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SUPEROXIDE MODULATES MYOGENIC CONTRACTIONS OF MOUSE AFFERENT ARTERIOLES

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Abstract

Reactive oxygen species enhance or impair autoregulation. Since superoxide is a vasoconstrictor, we tested the hypothesis that stretch generates superoxide that mediates myogenic responses. Increasing perfusion pressure of mouse isolated perfused renal afferent arterioles from 40 to 80 mmHg reduced their diameter by 13.3 ± 1.8 % ($p < 0.001$) and increased reactive oxygen species (ethidium: dihydroethidium fluorescence) by 9.8 ± 2.3 % ($p < 0.05$). Stretch-induced fluorescence was reduced significantly ($p<0.05$) by incubation with tempol (3.7 ± 0.8%), pegylated superoxide dismutase (3.2 ± 1.0%) or apocynin (3.5 ± 0.9%) but not by pegylated catalase, L-Nitroarginine methylester or Ca^{2+}-free medium, relating it to Ca^{2+}-independent vascular superoxide. Compared to vehicle, basal tone and myogenic contractions were reduced significantly ($p<0.05$) by pegylated superoxide dismutase (5.4±0.8), tempol (4.1 ± 1.0 %) apocynin (1.0 ± 1.3%;) and diphenyleneiodinium (3.9 ± 0.9%) but not by pegylated catalase (10.1 ± 1.6 %). L-Nitroarginine methylester enhanced basal tone but neither it (15.8 ± 3.3%), nor endolethlial nitric oxide synthase knockout (10.2 ± 1.8%) significantly changed myogenic contractions. Tempol had no further effect after superoxide dismutase but remained effective after catalase. H_2O_2 above 50μM caused contractions but at 25μM inhibited myogenic responses (7.4 ± 0.8%; $p< 0.01$). In conclusion, increasing the pressure within afferent arterioles led to Ca^{2+}-independent increased vascular superoxide production from nicotinamide adenine dinucleotide phosephate oxidase which enhanced myogenic contractions largely independent of nitric oxide whereas H_2O_2 impaired pressure-induced contractions but was not implicated in the normal myogenic response.

Keywords

Oxidative stress; reactive oxygen species; hydrogen peroxide; renal autoregulation; hypertension

Introduction

Autoregulation maintains renal blood flow (RBF), glomerular filtration rate (GFR) and tubular fluid delivery during changes in perfusion pressure (PP)[1]. Defects in the buffering of

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Conflict of Interest None.

arterial pressure by renal autoregulation have been implicated in renal barotrauma[2]. Renal autoregulation depends primarily on a rapid myogenic contraction of the afferent arteriole[3] followed by a tubuloglomerular feedback (TGF) response[1, 3].

Myogenic mechanisms in the afferent arteriole are incompletely understood[1]. Reactive oxygen species (ROS) have been implicated in the increased vascular reactivity of the renal afferent arterioles to angiotensin II (Ang II) in states of oxidative stress[4–6]. An increase in pressure in a large conduit artery increased vascular ROS generation by nicotine adenine dinucleotide phosphate (NADPH) oxidase, but conduit arteries have little myogenic reactivity[7]. Recently, ROS have been implicated in the enhanced myogenic contractions of renal afferent arterioles from spontaneously hypertensive rats (SHR)[8] although the more modest myogenic contractions of normotensive rates were independent of ROS[8]. On the other hand, ROS may impair autoregulation. Thus, rat kidneys with oxidative stress caused by transforming growth factor beta (TGF-β)[9] or by a high salt intake and Ang II infusion[10] had impaired myogenic responses that were preserved by the redox-cycling nitroxide tempol[11], while exposure of cerebral arterioles to ROS abolished autoregulation[12]. Therefore, it is presently unclear whether ROS contribute positively or negatively to myogenic responses[13]. This could indicate different effects of superoxide ($O_2^{\bullet -}$) which was a potent stimulator of vascular reactivity[4, 5] and hydrogen peroxide (H_2O_2) which had variable effects[14, 15].

The mouse isolated perfused renal afferent arteriole displayed a linear increase in active wall tension above a perfusion pressure of approximately 40 mmHg which defined the myogenic response[16]. We used this preparation to test the hypothesis that stretch increased ROS and that $O_2^{\bullet -}$ and/or H_2O_2 were required for the myogenic contraction. We loaded vessels with dihydroethidium which is a ROS-sensitive fluorophore to determine release of ROS by increased perfusion pressure. Tempol was added to the bath to metabolize ROS. Since tempol can metabolize both $O_2^{\bullet -}$ and H_2O_2, the ROS responsible was assessed from the effects of bath addition of pegylated superoxide dismutase (PEG-SOD) or pegylated catalase (PEG-CAT) which are taken up into cells and metabolize $O_2^{\bullet -}$ or H_2O_2 respectively[17]. Although we found no effects of PEG-CAT on myogenic contractions in afferent arterioles from normal mice, we investigated the effects of bath addition of H_2O_2 on basal contractility and myogenic responses to determine its potential role in states of vascular oxidative stress. The source of ROS was assessed from the effects of bath addition of apocynin, diphenyleneidinium or L-nitroarginine methyl ester (L-NAME). Apocynin inhibited NADPH oxidase in renal afferent arterioles[8] and L-NAME blocked ROS derived from uncoupled eNOS[18]. ROS have direct effects on vascular smooth muscle cell (VSMC) contractility[11] or indirect effects via bioinactivation of nitric oxide (NO) by $O_2^{\bullet -}$. NO blunted myogenic contractions in vivo in rat kidneys but this was ascribed to an indirect effect via tubuloglomerular feedback[20] and blunted contractions in rabbit afferent arterioles but only when NO generation was stimulated by vascular flow[21]. Therefore, we assessed the effects of NO on myogenic responses by blockade of NOS with L-NAME and in mice with a knockout of endothelial nitric oxide synthase (eNOS −/−). Ca^{2+} is essential for myogenic responses, but its relationship to VSMC ROS is not established[22]. Therefore, we assessed myogenic responses and PP-induced ROS in Ca^{2+}-free medium.

Methods and protocols

Male C57BL/6 mice, aged 3 to 5 months and weighing 25g to 28g (Jackson Laboratory, Bar Harbor, Maine) were fed a 0.4% NaCl (normal) control test diet (Harlan Teklad, CA) and allowed free access to tap water. Additional studies were undertaken in eNOS knockout mice from Jackson Labs, Maine. All procedures conformed to the Guide for Care and Use of Laboratory Animals prepared by The Institute for Laboratory Animal Research. Studies

were approved by the Georgetown University Animal Care and Use Committee. Details of methods appear in supplement (please see http://hyper.ahajournals.org).

Animal preparation, dissection and mounting of afferent arterioles and surgery

Mice were anesthetized with 2% isoflurane and oxygen, the kidneys were removed and a single renal afferent arteriole prepared as described in detail[16] and in the supplement (please see http://hyper.ahajournals.org).

Measurements of ROS and myogenic responses in afferent arterioles

These were as described previously[16, 22–24] and detailed further in the supplement (please see http://hyper.ahajournals.org).

Pharmacological agents

The drugs used were: 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (tempol), superoxide dismutase-polyethylene glycol, catalase-polyethylene glycol, apocynin, diphenyleneiodinium (DPI), L-nitroarginine methyl ester (L-NAME) and hydrogen peroxide (H_2O_2) from Sigma-Aldrich. Drugs were added to the bath 30 min prior to testing in the concentrations shown to be effective.

Statistics

Data were expressed as mean ± SEM. An analyses of variance (ANOVA) compared the effects of vehicle and drugs added to the bath. When appropriate, these calculations were followed by Bonferroni post hoc Student's *t* tests. Changes were analyzed using nonparametric statistics (GraphPad Prism, GraphPad Software). $P < 0.05$ was considered statistically significant.

Results

Data in arterioles from normal mice are shown in figure S1 in the supplement (please see http://hyper.ahajournals.org).

Increasing renal perfusion pressure from 40 to 80 mmHg increased the fluorescent signal for ROS, detected as the ratio of ethidium to dihydroethidium (E:DHE) by 9.8 ± 2.3% (Figure1). The ROS signal was significantly ($p< 0.01$) reduced by incubating with PEG-SOD (3.2 ± 1.0%), tempol (3.7 ± 0.8%) or apocynin (3.5 ± 0.9%) but it was not affected by PEG-CAT (10.2 ± 1.9%) or L-NAME (11.8 ± 2.8%) or removal of external Ca^{2+} with Ca-free bath and EGTA (11.7 ± 3.2%)[16]. The increase in vascular ROS with increased perfusion pressure detected with E:DHE was similar to that detected by tempo-9AC (n = 4) which was increased by 10.4 ± 1.6%.

The effects of bath additional of tempol on the diameter of isolated renal afferent arterioles perfused at 60 mmHg are shown in Figure 2. Tempol caused graded increases in vascular diameter that were maximal at approximately 10^{-4} M (14.4 ± 2.2%; Figure 2A). The relaxation occurred over the first 6 minutes, and was stable by approximately 15 minutes (Figure 2B). Therefore, a dose of 10^{-4} M tempol and an incubation time of 30 minutes were selected for these studies. The basal diameter also was increased by incubation of arterioles with 200 u·ml^{-1} PEG-SOD (12.4 ± 0.6% $p < 0.001$), apocynin (15.4 ± 3.1%; $p< 0.05$) or DPI (8.9 ± 2.5%; $p<0.05$). Following incubation with 200 u·ml^{-1} PEG-SOD, the addition of 10^{-4} M tempol for 15 minutes did not further increase the basal diameter (3.0 ± 1.5%; p =

ns). The basal diameter was not affected by incubation with 1000 u·ml^{-1} PEG-CAT (0.1 ± 0.1%; p = ns) but was reduced by L-NAME (−9.9 ± 2.7%; p<0.01). Following incubation with 1000 μ·ml^{-1} PEG-CAT, the addition of 10^{-4} M tempol for 15 minutes increased the diameter by 12.2 ± 2.2% (p < 0.05) which was similar to tempol alone.

There were no significant differences in the basal luminal diameter measured at 40 mmHg between the groups (table 1). Compared to vehicle, incubation of the vessels with 10^{-4} M tempol, 200 u·ml^{-1} PEG-SOD, 10^{-5} M apocynin or 10^{-5}M DPI all attenuated the pressure-induced reduction in diameter (Table 1 and Figure 3A and B) without affecting the passive wall tension (Figure 3C), resulting in significant reductions in active wall tension (Figure 3D; myogenic response, Table 1). Compared to vehicle, the myogenic response was reduced by 71% by tempol, by 58% by PEG-SOD, by 58% by apocynin and by 65% by DPI (table 1). After pre-incubation with PEG-SOD, the addition of tempol had no significant further effect.

Incubation of vessels with 1000 u·ml^{-1} PEG-CAT had no significant effects on the reductions in vessel diameter with increasing perfusion pressure (Figure 4A and 4B) or the passive or active wall tensions (Figure 4C and 4D) and did not modify the response to 10^{-4} M tempol (table 1).

Afferent arterioles were perfused at 60 mmHg to provide some basal tone and incubated with graded concentration of H_2O_2 for 15 minutes. H_2O_2 significantly reduced the diameter at concentrations > 50μM (Fig 5A). A subthreshold concentration of H_2O_2 of 25μM blunted the reduction in luminal diameter with PP (Fig 5B) and the myogenic response (table 1).

Bath addition of L-NAME or use of eNOS −/− mice had no significant effects on changes in vessel diameter with increasing perfusion pressure (Fig 6 A and B), passive or active wall tension (Fig 6 C and D) or myogenic responses (Table 1).

Discussion

The main new findings from this study of afferent arterioles from normal C57BL/6 mice were that increasing the perfusion pressure from 40 to 80 mmHg caused a myogenic contraction accompanied by an increase in ROS signal whether detected by dihydroethidium or tempo −9AC. The fluorescent ROS signal was predominately $O_2^{\bullet-}$ since it was reduced by incubation with PEG-SOD or tempol but not with PEG-CAT and was upstream from Ca^{2+} since it persisted in Ca^{2+}-free medium. Incubation of vessels with tempol, PEG-SOD, apocynin or DPI reduced basal and myogenic tone whereas PEG-CAT was not effective, indicating that the responses were enhanced by $O_2^{\bullet-}$ generated from NADPH oxidase. The moderation of myogenic contractions by tempol was prevented by preincubation with PEG-SOD but was preserved by preincubation with PEG-CAT. H_2O_2 caused contractions at concentrations above 50μM but inhibited myogenic responses at 25μM. L-NAME increased basal tone but did not affect pressure-induced ROS generation. Neither L-NAME nor eNOS knockout affected myogenic contractions.

Tempol added to the bath prevented the enhanced Ang II contractions of perfused renal afferent arterioles of SOD-1 −/− mice[6], moderated U-46,619-induced vasoconstriction[25] and prevented the endothelium dependent contractions in rabbits with oxidative stress[5]. Tempol is an SOD mimetic and reduced tissue $O_2^{\bullet-}$[11] but it might thereby increase tissue H_2O_2[11, 26–28]. However, the increase in H_2O_2 in blood vessels was modest and lasted less than 2 minutes[29]. Moreover, tempol is a redox cycling nitroxide[11] with catalase-like actions in tissues that prevented H_2O_2 accumulation[30, 31]. We found that the pressure-induced increase in fluorescence signal from the oxidation of DHE was reduced by tempol, similar to PEG-SOD, but was not reduced by PEG-CAT. This indicated that the ROS generated by

vascular stretch was superoxide and that this was inhibited by tempol. Moreover, tempol had no further effect on the myogenic response in vessels preincubated with PEG-SOD, but retained its full efficacy after PEG-CAT. This related the inhibitory effects of tempol on the myogenic response to metabolism of $O_2^{\bullet-}$ rather than to increased H_2O_2. Indeed, myogenic responses were blunted by PEG-SOD but not by PEG-CAT, indicating that the normal myogenic response was enhanced by $O_2^{\bullet-}$ rather than by H_2O_2. However, although H_2O_2 was not implicated in normal myogenic responses, it may contribute if it accumulated sufficiently in the vessels since 25μM H_2O_2 inhibited myogenic contractions. H_2O_2 also inhibited angiotension-induced contractions and intracellular calcium in rat afferent arterioles[22]. Therefore, the catalase-like activity of tempol might explain its improvement in myogenic responses in models of severe or prolonged oxidative stress if H_2O_2 accumulated sufficiently to blunt myogenic contractions in these circumstances.

Apocynin inhibited the enhanced myogenic responses of afferent arterioles from SHR. Apocynin is not a specific inhibitor of NADPH oxidase[32]. However, similar results were obtained by inhibition of NADPH oxidase with gp9lds-tat[8]. The finding that apocynin and another NADPH oxidase inhibitor, DPI had similar effects as tempol or PEG-SOD in reducing basal and myogenic tone in mouse afferent arterioles in this study confirmed that the source of $O_2^{\bullet-}$ was predominantly NADPH oxidase[7]. Since the perfusion-pressure-induced increase in contraction was abolished in Ca^{2+}-free medium, yet the increase in ROS was unaffected, we concluded that the myogenic response was entirely dependent on Ca^{2+} and that changes in Ca^{2+} concentration or sensitivity were downstream from increased $O_2^{\bullet-}$.

We found comparable effects of the drugs that blocked $O_2^{\bullet-}$ to reduce basal and active myogenic tone. This suggests that both depended on the generation of $O_2^{\bullet-}$, consistent with effects of NADPH oxidase to increase basal tone in SHR aortas[33]. Blockade of NOS by L-NAME increased the basal tone of the perfused afferent arteriole but did not change perfusion-pressure induced ROS or contractions. Thus, eNOS uncoupling did not contribute to $O_2^{\bullet-}$ generation and NO did not modulate the myogenic response. Moreover, prolonged deletion of the eNOS gene also did not affect myogenic responses (fig 6 and table 1). This is consistent with the conclusions of Juncos et al[21] that the stretch-induced contraction of rabbit isolated afferent arterioles was not dependent on NO although flow induced NO release affected basal tone.

The present conclusions differ from prior studies in normal rats where $O_2^{\bullet-}$ contributed to the enhanced myogenic response of SHR afferent arterioles but had little influence under normal conditions[8]. Sharma et al[9] reported that TGF-β blocked autoregulatory responses of the rat juxtamedullary nephron preparation by stimulating ROS. Saeed et al[10] reported a reduced myogenic response in intact kidney of rats given a high salt intake and infused with angiotension II for 14 days that was preserved by tempol. Clearly, ROS may have opposite modulating effects on myogenic responses that may relate to experimental conditions (isolated arterioles vs intact kidneys), species (rat vs mouse) or ROS ($O_2^{\bullet-}$ vs H_2O_2). The present study is the first to implicate $O_2^{\bullet-}$ in myogenic responses of afferent arterioles from normal mice.

Perspective

Glomerular filtration requires a uniquely high capillary pressure that renders the glomerular capillaries susceptible to baratrauma if there is a breakdown of renal myogenic responses and a rise in blood pressure, as in some models of chronic kidney disease[34]. Thus, if afferent arteriolar ROS enhance myogenic responses, they might have a renal protective effect. However, the effects of metabolism of ROS by tempol vary widely from inhibitory effects on acute myogenic responses seen in mice arterioles in this study and in SHR[8] to restorative

effects in some others[9, 10]. Further study will be needed to determine if variable effects of tempol could underlie some discordant reports of its effects on the kidneys in models of chronic kidney disease that range from renal protection in the reduced renal mass model in rats[35] or mice[36] to no effect in models of diabetes mellitus[28] or antiglomerular basement membrane nephritis[27].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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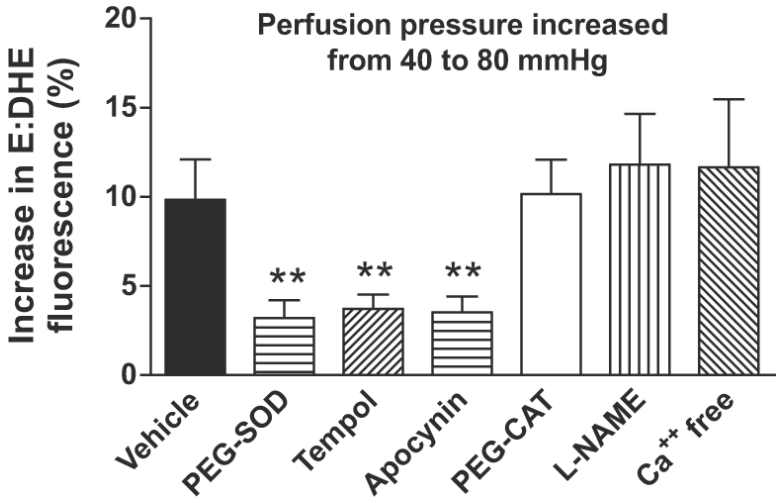

Figure 1.

Mean ± SEM values (n=5–6) for change in ethidium to dihydroethidium fluorescence of afferent arterioles after increasing perfusion pressure from 40 to 80 mmHg with 30 min incubation in vehicle, 1000 $\mu \cdot ml^{-1}$ pegylated catalase, 200 $\mu \cdot ml^{-1}$ pegylated superoxide dismutase, 10^{-4}M tempol, 10^{-5}M apocynin, 10^{-4}M L-NAME or in a calcium-free medium with EGTA. Compared to vehicle: **, $p < 0.01$

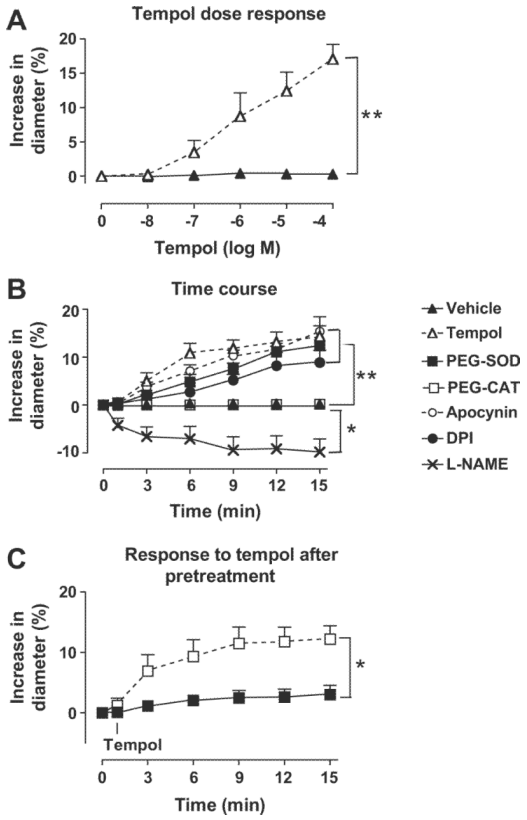

Figure 2.

Mean ± SEM values (n=4 to 8) comparing afferent arterioles perfused at 60 mmHg and incubated with vehicle (solid triangles and continuous lines) or with graded concentrations of tempol (open triangles and interrupted lines) for change in diameter (A) or, for time after addition of vehicle, 10^{-4} M tempol, 200 $\mu \cdot ml^{-1}$ PEG-SOD (solid squares and continuous lines), 1000 $\mu \cdot ml^{-1}$ PEG-CAT (open squares and interrupted lines), 10^{-5}M apocynin (open circles and interrupted lines), 10^{-5}M DPI (solid circles and continuous lnes) or 10^{-4}M L-NAME (crosses and continuous lines) (B), or for the effects of incubation with 10^{-4}M tempol for the times shown after preincubation for 30 minutes with PEG-SOD (solid triangles and continuous lines) or PEG-CAT (open triangles and interrupted lines)(C). Comparing differences: *p<0.05; **, p < 0.01;***, p < 0.005.

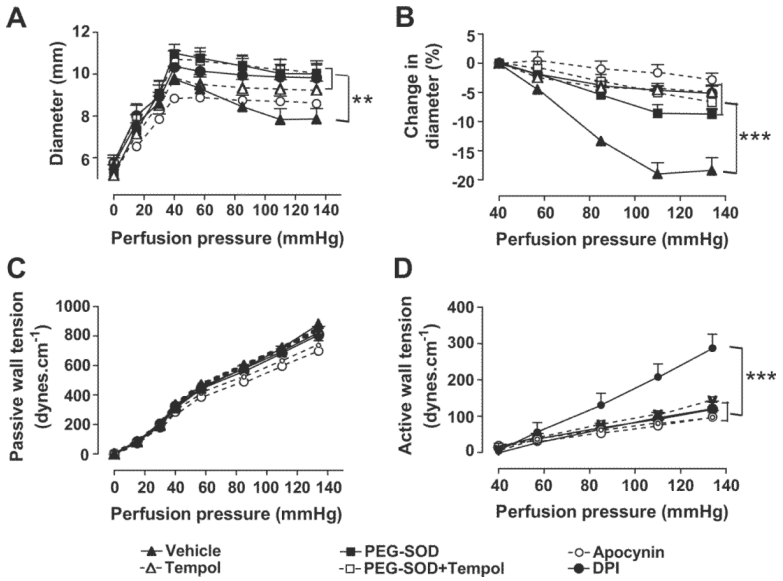

Figure 3.

Mean ± SEM values (n = 5 to 8) for vessels incubated with a vehicle (solid triangles and continuous lines), 10^{-4} M tempol (open triangles and interrupted lines), 200 $\mu \cdot ml^{-1}$ PEG-SOD (solid squares and continuous lines), 10^{-5}M apocynin (open circles and interrupted lines), 10^{-5}M DPI (solid circles and continuous lines) or PEG-SOD followed by tempol (open squares and broken lines). Data are shown for diameter (panel A), change in diameter (panel B), passive wall tension (panel C), and active wall tension (panel D). Comparing groups: **, $p < 0.01$;***, $p < 0.005$.

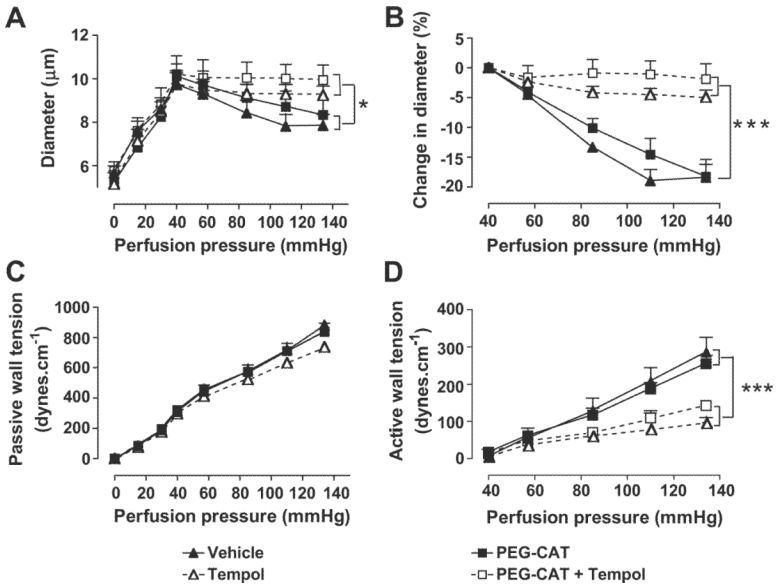

Figure 4.

Mean ± SEM values (n = 6 to 8) for vessels incubated with a vehicle (solid triangle and continuous lines), 10^{-4} M tempol, (open triangle and broken lines), 1000 $\mu \cdot ml^{-1}$ PEG-catalase (solid square and continuous lines) and tempol following PEG-catalase (open square and broken lines). Comparing groups: *, $p < 0.05$; ***, $p < 0.005$.

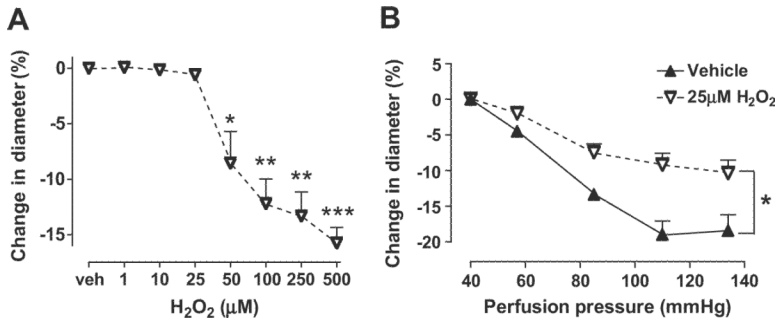

Figure 5.

Mean ± SEM values (n=10) in panel A for vessels perfused at 60 mmHg and incubated by 30 mins with vehicle (solid triangles and continuous lines) or graded concentrations of hydrogen peroxide (open triangles and broken lines). In panel B, vessels were incubated for 30 mins with vehicle (solid triangles and continuous lines) or 25μmol·l^{-1} H_2O_2 (open triangles and broken line) and perfused at graded pressures. Comparing groups: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

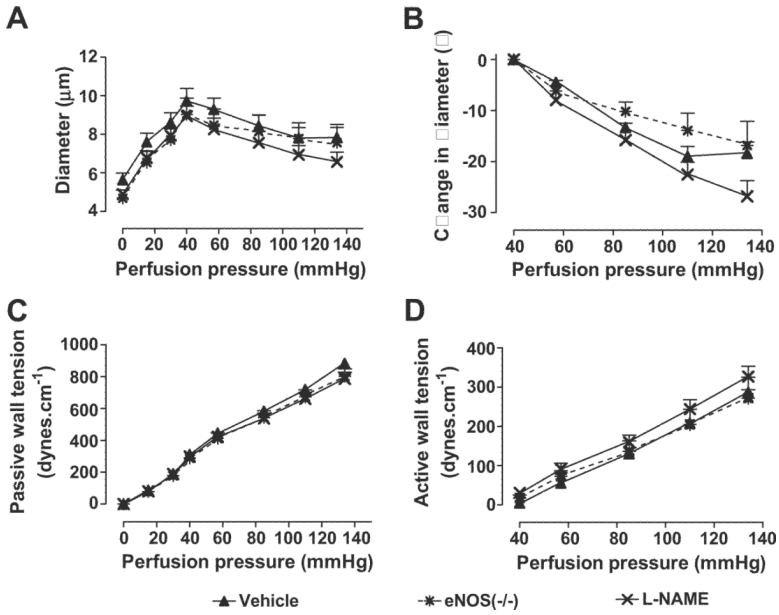

Figure 6.

Mean ± SEM values (n = 6) for vessels incubated with a vehicle (solid triangle and continuous lines) or 10^{-4} M L-NAME (crosses and continuous lines), or vessels from eNOS −/− mice (crosses and broken lines). Comparing groups: *, $p < 0.05$; **, $p<0.01$; ***, $p < 0.005$.

Table 1

Basal luminal diameter of afferent arterioles and characteristics of myogenic responses during increases in perfusion pressure from 40 to 135 mmHg after bath addition of vehicle or drugs

Addition to bath	Strain	Number	Basal diameter (μm)	Δ diameter from 40 to 135 mmHg (%)	Myogenic response (dynes · cm^{-1} /mmHg)
Vehicle	C57BL/6	8	10.1 ± 0.5	−18.1 ± 2.5	3.1 ± 0.3
Tempol	C57BL/6	8	9.8 ± 0.5	−4.9 ± 1.2*	0.9 ± 0.1*
PEG-SOD	C57BL/6	6	10.9 ± 0.5	−8.7 ± 0.8*	1.3 ± 0.2*
PEG-SOD then tempol	C57BL/6	5	10.7 ± 0.4	−6.6 ± 0.9*	1.4 ± 0.1*
Apocynin	C57BL/6	4	8.8 ± 0.2	−2.8 ± 1.2*	0.8 ± 0.2*
DPI	C57BL/6	5	10.3 ± 0.7	−5.1 ± 1.1*	1.1 ± 0.1*
PEG-CAT	C57BL/6	6	10.1 ± 0.6	−18.2 ± 2.9	2.5 ± 0.2
PEG-CAT then tempol	C57BL/6	6	10.2 ± 0.8	−1.9 ± 2.6*	1.3 ± 0.1*
H_2O_2	C57BL/6	10	9.3 ± 0.3	−10.4 ± 1.5*	1.6 ± 0.1*
L-NAME	C57BL/6	6	8.9 ± 0.5	−26.8 ± 3.4	3.1 ± 0.3
Vehicle	eNOS −/−	6	9.0 ± 0.8	−16.8 ± 4.7	3.2 ± 0.3

Mean ± SEM. Basal diameter was measured at 40 mmHg perfusion pressure before drugs. Compared to vehicle;

*, $p<0.05$.