Superoxide Modulates Myogenic Contractions of Mouse Afferent Arterioles

En Yin Lai, Anton Wellstein, William J. Welch, Christopher S. Wilcox

Abstract—Reactive oxygen species enhance or impair autoregulation. Because 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 mm Hg 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 Ca2+-free medium, relating it to Ca2+-independent vascular superoxide. Compared with 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 endothelial NO synthase knockout (10.2 ± 1.8%) significantly changed myogenic contractions. Tempol had no further effect after superoxide dismutase but remained effective after catalase. H2O2 > 50 μmol/L caused contractions but at 25 μmol/L inhibited myogenic responses (7.4 ± 0.8%; P < 0.01). In conclusion, increasing the pressure within afferent arterioles led to Ca2+-independent increased vascular superoxide production from nicotinamide adenine dinucleotide phosphate oxidase, which enhanced myogenic contractions largely independent of NO, whereas H2O2 impaired pressure-induced contractions but was not implicated in the normal myogenic response. (Hypertension. 2011;58:650-656.)

Key Words: oxidative stress ■ reactive oxygen species ■ hydrogen peroxide ■ renal autoregulation ■ hypertension

Autoregulation maintains renal blood flow, glomerular filtration rate, and tubular fluid delivery during changes in perfusion pressure (PP).1 Defects in the buffering of arterial pressure by renal autoregulation have been implicated in renal barotrauma.2 Renal autoregulation depends primarily on a rapid myogenic contraction of the afferent arteriole3 followed by a tubuloglomerular feedback 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 in states of oxidative stress.4–6 An increase in pressure in a large conduit artery increased vascular ROS generation by 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 (SHRs),8 although the more modest myogenic contractions of normotensive rats were independent of ROS.8 On the other hand, ROS may impair autoregulation. Thus, rat kidneys with oxidative stress caused by transforming growth factor-β9 or by a high salt intake and angiotensin II infusion10 had impaired myogenic responses that were preserved by the redox-cycling nitroxide Tempol,11 whereas 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 (O2−), which was a potent stimulator of vascular reactivity,4,5 and hydrogen peroxide (H2O2), which had variable effects.14,15

The mouse isolated perfused renal afferent arteriole displayed a linear increase in active wall tension above a PP of ≈40 mm Hg, which defined the myogenic response.16 We used this preparation to test the hypothesis that stretch increased ROS and that O2− and/or H2O2 were required for the myogenic contraction. We loaded vessels with dihydroethidium, which is a ROS-sensitive fluorophore, to determine release of ROS by increased PP. Tempol was added to the bath to metabolize ROS. Because Tempol can metabolize both O2− and H2O2, 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 metabolized O2− or H2O2, 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 H2O2 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 endothelial NO synthase (eNOS).18 ROS have direct effects on vascular smooth muscle cell contractility11 or indirect effects via bioinactivation of NO by O2−.19 NO blunted myogenic contractions in vivo in rat kidneys, but this was ascribed to an indirect effect via tubuloglomerular feedback20 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 NO synthase with L-NAME and in mice with a knockout of eNOS (eNOS−/−). Ca2+ is essential for myogenic responses, but its relationship to vascular smooth muscle cell ROS is not established.22 Therefore, we assessed myogenic responses and PP-induced ROS in Ca2+-free medium.

Methods and Protocols
Male C57BL/6 mice, aged 3 to 5 months and weighing 25 to 28 g (Jackson Laboratory, Bar Harbor, ME), were fed a 0.4% NaCl (normal) control test diet (Harlan Teklad) and allowed free access to tap water. Additional studies were undertaken in eNOS knockout mice from Jackson Laboratories. All of the 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 the online Data 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 was prepared as described in detail16 and in the online Data Supplement.

Measurements of ROS and Myogenic Responses in Afferent Arterioles
These were as described previously16,22–24 and detailed further in the online Data Supplement.

Figure 1. Mean±SEM values (n=5 to 6) for change in ethidium to dihydroethidium fluorescence of afferent arterioles after increasing perfusion pressure from 40 to 80 mm Hg with 30-minute incubation in vehicle, 1000 μ·mL−1 pegylated catalase, 200 μ·mL−1 pegylated superoxide dismutase, 10−4 M Tempol, 10−5 M apocynin, 10−4 M L-nitroarginine methyl ester (L-NAME), or in a calcium-free medium with EGTA. Compared with vehicle: **P<0.01.

Figure 2. Mean±SEM values (n=4 to 8) comparing afferent arterioles perfused at 60 mm Hg 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 μ·mL−1 PEG-SOD (solid squares and continuous lines), 1000 μ·mL−1 PEG-CAT (open squares and interrupted lines), 10−5 M apocynin (open circles and interrupted lines), 10−5 M diphenyleneiodinium (DPI; solid circles and continuous lines), or 10−4 M L-nitroarginine methyl ester (L-NAME; crosses and continuous lines; B), or for the effects of incubation with 10−4 M Tempol for the times shown after pre-incubation for 30 minutes with PEG-SOD (solid triangles and continuous lines) or pegylated catalase (PEG-CAT; open triangles and interrupted lines; C). Comparing differences: *P<0.05; **P<0.01; ***P<0.005.

Pharmacological Agents
The drugs used were as follows: 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (Tempol), superoxide dismutase-polyethylene glycol, catalase-polyethylene glycol, apocynin, diphenyleneiodinium (DPI), L-NAME, and H2O2 from Sigma-Aldrich. Drugs were added to the bath 30 minutes before testing in the concentrations shown to be effective.

Statistics
Data were expressed as mean±SEM. An ANOVA compared the effects of vehicle and drugs added to the bath. When appropriate, these calculations were followed by Bonferroni post hoc Student 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 online Data Supplement (please see http://hyper.ahajournals.org). Increasing renal PP from 40 to 80 mm Hg increased the fluorescent signal for ROS, detected as the ratio of ethidium:dihydroethidium by 9.8 ± 2.3% (Figure 1). The ROS signal was significantly increased (∗P < 0.01) by incubation 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 Ca²⁺ with Ca-free bath and EGTA (11.7 ± 3.2%). The increase in vascular ROS with increased PP detected with ethidium-dihydroethidium was similar to that detected by tempol-9AC (n = 4), which was increased by 10.4 ± 1.6%.

The effects of addition of Tempol to the bath on the diameter of isolated renal afferent arterioles perfused at 60 mm Hg are shown in Figure 2. Tempol caused graded increases in vascular diameter that were maximal at 10⁻⁴ m Tempol (Figure 2A). The relaxation occurred over the first 6 minutes and was stable by 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 was also 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). After incubation with 200 u • mL⁻¹ of PEG-SOD, the addition of 10⁻⁴ m Tempol for 15 minutes did not further increase the basal diameter (3.0 ± 1.5%; ∗P value not significant). The basal diameter was not affected by incubation with 1000 u • mL⁻¹ of PEG-CAT (0.1 ± 0.1%; ∗P value not significant) but was reduced by l-NAME (−9.9 ± 2.7%; ∗P < 0.01). After incubation with 1000 µ • mL⁻¹ of 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 mm Hg between the groups (Table). Compared with vehicle, incubation of the vessels with 10⁻⁴ m Tempol, 200 u • mL⁻¹ of PEG-SOD, 10⁻⁵ m apocynin, or 10⁻⁵ m DPI all attenuated the pressure-induced reduction in diameter (Table and Figure 3A and 3B) without affecting the passive wall tension (Figure 3C), resulting in significant reductions in active wall tension (Figure 3D; myogenic response, Table). Compared with 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). After preincubation with PEG-SOD, the addition of Tempol had no significant further effect.

Incubation of vessels with 1000 u • mL⁻¹ of PEG-CAT had no significant effects on the reductions in vessel diameter with increasing PP (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). Afferent arterioles were perfused at 60 mm Hg to provide some basal tone and incubated with graded concentration of H₂O₂ for 15 minutes. H₂O₂ significantly reduced the diameter at concentrations >50 µmol/L (Figure 5A). A subthreshold concentration of H₂O₂ of 25 µmol/L blunted the reduction in luminal diameter with PP (Figure 5B) and the myogenic response (Table). Bath addition of l-NAME or use of eNOS⁻/⁻ mice had no significant effects on changes in vessel diameter with increasing PP (Figure 6A and 6B), passive or active wall tension (Figure 6C and 6D), or myogenic responses (Table).

Discussion

The main new findings from this study of afferent arterioles from normal C57BL/6 mice were that increasing the PP from 40 to 80 mm Hg caused a myogenic contraction accompanied by an increase in ROS signal whether detected by dihydroethidium or tempol-9AC. The fluorescent ROS signal was predominately O₂⁻, because it was reduced by incubation with PEG-SOD or Tempol but not with PEG-CAT and was upstream from Ca²⁺ because it persisted in Ca²⁺-free medium. Incubation of vessels with Tempol, PEG-SOD, apoc-
ynin, or DPI reduced basal and myogenic tone, whereas PEG-CAT was not effective, indicating that the responses were enhanced by $O_2$ \textsuperscript{...} 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 $>$50 $\mu$mol/L but inhibited myogenic responses at 25 $\mu$mol/L. 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 angiotensin II contractions of perfused renal afferent arterioles of superoxide dismutase 1 \textsuperscript{-/-} mice, \textsuperscript{6} moderated U-46 619-induced vasoconstriction, \textsuperscript{25} and prevented the endothelium-dependent contractions in rabbits with oxidative stress. \textsuperscript{5} Tempol is a superoxide dismutase mimetic and reduced tissue $O_2$ \textsuperscript{...}, \textsuperscript{11} but it might thereby increase tissue $H_2O_2$. \textsuperscript{11,26–28} However, the increase in $H_2O_2$ in blood vessels was modest and lasted <2 minutes. \textsuperscript{29} Moreover, Tempol is a redox cycling nitroxide \textsuperscript{11} with catalase-like actions in tissues that prevented $H_2O_2$ accumulation. \textsuperscript{30,31} We found that the pressure-induced increase in fluorescence signal from the oxidation of dihydroethidium 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$_2^{-}$ rather than to increased H$_2$O$_2$. Indeed, myogenic responses were blunted by PEG-SOD but not by PEG-CAT, indicating that the normal myogenic response was enhanced by O$_2^{-}$ rather than by H$_2$O$_2$. However, although H$_2$O$_2$ was not implicated in normal myogenic responses, it may contribute if it accumulated sufficiently in the vessels, because 25 µmol/L of H$_2$O$_2$ inhibited myogenic contractions. H$_2$O$_2$ also inhibited angiotensin-induced contractions and intracellular calcium in rat afferent arterioles. Therefore, the catalase-like activity of Tempol might explain its improvement in myogenic responses in models of severe or prolonged oxidative stress if it accumulated sufficiently in the vessels, because 25 µmol/L of H$_2$O$_2$ inhibited myogenic contractions. H$_2$O$_2$ also inhibited angiotensin-induced contractions and intracellular calcium in rat afferent arterioles. Therefore, the catalase-like activity of Tempol might explain its improvement in myogenic responses in models of severe or prolonged oxidative stress if H$_2$O$_2$ accumulated sufficiently to blunt myogenic contractions in these circumstances.

Apocynin inhibited the enhanced myogenic responses of afferent arterioles from SHRs. Apocynin is not a specific inhibitor of NADPH oxidase. However, similar results were obtained by inhibition of NADPH oxidase with gp9lds-tat. 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$_2^{-}$ was predominantly NADPH oxidase. Because 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$_2^{-}$.

We found comparable effects of the drugs that blocked O$_2^{-}$ to reduce basal and active myogenic tone. This suggests that both depended on the generation of O$_2^{-}$ consistent with effects of NADPH oxidase to increase basal tone in SHR aortas. 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$_2^{-}$ generation, and NO did not modulate the myogenic response. Moreover, prolonged deletion of the eNOS gene also did not affect myogenic responses (Figure 6 and Table). This is consistent with the conclusions of Juncos et al that the stretch-induced contraction of rabbit isolated afferent arterioles was not dependent on NO, although flow-induced NO release affected basal tone.

**Figure 5.** Mean±SEM values (n=10) in A for vessels perfused at 60 mm Hg and incubated by 30 minutes with graded concentrations of hydrogen peroxide (open triangles and broken lines). In B, vessels were incubated for 30 minutes with vehicle (solid triangles and continuous lines) or 25 µmol·L$^{-1}$ H$_2$O$_2$ (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-nitroarginine methyl ester (L-NAME; crosses and continuous lines), or vessels from endothelial NO synthase (eNOS$^{-/-}$) mice (crosses and broken lines). Comparing groups: *P<0.05; **P<0.01; ***P<0.005.
The present conclusions differ from previous studies in normal rats where $O_2^{-}$ contributed to the enhanced myogenic response of SHR afferent arterioles but had little influence under normal conditions. Sharma et al reported that transforming growth factor-$\beta$ blocked autoregulatory responses of the rat juxtamedullary nephron preparation by stimulating ROS. Saeed et al reported a reduced myogenic response in intact kidney of rats given a high-salt intake and infused with angiotensin 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 versus intact kidneys), species (rat versus mouse), or ROS ($O_2^{-}$ versus $H_2O_2$). The present study is the first to implicate $O_2^{-}$ in myogenic responses of afferent arterioles from normal mice.

**Perspectives**

Glomerular filtration requires a uniquely high capillary pressure that renders the glomerular capillaries susceptible to barotrauma if there is a breakdown of renal myogenic responses and a rise in blood pressure, as in some models of chronic kidney disease. 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 SHRs to restorative effects in some others. Further study will be needed to determine whether these variable effects of Tempol on myogenic responses 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 or mice to no effect in models of diabetes mellitus or antiglomerular basement membrane nephritis.

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**Disclosures**

None.

**References**


ONLINE DATA SUPPLEMENT

SUPEROXIDE MODULATES MYOGENIC CONTRACTIONS OF MOUSE AFFERENT ARTERIOLES

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Methods

Animal preparation, dissection and mounting of afferent arterioles

The kidneys were sliced along the corticomedullary axis immediately after sacrifice, placed in 4°C dissection solution and an afferent arteriole with glomerulus attached was microdissected using sharpened forceps under a stereomicroscope (model SZ40; Olympus Corp., Melville, NY) as described 1. The afferent arterioles were identified in the cortex from the interlobular arterial tree. The arteriole with its glomerulus was transferred to a thermoregulated chamber on the stage of an inverted microscope (Olympus IX70, Olympus America, Inc., NY). Arterioles were perfused using a micromanipulator system (Vestavia Scientific, Vestavia Hills, AL) with concentric holding and perfusion pipettes made of custom glass tubes (Drummond Scientific Company, PA). The holding pipette had a tip aperture of approximately 24 µm. The arteriole was aspirated into this pipette. The inner perfusion pipette had a tip diameter of 6 µm. It was advanced into the lumen of the arteriole. The pressure at its tip was calibrated using a closed chamber connected to a DPM-1B pneumatic transducer calibrator (Bio-Tek Instruments, INC., Winooski, VT). Microdissection and cannulation were completed within 120 min, after which the bath was gradually warmed to 37°C and the arteriole stabilized for 20 minutes. The cannulated afferent arteriole was perfused with Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham (DMEM, Sigma, St. Louis, MO) at 60 mmHg. The bath was perfused at 1 ml·min⁻¹. DMEM bubbled with 95% O₂ and 5% CO₂ and pH adjusted to 7.4 was used for dissection, bath and perfusion. The microperfused arteriole was displayed at ×400 magnification (Nomarski optics; Olympus Corp., Melville, NY) on a video monitor via a black and white camera (model NC 70; Dage-MTI, Inc., Michigan City, IN, USA) on an inverted microscope and recorded on VHS tape. Arterioles were selected according to the criteria of basal tone and a rapid constriction with KCl (100 mmol·l⁻¹) as described previously 2,3.

Measurement of myogenic tone in afferent arterioles

The experiments were recorded by a video system, digitized, and monitored in real time. A full set of 20 mmHg pressure steps from zero to 135 mmHg were undertaken in each arteriole in physiologic solution and in a perfusate without Ca²⁺ and containing 5 x 10⁻³ M ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA, Sigma, St. Louis, MO) to abolish active tone. The active wall tension (AWT) was calculated as the difference between the tension measured during perfusion with these two solutions, as described (ref 1 of this section).

ROS Determination

ROS generation was assessed by fluorescence microscopy of perfused afferent arterioles with dihydroethidium (DHE) (Invitrogen, Carlsbad, CA). DHE is a cell-permeable ROS-sensitive fluorophore that is oxidized by O₂⁻ to the highly fluorescent compound ethidium, which is trapped intracellularly and intercalated into DNA. This
method has been shown to also detect an oxidation product that differs from ethidium. Therefore additional studies were undertaken using another O$_2$\textsuperscript{·−} sensitive fluorophore, tempo-9-AC (Invitrogen, Carlsbad, CA).\textsuperscript{4}

Single-agent signal capture was achieved by cycling at 3 sec intervals between a 460- and 605-nm filter. Changes in O$_2$\textsuperscript{·−} were expressed as the ratio of ethidium:DHE fluorescence. The system used an Olympus IX70 fluorescence microscope equipped with dual photomultipliers (PMT, Photon Technology Int., Lawrenceville, NJ). Excitation was provided by a 75-W xenon arc lamp using a 380/460 nm wavelength combination isolated with a computer-controlled monochromator. Ethidium and DHE emit blue and red light, respectively, that were directed to a dual PMT assembly by a beam splitter that directed light to the two separate PMT using a 400-nm dichroic mirror and barrier filters centred at 460 and 605 nm, respectively. The ratio of ethidium:DHE was monitored in real time and recorded by software (Felix32; Photon Technology Int.).

Results

As in a prior study\textsuperscript{1}, graded increases in perfusion pressure above 40 mmHg reduced luminal diameter progressively (Supplement Figure S1A) with a maximum response of 18.4 ± 5.3\% (Figure S1B). There were linear increases in wall tension with pressure of vessels in a physiologic solution and in passive wall tension of vessels in a calcium-free solution containing EGTA (Figure S1C) and in active wall tension which was the difference between these two (Figure S1D).
Reference List


Figure Legends

Figure S1: Mean ± SEM values (n = 6) for responses of afferent arterioles to graded increases in perfusion pressure for diameter (A), change in diameter (B), wall tension with vehicle in physiology solution or with EGTA in calcium free solution (C) and active wall tension (D).