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Shear stress attenuates inward remodeling in cultured mouse thoracodorsal arteries in an eNOS-dependent, but non-hemodynamic manner, and increases Cx37 expression

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Abstract

Background: Arteries chronically constricted in culture, remodel to smaller diameters. Conversely, elevated luminal shear stress (SS) promotes outward remodeling of arteries *in vivo* and prevents inward remodeling in culture in a nitric oxide synthase (NOS)-dependent manner.

Objectives: Determine whether SS-induced prevention of inward remodeling in cultured arteries is specifically eNOS-dependent and requires dilation, and whether SS alters expression of eNOS and other genes potentially involved in remodeling.

Methods: Female mouse thoracodorsal arteries were cannulated, pressurized to 80 mmHg, and cultured 2 days with Low SS (<7 dynes/cm^2), High SS (≥15 dynes/cm^2), High SS + L-NAME (NOS inhibitor, 10^{-4} M), or High SS in arteries from eNOS −/− mice. In separate arteries cultured 1 day with Low or High SS, eNOS, Cx37, Cx40, Cx43 mRNA were measured with real-time PCR.

Results: High SS caused little change in post-culture passive diameters (−4.7±2.0 %), which was less than Low SS (−18.9±1.4 %; p<0.0001), high SS eNOS−/− (−18.0±1.5; p<0.001), or High SS +L-NAME (−12.0±0.6 %; NS), despite similar constriction during culture. Cx37 mRNA was greater (p<0.05) with High SS, but other genes were not different.

Conclusions: eNOS is involved in SS-induced prevention of inward remodeling in cultured small arteries. This effect does not require NO-mediated dilation. SS increased Cx37.

Keywords

flow; remodeling; eNOS; connexins; gap junctions

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DISCLOSURE STATEMENT

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INTRODUCTION

Arteries remodel in response to chronic hemodynamic or vasomotor changes. For example, chronic increases in flow *in vivo* promote reorganization of cellular constituents to form a larger luminal diameter, called outward remodeling, and chronic decreases in flow promote a smaller luminal diameter, called inward remodeling (1–7). Additionally, chronic vasoconstriction, as typically occurs with essential hypertension, leads to inward remodeling (8–11), and sustained vasodilation leads to outward remodeling (11). Increased flow, and the resulting increased shear stress, is well known to stimulate production of nitric oxide (12), but the role of nitric oxide in remodeling is less clear. *In vivo* inhibition of nitric oxide synthase has been reported to prevent flow-induced remodeling in many (13–15), but not all cases (16, 17). Moreover, nitric oxide is known to have anti-proliferative effects that could potentially counter the remodeling process (18–21). It is also unclear from *in vivo* studies whether nitric oxide's role in shear-induced remodeling is based on its effect on vasomotor tone and diameter or its effect on growth processes.

Isolated arteries maintained in organ culture have been useful in helping to dissect the stimuli that can produce remodeling, because this method allows precise control of hemodyamic parameters such as pressure and flow, and eliminates neuro-humoral influences and remote factors such as tissue ischemia. This approach has been most useful for studying inward remodeling. For example, chronic exposure to a variety of vascoconstrictors under constant luminal pressure has been shown to induce inward remodeling of cultured arterioles over a timecourse of 4 hrs to 3 days (22–25). Adding luminal flow in the physiological range was shown in a study by Pistea, et al. (26) to prevent this inward remodeling in porcine coronary arteries. This effect was eliminated with inhibition of nitric oxide synthases (NOS) (using L-NNA), indicating that flow-induced prevention of inward remodeling is NOS-dependent.

The purpose of this study is to extend the findings of Pistea, et al. into the mouse, which allows use of a genetically deficient model. The objective was to examine whether elevated luminal shear stress would prevent inward remodeling in cultured thoracodorsal arteries (a small artery) (27) and whether NOS and specifically eNOS is involved, using arteries from eNOS −/− mice. The second objective was to examine expression of select genes during acute increased shear stress (20 hours in culture). We measured eNOS mRNA expression to determine whether shear stress alters this gene in cultured arteries as it does in cultured endothelial cells (28–33). We also measured the vascular gap junction genes Cx37, Cx40, and Cx43 as these are involved in vasomotor control through mediation of endothelial-dependent hyperpolarization (EDH) (34) and are involved in arterial response to shear stress and implicated in remodeling (35–40). In addition, comparing expression of these genes between high and low shear stress conditions provided a way to determine whether low shear stress conditions lead to deterioration of vasomotor control pathways in culture. This study finds that shear stress-induced prevention of inward remodeling is eNOS dependent, but does not require chronic dilation. It also finds that Cx37 mRNA is increased, while the other select genes are not altered.

METHODS

This study had two parts. The objective of Part 1 was to test the effect of luminal flow, and the calculated shear stress, on inward remodeling in mouse thoracodorsal arteries after 2 days of culture and the contribution of eNOS to this process. The objective of Part 2 was to measure the effect of flow on expression of genes responsive to shear stress after 1 day of culture.

Part 1: Remodeling Response to Shear Stress and Contribution of eNOS.

All procedures were approved by the Yale Animal Care and Use Committee. Female mice (14.8 ± 0.5 wk old; range: 7 – 26 wk old) of wild-type (chimeric C57BL6/129 or C57BL6) and eNOS − /− mice (Jackson Labs) genotypes were used in this study. Most of the wild-type mice were chimeric C57BL6/129, the same background as the eNOS−/− mice, but due to limited populations, these were supplemented with C57BL6. Four different treatments were designed to test the effects of high and low shear stress on inward remodeling and the contributions of NOS and eNOS on the effects of high shear stress. The treatment groups were 1) arteries from wild-type mice with low flow and shear stress <7 dynes/cm^2 (Low SS), 2) arteries from wild-type mice with high flow and shear stress ≥15 dynes/cm^2 (High SS), 3) arteries from wild-type mice with high shear stress and L-NAME (10^{-4} M), a general NOS inhibitor, in the superfused culture medium (High SS + L-NAME), and 4) arteries from eNOS −/− mice with high shear stress (High SS eNOS −/−).

Mice were anesthetized with Xylazine/Ketamine (3.2 mg/kg and 34 mg/kg, respectively, i.p. or i.m.) and thoracodorsal arteries were isolated and placed in an organ chamber (Culture Myograph, 202 EvB, Danish Myo Technology, Aarhus, Denmark) containing fresh, chilled MOPS-buffered physiological salt solution (PSS) (in mM: 145 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 1.2 NaH_2PO_4, 2.0 MOPS, 0.02 EDTA, 5.0 Glucose, 2.0 Pyruvate, pH 7.4, sterile filtered). Mice were euthanized by overdose of Xylazine/Ketamine (i.p.). A total of 84 mice were used, with one thoracodorsal artery isolated from each mouse, except for 5 mice in which 2 arteries were isolated, for a total of 89 arteries.

Arteries were cannulated and secured with 8–0 nylon suture on one end, blood was gently cleared from the lumen, and the other end was cannulated and secured. Arteries were pressurized to 80 mmHg using a pressure manometer and equilibrated for approximately 30 min at 37°C in MOPS-buffered PSS. Pre-culture artery integrity was tested by vasoconstriction to phenylephrine (10^{-5} M) to approximately 50% baseline diameter, followed by ≥ 80% vasodilation to acetylcholine (ACh; 10^{-5} M generally, with 10^{-6} or 10^{-4} M used in a few arteries) [% dilation = (diameter with ACh – preconstricted diameter)/(maximal diameter – preconstricted diameter)*100]. Diameters were measured using a 10X objective, CCD camera, and Vediview software (DMT-USA, Inc., Atlanta, GA). The artery was fully dilated with 10^{-4} M papaverine (Passive Diameter). In some arteries, passive diameters were determined from the dilation to ACh, which resulted in full dilation to baseline diameter and was found to be nearly identical to diameters with papaverine. Passive diameters were also measured at 20, 40, 60, 80, 100 and 120 mmHg in the presence of papaverine (10^{-4} M) in most arteries before and after culture. The chamber was then flushed

for at least 10 min. In general, only single dose-responses to drugs were performed in order to minimize the exposure of arteries to vasoactive substances before culturing (41).

Arteries were then cultured for 2 days in Lebovitz medium (L15, JRH Biosciences, Lenexa, KS) supplemented with L-glutamine (2.8 mM, Mediatech, Inc., Herndon, VA), penicillin/streptomycin (10 IU/ml and 10 ug/ml, respectively; Mediatech, Inc.), and 10% fetal bovine serum (characterized FBS, Hyclone, Logan, UT), sterile-filtered, which was superfused around the artery and perfused through the lumen and maintained at room temperature (19 – 24 °C) to minimize microbial growth and optimize long-term viability and vasomotor function, as determined in pilot experiments. The lower temperature may be optimal for this artery given that it is a superficial artery. In support of this idea, another superficial artery, the gracilis muscle feed artery, has been successfully cultured at room temperature (42). The introduction of medium caused vasoconstriction to ~50% of baseline diameter in all arteries, which continued throughout the culture period. Superfusion medium was re-circulated from a 100 ml reservoir and was refreshed after ~24 hr. Flow through the lumen was controlled by gravity. Inflow and outflow reservoirs (large reservoirs were used to minimize changes in pressure gradient with chronic flow) were raised to 109 cmH_20 (equivalent to 80 mmHg) and flow was initiated by raising the inflow reservoir and lowering the outflow reservoir equal distances. Because the cannula resistances were matched, the average pressure would be maintained at 80 mmHg. A micro-flowmeter (model GF-3060, Gilmont Instruments, Barrington, IL) was placed between the outflow cannula and the outflow reservoir. Flow was set to produce a shear stress of <5 dynes/cm^2 (low shear stress group) or >15 dynes/cm^2 (high shear stress groups). Shear stress (dynes/cm^2) was calculated by: $(4*viscosity*flow*10^9/\pi*radius^3)$; with viscosity = 0.007 dynes*s/cm^2, flow in μl/s, the 10^9 factor to correct for using μl/s for flow (1 μl = 10^9 μm^3), and radius in μm. The pressure gradient set to achieve the desired shear stress at the beginning of the experiment (based on the initial artery diameter in the presence of culture medium) was maintained throughout the rest of the culture period. High SS generally produced an acute dilation, but this was very transient and not routinely measured. Initial culture diameter was measured after a steady state diameter was reached (>5 min after initiation of flow). The flow and artery diameter (in the presence of culture medium) were recorded daily (at 0, 24, and 48 hrs) and the average value was used to calculate the average shear stress over the culture period (Table 2). Flow rates over the first and second 24 hr periods were similar (Table 2) and at a level to maintain the desired shear stress range. The average physiological shear stress in this sized artery is estimated to be between 10–55 dynes/cm^2 (43, 44). Average shear stress over the culture period was significantly greater in all the High SS groups compared to the Low SS group, when compared by Student's T-test (Table 2), but only in the High SS eNOS −/− when compared by ANOVA. However, average shear stress levels remained in the target ranges for each treatment over the culture period.

At the end of the 2-day culture period, the chamber and artery lumen were flushed with MOPS-buffered PSS at 37 °C, and equilibrated for at least 30 min with luminal pressure of 80 mmHg generated by a pressure manometer, without luminal flow. Responses to phenylephrine (10^{-5} M) and ACh (10^{-5} M) were again measured, and maximal diameter was measured either in the presence of sodium nitroprusside (10^{-4} M) or Ca^{++}-free MOPS-buffered PSS with EGTA (1 mM). The change from sodium nitroprusside to Ca^{++}-free PSS

occurred half-way through the project with the rationale that it might more fully relax the arteries, however, we found no significant differences within any of the groups in final passive or % change in passive diameter with the different methods of measuring final passive diameter so the data with the different methods are combined (data not shown).

Many arteries began the culture protocol (89 arteries), but were discarded for technical or viability reasons (57 arteries). The technical issues included were clogs that developed in the perfusion line that stopped flow (most common in the High SS group), arteries leaking or dislodging from the cannula, and chamber leaks. In addition, arteries that did not respond to PE at the beginning or end of the culture period were considered dead and were discarded. Arteries were typically very constricted during culture, but some spontaneously dilated sometime during culture. We found that these arteries did not respond to PE, indicating loss of viability. The arteries used in analyses (32 arteries) originated from mice that were 14.6 ± 0.5 wk old (range: 7 – 25 wk old). Ages of mice were similar in each group [mean (wks) ± SEM: Low SS = 17.3 ± 2.0, High SS = 16.1 ± 1.5, High SS + L-NAME = 15.4 ± 1.8], with the exception of slightly younger mice in the High SS eNOS −/− group (9.8 ± 1.0; $p<0.05$ compared to Low SS and High SS).

Part 2: Gene Expression with Differential Shear Stress in Cultured Arteries.

All procedures were approved by the William & Mary Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council). Female C57BL/6 mice (Charles River Laboratories, Inc., Wilmington, MA) were housed in standard cages in limate-controlled, 12-hr light/12-hr dark environment, with free access to food and water and used in the experiment at 19.0 ± 1.0 wks of age (range: 10.4 – 22.0 wks of age). The treatment groups were low and high shear stress, with no difference in the average ages of mice used in the two groups (Low SS: 20.4 ± 0.4 wks, High SS: 17.7 ± 1.9).

Arteries were cultured as described in Part 1, with some differences. Mice were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg body weight, i.p.). Arteries were initially cannulated with superfusion of L15 medium (with L-glut and pen/strep as in Part 1) without serum and perfusion of L15 medium with serum (newborn calf serum, HyClone). Serum was omitted from the superfusate only during reactivity measurements before and after culture to reduce the amount of pre-constriction, which is near maximal when included, but then added before inducing flow and undergoing culture. After initial equilibration of the artery in serum-free culture medium, which induced ~50% constriction (Table 2), peak response to 10^{-5} M ACh (3–5 min after addition) was measured to ensure endothelial viability. The artery chamber was then flushed with fresh medium containing serum and allowed to equilibrate to the smaller diameter (~65% constriction; Table 2). Luminal flow was then increased to either: 1) induce low shear stress (Low SS) (<10 dynes/cm^2 based on diameter immediately before flow; generally, <10 μl/min flow), or 2) high shear stress (High SS) (>20 dynes/cm^2; generally, <20 μl/min flow). After equilibration (~20 min), pre-culture diameter was measured, and the artery was cultured ~20 hr (19.8 ±3.2 hr) with 100 ml of recirculating medium with serum. Post-culture diameter and total flow was then measured, and the artery was flushed with medium without serum to measure post-culture ACh

reactivity (10^{-5} M followed by 10^{-4} M ACh in all arteries except one from each group that showed a robust response with 10^{-5} M). Arteries were then immediately placed in RNAlater and stored at −80 °C to preserve mRNA.

RNA was isolated using the RNeasy fibrous tissue MiniKit (Qiagen, Valencia, CA), and real-time PCR was performed at the Nucleic Acids Research Facilities at Virginia Commonwealth University using TaqMan one-step RT-PCR Master Mix reagent kit (Applied Biosystems). Samples were tested in triplicate with the following cycling conditions: 48°C for 30 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. Expression was quantified with a relative standard curve generated with control mouse aorta RNA. Primers and TaqMan probes for eNOS, Cx37, Cx40, Cx43, and smooth muscle alpha actin (SMAA) were previously published (45), except for PECAM-1 (Accession #: NM_008816; Forward Primer: GGCACACCTGTAGCCAACTTC, Reverse Primer: CGGCGATCTTGCTGAAATTC, TaqMan Probe: ACTGCGACAAGACCGTCTCTTCCT). No significant genomic DNA contamination was detected and optimal assay efficiency was observed. mRNA expression for each gene (eNOS, Cx37, Cx40, Cx43) was normalized to PECAM-1 mRNA to control for relative endothelial content and SMAA mRNA expression to control for relative smooth muscle content.

Statistical Analysis.—Comparisons between groups were performed using a one-way ANOVA, or two-way repeated measures ANOVA when before and after comparisons were made, both with Bonferroni's post-hoc analysis (Prism 4, GraphPad Software, Inc., San Diego, CA). Artery parameters between two treatment groups were compared by two-tailed, Student's t-test, and within each group before and after culture were compared by two-tailed, paired Student's t-test (Excel, Microsoft, Redmond, WA). Each figure and table indicates the specific statistical test used.

RESULTS

Part 1: Remodeling Response to Shear Stress and Contribution of eNOS.

Baseline diameters (diameter after 30 min equilibration in MOPS-buffered PSS) and passive diameters were similar before culture, indicating that there was no significant myogenic (basal) tone in thoracodorsal arteries in any group (Table 1), consistent with a previous study of this artery (27). However, after culture, baseline diameters were significantly smaller than passive diameters in all groups, indicating significant basal tone (Table 1), or perhaps a residual effect of the culture medium after wash-out. Also, arteries from eNOS −/− mice were significantly smaller than arteries from wild-type mice (Table 1). After culture, arteries constricted similarly to phenylephrine as they did before culture (Table 1). Dilation responses to acetylcholine were more variable within a group and were significantly reduced in all groups after culture (Table 1). There were, however, no significant differences in acetylcholine response between groups before culture or after culture.

After culture, passive diameters were reduced in arteries with low luminal shear stress, indicating inward remodeling (Fig. 1). The magnitude of this remodeling is similar to that found in a previous study of cultured rat cremaster arterioles with low flow (22). Increased

shear stress prevented this remodeling and this attenuation was most pronounced with shear stress > 15 dynes/cm^2 (Fig. 1), the predicted physiological shear stress (43, 44). When artery responses were grouped by shear stress, the magnitude of inward remodeling after culture was significantly greater in the Low SS group (SS < 7 dynes/cm^2) compared to the High SS group (SS > 15 dynes/cm^2), which had minimal remodeling (Fig. 2). Chronic inhibition of NOS with L-NAME resulted in inward remodeling despite elevated shear stress (High SS + L-NAME group; Fig. 2). Similarly, absence of eNOS in knockout mice also resulted in inward remodeling despite elevated shear stress (High SS eNOS −/− group; Fig. 2), that was significantly greater than High SS. The differences in passive diameters before and after culture with stepped-increases in pressure, measured in most but not all vessels, showed similar differences as observed at 80 mmHg (Fig. 3). Significantly smaller post-culture passive diameters were observed in Low SS and High SS eNOS −/− groups at all pressures except 20 mmHg, and there were no differences in post-culture diameters in High SS group. The magnitude of inward remodeling in the High SS + L-NAME group was not significantly different from the High SS group (Fig. 2) and a significantly smaller post-culture diameter was only observed at 80 mmHg (Fig. 3).

Arteries in all groups immediately constricted in response to addition of culture medium and remained constricted throughout the culture period (Fig. 4), with a similar average daily magnitude of constriction (Fig. 4), and similar overall average constriction magnitudes (% of pre-culture passive diameter) over the entire culture period (Table 2).

Within the treatment groups using wild-type mice, most of the mice were chimeric C57BL6/129 mice (Low SS: 5/7 mice, High SS: 8/12, High SS + L-NAME: 3/5) with some C57BL6 mice. There were no differences in passive diameter, remodeling response, phenylephrine and acetylcholine reactivity, or average culture diameter between these strains within each treatment group (t-tests; data not shown).

Part 2: Gene Expression with Differential Shear Stress in Cultured Arteries.

Pre-culture, artery constriction to culture medium (without serum) and subsequent dilation to ACh were similar before culture between Low SS and High SS groups. However, Low SS arteries constricted significantly by the end of the culture period (Final Culture Diameter compared to Initial Culture Diameter) and exhibited a smaller post-culture dilation response to ACh compared to the High SS arteries (Table 3). Both groups dilated to a smaller final diameter with ACh at post-culture (Table 3). These functional responses show a similar to pattern to those in Part 1. PECAM-1 mRNA was similar between groups when normalized to the smooth muscle cell marker, SMAA, indicating similar endothelial cell content between groups (Fig. 5). Because constriction responses during the post-culture reactivity test were similar, it is logical to conclude that smooth muscle cell content is similar between the groups, and therefore, SMAA is a valid normalizing gene. eNOS, Cx40, and Cx43 mRNA were similar between groups whether normalized to SMAA or PECAM-1 (Fig. 5). However, Cx37 mRNA expression was significantly greater in the High SS group whether normalized to SMAA or PECAM-1 (Fig. 5).

DISCUSSION

This study shows that shear stress in the physiological range can nearly eliminate inward remodeling in pressurized, cultured mouse small arteries. eNOS is shown to be essential to this process, and increased Cx37 was coincident. This finding extends those of Pistea, et al. (26) indicating involvement of nitric oxide in the ability of shear stress to counter inward remodeling in culture, with some differences between the studies. Their porcine coronary arteries that were treated with high flow were relatively dilated (i.e., had less constriction) during culture, and addition of L-NAME did not prevent this dilation but did prevent flow-induced attenuation of inward remodeling. So, while our study did not demonstrate a sustained vasomotor effect of flow as theirs did, both studies indicate that vasodilation is not necessarily coupled to flow-induced prevention of inward remodeling. It is also notable that while the ACh-induced vasodilation in the mouse thoracodorsal arteries used in the present study is only ~50% dependent on nitric oxide (the remainder due to cyclooxygenase and EDH) (27), we found that the shear-induced prevention of remodeling is largely nitric oxide- and eNOS-dependent.

The most important contribution of the present study is the use of eNOS −/− mice because it specifically tests the involvement of one of the NOS isoforms. This is important given that there is evidence that nNOS and iNOS are also involved in remodeling *in vivo* (46–50). It also offers a technical advantage because it avoids non-specific effects of NOS inhibitors (51–53), or incomplete inhibition. Based on the data in the present study, we can conclude that eNOS is essential to shear stress-induced prevention of inward remodeling in cultured arteries. This approach allows for control of hemodynamic parameters and vasoactive agonists and assessment of their effects on remodeling without the influence of neuro-humoral factors. However, the role of eNOS in shear stress-induced remodeling *in vivo* is likely to be more complex, as suggested by evidence from eNOS −/− mice. These mice exhibit the expected attenuated outward remodeling to high flow (54, 55). However, the response to low flow is more complicated, with evidence for paradoxically reduced inward remodeling in some vessels (13, 54, 56), increased inward remodeling in others (49), and exaggerated neo-intima formation in large arteries (13, 49). *In vivo*, recruited bone marrow-derived cells expressing various NOS isoforms are involved in suppression of inward remodeling after carotid ligation (48). Thus, the use of eNOS −/− arteries in culture in this study eliminates these systemic factors and identifies the ability of resident eNOS in endothelium to counter inward remodeling processes intrinsic to the vessel.

Nitric oxide is well known to be involved in remodeling responses to flow (14, 15, 57), but it has not been clear whether the effects of nitric oxide occur via regulation of vasomotor tone or cellular growth/remodeling processes. Our results argue against a vasomotor role for nitric oxide (given that artery diameters were similar at different shear stresses) and support the idea that nitric oxide regulates remodeling. Inward remodeling of small arteries is likely to involve remodeling of the extracellular matrix and smooth muscle cell cytoskeletal structure and contacts (8, 58, 59). An important mediator in the acute phase of inward remodeling has been found to be the cross-linking enzyme, tissue-type transglutaminase (tTG, or TG2) (60). *In vivo*, pharmacological inhibition or genetic knock-out of tTG delays low-flow inward remodeling (61, 62), and pharmacological inhibition eliminates

vasoconstrictor-induced inward remodeling (11). In cultured arteries inhibition of tTG eliminates inward remodeling without inhibiting constriction (62). This enzyme is of particular relevance to the present study because it is inhibited by nitric oxide (63, 64). Thus, the likely increase in nitric oxide with high shear stress may have prevented the action of tTG, and therefore, the inward remodeling. This mechanism would need to be specifically tested.

With 1-day culture, we found an increase in Cx37, without change in the other genes examined. Cx37 is primarily expressed in endothelial cells (65) and the increase in Cx37 is in line with a majority of the findings in cultured cells and *in vivo*. In cultured endothelial cells, Cx37 has been reported to increase in response to high shear stress (5–25 dynes/cm^2, 30 min – 24 hr treatment) vs. static flow in many studies (37, 40, 66, 67), but not all (68–70). One of these studies found, that Cx37 was increased when co-cultured with smooth muscle cells (68), but other studies did not (66, 70). Elevating shear stress from normal levels (15 – 20 dynes/cm^2) to very high levels (75 – 100 dynes/cm^2) in cultured endothelial cells (24 hrs) has not been shown to increase Cx37 (71). *In vivo*, Cx37 is more prominently expressed in arterial endothelial cells in areas of high shear stress (37), in developing/remodeling arteries (40), and in arterial smooth muscle (where it's not normally expressed) during collateral growth (72), but decreased in areas of disturbed flow (37). The present study shows that Cx37 also increases in cultured arteries exposed to high shear stress without exposure to *in vivo* neural-humoral factors, indicating that the hemodynamic factors are sufficient for, or at least participate in, the increase. It is not clear what the role of the increased Cx37 is in this study, but *in vivo* studies indicate that endothelial Cx37 expression is involved in flow-induced endothelial cell arrest and limiting angiogenesis and blood flow recovery during ischemic occlusion (35, 36).

We found no change in Cx40 or Cx43 mRNA expression after one-day treatment with shear stress. This is not surprising given that the role of these connexins in mechanotransduction and remodeling is unclear. Cx40 is expressed in endothelial cells (65), but its response to shear stress is in cultured endothelial cells is variable (39, 68, 70, 73) and *in vivo* its expression does not seem to be affected by chronic variations in shear stress in large arteries (37, 74). While its role in shear stress-induced remodeling is unclear, it is reported to have a protective role against atherosclerotic remodeling with low shear stress (39). Cx43 is expressed in endothelial and smooth muscle cells (65). It increases in cultured endothelial cells in response to changes in shear stress, both laminar and oscillatory, but the increase appears to be very transient, except with oscillatory shear stress (68, 70, 75–79). *In vivo*, greater Cx43 is expressed in aortic endothelium in areas of disturbed flow (74). Our results indicate no effect of shear stress on Cx43 at 20 hr of culture, but this does not rule out a transient early change.

The lack of change in eNOS with high shear stress is not entirely unexpected given evidence from other studies. It is well established that shear stress acutely (3–24 hr) increases eNOS mRNA expression in cultured endothelial cells (28–33). However, evidence in isolated arteries indicates both increase (80) or no change (81) in eNOS mRNA with acute (2–4 hr) shear stress, and evidence *in vivo* indicates that 24 hr of either high or low shear stress decreases eNOS mRNA in rat mesenteric arteries (82). The lack of change in our

experiments may be due to the chronic constriction caused by the serum added to the culture medium, as a previous study found that attenuating this constriction with a ROCK inhibitor prevented the decrease in eNOS mRNA that occurred at 7 days of culture with fetal bovine serum (83).

Limitations.

A limitation of this study is that endothelial integrity at the end of the culture period was not confirmed by a measure other than acetylcholine response, which was variable between arteries. Post-culture acetylcholine response significantly decreased in both 1-day and 2-day cultured arteries and similarly across the groups. This is not unexpected. Loss of agonist-induced endothelial dependent vasodilation is a common finding in chronically constricted arteries in culture, even with a confirmed intact endothelium (by electron microscopy) (22, 23, 26), and is not dependent on whether there is luminal flow in the artery (26). It is unlikely that the decline in acetylcholine response in the present study affected the remodeling response because there was no correlation between these two parameters in any of the groups (Fig. 6). Moreover, neither PECAM-1 nor eNOS expression was different between High and Low SS groups, suggesting that endothelial cell content was similar between the groups at day 1. Therefore, with similar acetylcholine responses at 1 day and 2 days, it's likely that a decreased acetylcholine response does not indicate endothelial cell loss but may instead be a long-term effect of the chronic constriction and/or culture conditions. Even if there was some endothelial cell loss in some arteries over the course of the culture, it may not affect the remodeling response because there is evidence that inward remodeling can occur as early as the first 4 hours of culture during chronic constriction (24, 84).

This study did not specifically test the effects of low flow on remodeling with NOS inhibition or eNOS knock-out for two reasons. The previous study by Pistea, et al (26), showed that low flow and high flow with NOS inhibition produced the same amount of inward remodeling, and was also similar to low flow alone. In addition, we saw no differences in culture diameters in the NOS inhibition or eNOS knockout groups to suggest that there would be enhanced remodeling beyond the Low SS group. Therefore, these experiments did not seem to be critical, but could have provided additional confirmatory data. Notably, the magnitude of inward remodeling with L-NAME was not as great as in the eNOS −/− group and only observed at 80 mmHg. This might indicate that L-NAME did not completely block nitric oxide production.

Another potential limitation is the difference in age between the groups in Part 1 of the study. High SS eNOS −/− were slightly younger than two of the other groups. However, all mice were at the same life stage, mature adults and pre-middle-age for this strain (C57Bl6), and therefore expected to all be at an age of peak physiological function (85).

Summary & Conclusions.

Shear stress in the physiological range prevents inward remodeling in cultured arteries due to chronic constriction through a nitric oxide-, and specifically an eNOS-dependent mechanism. The effects of shear stress require nitric oxide but do not require vasodilation.

Thus, nitric oxide induced by shear stress prevents inward remodeling independent of changes in artery diameter. While eNOS expression is not acutely changed in response to flow in culture, the increase in Cx37 expression indicates that it is responsive to increased shear stress under these conditions.

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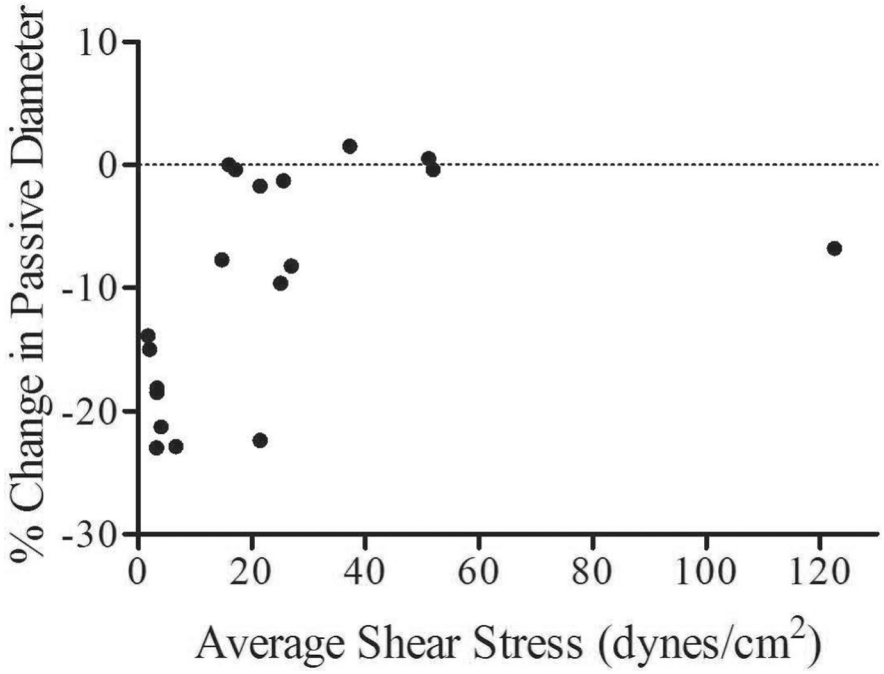

Figure 1. Greater luminal shear stress prevents inward remodeling (% Change in Passive Diameter) in mouse thoracodorsal arteries cultured for 2 days.

Passive diameter was measured at 80 mmHg luminal pressure before culture in MOPS-buffered PSS with addition of papaverine (10^{-4} M), and after culture with SNP (10^{-4} M) or Ca^{++}-free MOPS-buffered PSS with EGTA (1mM). Artery reactivity of each treatment group is shown in Table 1 and culture parameters are shown in Table 2 (first two treatment columns in each table).

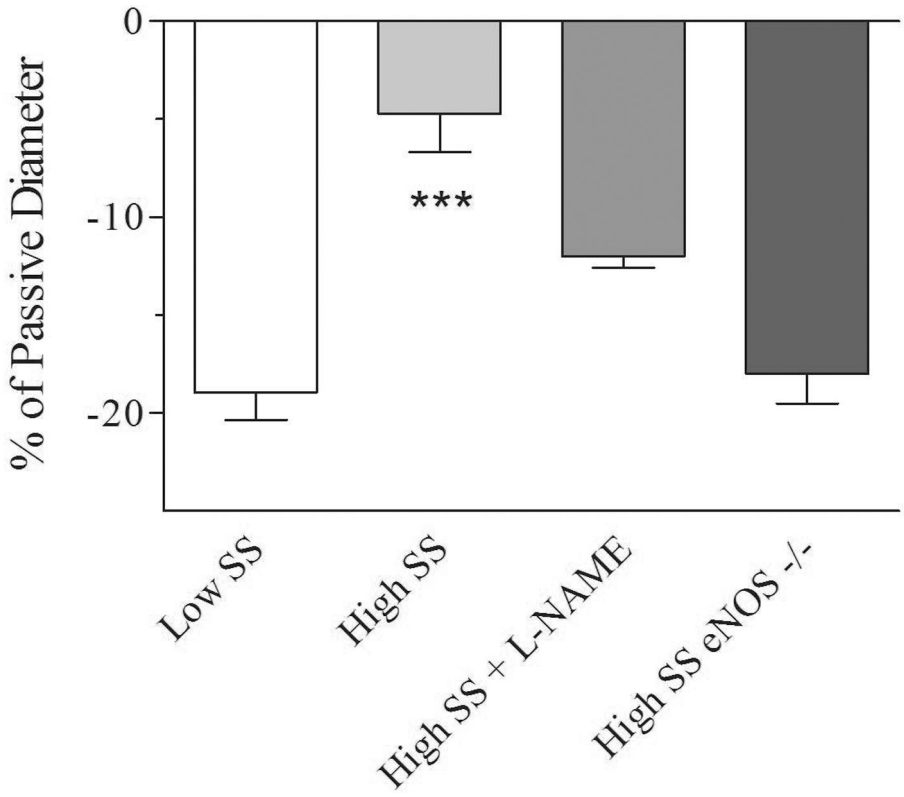

Figure 2. The magnitude of inward remodeling (mean ± SEM) in 2-day cultured arteries is determined by the levels of luminal shear stress and presence of nitric oxide and eNOS.

Artery reactivity of each treatment group is shown in Table 1 and culture parameters are shown in Table 2. *** $p < 0.001$ vs. Low SS and High SS eNOS −/− (one-way ANOVA with Bonferroni post-hoc tests).

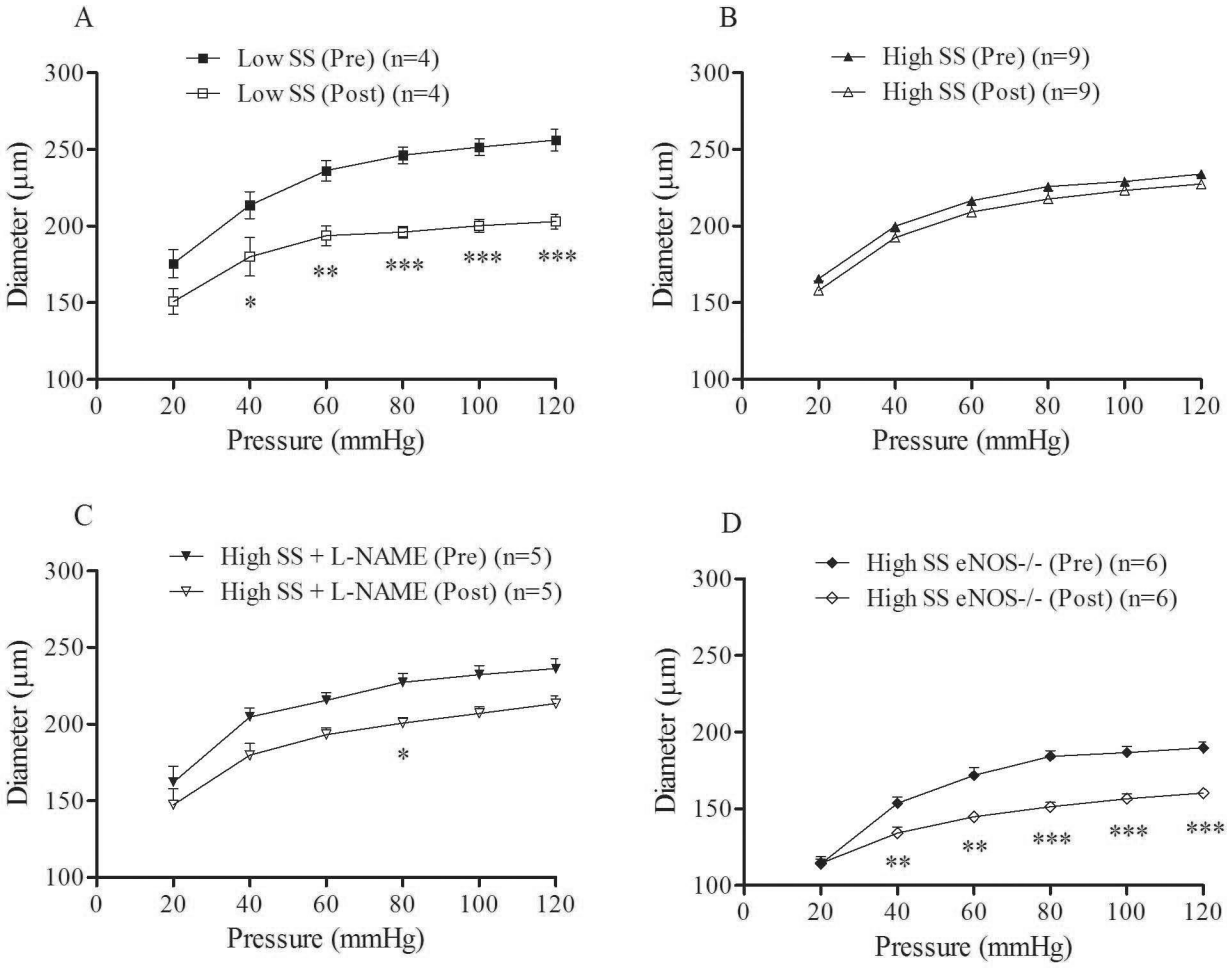

Figure 3. Passive diameters (mean ± SEM) are smaller post-culture compared to pre-culture in Low SS, High SS + L-NAME, or High SS eNOS −/− groups at various pressures.

There were no differences in pre- vs. post-culture diameters in High SS group at any pressure. Pressures were increased step-wise in ~5 min intervals. * $p < 0.05$. ** $p < 0.01$, *** $p < 0.001$ compared to pre-culture passive diameter (2-way repeated measures ANOVA with Bonferroni post-hoc tests).

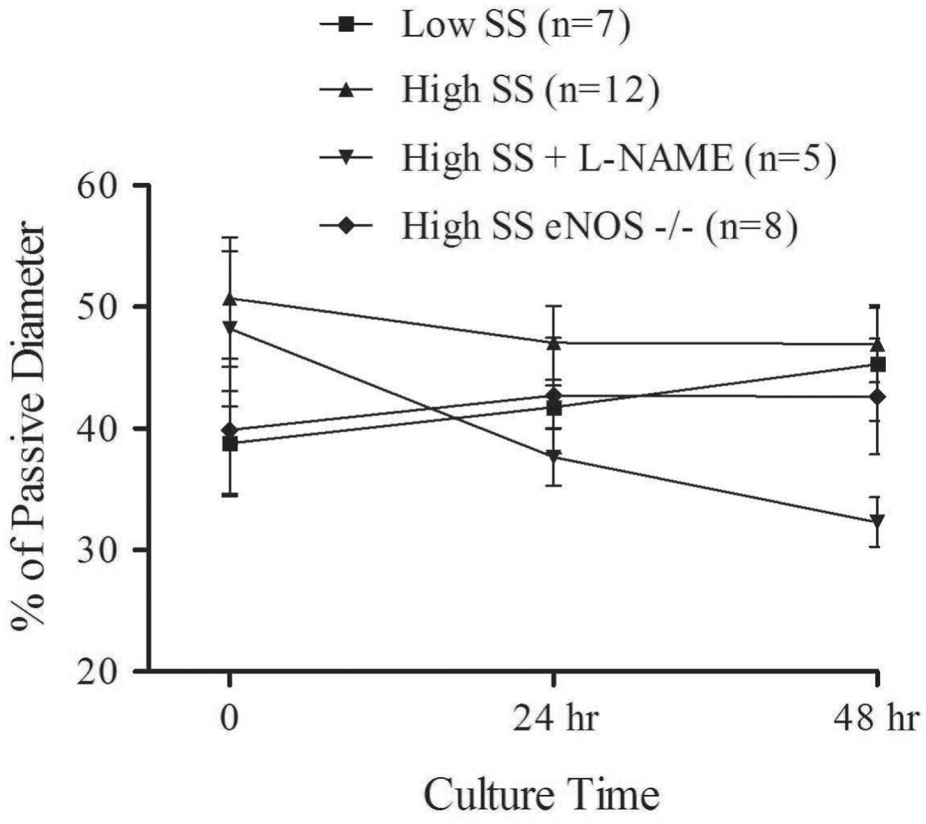

Figure 4. The levels of shear stress do not regulate the magnitude of constriction (mean ± SEM) during 2-day culture.

Arteries were then cultured at 80 mmHg luminal pressure in Lebovitz medium + FCS for 2 days. Arteries stably constricted (% of Passive Diameter = diameter / initial passive diameter * 100) in the presence of culture medium throughout the culture period with no differences between the groups. Artery reactivity of each treatment group is shown in Table 1 and culture parameters are shown in Table 2.

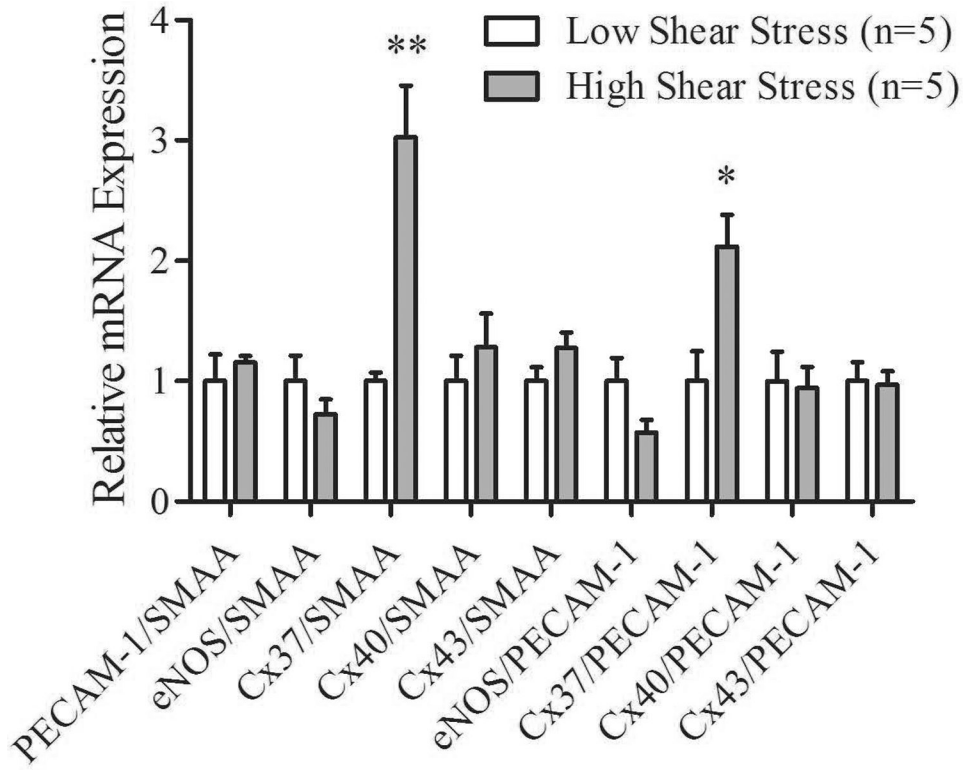

Figure 5. Relative mRNA expression normalized to mRNA of smooth muscle cell marker (SMAA) or endothelial cell marker (PECAM-1) in arteries cultured for 1 day with low or high luminal shear stress (SS).

Ratios (mean ± SEM) are expressed relative to the average value for the Low SS group. * $p < 0.05$, ** $p < 0.01$ vs. Low SS (two-tailed Student's t-test). Artery reactivity in each treatment group is shown in Table 3.

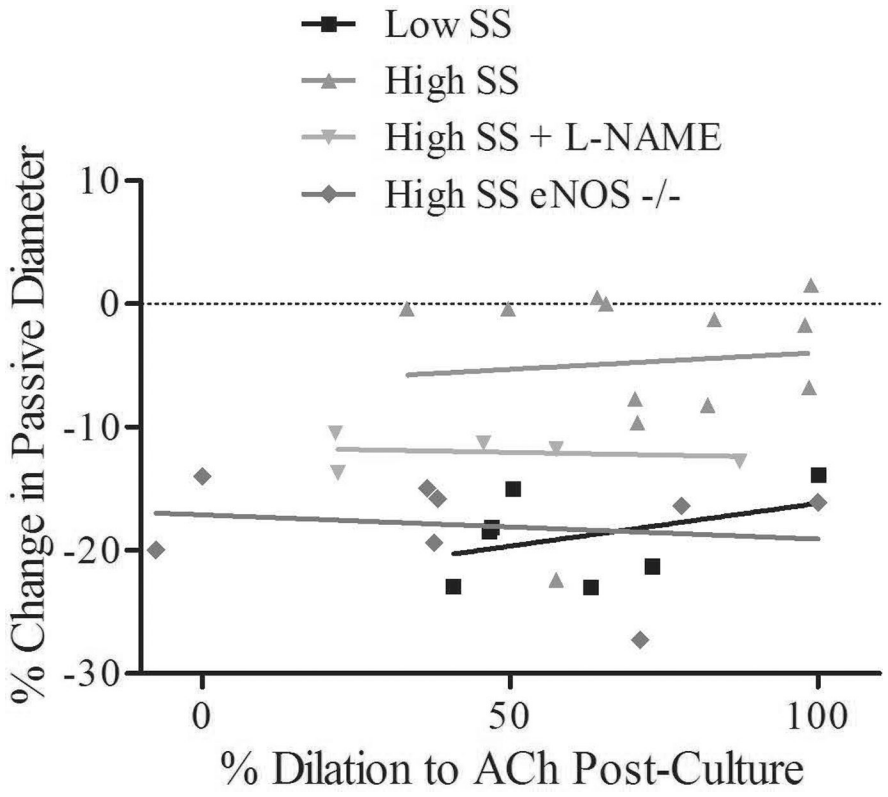

Figure 6. Responsiveness to acetylcholine at the end of 2-day culture is not correlated with the magnitude of inward remodeling.

Arteries from each treatment group displayed a range of reactivity to acetylcholine at the end of culture (x-axis) that did not correlate with the magnitude of remodeling (y-axis). Linear regression lines shown for each treatment group are not significant.

Table 1.

Artery diameters and pharmacological responses (mean ± SEM) before (pre) and after (post) 2-day culture.

		Low SS (n=7)	High SS (n=12)	High SS + L-NAME (n=5)	High SS eNOS −/− (n=8)
Baseline Diameter (μm)	pre	232 ± 7	222 ± 4	220 ± 8	178 ± 8 **
	post	158 ± 7 †††	130 ± 9 †††	154 ± 13 ††	118 ± 7 §††
PE (% of passive diameter)	pre	39.0 ± 2.8	43.2 ± 3.5	45.0 ± 6.6	38.9 ± 5.2
	post	38.8 ± 5.2	29.9 ± 5.1	30.0 ± 3.4	31.4 ± 5.3
Ach (% dilation)	pre	90.0 ± 8.1	98.5 ± 0.9	96.4 ± 2.5	88.5 ± 3.7
	post	60.1 ± 7.9 †	72.7 ± 5.9 †††	46.8 ± 12.3 †	44.3 ± 13.2 †
Passive Diameter (μm)	pre	243 ± 5	227 ± 5	230 ± 5	187 ± 3 ***
	post	196 ± 2 †††	216 ± 6 §†	203 ± 5 †††	153 ± 2 ***†††
Basal Tone (% of passive diameter)	pre	95.5 ± 1.7	98.0 ± 0.9	95.4 ± 2.9	94.8 ± 3.3
	post	80.5 ± 4.1 †	60.4 ± 4.1 §†††	75.8 ± 5.3 ††	77.8 ± 5.6 †

Diameters were measured at 80 mmHg intraluminal pressure with PSS-MOPS superfusion and PSS-MOPS presence in the lumen without flow at 37 °C.

PE = phenylephrine.

% of baseline diameter = diameter with 10^{-5} M PE/baseline diameter × 100.

Ach = acetylcholine.

% dilation = (diameter with 10^{-5} M Ach – preconstricted diameter)/(passive diameter – pre-constricted diameter) × 100.

One or two arteries in each group were measured with 10^{-6} M or 10^{-4} M.

** $p < 0.01$ vs. all other groups (one-way ANOVA).

*** $p < 0.001$ vs. all other groups (one-way ANOVA).

§ $p < 0.05$ vs. Low SS (one-way ANOVA).

† $p < 0.05$,

†† $p < 0.01$,

††† $p < 0.001$ vs. corresponding “pre” value (paired t-test).

4 arteries in High SS group, post-culture PE response were missing data, and 1 artery in High SS + L-NAME pre-culture PE response is missing data because baseline diameter was very constricted.

Table 2.

Artery luminal flow, diameter and luminal shear stress (mean ± SEM) during 2-day culture.

		Low SS (n=7)	High SS (n=12)	High SS + L-NAME (n=5)	High SS eNOS −/− (n=8)
Average Culture Diameter (μm) (% of pre-culture passive diameter)		101 ± 6	109 ± 4	91 ± 5	76 ± 7 **†
		42.0 ± 2.8	48.2 ± 2.1	39.4 ± 2.2	41.3 ± 4.1
Average Flowrate (μl/min)	day 1	4 ± 1	35 ± 6	23 ± 3	25 ± 3
	day 2	3 ± 1	37 ± 8	23 ± 6	18 ± 3
	overall	3 ± 1	36 ± 7 †††	23 ± 4	21 ± 3
Average Luminal Shear Stress (dynes/cm^2)		3.4 ± 0.6	36.3 ± 8.5 $^{++}$	37.5 ± 5.9 $^{+++}$	76.8 ± 27.2 $^{\dagger+}$
	range	1.7 – 6.6	14.7 – 121.2	15.2 – 47.3	15.0 – 256.8
Culture Time (hr)		44.5 ± 0.7	44.2 ± 0.5	45.1 ± 0.6	44.1 ± 0.5

Diameters were measured at 80 mmHg intraluminal pressure with culture medium + FBS superfusion and luminal perfusion at room temperature.

** $p < 0.01$ vs. High SS (one-way ANOVA).

$^{+}$ $p < 0.05$,

$^{++}$ $p < 0.01$,

$^{+++}$ $p < 0.001$ vs. Low SS (t-test).

† $p < 0.05$,

††† $p < 0.001$ vs. Low SS (one-way ANOVA).

Individual Average Flowrate values for each day were missing from one artery in Low SS group.

Table 3.

Artery Responses (mean ± SEM) to Culture (~20 hr) with Luminal Flow.

	Low Flow (n=5)	High Flow (n=5)
Pre-Culture Reactivity		
Baseline Diameter	149 ± 21	120 ± 18
+ACh(10^{-5})	267 ± 11	252 ± 10
Dilation (μm)	118 ± 13	132 ± 20
Post-Culture Reactivity		
Baseline Diameter (μm)	137 ± 22	106 ± 12
+ACh(10^{-5} - 10^{-4})(μm)	168 ± 27†	183 ± 10†
Dilation (μm)	31 ± 8†	77 ± 7**
Initial Culture Diameter (μm)	82 ± 4	95 ± 7
Final Culture Diameter (μm)	65 ± 5†	70 ± 8
Average Diameter during Culture	74 ± 4	83 ± 5
Average Flowrate (μl/min)	2.1 ± 0.4	29 9 ± 1 7****
Average Shear Stress (dynes/cm^2)	5.9 ± 0.9	69.7 ± 14.2**

These arteries were used in the gene expression analysis in Figure 5.

Arteries were maintained in L-15 medium + newborn calf serum (NCS) in perfusate and superfusate during culture.

Pre- and post-culture reactivity was measured in superfusate without NCS.

** $p < 0.01$ and

**** $p < 0.0001$ compared to Low Flow (Student's t-test).

† $p < 0.01$ compared to corresponding Pre-Culture value or Initial Culture Diameter (paired Student's t-test).