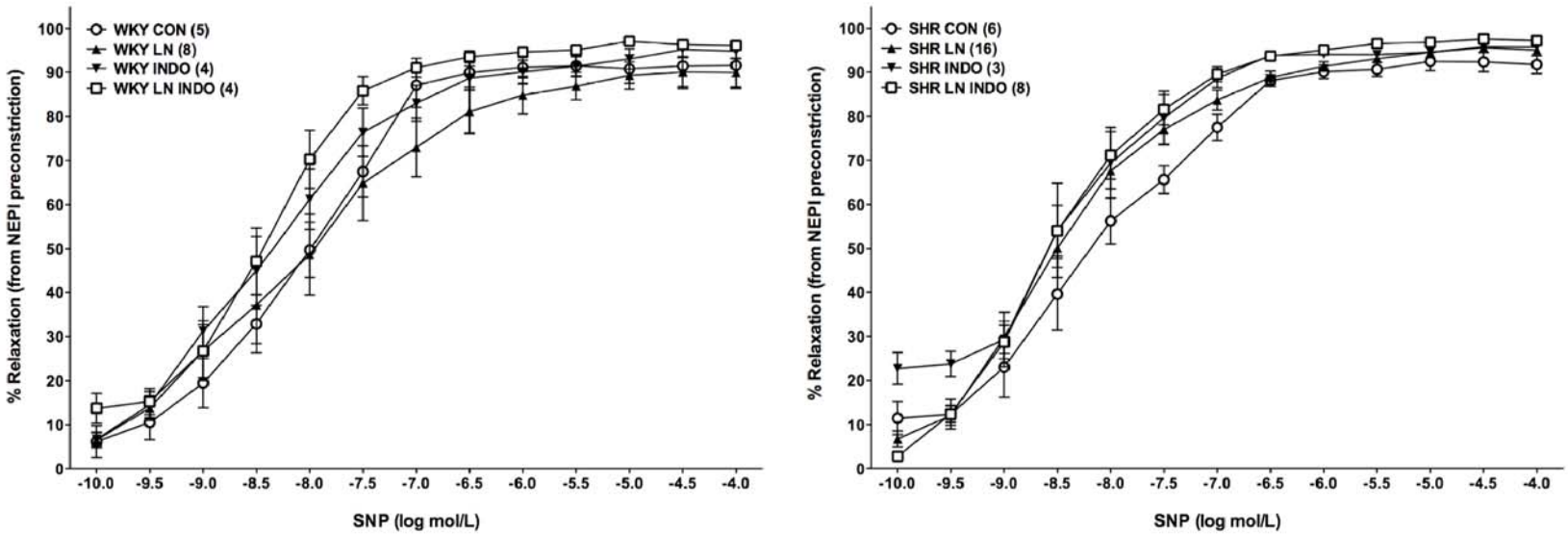

Figure 5. *Endothelium-independent dilation to SNP in the presence of LN and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. SNP, sodium nitroprusside; NEPI, norepinephrine; CON, untreated; LN, L-NAME; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details.

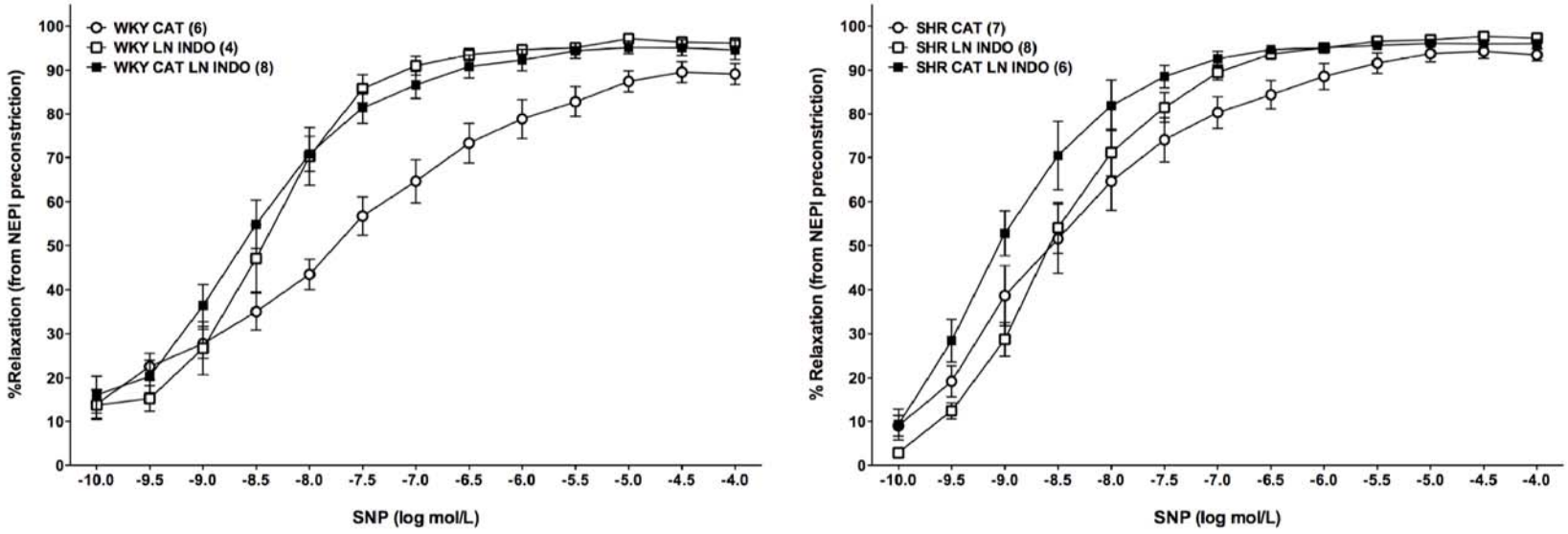

Figure 6. *Endothelium-independent dilation to SNP in the presence of CAT, LN, and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. SNP, sodium nitroprusside; NEPI, norepinephrine; CAT, catalase; LN, L-NAME; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details.

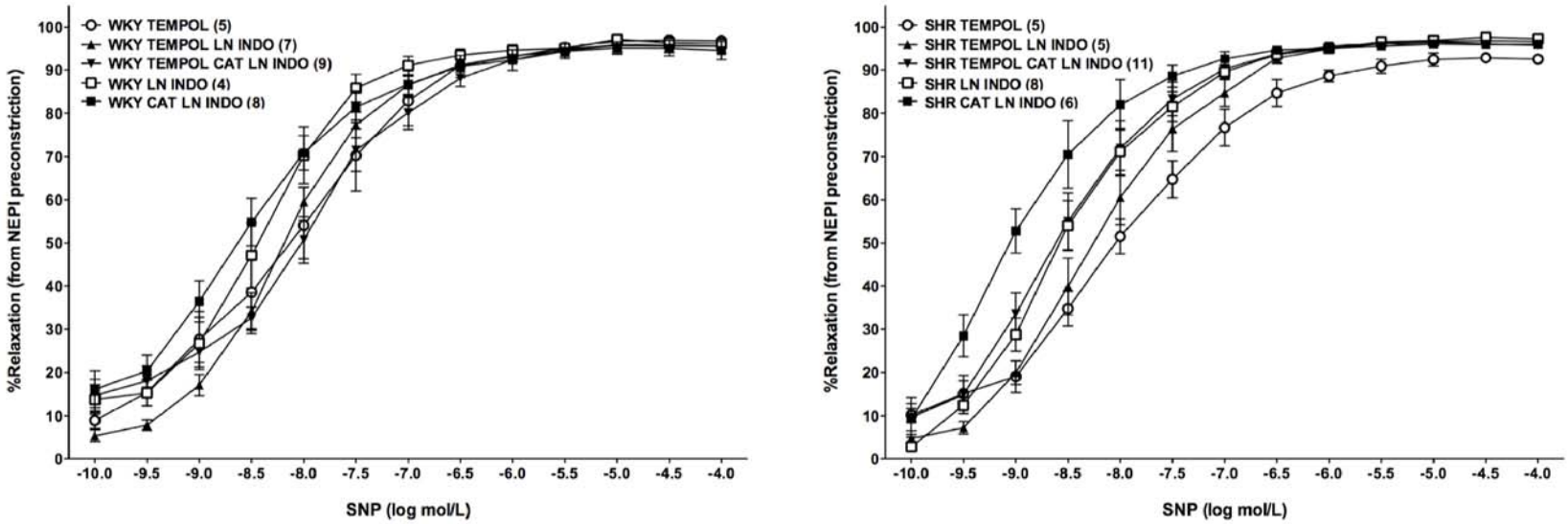

Figure 7. *Endothelium-independent dilation to SNP in the presence of TEMPOL, CAT, LN, and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. SNP, sodium nitroprusside; NEPI, norepinephrine; CAT, catalase; LN, L-NAME; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details.

***Table 4**. Receptor-mediated Constriction to NEPI*

A

NEPI	P values		
	Strain	Drug	Strain*Drug
MC (mN)	0.015	0.028	0.982
$logEC_{50}$ (mol/L)	0.877	0.256	0.040
AUC (AU)	0.010	0.025	0.836

B

NEPI	WKY			SHR		
	CON	INDO	LN INDO	CON	INDO	LN INDO
MC (mN)	10.1±0.8	10.4±2.1	10.7±2.5	12.5±0.7	12.5±1.5	15.4±2.5
$logEC_{50}$ (mol/L)	-7.6±0.0	-7.3±0.1	-7.3±0.2	-7.4±0.0	-7.3±0.1	-7.5±0.1
AUC (AU)	37.1±2.9	34.6±7.2	35.8±9.3	42.7±2.3	41.4±4.8	54.6±9.2

C

NEPI	WKY			SHR		
	TEMPOL	CAT LN INDO	TEMPOL CAT LN INDO	TEMPOL	CAT LN INDO	TEMPOL CAT LN INDO
MC (mN)	12.9±1.9	12.2±2.3	15.3±1.1	15.0±2.5	14.8±2.1	19.3±1.6
$logEC_{50}$ (mol/L)	-7.5±0.2	-7.4±0.1	-7.5±0.0	-7.3±0.2	-7.6±0.1	-7.5±0.2
AUC (AU)	44.2±5.4	41.0±6.7	53.7±4.1	50.7±8.4	54.1±7.1	69.1±8.5

Values are mean±s.e.m. *A:* p-values from ANOVA; *B.* Effects of L-NAME and INDO; *C.* Effect of CAT and TEMPOL. NEPI, norepinephrine; MC, maximal constriction; AUC, area under the dose-response curve; LN, L-NAME; INDO, indomethacin; CAT, catalase; Strain, main effect of rat strain; Drug, main effect of drug condition; Strain*Drug, interaction effect of rat strain and drug condition.

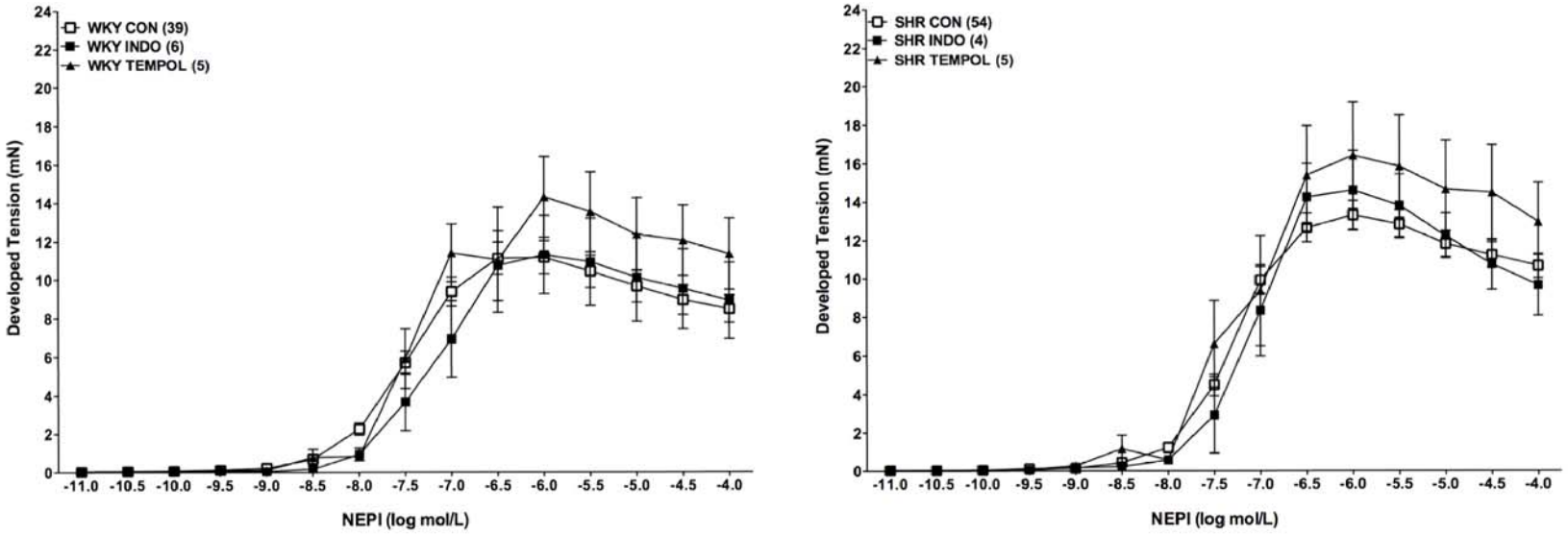

Figure 8. *Receptor-mediated constriction to NEPI in the presence of TEMPOL and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. NEPI, norepinephrine; CON, untreated; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details

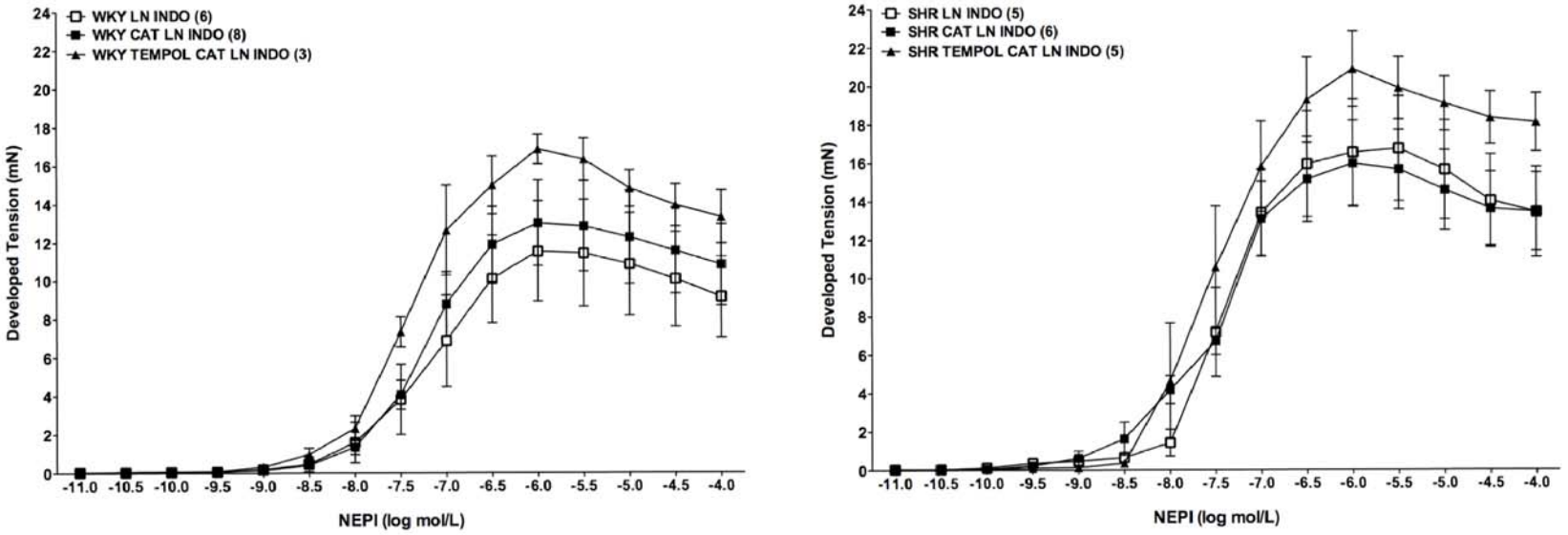

Figure 9. *Receptor-mediated constriction to NEPI in the presence of TEMPOL, CAT, LN, and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. NEPI, norepinephrine; CAT, catalase; LN, L-NAME; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details

***Table 5**. Electromechanical coupling to KCl*

A

KCl	P values		
	Strain	Drug	Strain*Drug
MC (mN)	0.123	0.160	0.685
EC_{50} (mol/L)	0.845	<0.001	0.142
AUC (AU)	0.109	0.025	0.424

B

KCl	WKY			SHR		
	CON	INDO	LN INDO	CON	INDO	LN INDO
MC (mN)	11.3±1.0	9.4±2.2	13.5±2.2	11.2±1.3	13.7±2.2	16.2±2.9
EC_{50} (mol/L)	46.4±0.8	49.7±2.5	40.3±1.7	49.0±1.3	44.4±2.0	39.8±0.6
AUC (AU)	605.4±53.1	483.8±120.0	786.0±121.2	581.5±72.5	770.8±146.8	972.3±176.2

C

KCl	WKY		SHR	
	TEMPOL	CAT LN INDO	TEMPOL	CAT LN INDO
MC (mN)	11.3±2.5	13.1±2.2	11.7±2.8	16.9±2.7
EC_{50} (mol/L)	48.5±2.7	44.4±4.1	54.4±2.8	43.0±2.1
AUC (AU)	606.1±141.7	723.4±130.5	554.0±147.7	978.3±168.7

Values are mean±s.e.m. *A:* p-values from ANOVA; *B.* Effects of L-NAME and INDO; *C.* Effect of CAT and TEMPOL. KCl, potassium chloride; MC, maximal constriction; AUC, area under the dose-response curve; LN, L-NAME; INDO, indomethacin; CAT, catalase; Strain, main effect of rat strain; Drug, main effect of drug condition; Strain*Drug, interaction effect of rat strain and drug condition.

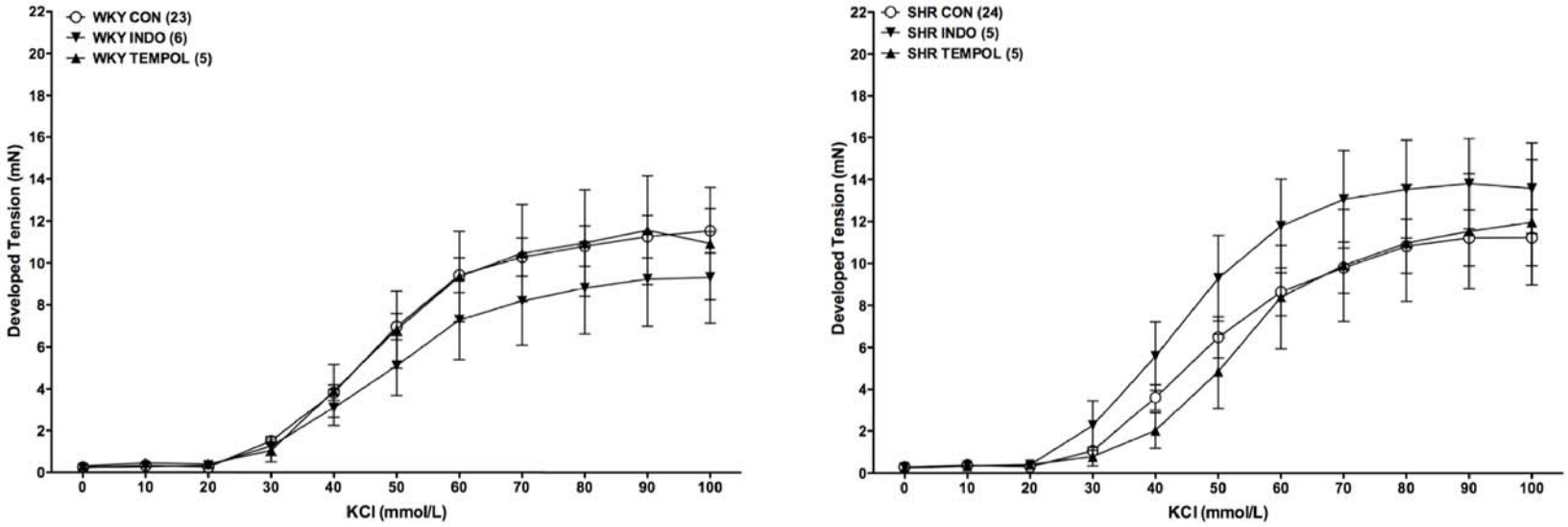

Figure 10. *Electromechanical constriction to KCl in the presence of TEMPOL and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. KCl, potassium chloride; CON, untreated; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details

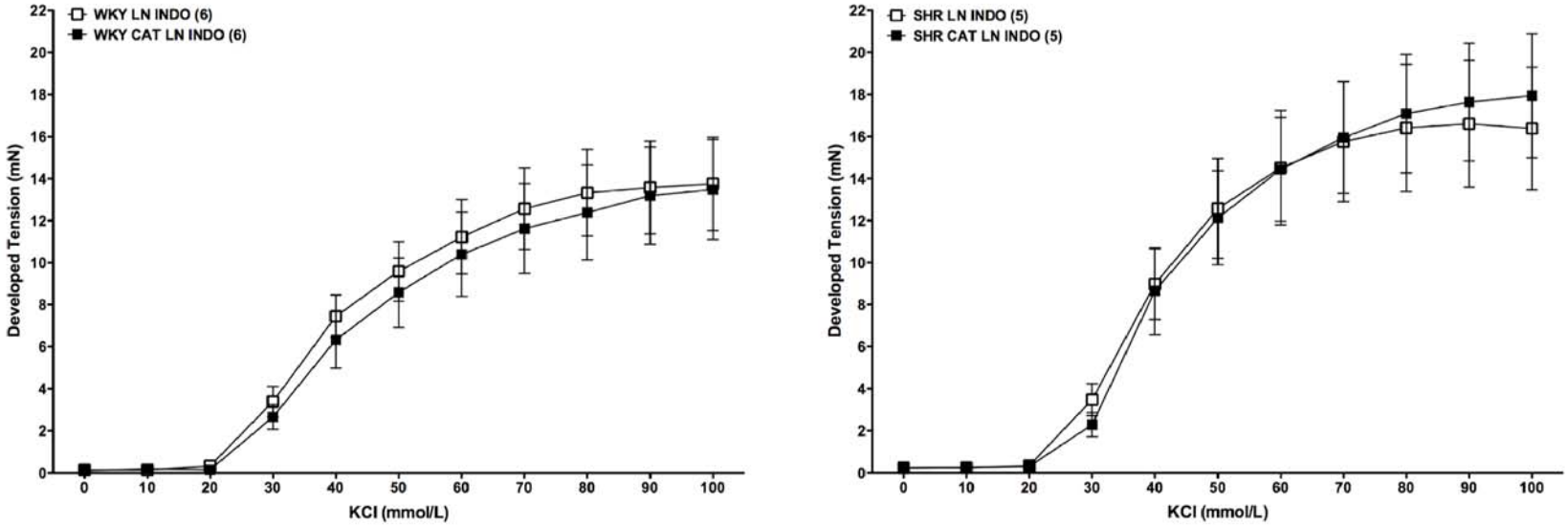

Figure 11. *Electromechanical constriction to KCl in the presence of CAT, LN, and INDO. Left:* WKY; *Right:* SHR. Values are means±s.e.m., n per group in parenthesis. KCl, potassium chloride; CAT, catalase; LN, L-NAME; INDO, indomethacin. Symbols for statistical difference were omitted for clarity. Please refer to text for details

***Table 6**. Effects of Exogenous H_2O_2*

	Parameter	WKY	SHR	P value
H_2O_2 dilation from preconstricted vessel	PC (mN)	13.9±1.3	7.5±2.6	0.059
	MR (%)	85.6±2.5	78.3±6.2	0.310
	$logEC_{50}$ (mol/L)	-5.6±0.1	-5.5±0.3	0.815
	AUC (AU)	327.9±10.2	295.1±36.2	0.408
H_2O_2 constriction from quiescent vessel	MC (mN)	4.4±0.7	5.6±1.0	0.338
	$logEC_{50}$ (mol/L)	-3.4±0.1	-3.2±0.0	0.146
	AUC (AU)	7.0±1.5	7.9±1.1	0.759
Post H_2O_2 reconstriction	MC (mN)	-0.011±0.009	0.010±0.063	0.744

Values are mean±s.e.m. PC, norepinephrine preconstriction; MR, maximal relaxation; MC, maximal constriction; AUC, area under the dose-response curve. For H_2O_2 dilation, vessels were preconstricted with 10^{-5}mol/L NEPI, followed by a dose response curve with 10^{-9}-10^{-2}mol/L H_2O_2. For H_2O_2 constriction, vessels were not preconstricted and a dose response curve with 10^{-9}-10^{-2}mol/L H_2O_2 was completed from a quiescent vessel. Following either the H_2O_2 dilation or H_2O_2 constriction dose response curves, the bath was exchanged and the vessels were reconstructed with 10^{-5}mol/L NEPI. p-value from a Student T-test for independent means.

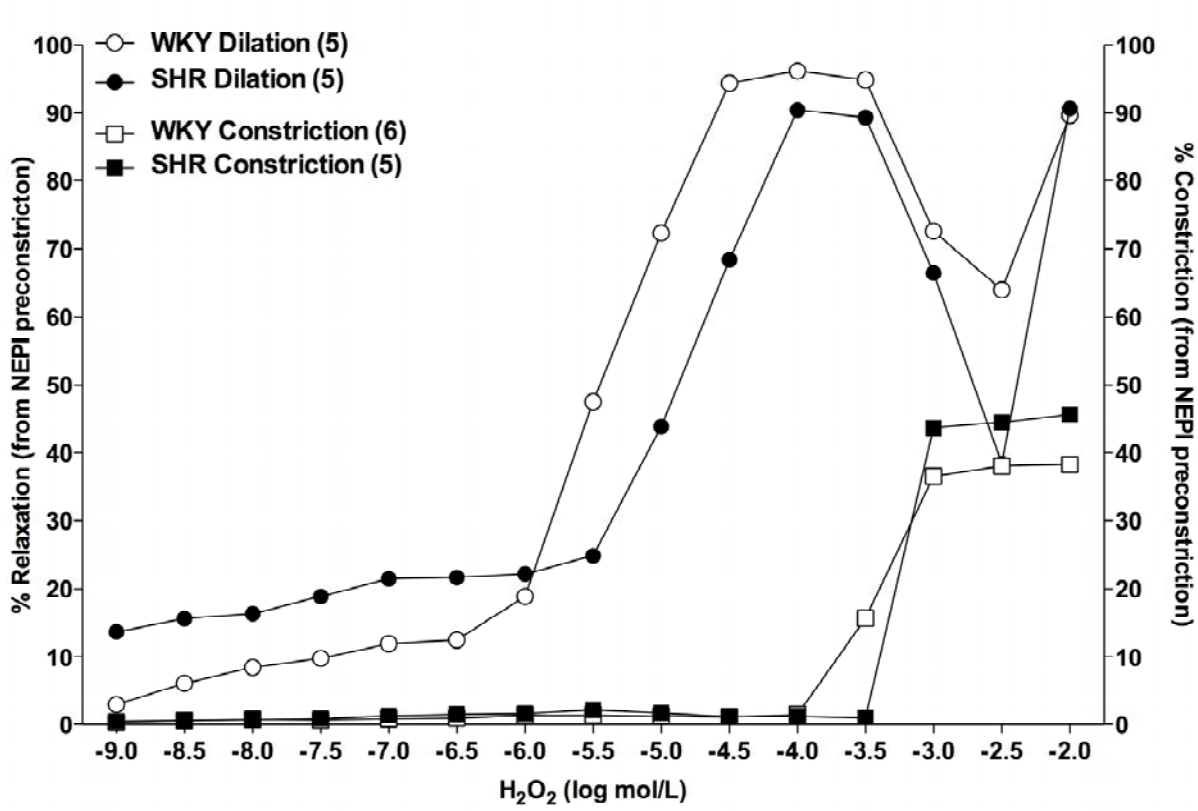

Figure 12. *H_2O_2-induced dilation and constriction.* Values are means, n per group in parenthesis. NEPI, norepinephrine; Dilation, relaxation from NEPI preconstriction; Constriction, developed tension from quiescent vessel expressed relative to NEPI preconstriction from endothelial function test. Symbols for statistical difference, and error bars, were omitted for clarity. Figure 21 contains data with error bars. Please refer to text for details.

Table 7. *H_2O_2 Accumulation in Isolated Arteries*

A

	WKY				SHR			
	Basal		Stimulated		Basal		Stimulated	
	PSS	LN INDO	ACh	ACh LN INDO	PSS	LN INDO	ACh	ACh LN INDO
H_2O_2 (pmol/ mg)	17.9±2.7	21.6±4.8	15.2±2.6	11.8±2.8	20.4±3.3	17.3±5.1	18.0±2.7	20.6±4.2

B

	WKY				SHR			
	Basal		Stimulated		Basal		Stimulated	
	CAT	CAT LN INDO	ACh CAT	ACh CAT LN INDO	CAT	CAT LN INDO	ACh CAT	ACh CAT LN INDO
H_2O_2 (pmol/ mg)	5.8±2.4	0.4±0.4	9.5±5.6	7.9±4.0	1.0±0.8	1.5±0.5	5.1±2.3	0.5±0.5

C

	WKY					SHR				
	Basal			Stimulated		Basal			Stimulated	
	TEMPOL	TEMPOL CAT	ACh TEMPOL	ACh TEMPOL LN INDO	ACh TEMPOL CAT LN INDO	TEMPOL	TEMPOL CAT	ACh TEMPOL	ACh TEMPOL LN INDO	ACh TEMPOL CAT LN INDO
H_2O_2 (pmol/ mg)	9.7±1.7	2.6±1.6	11.0±3.8	27.2±9.2	3.5±1.7	23.0±5.0	2.2±1.1	26.3±5.8	27.0±5.4	4.2±2.7

Values are mean±s.e.m, n=5-8 per group. *A:* Comparison of control responses with L-NAME and INDO; *B*: Effect of CAT *C:* Effect of TEMPOL. Basal, vessels not stimulated by ACh; Stimulated, vessels treated with ACh; LN, L-NAME; INDO, indomethacin; CAT, catalase. Please refer to text for details.

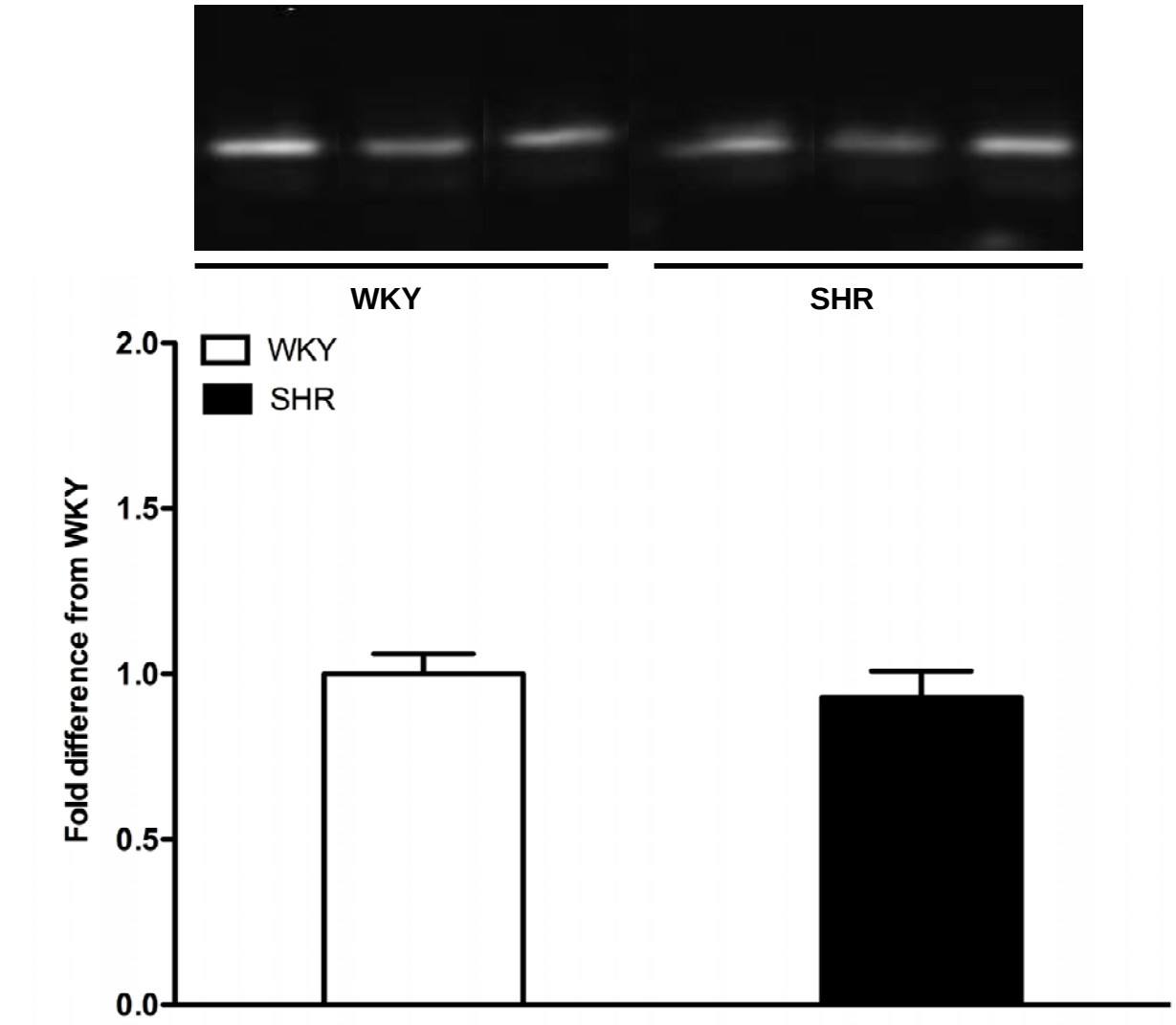

Figure 13. *Protein levels of catalase.* Values are means±s.e.m, n = 10-12 per strain. *Top:* representative immunoblots *Bottom:* Expression differences between rat strains. Please refer to text for details.

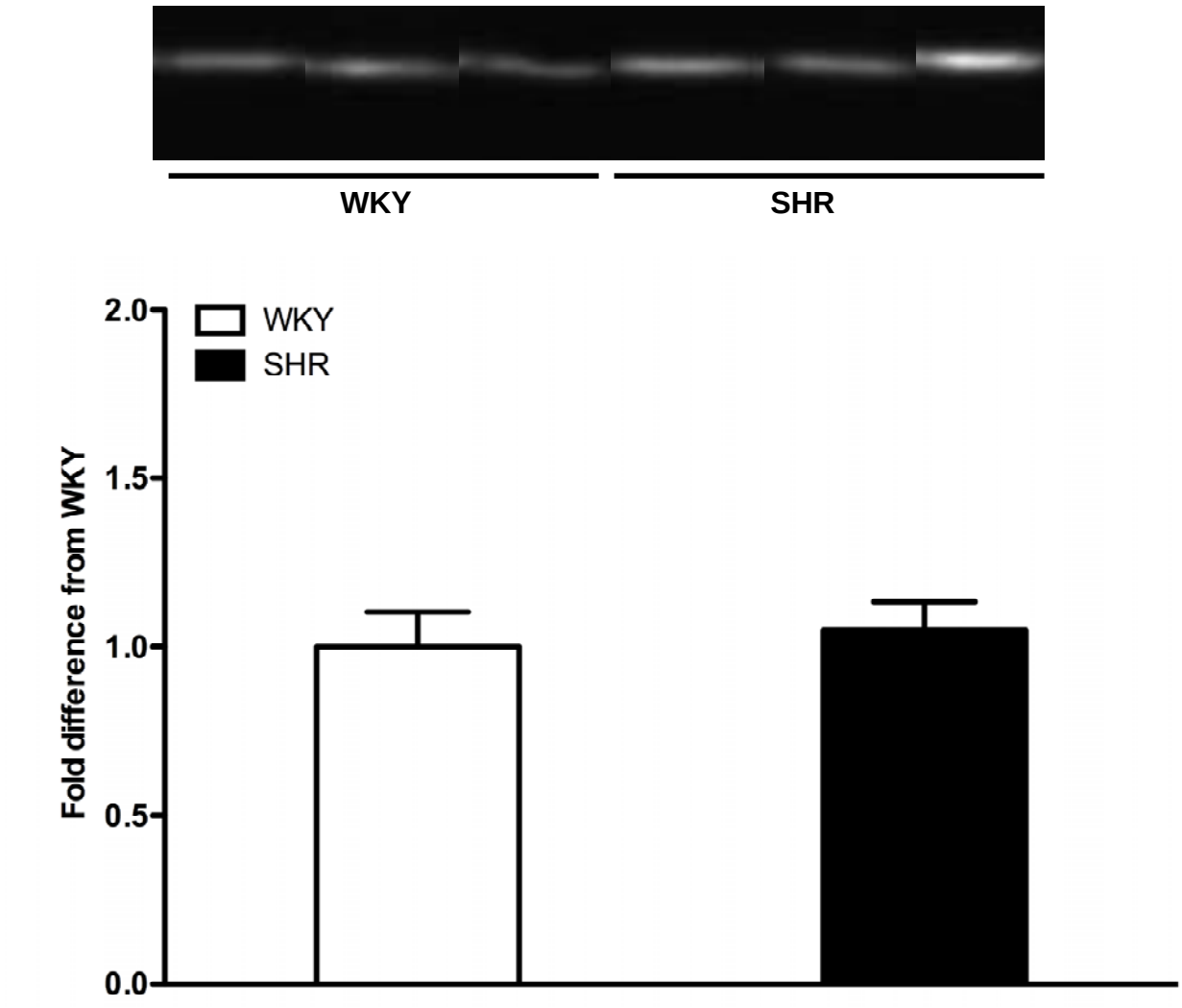

Figure 14. *Protein levels of SOD-1*. Values are means±s.e.m, n = 10-14 per strain. *Top:* representative immunoblots *Bottom:* Expression differences between rat strains. Please refer to text for details.

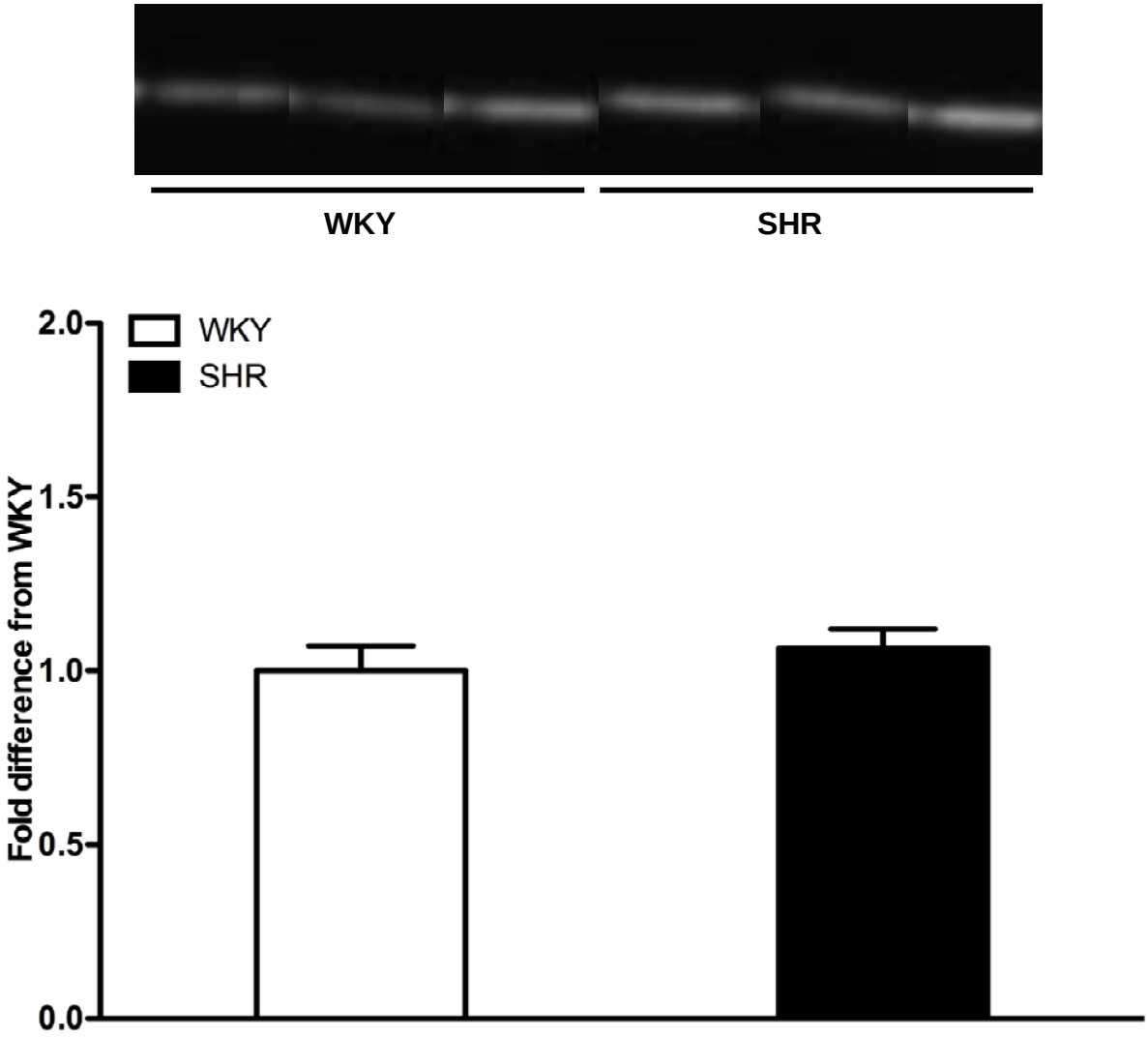

Figure 15. *Protein levels of SOD-2.* Values are means±s.e.m., n = 10-14 per strain. *Top:* representative immunoblots *Bottom:* Expression differences between rat strains. Please refer to text for details.

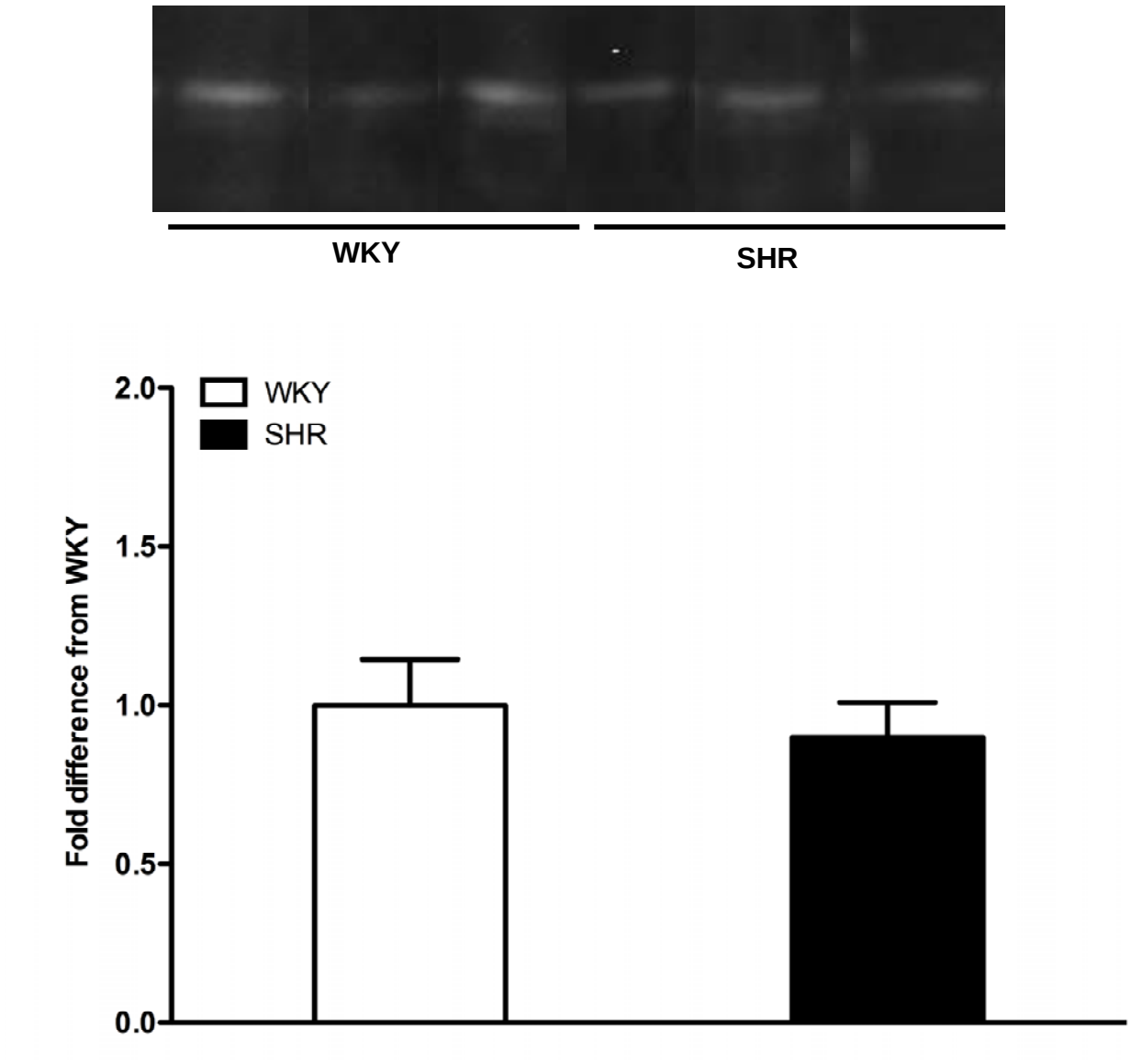

Figure 16. *Protein levels of p47phox.* Values are means±s.e.m., n = 8-14 per strain. *Top:* representative immunoblots *Bottom:* Expression differences between rat strains. Please refer to text for details.

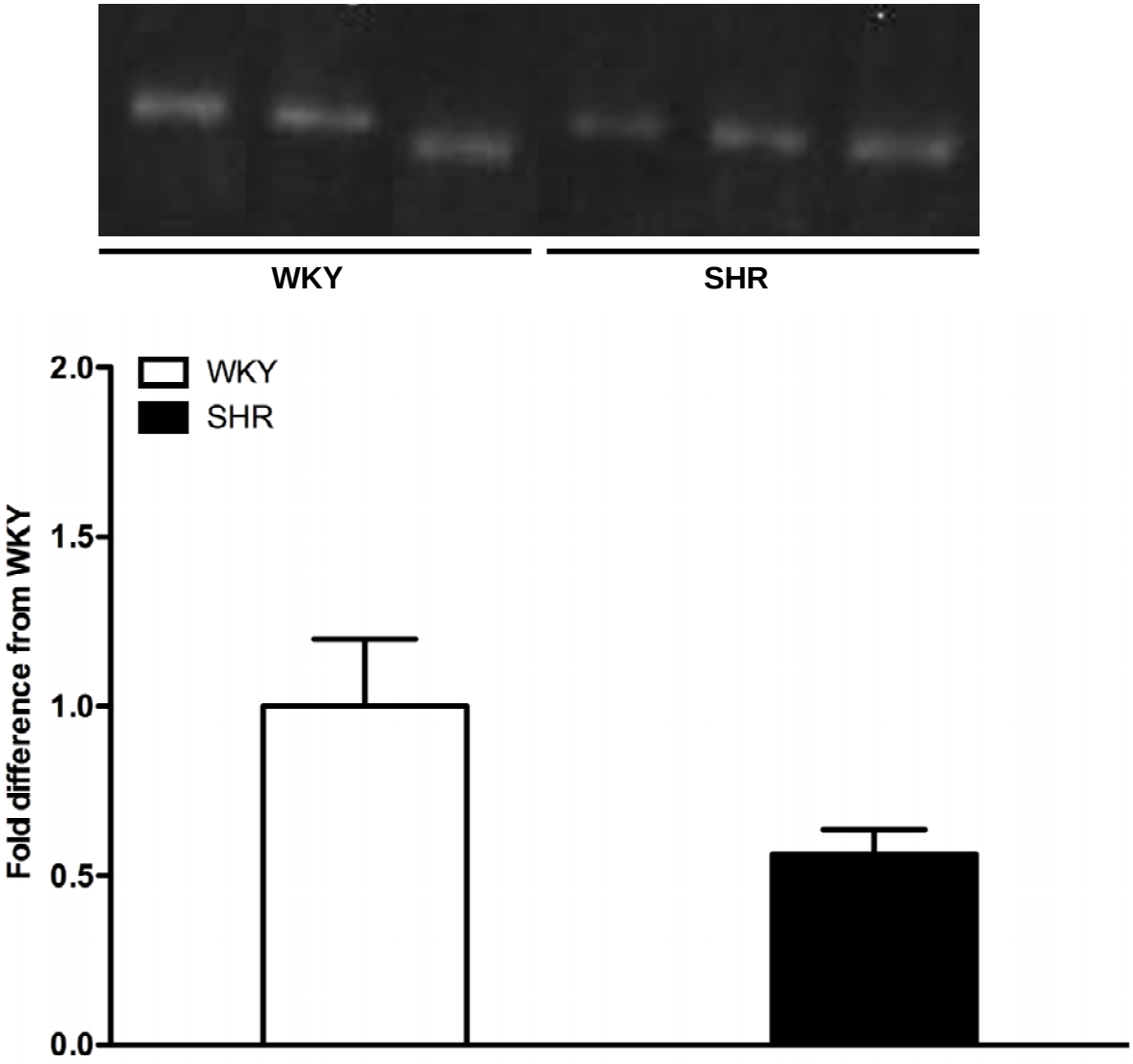

Figure 17. *Protein levels of eNOS.* Values are means±s.e.m., n = 10-14 per strain. *Top:* representative immunoblots *Bottom:* Expression differences between rat strains. Please refer to text for details.

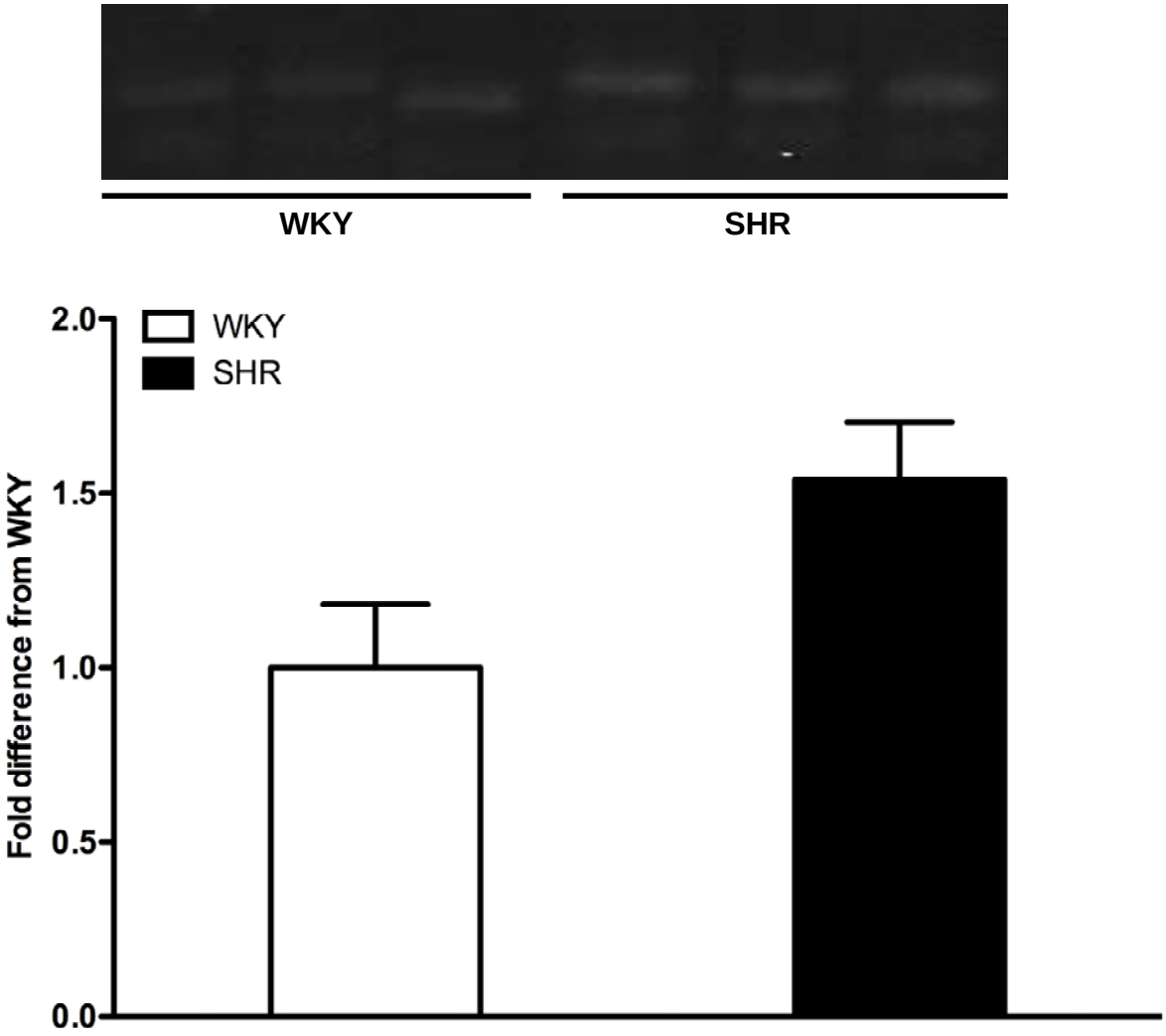

Figure 18. *Protein levels of COX-1.* Values are means±s.e.m., n = 9-13 per strain. *Top:* representative immunoblots *Bottom:* Expression differences between rat strains. Please refer to text for details.

DISCUSSION.

The aim of this study was to investigate the role of endogenous H_2O_2 in 3NP-mediated endothelium-dependent dilation of rat mesenteric resistance arteries, and the changes that occur in these vessels with essential hypertension. The main findings of this study are:

1. SHR rats exhibit severe hypertension that coincides with impaired endothelium-dependent dilation and COX products mediate the impaired vasomotor function.
2. When NO and PG are inhibited, H_2O_2 is an endogenous endothelium-mediated dilator in both WKY and SHR while exogenous H_2O_2 can both dilate and constrict mesenteric vessels.
3. The proportion of ACh-induced dilation that is mediated by 3NP is reduced in SHR compared to WKY mesenteric arteries.
4. SHR and WKY mesenteric vessels express similar levels of both anti- and pro-oxidant enzymes; however, SHR expresses elevated COX-1 and reduced eNOS.
5. TEMPOL may improve dilation through scavenging of O_2^-.

VASODILATION.

A) Comparison of control responses

As expected, SHR rats had an elevated blood pressure compared to WKY, and their mesenteric arteries demonstrated an impaired endothelium-dependent dilation to ACh. SHR mesenteric arteries achieved only ~79% of the maximal relaxation and total response in the ACh dose-response curve, and less than 50% of the dilation in the endothelial function test

compared to age-matched WKY (Figure 2). Notably, the maximal response to SNP was no different between SHR and WKY when collapsed across drug condition but SHR had a ~6% greater AUC and an 85% increase in sensitivity to SNP compared to WKY. This increased AUC and sensitivity in the SHR was not conserved in the comparison of the CON responses, suggesting that, when collapsed across drug conditions, there were other strain-dependent differences revealed by the drug treatments that increased the responses SHR arteries over WKY. The similarity in SNP responses in both WKY and SHR CON groups suggest that the reduced dilation to ACh in the SHR is due to impaired endothelial vasomotor function, and not due to impaired responses of the VSM to NO, a finding that is in agreement with previous works in the SHR mesenteric arteries (Diederich *et al,* 1990; Jameson *et al,* 1993). However, the similarity in responses to SNP does not rule out the possibility that the sensitivity of the VSM to 3NP-mediated dilators may be different between SHR and WKY, as 3NP-mediated dilation may employ mechanisms distinct from the PKG/sGC/cGMP pathway, such as H_2O_2-induced hyperpolarization, to relax the VSM (Lucchesi *et al,* 2005; McGuire *et al,* 2001). Further investigations with a K-channel activator to induce hyperpolarization at the VSM may have revealed strain dependent differences in the VSM sensitivity to 3NP dilators (Lucchesi *et al,* 2005; McGuire *et al,* 2001). However, in the present study, treatments with LN, INDO and CAT were capable of demonstrating the contributions of NO, PGs, and endogenous H_2O_2 to endothelium-dependent dilation, and exposed the pathways responsible for vasomotor dysfunction in SHR compared to WKY.

B) Effect of LN on ACh- and SNP-induced dilation

Incubation with 300μmol/L LN inhibited maximal dilation to ACh by ~69% in SHR mesenteric arteries but only caused a ~12% inhibition in WKY, suggesting that the hypertensive vessels may have a greater dependency of NO for endothelium-dependent dilation despite a reduced total endothelium-dependent dilation (Figure 2). Previous work supports an increased role for NO in the mesenteric vasculature of SHR over WKY, revealed by differences between LN INDO and INDO alone drug treatments. LN, in the presence of INDO, inhibited ACh-induced dilation of WKY mesenteric arteries compared to incubation with INDO alone, but only at the lowest concentrations of ACh ($10^{-7.5}$mol/L) (Li and Bukoski, 1993). Conversely, in SHR, administration of LN and INDO reduced the ACh response by more than 90% from INDO treated vessels. The administration of methylene blue, an inhibitor of sGC strengthened these findings, where SHR dilation decreased ~80% while WKY responses were unchanged. Shimokawa *et al* (1996) provided further support for the present findings, reporting that WKY arteries become proportionally less reliant on NO, and more reliant on 3NP in response to ACh as the vessel size decreases, an effect that coincides with reduced eNOS expression in smaller vessels, and a reduced eNOS content per endothelial cell in coronary resistance arteries (Laughlin *et al*, 2003). This previous work, together with the present study, supports the decreased role of NO as a dilator in WKY and suggests that NO may be proportionally more important for dilation in the SHR mesenteric vessels.

Consistent with the SNP responses of WKY and SHR arteries under CON conditions, the SNP responses following LN treatment were similar between strains (Figure 5), suggesting that the effect of LN on ACh-induced dilation in this preparation was endothelium-dependent and not likely related to increased VSM sensitivity for NO. This finding is

supported by previous studies where sGC expression is similar in the mesenteric arteries of 20wk old SHR and WKY (Ndisang *et al*, 2002), suggesting that the sensitivity of the VSM to NO is not altered. In contrast, other work has demonstrated an increased sensitivity and maximal dilation to SNP in the mesenteric arteries of 4wk old SHR and 35wks old SHRSP compared to age-matched WKY (Jameson *et al*, 1993; Diederich *et al*, 1990). However, the latter studies used SHR rats that were younger with a lower SBP (120mmHg; Jameson *et al*, 1993) and older with a higher SBP (210mmHg; Diederich *et al*, 1990) than the 16-20wks old rats in the present study (SBP: 198mmHg), suggesting an age and hypertension related difference in the sensitivity of SHR for SNP compared to age-matched WKY. In addition, the 4wk old SHR did not have the elevated dependence on NO for ACh-induced dilation found in the present study (Jameson *et al*, 1993), suggesting that the improvement in VSM sensitivity to NO in SHR may be a compensatory adaptation at 4wks, which is no longer necessary at 16-20wks as NO bioavailability may be increased in the endothelial layer.

eNOS expression was reduced by ~44% in the SHR mesenteric arteries compared to WKY, which seems to contrast with the increased LN-sensitive component in the ACh response of SHR; however, preliminary work suggests that eNOS may have been post-translationally activated to provide the increased NO for dilation in the SHR. Preliminary western blots from SHR and WKY mesenteric arteries indicated that the expression of the phosphorylated eNOS protein (phospho-eNOS) was similar between strains (data not shown). The reduced eNOS expression in the SHR coupled with the consistent level of phopsho-eNOS suggests that the phospho-eNOS:eNOS ratio, an indicator of eNOS activation, was elevated. Since phosphorylation of eNOS at Ser^{1179} increases both basal and stimulated NO production (Boo *et al*, 2003), the potential increase in the phospho-eNOS:eNOS ratio in the SHR

mesenteric vessels may have allowed for the increased provision of NO in the SHR. In addition, the lack of differences in the expression of the pro-oxidant enzyme p47phox, and antioxidant enzymes SOD-1, SOD-2, and CAT in the SHR and WKY mesenteric arteries may indicate that ROS stress did not contribute to increased NO scavenging in these vessels, as increased pro-oxidant and decreased anti-oxidant enzymes in the SHR aorta are often associated with increased oxidative stress and reduced NO bioavailability (Graham and Rush, 2004). Thus, the increased NO component of endothelium-dependent dilation in the SHR may be related to increased post-translational phosphorylation of eNOS and a level of oxidative stress that could have been managed by the endogenous antioxidant enzymes.

C) Effect of INDO on ACh- and SNP-induced dilation

Treatment with INDO improved the AUC of the ACh response by 23% in SHR, achieving the same level as WKY CON, and despite an increase maximal dilation in SHR, maximal dilation remained higher in WKY CON compared to SHR INDO (Figure 2). In WKY, AUC was augmented by 12% with INDO over CON; however, INDO did not improve the maximal response in the WKY (Figure 2). The present findings suggest that constrictory PGs may be responsible for the impaired endothelium-dependent dilation in the SHR mesenteric arteries, and contribute to reduced dilation at submaximal, but not maximal doses of ACh in the WKY. This result is in agreement with early work where INDO improved ACh-induced peak relaxation in mesenteric arteries of 4-34wks old SHR but not in age matched WKY (Luscher *et al,* 1990; Jameson *et al,* 1993; Diederich *et al,* 1990). ACh-induced relaxation is also increased by INDO at higher doses of ACh (10^{-6}-10^{-4}mol/L) in renal arteries of 24wks old WKY (Luscher *et al*, 1988), supporting the involvement of constrictory PGs.

The improvement with INDO at high doses of ACh in WKY renal arteries (Luscher *et al*, 1988), but not in the WKY mesenteric arteries in the present study, may be due to differences in the maximal dilation to ACh. In contrast to the 95% dilation in WKY CON, the renal arteries dilated only ~70% allowing for improvements in maximal dilation in the latter.

Consistent with Diederich *et al* (1990), INDO did not alter the AUC or maximal response to SNP in the mesenteric arteries of either SHR or WKY, suggesting that the improvements in ACh-induced dilation by INDO treatment is not due to changes in the dilatory response of the VSM to NO. Since the use of SNP would not reveal differences in the sensitivity of the VSM for PGs, due to the separate molecular pathways that control PG and NO-induced dilation in the VSM, the large improvement in ACh-induced dilation in the SHR could still be a consequence of greater sensitivity of the VSM to constrictory PGs in SHR. Previous work has demonstrated that the responses of mesenteric arteries to the TXA_2 analogue U-46619 is no different between 12wk old SHR and WKY (Lang *et al*, 1995), suggesting that the sensitivity of the VSM for TXA_2, one of the constrictory PGs, is not altered with hypertension; however, the SHR aorta does exhibit greater sensitivity to $PGF_{2\alpha}$ (Ge *et al*, 1995). Together, these findings suggest that the improvement in the response to INDO in the present study was likely due to reduced release of constrictory PGs from the endothelium following ACh stimulation, but the SHR arteries may also have increased sensitivity to some, but not all, constrictory PGs.

The importance of COX-1 to vasomotor dysfunction in the SHR mesenteric arteries is supported by the 54% increase in COX-1 expression over WKY. Preliminary work did not detect COX-2 in the mesenteric arteries of SHR or WKY (although COX-2 was highly expressed in the SHR thoracic aorta standard -- data not shown), suggesting that the

dysfunction in endothelium-dependent dilation in the SHR is mediated by a metabolite of COX-1. In support of the COX-1 expression data from the present study, Ge *et al* (1995) detected a ~2-fold greater expression of COX-1 mRNA and protein in the aorta of SHR compared to WKY. In the quiescent aortas of 35wks old SHR, ACh invokes a potent constriction that is inhibited with removal of the endothelium and with exposure COX-1 specific inhibitor tenidap, but not the COX-2 specific inhibitor NS-938 (Ge *et al*, 1995). ACh also causes an increased release of the constrictory PGH_2 and $PGF_{2\alpha}$ from the SHR aorta compared to WKY, an effect that was sensitive to INDO. The direct provision of $PGF_{2\alpha}$ to denuded SHR aorta caused increased constriction compared to WKY, while PGH_2 caused comparable constriction between strains, suggesting that the aortic VSM in SHR has altered sensitivity to $PGF_{2\alpha}$. A similar effect is noted in the murine mesenteric arteries in the angiotensin-II (AII) model of induced hypertension, where treatment of the arteries with a COX-1 inhibitor or a thromboxane-prostanoid receptor antagonist SQ-29548, but not a COX-2 or a thromboxane sythetase inhibitor ozagrel, improves vasodilation to ACh in the AII-treated mice but not in controls (Virdis *et al*, 2007). Furthermore, AII-treatment increased COX-1 and decreased COX-2 mRNA and protein expression in the mesenteric arteries (Virdis *et al*, 2007), confirming a role for COX-1 in the vasomotor dysfunction associated with hypertension. However, the pivotal role of the TP receptor outlined by Viridis *et al* (2007) is not consistent with results in SHR mesenteric arteries since pretreatment with the thromboxane-prostanoid receptor antagonist SQ-29548, or the thromboxane synthetase inhibitor CGS-13080, failed to improve ACh-induced dilation (Jameson *et al*, 1993, Diederich *et al*, 1990). Taking these findings into account, it is possible to speculate that, in the present study, the reversal of the impaired ACh-mediated dilation in the SHR mesentery by INDO

treatment may have been due to an increased expression of COX-1, leading to the increased release of PGs, and a possible increase in sensitivity to $PGF_{2\alpha}$; however, the involvement of the TP receptor in this response remains unclear.

D) Effect of L-NAME and INDO on ACh- and SNP-induced dilation

Co-incubation with LN and INDO revealed the 3NP component of ACh-induced dilation in the mesenteric arteries of SHR and WKY (Figure 2). In WKY, the 3NP component accounted for the entire CON response, but was only responsible for 61% of the SHR response, suggesting a lower proportion of SHR dilation is mediated by 3NP compared to WKY. The proportion of 3NP involvement in ACh-induced dilation in the present study is supported by the literature, since, when NO and PG are inhibited, normotensive SD mesenteric arteries dilate 95% (Chauhan *et al,* 2003) and 15wk old WKY femoral arteries dilate 70%, while SHR femoral arteries dilate only 50% to endothelium-dependent agonists (Mori *et al,* 2006). The comparison of the 3NP contribution between strains in the present study indicates that SHR do not compensate for impaired vasomotor function by augmenting the 3NP proportion of their endothelium-dependent dilation, relying instead on an increased level of NO for dilation. Indeed, Feletou and Vanhoutte (2006) state that NO levels are generally maintained in mesenteric arteries but the 3NP-component is impaired in hypertension.

Co-incubation with LN and INDO increased the maximal response to SNP by 4% in both SHR and WKY (Figure 5-6), suggesting that endothelium-derived factors may modulate the response of mesenteric arteries to SNP. This finding is supported by other work where the SNP response in endothelium-denuded mesenteric arteries of SHRSP and WKY are no

different, while the responses of endothelium-intact SHRSP arteries are greater than WKY (Diederich *et al,* 1990). These results in the endothelium-intact vessels contrast with the similarity in SHR and WKY CON responses to SNP found in the present study; however, this difference may be explained by the much older rats used in the aforementioned study.

As the ACh responses integrate the release of factors from the endothelium with the sensitivity of the smooth muscle for those factors, it is possible that the increase in SNP responses following LN INDO treatment would exaggerate the apparent contribution of 3NP components to the ACh response. The implication of the improved SNP response with LN INDO could also magnify the component of endothelium-dependent dilation mediated by H_2O_2, which may operate through the same sGC/cGMP/PKG mechanism stimulated by SNP (Cohen, 2000; Itoh *et al,* 2003; Leung *et al,* 2006). However, endogenous H_2O_2 may also dilate through mechanisms unrelated to the SNP response, including direct or indirect hyperpolarization of the VSM (Hattori *et al,* 2003; Lucchesi *et al,* 2005). Taking these findings into consideration along with the results of the present study, the apparent contribution of the 3NP pathway to endothelium-dependent dilation, and the role of endogenous H_2O_2 in this pathway, may be exaggerated in the mesenteric arteries of SHR and WKY by increased sensitivity of the VSM to NO in 3NP conditions, assuming that H_2O_2 operates through a similar pathway to SNP. The influence of H_2O_2 on mechanisms separate from sGC/cGMP/PKG at the VSM could not be assessed by SNP, suggesting that the addition of a hyperpolarization dose-response curve would be important to further test the sensitivity of the VSM for mechanisms employed by endogenous H_2O_2. Nevertheless, the similar improvements in SNP responses following LN INDO between strains suggest that changes in

the endothelium cause differences in the contribution of 3NP between strains, although 3NP may operate through different pathways at the VSM that were not investigated.

E) Effect of CAT on 3NP-mediated dilation

A major finding of this study is the greater influence of endogenous H_2O_2 on endothelium-dependent dilation in SHR mesenteric arteries over WKY. This finding is based on the observation that incubation with CAT LN INDO caused a 36% decrease in maximal dilation from LN INDO in SHR but only a 7% decrease in WKY. It should be noted that the large decrease in dilation in the SHR reached significance in the Student T-test ($p=0.041$) but not in the ANOVA ($p=0.062$). These differences in statistics are likely attributable to the variance in the responses of groups unrelated to SHR LN INDO or CAT LN INDO that were included in the ANOVA model. These data show that endogenous H_2O_2 may contribute up to 22% of maximal dilation in SHR but only 3% of maximal dilation in WKY, suggesting that H_2O_2 may be a greater mediator of endothelium-dependent dilation in SHR when LN and INDO are present.

Since CAT only inhibited ACh-induced dilation in combined incubations with LN and INDO, it seems that endogenous H_2O_2 is vasoactive when NO and PG are inhibited. In support of this finding, incubation with CAT alone or a CAT and INDO co-incubation did not alter ACh-induced dilation in mouse mesenteric arteries (Matoba *et al*, 2000). In addition, this effect is likely attributable to alterations in the endothelial layer since all preconstrictions, and the KCl, NEPI, and SNP responses, were similar between the CAT LN INDO and LN INDO conditions. The finding that H_2O_2 acts as an endogenous vasodilator through a 3NP pathway is supported by studies in mouse mesenteric arteries (Matoba *et al*, 2000; Morikawa *et al*,

2003), pig and dog coronary arteries (Matoba *et al*, 2003; Yada *et al*, 2003) where CAT, in the presence of LN and INDO inhibited ACh- or BKY-induced dilation. Contrary to the present study, in diseased human coronary arterioles, CAT alone has been shown to reduce dilation to flow (Miura *et al*, 2003). Importantly, in Miura *et al* (2003), all experiments were performed without the addition of NOS or COX inhibitors and normal dilatory responses of the vessels were not established, so it is possible that NO and PG were inhibited pathophysiologically thereby exhibiting an inhibitory effect of CAT in the absence of LN and INDO. Nevertheless, other work in mouse mesenteric arteries (Ellis *et al*, 2003), mouse aorta (Suvorava *et al*, 2005), human radial arteries (Hamilton *et al*, 2001), and WKY coronary arteries (Fulton *et al*, 1997) suggest that H_2O_2 is not involved in the 3NP component of endothelium-dependent dilation. The present study does not rectify these divergent findings, as differences in species, vessel bed, endothelium-dependent agonist, and method to quantify vasomotor function have not been addressed. However, this study is the first to demonstrate that H_2O_2 acts as an endogenous vasodilator in the mesenteric arteries of both rat strains, but to a greater extent in SHR.

Treatment with CAT alone did not alter the ACh or SNP responses from CON in either strain; however, pre-incubation with CAT alone reduced NEPI preconstriction by 41% in WKY and 21% in SHR. This reduction in preconstriction could have hidden an impairment in ACh dilation responses with CAT alone, as a lower preconstriction would have exaggerated the relative % dilation for the same absolute change in tension, assuming that ACh responses are unaffected by the magnitude of the preconstriction. However, previous work in SHR and WKY aorta, which also demonstrated different preconstrictions to PE, show a similar interpretation in ACh data when expressed either as % dilation or as change in

absolute tension (Graham and Rush, 2004). This previous work suggests that despite the differences in preconstriction, CAT likely did not affect ACh dilation either expressed relative to NEPI, or as change in absolute tension. Additionally, if the loss in tension with ACh was proportional to, rather than independent of preconstricted tension, the effect of CAT would not be altered from the present results since the ACh responses were expressed relative to the preconstricted tension. Importantly, the calculations of the 3NP component of ACh-induced dilation were not affected by the method of expressing the data, as NEPI preconstriction was consistent between LN INDO and CAT LN INDO conditions.

One potential relationship revealed by the present study is the association between COX-1 and the role of H_2O_2 as a 3NP-mediated dilator in the SHR. In the SHR response to ACh, LN reduced maximal dilation to 23%, which was increased to 46% with LN INDO, and subsequently reduced to 30% with CAT LN INDO (Figure 3). This relationship was conserved in WKY, but the magnitude of the effects were reduced, where LN reduced dilation to 83% of maximum, LN INDO increased dilation to 98%, and CAT LN INDO reduced maximal dilation to 92%. Statistically, LN and CAT LN INDO maximal dilation responses were no different, but LN INDO was greater than both LN and CAT LN INDO; a relationship that was consistent between strains. This relationship suggests that COX inhibition may allow H_2O_2 to become available for dilation, an effect that is sensitive to CAT, and is greater in SHR than WKY. This association is reinforced by the greater role of COX-1 in the SHR than WKY, indicated by the larger improvement in AUC following INDO treatment in SHR, and the overexpression of COX-1 in SHR mesenteric arteries. The apparent relationship between COX-1 and H_2O_2 is not revealed in the responses to SNP, NEPI, or KCl, suggesting that this effect is not occurring through modulation of the VSM.

In support of the relationship between COX and H_2O_2 availability, H_2O_2 is required for activation of the COX in purified enzyme preparations (Davidge, 2001). This interaction is illustrated by the inhibition COX-1 and COX-2 activity in the presence of the H_2O_2 scavenger GPx (Mashall *et al*, 1987; Kulmacz and Wang, 1995) and with increased activation of the COX enzymes with greater doses of ethyl hydrogen peroxide or a fatty acid hydroperoxide (Lu *et al*, 1999). In addition, COX enzymes are known peroxidases that consume H_2O_2 in the conversion of PGG_2 to PGH_2 in purified cell extracts (Lu *et al*, 1999; Liu *et al*, 2007). Interestingly, COX-1 metabolism is inactivated at lower doses of GPx than COX-2, suggesting that COX-1 requires almost 10-fold more H_2O_2 to become activated compared to COX-2 (Kulmacz and Wang, 1995). In light of the different levels of H_2O_2 required for activation of COX-1 and COX-2, this mechanism may allow for regulation of PG production, especially considering the ease of movement of H_2O_2 across cell membranes (Kulmacz and Wang, 1995; Antunes and Cadenas, 2000). In support of the relationship of H_2O_2 and COX in vascular cells, bovine aortic VSM cells induced with COX-2 and stimulated by endotoxin, H_2O_2, and the NO donor SIN-1 increase PG synthesis (Schildknecht *et al*, 2005). Futhermore, LN, SOD, and the NADPH oxidase inhibitor apocynin each caused dose-dependent decreases in PG production from these same cells, suggesting that while H_2O_2 can directly stimulate PG production in VSM cells, $ONOO^-$ may also be involved (Schildknecht *et al*, 2005). In addition, in perfused porcine coronary arteries, arachidonic acid (AA)-induced dilation is inhibited by INDO, by PEG-CAT, and by PEG-SOD, suggesting that ROS mediate the metabolism of COX to produce dilatory PGs (Oltman *et al*, 2003). Importantly, AA alone increased ROS formation in coronary vessels, an effect that is inhibited by pretreatment of the vessels with PEG-SOD and PEG-CAT co-incubation, or with INDO alone (Oltman *et al*,

2003). Together, the findings of Oltman *et al* (2003) suggest that ROS are required for COX to metabolize AA and cause dilation but AA metabolism by COX can also stimulate the release of ROS in intact vessels. The above study also implies that ROS do not become more bioavailable when INDO is present, seemingly refuting the theory proposed by the present study. However, the direct provision of AA was used to stimulate endothelium-dependent dilation through COX, whereas the present study administered ACh to augment intracellular Ca^{2+} to increase AA liberation for COX metabolism. The differences in the agonists, as well as a difference in species, vessel bed, and level of hypertension may allow for INDO to increase H_2O_2 bioavailability, possibly through inhibition of COX-1 peroxidase activity, but further investigation is required to confirm this theory.

F) Direct actions of exogenous H_2O_2 on vasomotor function

Further evidence supports a role for H_2O_2 as an endogenous vasodilator. Following NEPI preconstriction, exogenous H_2O_2 caused dilation from 10^{-9}-10^{-4}mol/L in WKY and SHR mesenteric arteries (Figure 12). In addition, pilot work in SD mesenteric vessels indicated that dilation to exogenous H_2O_2 depends upon the endothelium from ~$10^{-9.0}$-$10^{-4.5}$mol/L, but becomes less dependent on the endothelium thereafter (Figure 21). In the H_2O_2 dilation curve, SD E- vessels experienced a 40% decrease in AUC from E+; however, the VSM was still capable of dilating to higher concentrations of H_2O_2, possibly due to irreversible cellular damage (Gil-Longo and Gonzalez-Vazquez, 2005). The H_2O_2 dilation data in all strains, taken together with the ACh responses in the WKY and SHR suggest that endogenous H_2O_2 may act at the endothelium to either directly or indirectly cause dilation at the vascular smooth muscle. Since the endothelium potentiated the dilatory response of the VSM to exogenous

H_2O_2 in SD, it is possible that endogenous H_2O_2 may act at the endothelium to release a second messenger distinct from H_2O_2 to cause relaxation at the VSM. However, at higher concentrations, exogenous H_2O_2 can directly relax the VSM, possibly through irreversible cellular damage.

In both the SHR and the WKY, the H_2O_2 dilation curve developed a reconstriction from ~90% dilation to 40-60% dilation at $10^{-3.5}$-$10^{-2.5}$mol/L. Interestingly, the H_2O_2 constriction curve in quiescent vessels showed a similar constriction at $10^{-3.5}$-$10^{-2.0}$mol/L to ~35-45% of NEPI preconstriction values (Figure 12). These data suggest that H_2O_2 causes a constriction at mmol/L concentrations that is not dependent upon the level of baseline tone. In addition, both SD E+ and E- mesenteric arteries constricted to approximately the same tension (Figure 21), suggesting that the H_2O_2 induced constriction was endothelium-independent.

In response to exogenous H_2O_2, previous studies have shown that dilation occurs through the stimulation of COX metabolites leading to hyperpolarization of the VSM, reinforcing the theory that H_2O_2 is required for COX activity in the mesenteric arteries of SHR. Endothelium-intact and -denuded internal thoracic arteries from patients undergoing coronary artery bypass graft show a dose-dependent relaxation to H_2O_2 that is inhibited by ~50% with INDO, and by ~90% with the voltage gated K^+-channel blocker 4-aminopyridine (Nacitarhan *et al*, 2007). In addition, evidence from rabbit mesenteric VSM demonstrates a reduction of H_2O_2-induced hyperpolarization that is additive with COX and 5-LOX inhibition, suggesting that these lipids may lead to the hyperpolarization and subsequent relaxation of VSM to H_2O_2 (Hattori *et al*, 2003). However, COX activation and hyperpolarization of the VSM may independently cause dilation in response to H_2O_2 administration. INDO and COX-1 specific inhibition in intact pig coronary arterioles provides the same reduction in H_2O_2

dilation as denudation, likely through inhibition of PGE_2, although PGE_2-mediated dilation was not sensitive to blockade of K_{Ca}-channels (Thengchaisri and Kuo, 2003). Similarly, preconstriction of mouse aorta with KCl eliminates dilation to 10^{-4}mol/L H_2O_2, but the vessel still exhibits an endothelium-independent dilation of nearly 80% at $10^{-3.5}$mol/L H_2O_2, an effect that is not altered by incubation with INDO (Gil-Longo and Gonzalez-Vazquez, 2005). Together these studies suggest that H_2O_2 could cause dilation either through direct hyperpolarization of the vascular smooth muscle, or through the provision of PGs to dilate the VSM, independent of hyperpolarization. This body of evidence supports a role for H_2O_2 in the production of vasoactive PG by COX in the mesenteric arteries of the present study, and allows for the possibility that INDO may inhibit COX-1 allowing H_2O_2 to become bioavailable for dilation.

Reconstriction with NEPI in both SHR and WKY mesenteric arteries was completely abolished following both the H_2O_2 constriction or dilation curves, suggesting that the constrictory mechanisms of the arteries were irreversibly damaged by the previous exposure to H_2O_2. This finding is supported by studies in denuded mesenteric arteries of SHR and WKY where repeated 30min administrations of $5x10^{-4}$mol/L H_2O_2 caused an apparent tachyphylaxis in response to subsequent bolus injections of H_2O_2 that is consistent between strains (Gao and Lee, 2001). Similarly, exposure of WKY aorta to $3x10^{-4}$mol/L H_2O_2 for 1hr, followed by a 1-2hr washout eliminates constriction to PE and KCl (Gil-Longo and González-Vázquez, 2005) suggesting that the tachyphylaxis noted in the Gao and Lee (2001) study may have been due to damage of the constrictory mechanism rather than sensitization to the H_2O_2 stimulus. Consistent with these findings, and the present study, the loss of contractile function following the H_2O_2 dose-response curve may have been caused by the application of H_2O_2

greater than 10^{-5}mol/L, leading to oxidative stress-induced cell death (Stone and Yang, 2006; Antunes and Cadenas, 2001). Curiously, in the present study, both WKY and SHR mesenteric vessels were still capable of constricting at the maximal dose of 10^{-2}mol/L H_2O_2; however, they were not able to constrict in the subsequent NEPI administration after 15min incubation in PSS. This observation suggests that the effects of high doses of H_2O_2 may not be immediate, but may stimulate a cascade of events leading to damage of the VSM contractile mechanisms.

The theory that endogenous H_2O_2 acts as a vasodilator in our preparation is largely dependent upon the effect of CAT on dilation in the presence of LN and INDO. As a result, the efficacy of the scavenging of H_2O_2 with CAT was determined using vascular myography and in measures of H_2O_2 accumulation by Amplex Red. Pre-incubation of both E+ and E- SD mesenteric arteries with 860U/mL CAT showed a 55-74% reduction in AUC of the H_2O_2 dilation curve that was not affected by the absence of the endothelium (Figure 21). In addition, administration of CAT alone, or in the presence of ACh, LN, INDO, or TEMPOL caused a reduction in H_2O_2 accumulation, measured by the extracellular dye Amplex Red in suspensions of the mesenteric vessels of both SHR and WKY. Taken together, these data support the theory that the 860U/mL CAT, in the conditions employed, was capable of scavenging the H_2O_2 released endogenously to cause dilation in the mesenteric arteries of SHR and WKY. In addition, the absence of a change in ACh- or SNP-induced dilation in the presence of CAT alone suggests that the enzyme did not injure the vessel, or cause a loss of tension unrelated to the level of H_2O_2. The efficacy of CAT is supported by previous studies in mouse mesenteric arteries where CAT, along with LN and INDO, greatly inhibited 3NP-mediated dilation to ACh, an effect that was prevented with the inactivation of CAT with AT

(Matoba *et al*, 2000). In addition, BKY-induced hyperpolarization of porcine coronary microvessels is inhibited by CAT alone and the inactivation of CAT with AT reverses this effect suggesting that CAT is not damaging the ability of the arteries to hyperpolarize (Matoba *et al*, 2003), confirming that the reduction of ACh-induced dilation with CAT is likely due to scavenging of H_2O_2, not vessel damage. Therefore, CAT decreased H_2O_2-induced dilation, decreased H_2O_2 accumulation, and decreased the 3NP component of ACh-induced dilation, effects that were likely caused by the peroxidase activity of CAT.

VASOCONSTRICTION

A) Comparison of control responses

The SHR maximal response to the receptor-mediated agonist NEPI was increased by 25% over WKY when collapsed across drug conditions, and SHR CON had a 24% increase in NEPI constriction over WKY CON (Figure 8). This finding coincides with a 25% increase in SHR preconstriction to NEPI prior to the SNP dose-response curve, when collapsed across drug conditions. Interestingly, no effect of strain was noted for KCl responses when collapsed across drug condition, or when compared between CON groups (Figure 10). Taken together, these constriction results suggest that the increased responses in SHR compared to WKY may be due to increased expression, activity, or sensitivity of the α_1-adrenergic receptor (α_1-AR), rather than differences in the capacity of the VSM to constrict to electrochemical stimuli. The increased α_1-AR-mediated responses in SHR seemingly contrasts with several studies where, in response to NEPI, and in the absence of PRO, constriction in mesenteric vessels is no different between 4wk old WKY and SHR (Jameson *et al*, 1993) or between 35wk old WKY and SHRSP (Diederich *et al*, 1990). However, in the presence of PRO and cocaine, the

mesenteric arteries of 25wks old SHR show a 10% greater maximal NEPI constriction compared to WKY (Yang *et al*, 2005), which supports the findings of the present study. NEPI re-uptake likely did not account for differences between SHR and WKY mesenteric arteries in the present studies as NEPI constriction of SHR and WKY femoral arteries is not altered by the presence of cocaine (Mulvany *et al*, 1982) In support of the present study, SHR generate 50-70% greater pressure to a maximal dose of the selective α_1-AR receptor agonists methoxamine and phenylephrine (PE), compared to age-matched WKY (Husken *et al*, 1994; Girouard and Champlain, 2005). Furthermore, the α_1-AR has greater affinity for NEPI in the mesenteric arteries of SHR compared to WKY (Nyborg and Bevan; 1988). Thus, the results of the present study, together with the literature, suggest SHR mesenteric arteries have augmented responses to α_1-AR stimulation, an effect that may be due to increased sensitivity of the α_1-AR for NEPI in SHR.

In contrast to the NEPI results, the KCl responses were no different between SHR and WKY CON groups (Figure 10). These findings are supported by work using a similar method of isolated vessel myography where mesenteric arteries of 12wk SHR had a similar constriction to 100mmol/L KCl as age-matched WKY (Lang *et al*, 1995). Similarly, the aortas of 15-16wk old SHR and WKY have a similar constriction to 60mmol/L KCl (Graham and Rush, 2004). In perfused mesenteric arterial beds, the responses of 15wk old SHR and WKY to KCl are also similar (Girouard and Champlain, 2005). However, several findings contrast with the findings of the present study by demonstrating an increase in KCl constriction in SHR over WKY. SHR mesenteric arterial beds produce greater peak perfusion pressure at 100mmol/L KCl compared to WKY (Le Marquer-Domagala and Finet, 1997); however, the SBP of the SHR in this study were markedly lower than in the present study (146 vs.

198mmHg). Indeed, the MAP of the SHR in the Girouard and Champlain (2005) study, where KCl responses were no different between strains, was 152mmHg, and approximates the MAP of 168mmHg in the present study, suggesting that the differences in KCl constriction may be blunted as blood pressure increases in SHR. Dickhout and Lee (1997) demonstrated a greater peak and total response to KCl in the perfused mesenteric arteries of SHR rats at 4wks of age compared to age-matched WKY whereas at 74wk SHR mesenteric arteries had a reduced KCl constriction compared to age-matched WKY and 12wk SHR (Lang *et al*, 1995). Together with the findings of the present study, these results suggest that the KCl responses in SHR may be increased in youth, equivalent in adulthood, and reduced in senescence. Nevertheless, other work indicates that the response to direct VGCC stimulation by Bay k 8644 is augmented in 12wk SHR compared to WKY, and harvested myocytes from these vessels demonstrate a greater increase in Ca^{2+} current in SHR than WKY, a difference that was maintained with additional stimulation by Bay k 8644 (Matsuda *et al*, 1997). Differences in VGCC function likely exist between SHR and WKY; however, the vascular responses to depolarization by high K^+ in the present study may involve the non-specific activation of ion channels on the membrane and intracellular systems that could affect the function of the VGCC in a complex interaction, and could obscure differences in specific VGCC function between SHR and WKY.

B) Effect of LN and INDO on constriction

In response to INDO incubation, NEPI and KCl responses were no different from CON when collapsed across strain, suggesting that PGs do not alter either α_1-AR-mediated or electromechanical contraction in mesenteric arteries. In contrast to the present study, INDO

treatment alone has been shown to reduce NEPI-induced constriction in 30-34wk SHR mesenteric vessels but not WKY, an effect that implies the impairment of dilatory PGs, or the involvement of constrictory PGs in NEPI constriction (Luscher *et al*, 1990). However, in the Luscher *et al* (1990) study, PRO was not administered along with NEPI, and the rats were significantly older, suggesting that differences in the responses may be due to an increased release of constrictory PGs in the older rats, possibly modulating the dilatory effects of NEPI, although no known study has addressed this hypothesis.

Co-incubation with LN and INDO in the KCl dose-response curve increased AUC by 67% in SHR and by 30% in WKY over respective CON groups, while the NEPI responses to LN INDO were similar between strains (Figures 9 and 11). In the KCl response, there was also a trend towards an improvement in AUC in LN INDO over INDO alone ($p=0.063$), suggesting that the additional treatment with LN improved AUC over INDO alone. In support of the present study, LN has been previous shown to improve the response to KCl, where two NOS inhibitors (LN and N^G-nitro-L-arginine) increased the KCl response in both WKY and SHR perfused mesenteric beds while two COX inhibitors (INDO and mefenamic acid) did not alter KCl response in SHR (Le Marquer-Domagala and Finet, 1997). However, in the present study, LN INDO did not alter α_1-AR-mediated constriction, a result that has been previously shown in 12wk old WKY where the NEPI dose response curve was not altered by LN or LN INDO (Lang *et al*, 1995). Conversely, in SHR, Lang *et al* (1995) demonstrated a 23% greater NEPI AUC with LN over CON, while LN INDO showed no difference, suggesting that NEPI releases dilatory NO which is somehow reversed by COX inhibition. In the present study, INDO did not alter NEPI responses, so the lack of change with LN INDO co-incubation is not likely due to a reversal of the effects of LN with additional INDO. Taking the constriction

responses together, the results of the present study suggest that, in both SHR and WKY, KCl but not NEPI, stimulates the release of NO, whereas PGs are not released by either KCl or NEPI.

MODULATION OF ENDOGENOUS H_2O_2 BY TEMPOL

An additional component of the present study addressed the effect of supplementation with the antioxidant TEMPOL on the level of endogenous H_2O_2 available for endothelium-dependent dilation. As a membrane-permeable SOD mimetic, TEMPOL could convert O_2^- to H_2O_2, thereby releasing H_2O_2 for dilation. Pilot work demonstrated that TEMPOL administration to SHR or WKY preconstricted mesenteric vessels caused a 32-52% dilation over 20-30min that was partially reversed by the subsequent addition of CAT over 10-20min, an effect that was no different between SHR and WKY (Figure 19). This H_2O_2-dependent effect of TEMPOL is supported by evidence from SD mesenteric arteries where preconstricted vessels had a transient dilation to 1mmol/L TEMPOL that was reversed by CAT (Chen *et al*, 2007). This data, taken in isolation, could seemingly illustrate the separate effects of TEMPOL and CAT on α_1-AR-mediated tone, rather than the action of CAT to reverse the H_2O_2 provided by TEMPOL. However, in the present study, CAT pre-incubation causes a reduction, rather than an increase, in NEPI preconstriction prior to the ACh curve, suggesting that CAT alone does not potentiate α_1-AR-mediated constriction. Furthermore, the TEMPOL alone or TEMPOL LN INDO treatments tended to increase H_2O_2 accumulation in the Amplex Red assay by 24-60% isolated mesenteric arteries of both strains over PSS (SHR ACh TEMPOL p=0.085, SHR ACh TEMPOL LN INDO p=0.064, WKY TEMPOL p=0.063), an effect that was inhibited by the addition of CAT, and supported by similar experiments in

SD mesenteric arteries (Chen *et al*, 2007). Thus, direct administration of TEMPOL caused dilation in SHR and WKY vessels that was partially reversed with CAT, suggesting that TEMPOL released H_2O_2 for dilation.

In the mesenteric arteries of SHR and WKY, TEMPOL alone did not alter responses to ACh, SNP, NEPI, or KCl, from CON suggesting that changes in O_2^- or H_2O_2 were not enough to alter vasomotor tone following stimulation by an agonist. In addition, co-incubation with TEMPOL LN INDO did not alter ACh or SNP responses from LN INDO, suggesting that even in the absence of NO and PGs, TEMPOL did not release adequate H_2O_2 to affect dilation. However, in SHR but not WKY, the co-administration of TEMPOL CAT LN INDO prevented the decrease in ACh dilation occurring with CAT LN INDO compared to LN INDO (Figure 4), and in the SNP dose response curve, TEMPOL CAT LN INDO incubation reduced AUC by 7-8% below CAT LN INDO (Figure 7). When the SNP and ACh results are taken together, TEMPOL may maintain endothelium-dependent dilation in SHR by scavenging O_2^-, rather than through the provision of H_2O_2, an effect made even more important by a decreased dilatory response of the VSM with the TEMPOL CAT LN INDO treatment. Previous studies contrast with the present findings and support the release of H_2O_2 by SOD mimetics to stimulate vasodilation (Morikawa *et al*, 2003; Matoba *et al*, 2003). However, PEG-SOD has also been shown not to improve the 3NP component of ACh-induced dilation in porcine coronary arteries (Matoba *et al*, 2003), and to have no effect on the 3NP-mediated component of ACh-induced dilation in mouse mesenteric arteries (Ellis *et al,* 2003), although this latter study also failed to demonstrate a role for H_2O_2 as an endogenous vasodilator in the absence of PEG-SOD. In additional support for role of TEMPOL as an O_2^- scavenger in the present study, SOD, and its mimetic Tiron, reduce O_2^-

measured by lucigenin chemiluminescence in renal cortical microsomes (Fulton *et al*, 1997). Similarly, through some undefined mechanism, increased O_2^- buffering by TEMPOL in SHR may improve the function of calcium-activated K^+-channels or myoendothelial gap junctions, both factors implicated in 3NP mediated dilation (Feletou and Vanhoutte, 2006; Griffith *et al*, 2005), reinforcing the present findings where that TEMPOL may have preserved ACh-induced dilation in SHR through scavenging O_2^- rather than by providing H_2O_2, but only in the presence of LN and INDO.

In the present study, incubation in TEMPOL CAT LN INDO increased maximal NEPI constriction by 25-30%, increased AUC by 28-31%, and increased NEPI preconstriction for the ACh curve by 33-63% compared to CAT LN INDO, effects that were consistent across strains. Together, these NEPI data imply a possible impairment of the α_1-AR-mediated response by O_2^-. It is unlikely that the improvement in NEPI response could have been mediated by impairment of the endothelium dependent dilatory pathways as the ACh response parameters were either improved or unchanged by TEMPOL CAT LN INDO when compared to CAT LN INDO. Similarly, the endothelium-independent response to SNP was slightly but significantly impaired by TEMPOL when combined with CAT LN INDO, but not impaired enough to explain the improvement in the NEPI response. In mesenteric arteries, endogenous OH^- inhibits α_1-AR mediated constriction (Girouard and Champlain, 2005), and may lend support to the present finding as O_2^- can quickly dismutate into OH^- in the presence of iron or copper through the Haber-Weiss reaction (Feletou and Vanhoutte, 2006). The results of the present study, combined with other findings, suggest that TEMPOL may scavenge the O_2^- that is being converted to OH^-, thereby improving NEPI-induced constriction.

BODY WEIGHT AND HEMODYNAMICS

As expected, SHR rats had an elevated blood pressure compared to WKY. SHR also had an increased left kidney-to-body weight ratio; however, this parameter was not correlated to endothelial function or the hemodynamic measures, suggesting that the kidney weight was not related to the degree of hypertension. The heart mass was increased in SHR in the present study, which is supported by previous work where, LV and HM were increased in the SHR when expressed in absolute mass or relative to body mass, indicating that hypertrophy of heart has occurred (Graham and Rush, 2004; Quadrilatero and Rush, 2006). However, the increased heart mass in the present study was not conserved when expressed relative to body weight, which suggests that, although the heart is larger in the SHR, it was related to the greater body weight of this strain, and not due to the establishment of LV hypertrophy.

LIMITATIONS.

The present study was limited by methodological and technical considerations. Firstly, the 860U/mL CAT purified from bovine liver used in the study was at a lower dose than other studies where the role of exogenous H_2O_2 was confirmed (1000-6250U/mL: Miura *et al,* 2003; Morikawa *et al,* 2003; Matoba *et al,* 2000, 2003) or refuted (2500U/mL: Ellis *et al,* 2003). However, the present study did demonstrate the expected effect of CAT by 1) an impairment of 3NP-mediated dilation with CAT LN INDO, 2) a rightward shift in the SD H_2O_2 dilatory curves following 860U/mL CAT pre-incubation, and 3) a complete reduction in the Amplex Red measures of H_2O_2 accumulation. These findings suggest that the 860U/mL

dose of CAT was adequate to scavenge H_2O_2 in multiple experiments, thereby allowing an investigation of the role of endogenous H_2O_2 in 3NP dilation.

The present study used cell-impermeable CAT and cell-permeable TEMPOL; thus, the effects of TEMPOL as an O_2^- scavenger or a H_2O_2 producer, and the implications on vasomotor function, may have been obscured. Exogenous H_2O_2 is known to cross cell membranes freely, creating a 7-fold concentration gradient between extracellular and intracellular spaces (Antunes and Cadenas, 2000), and suggests that the endogenously produced H_2O_2 could diffuse to the extracellular space to be scavenged by the extracellular CAT. As such, CAT would act as an extracellular "sink" for exogenous H_2O_2 by causing a concentration gradient that favoured diffusion of H_2O_2 to the extracellular space. However, the H_2O_2 produced by TEMPOL may have had an intracellular effect before being scavenged by extracellular CAT, thereby underestimating the effects of TEMPOL as a H_2O_2 producer and overemphasizing the O_2^- scavenging properties. Through a similar mechanism, the extracellular Amplex Red reagent may have undestimated the H_2O_2 produced by TEMPOL. The effectiveness of 6250U/mL CAT on the reversal of TEMPOL-induced H_2O_2 production has been shown in the mesenteric arteries of SOD-$1^{-/-}$ mice (Morikawa *et al*, 2003); however, it is uncertain whether the H_2O_2 accumulation from TEMPOL was completely scavenged by the 860U/mL CAT used in the present study.

The NO donor SNP assessed the sensitivity of the VSM for NO, and changes in endothelium-independent dilation with the various drug treatments. However, it was not possible to assess differences in the sensitivity of the VSM for PGs or 3NP-mediated dilators using SNP, as these pathways were not directly stimulated. Previous work suggests that endogenous H_2O_2 may operate through a PKC/sGC/cGMP mechanism activated by SNP (Itoh

et al, 2003; Leung *et al*, 2006), although no differences in the SNP responses were observed between strains in the current study. The use of SNP alone, and not VSM hyperpolarizing drugs, limited the ability of the present study to uncover the mechanisms at the VSM activated in response to endogenous H_2O_2.

CONCLUSION.

This study demonstrated the involvement of endogenous H_2O_2 in endothelium-dependent dilation of rat mesenteric arteries when NO and PG were inhibited. The role of endogenous H_2O_2 was also exaggerated in the SHR rat resistance arteries, suggesting that this pathway may contribute to the maintenance of some, albeit limited, endothelial vasomotor function in SHR. Exogenous H_2O_2 was able to dilate mesenteric arteries and CAT reduced H_2O_2 accumulation from isolated mesenteric vessels, supporting the role for H_2O_2 in the vasomotor function of SHR and WKY. This study also provided evidence for a potential role for COX-1 inhibition in the provision of H_2O_2 for dilation in SHR mesenteric arteries, demonstrated by the improvement of endothelium-dependent dilation with INDO, the relationship between COX inhibition and the CAT effect, and the upregulation of COX-1 expression in the SHR mesenteric arteries. This is the first study to demonstrate the role of endogenous H_2O_2 in rat resistance vessels, its elevated contribution to dilation in SHR, and a potential role for COX inhibition on the provision of endogenous H_2O_2 for dilation.

The present study also demonstrated an increased reliance on NO for dilation in SHR compared to WKY, which may have been related to increased activation of eNOS in mesenteric arteries. In addition, the 3NP component of dilation was larger in the mesenteric arteries of WKY compared to SHR, suggesting that reduced 3NP may be involved in impaired

vasomotor function in hypertension, and that factors other than NO, PGs, or endogenous H_2O_2 likely contribute to vasomotor function in resistance arteries. Finally, TEMPOL, in the presence of CAT LN INDO provided improvement in the responses to α_1-AR-mediated constriction, suggesting that O_2^- may impair constriction in this preparation.

FUTURE DIRECTIONS.

Future studies will aim to define the role of COX-1 in the bioavailability of endogenous H_2O_2, and the particular mechanism involved in dilation of the VSM by endogenous H_2O_2. Firstly, COX-1 and COX-2 specific inhibitors would confirm the primary involvement of COX-1 in the function of hypertensive mesenteric arteries, and the co-incubation of LN with CAT could confirm the inhibition of COX-1 and the availability of H_2O_2 for dilation when compared to the other drug treatments. Furthermore, simultaneous measures of ROS and vasomotor function, using confocal microscopy, could verify the accumulation of H_2O_2 in the various drug treatments and the possibility of cellular compartmentalization. In addition, the use of hyperpolarizing factors and exogenous PGs could clarify the VSM pathway involved in the H_2O_2 response, and the influence of hypertension on this pathway. Further studies could also assess the responses of resistance vessels following AII-induced hypertension, or long term treatment with COX-1 inhibitors to maximize endogenous H_2O_2 and to investigate the tipping point between vasoactive mediation by endogenous H_2O_2 and potential cellular damage.

APPENDIX A- SUPPLEMENTARY METHODS

KCl CURVE SOLUTIONS

To achieve the required dose of KCl, a volume of stock oxygenated 37C PSS was created in the absence of NaCl or KCl (NaK-free-PSS concentration (mmol/L): 1.17$MgSO_4 \circ 7H_2O$, 1.18KH_2PO_4, 2.50$CaCl_2 \circ 2H_2O$, 25.00$NaHCO_3$, 0.03EDTA, 5.50 D-Glucose). For a given dose of KCl, 10mL of NaK-free-PSS was aliquoted and a volume of NaK-free-PSS was replaced with the required volume of stock NaCl (4.123mol/L dissolved in NaK-free-PSS) and KCl (3.3mol/L dissolved in NaK-free-PSS) (Appendix A, Table) and the appropriate in bath concentration of inhibitors.

Table: *KCl and NaCl Volumes for KCl Dose-Response Curve*

[KCl] in bath (mmol/L)	Volume of stock KCl/10mL bath (μL)	[NaCl] in bath (mmol/L)	Volume of stock NaCl/10mL bath (μL)
0	0	124	300
10	30	114	276
20	60	104	252
30	90	94	227
40	120	84	203
50	150	74	179
60	180	64	155
70	210	54	130
80	240	44	106
90	270	34	82
100	300	24	58

WESTERN BLOTTING SOLUTIONS.

SOURCES OF CHEMICALS.

Sigma

Bioshop

SAMPLE BUFFER

Concentration	Compound	Company
1.46M	Sucrose	Sigma-Aldrich
7.5% wt/vol	SDS	Bioshop
62.5mM	Tris-HCl	Sigma-Aldrich
2mM	EDTA	Sigma-Aldrich
0.2M	DTT	Bioshop
0.01% wt/vol	Bromophenol Blue	Sigma-Aldrich

TRANSFER BUFFER

Concentration	Compound	Company
25mM	Tris Base	Bioshop
0.2M	Glycine	Sigma-Aldrich
20% vol/vol	Methanol	Sigma-Aldrich

* Methanol not added for high MW transfers

APPENDIX B – SUPPLEMENTARY RESULTS

ACUTE TREATMENT WITH ANTIOXIDANTS.

The direct effects of TEMPOL as a potential H_2O_2 producer, and its possible reversal by CAT, were tested in both SHR (n=7) and WKY (n=5) following a NEPI preconstriction (Figure 20). The preconstrictions were no different between strains (plateau preconstriction; SHR: 15.6±1.2 vs WKY: 15.3±1.2nM, p=0.829). Similarly, the dilation with 1mM TEMPOL, which occurred over 20-30min, was no different between strains (SHR: 8.1±1.2 vs WKY: 5.0±1.8nM, p=0.196). In addition, the reconstriction with the subsequent addition of 860U/mL CAT, which also occurred over 20-30min, was no different between strains (SHR: 2.1±1.0 vs WKY: 1.4±0.7, p=0.261). Importantly, CAT did cause a reversal of a component of the TEMPOL effects, irrespective of strain (p=0.014). When data was expressed relative to preconstriction, there are no differences in the trends mentioned above.

VASOMOTOR RESPONSES IN ENDOTHELIUM-DENUDED ARTERIES

I) Rat and Ring Baseline Parameters

A series of experiments were completed on endothelium intact (E+) and endothelium-denuded (E-) SD mesenteric vessels to assess the effectiveness of the denuding procedure prior to assessing the role of the endothelium in the vasomotor responses to exogenous H_2O_2. No differences were found in the normalized diameter of E+ and E- vessels (E+: 180.3±12.4μm (n=29) vs E-: 208.5±11.5μm (n=24); p=0.079). To ensure the removal of the endothelium, an endothelium function tests was completed on both E+ and E- vessels (n=23-

24), which developed the same tension to NEPI preconstriction (E+: 9.7±1.2mN vs E-: 10.2±1.1mN; p=0.677), but the dilation to 10^{-6}mol/L ACh was greatly impaired in E- (E+: 35.4±3.9% vs E-: 5.9±1.0%; p<0.001).

II) Vascular Responses in E+ and E- Vessels

In the ACh dose-response curve, E- vessels (n=9) only dilate to ~25% of the E+ vessel (n=10) maximum (E+: 61.0±7.1% (n=10) vs E-: 15.4±1.9% (n=9); p<0.001) and AUC was reduced by ~81% in E- compared to E+ (E+: 173.9±20 vs E-: 32.5±3.5; p<0.001). Similarly, the sensitivity of the E- vessels was reduced compared to E+ (E+: -8.9±0.2 vs E-: -8.1±0.3;, p=0.025). In the SNP dose responses curve, E- have an increased AUC compared to E+ (E+: 273.0±30.4 (n=5) vs E-: 353.4±17.0 (n=5); p=0.050); however, maximal relaxation response and $logEC_{50}$ remained unchanged (E+: 75.8±5.9% vs E-: 86.9±1.5% p=0.104; E+: -7.6±0.2 vs E-: -8.1±0.2; p=0.081). Finally, in the NEPI dose-response curve, E- vessels maximally constrict ~2-fold more than E+ (E+: 5.96±0.69mN (n=9) vs E-: 11.69±1.75mN (n=11); p=0.012) with a ~2-fold greater AUC in E- (E+: 18.9±2.0 vs E-: 37.1±5.6; p=0.011) and an unchanged $logEC_{50}$ (E+: -7.2±0.1 vs E-: -7.2±0.1; p=0.872).

III) Responses of E+ and E- vessels to exogenous H_2O_2 and CAT pre-incubation

For the H_2O_2 dilatory curves, E+ and E- vessels were administered H_2O_2 following a preconstriction (n=3-5) (Table 8, Figure 21). Additional experiments involved pre-incubation of E+ and E- with 860U/mL CAT prior to the H_2O_2 dilation curve (n=2/group). The NEPI preconstrictions were no different between denuding conditions or the presence of CAT (2-way ANOVA: Main effect of denuding: p=0.196, main effect of CAT pre-incubation,

p=0.229, interaction effect denuding*pre-incubation: p=0.826). Due to the non-sigmoidal profile of the CAT pre-incubation curves, the AUC was the best measure of differences with endothelium and with the treatment (2-way ANOVA: Main effect of denuding: p=0.019, main effect of CAT pre-incubation, p=0.003, interaction effect denuding*pre-incubation: p=0.652). Thus, denuded vessels demonstrated a reduced total response to H_2O_2 when compared to intact vessels collapsed, across both drug condition (E+CON: 264.0±35.2 E+CAT: 119.1±23.3 vs E- CON: 157.9±1.6 E-CAT: 41.5±9.5). In addition, pre-incubation with 860U/mL CAT greatly reduced the AUC in both E+ and E- groups by ~55-74%. When assessing the H_2O_2 dilatory responses in E+ and E- vessels in the CON condition alone, no difference was observed between maximal dilation (E+ CON: 89.4±5.9% vs E-CON: 82.6±3.5%; p=0.413) or $logEC_{50}$ (E+ CON: -4.9±0.6 vs E- CON: -3.7±0.0; p=0.190); however, there was a trend towards a ~40% decrease in AUC in E- vessels (E+ CON: 264.0±35.2 vs E- CON: 157.9±1.6; p=0.052). Importantly, both E+ and E- vessels constrict to H_2O_2 to the same extent at 10^{-2}mol/L H_2O_2 achieving a maximum of 2-3mN (n=2/group) (Figure 21).

Table 8: *Endothelium-Dependent Dilation of SD Intact and Denuded Arteries*

ACh	P values		
	Strain	Drug	Strain*Drug
MR (%)	<0.001	0.002	0.013
$logEC_{50}$ (mol/L)	0.291	0.029	0.004
AUC (AU)	<0.001	0.002	0.003

p-values from ANOVA. ACh, acetylcholine; MR, maximal relaxation; AUC, area under the dose-response curve. Endothelium, main effect of denuding; Drug, main effect of CAT pre-incubation; Endothelium*Drug, interaction of denuding and CAT pre-incubation.

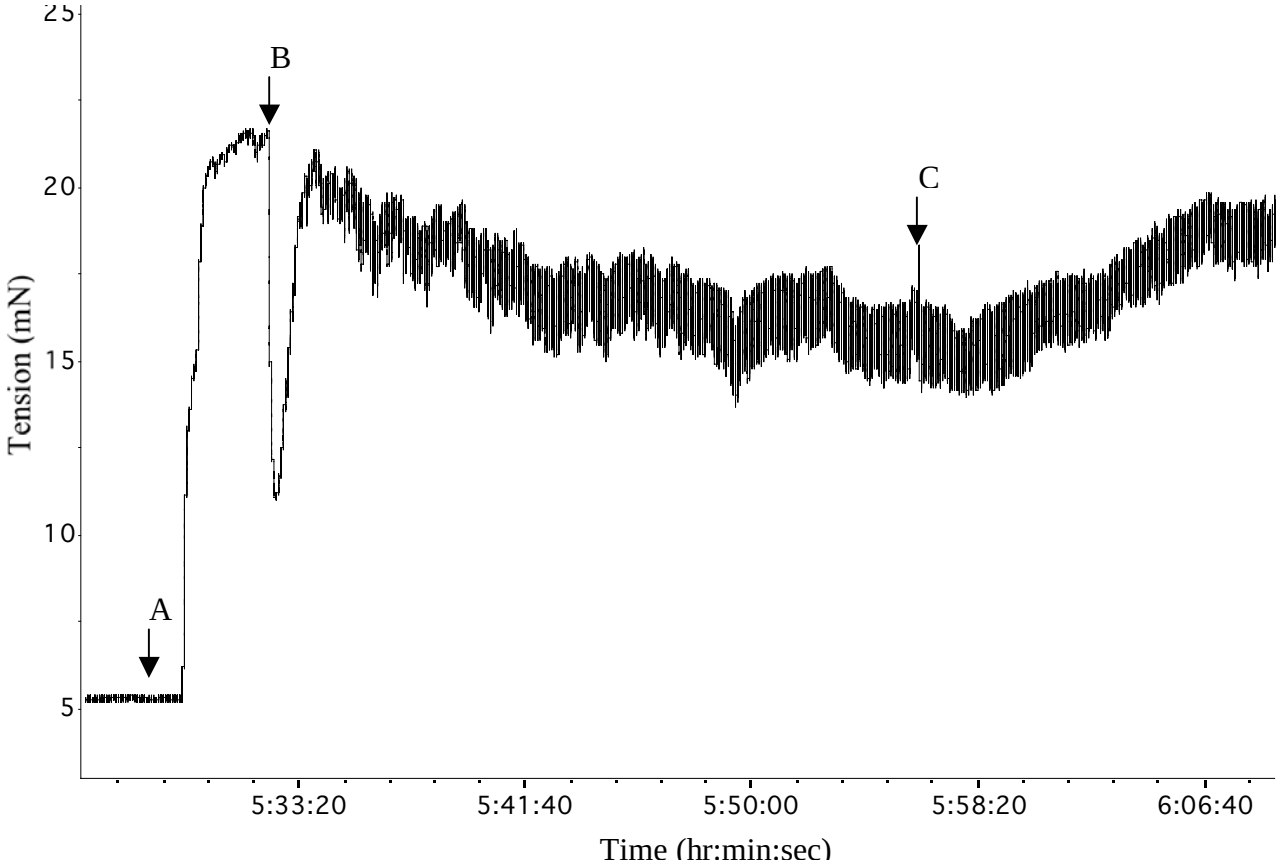

Figure 19. *Representative tracing of TEMPOL and CAT experiment.* A, Preconstriction with 10^{-5}mol/L NEPI with PRO present; B, Injection of 10mmol/L TEMPOL; C, Addition of 860U/mL CAT.

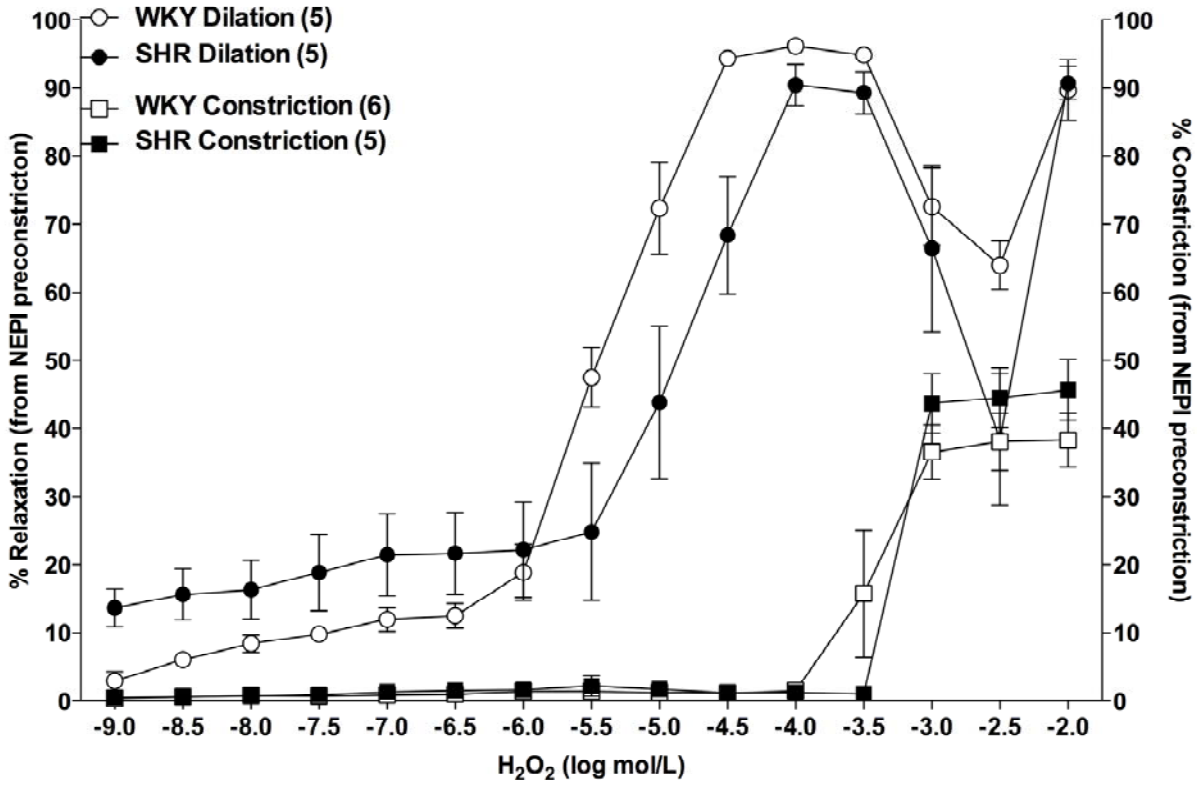

Figure 20. *H_2O_2-induced dilation and constriction.* Values are mean±s.e.m., n per group in parenthesis. NEPI, norepinephrine; Dilation, relaxation from NEPI preconstriction; Constriction, relative developed tension from quiescent vessel expressed relative to NEPI preconstriction in endothelial function test. Symbols for statistical difference were omitted for clarity. Identical to Figure 13 from text, error bars present**.** Please refer to text for details.

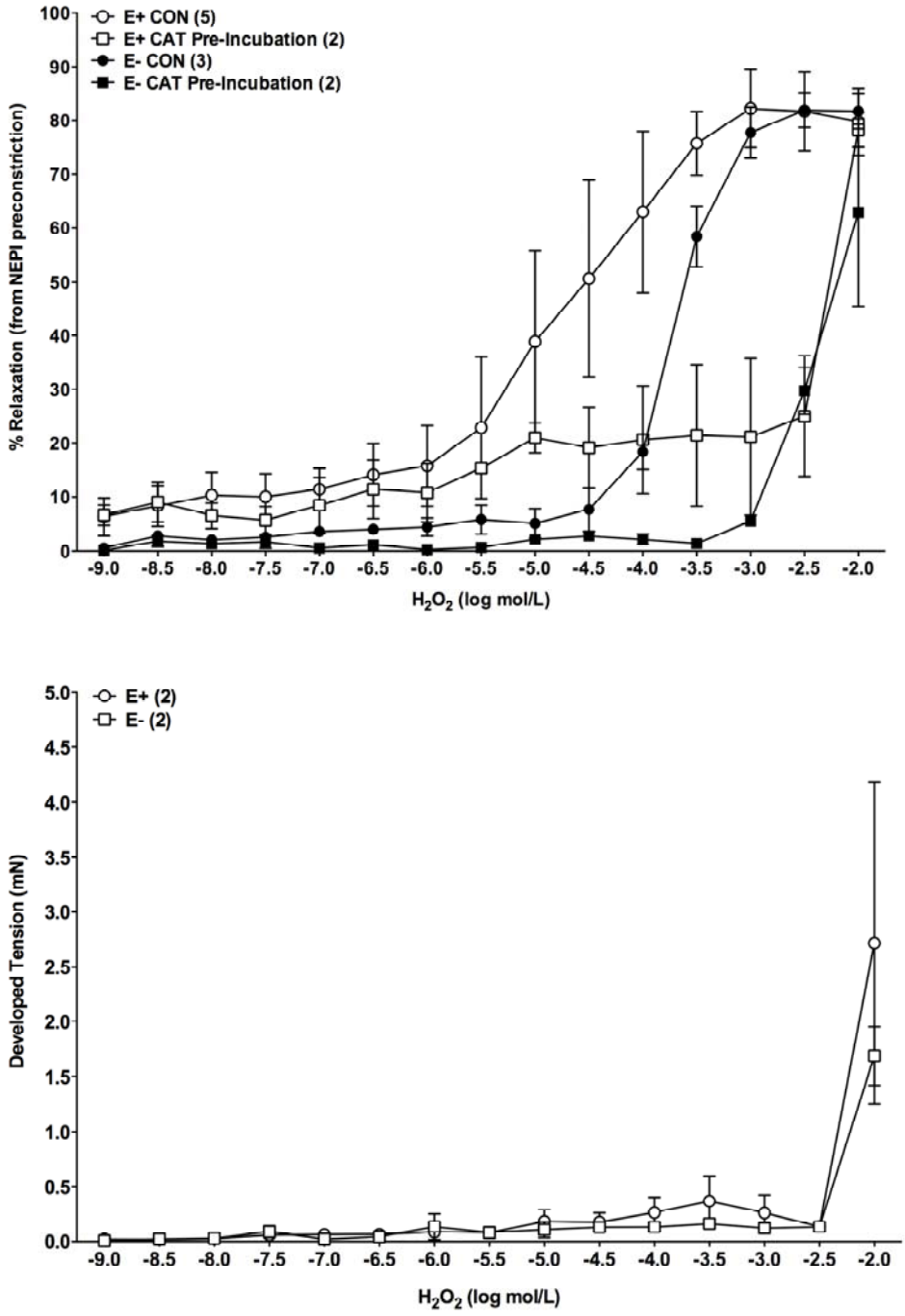

Figure 21. *H_2O_2-induced dilation and constriction in SD rats.* Values are means±s.e.m., n per group in parenthesis. *Top:* H_2O_2-induced dilation. *Bottom:* H_2O_2-induced constriction. E+, endothelium-intact; E-, endothelium-denuded; NEPI, norepinephrine; CON, untreated; CAT, catalase. Symbols for statistical difference were omitted for clarity. Please refer to text for details.

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