

# Differential Effects of Reduced Cyclic Stretch and Perturbed Shear Stress Within the Arterial Wall and on Smooth Muscle Function

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## BACKGROUND

Cyclic circumferential stretch and shear stress act in concert and yet are capable of independently mediating arterial smooth muscle function, modulating the production of superoxide and stimulating arterial remodeling.

## METHODS

Porcine carotid arteries were perfused *ex vivo* for 72 h. Groups combining normal (5%) and reduced (1%) stretch with high shear ( $6 \pm 3$  dyn/cm<sup>2</sup>) and oscillatory shear ( $0.3 \pm 3$  dyn/cm<sup>2</sup>) stress were created, while maintaining a pulse pressure of  $80 \pm 10$  mm Hg.

## RESULTS

Total superoxide production, fibronectin expression, and gelatinase activation were mediated by shear stress, but expression in the endothelial region was mediated by reduced cyclic stretch. By plotting intensity vs. radius, we saw that superoxide and gelatinase activity were in part mediated by stress distributions throughout

the vascular wall, whereas fibronectin and p22-phox were much less or not at all. These findings, when coupled with our results from tissue reactive studies, suggest that the arterial remodeling process triggered in the endothelial region due to reduced stretch causes the most significant changes in arterial smooth muscle function.

## CONCLUSIONS

We have found that the remodeling process triggered by reduced compliance in the endothelial region of large conduit arteries has a more profound detrimental effect to smooth muscle function than that brought on by perturbed shear stress. This work provides new insight by suggesting that although mechanical stimuli such as cyclic stretch and shear stress are known to augment similar markers of vascular remodeling, the location of their expression throughout the vascular wall differs greatly and this can have dramatic effects on vascular function.

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Arteries are constantly exposed to dynamic mechanical forces comprising shear stress and cyclic circumferential stretch. These stresses determine arterial structure and morphology, modulation of which leads to chronic adaptive remodeling within the arterial wall, a situation which has been linked to the progression of vascular diseases such as hypertension<sup>1</sup> and atherosclerosis.<sup>2,3</sup>

It has been documented that areas of the vasculature exposed to low mean shear stress and cyclic reversal of flow direction also called oscillatory flow, (OSC) are at higher risk of aggressive inward vascular remodeling, marked by matrix reorganization and cellular proliferation,<sup>4</sup> than those exposed to a high unidirectional shear stress (HSS). Similarly, it has been suggested that reduced arterial compliance may also promote

vascular remodeling,<sup>5</sup> in which the mechanisms also involve matrix reorganization and intimal cellular proliferation. In most previous works, these two stimuli have been simultaneously coupled *in vivo*, making it very difficult to understand the individual contributions of cyclic stretch and shear stress to vascular remodeling. A recent *in vivo* study has addressed how altered circumferential wall tension and shear stress affect aortic remodeling in rats,<sup>6</sup> yet how these mechanical forces are perceived and transformed into cellular signals throughout the vascular wall are still unclear. Some attempts have also been made to study the individualistic effects of these stimuli *in vitro*; however, the use of different cell lines in a wide variety of stretch systems and flow chambers has made it very difficult to draw definitive conclusions. In the present study, we found a compromise between the *in vivo* and *in vitro* works of the past by studying the autonomous effects of shear stress and cyclic stretch, and their relation to remodeling throughout the arterial wall.

Mechanisms implicated in the initiation of chronic adaptive arterial remodeling are numerous, but recent evidence has implicated the production of reactive oxygen species (ROS) to be an important mediator of hypertensive vascular remodeling.<sup>7</sup> All vascular cells are capable of producing ROS,

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chronic increases of which have been shown to be mediated by the membrane-bound NADPH oxidase subunit p22-phox.<sup>8</sup> Vascular remodeling is a process that requires the breakdown and reorganization of extracellular matrix (ECM), allowing enhanced cellular migration and proliferation in the intima region. Matrix metalloproteinases (MMPs) are a family of enzymes capable of degrading the ECM, of which MMP-2 has been shown to play a principal role.<sup>9</sup> Another important mediator of remodeling is fibronectin, which is deposited by a cellular-mediated process and helps rebuild the ECM. Fibronectin has also been shown to be an important regulator of cell proliferation and migration in vascular tissue.<sup>10</sup>

The first goal of this study is to understand how shear stress and cyclic stretch individually modulate crucial components of vascular remodeling, such as superoxide production, fibronectin expression, MMP expression/activation, and relate this to arterial smooth muscle cell functional changes. The second goal of the study is to see how the expression of these factors is modulated regionally throughout the vascular wall.

## METHODS

**Arterial preparation.** Left internal carotid arteries of 6-month-old pigs weighing between 120 and 150 kg were taken from a local slaughter house (Bell, Cheseaux-sur-Lausanne, Switzerland). Loosely attached adventitial tissue was removed, and a 3.5 cm segment, 1 cm distal to the primary bifurcation, was obtained. Arterial segments were then mounted on to the *ex vivo* arterial support system (see details below). The longitudinal stretch ratio was determined to be 1.3 times the unpressurized unstretched length. To reduce arterial compliance, a silicon cuff (Statice Sante, Besançon, France) of 6–8 mm (depending on the outer diameter) and of  $0.2 \pm 0.05$  mm thick was placed around the arterial segment. Reduction of cyclic circumferential stretch obtained with the cuff was roughly 80%, when compared to the uncuffed segments.

**Ex vivo arterial support system.** The *ex vivo* arterial support system used in this study offers a nice compromise between *in vitro* and *in vivo* setups, allowing the perfusion of arterial tissue segments while allowing precise control of perfusion pressure and flow. More precise details on *ex vivo* arterial support system have been given previously.<sup>11</sup> The arterial perfusions were maintained for 72 h with a medium cocktail, as previously described.<sup>5</sup> Perfusion flow was adapted to create either a pulsatile HSS with a mean value of 6 dyn/cm<sup>2</sup> and amplitude of 3 dyn/cm<sup>2</sup>, or an oscillatory shear stress (OSC) with a mean value of 0.3 dyn/cm<sup>2</sup> and amplitude of 3 dyn/cm<sup>2</sup>, both with a frequency of 1 s. Perfusion pressure was fixed at 80 mm Hg with a pulse pressure amplitude of  $\pm 10$  mm Hg. Resulting strains were 4–5% for the uncuffed segment, which is in the physiologic range of pulsatile stretch for the porcine carotid, and <1% for the cuffed segment, mimicking a less compliant arterial segment.

**Arterial function analysis.** Arterial rings were tested before and after the perfusion to determine their capacity to achieve

smooth muscle-mediated contraction. Arterial rings were mounted in an organ bath (Emka Technology, Paris, France), equilibrated in a Krebs buffer solution at 37°C, infused with 5% CO<sub>2</sub> and 95% O<sub>2</sub>. During the equilibration period, resting tension was repeatedly adjusted to 2 g. Arterial rings were precontracted with 80 mmol/l KCl until a constant maximum contraction was achieved. Next, tissues were rinsed with Krebs solution until baseline tension was achieved; doses from 10<sup>-8</sup> to 10<sup>-4</sup> mol/l of norepinephrine were introduced, and dose-response curves were obtained. Endothelial functionality was a criteria for inclusion in our study and was assessed using bradykinin (data not shown).

**Immunofluorescence, DHE, and gelatinase.** After the perfusion, a 5 mm long segment of the artery was rinsed with 0.9% NaCl, frozen in OCT compound (Tissue-Tek; Sakura Finetek Europe, Zoeterwoude, the Netherlands) and stored at -80°C for further analysis. For p22-phox and fibronectin staining, sections of 5 µm were cut, air dried and fixed in 100% acetone for 5 min at -20°C. Sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min, then incubated for 60 min with rabbit anti-p22<sup>phox</sup> (p22-phox) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit antifibronectin (fibronectin) (1:500; Sigma-Aldrich, St Louis, MO) in 10% normal goat serum in PBS. The p22-phox sections were then incubated with Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) as a secondary antibody for 45 min. Sections were examined on a Leica DMI 4000 microscope (Leica, Wetzlar, Germany) at a magnification of  $\times 10$ . All steps were performed at room temperature.

For gelatinase activity detection, 5 µm thick arterial cryosections (as described above) were incubated for 4 h at 37°C with 25 µg/ml of DQ gelatin (EnzChek gelatinase assay kit; Molecular Probes, Carlsbad, CA) in 10% agarose (Sigma-Aldrich) in PBS. Sections were examined on a Leica DMI 4000 microscope at a magnification of  $\times 10$ . All steps were performed in the dark.

Dihydroethidium (DHE) is a cell permeable compound that forms ethidium upon reacting with superoxide anion. For detection, 5 µm arterial cryosections (as described above) were incubated for 30 min at 37°C with 5 µmol/l DHE (FluoProbes; Interchim, Montluçon, France) in PBS, then rinsed for 1 min in PBS. Sections were examined on a Leica DM5500 at 20 $\times$ . All steps were performed in the dark.

**Protein extraction and western blot analysis.** Protein expression was assessed using standard western blot techniques. Protein was extracted from the samples with a Brij-35 lyses buffer (50 mmol/l Tris pH 7.5, 1 mol/l NaCl, 2 mol/l urea, 0.1% Brij-35, and 1 protease inhibitor cocktail (Roche, Basel, Switzerland)). Twenty micrograms of protein was electrophoresed, after which the proteins were transferred to a nitrocellulose filter (Amersham, Succursale, Switzerland). Filters were incubated with mouse anti-MMP-2 (MMP-2) (1:500, Millipore, Billerica, MA) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (1:1,000, Chemicon

International, Temecula, CA) followed by enhanced chemiluminescence-peroxidase-labeled anti-mouse secondary antibodies (1:5,000, Amersham). MMP-2 protein expression was normalized to GAPDH.

**Image analysis.** Images were analyzed using MetaMorph (Meta Imaging Series 7.0; Molecular Devices, Sunnyvale, CA). Autofluorescence of elastin was subtracted from each image so as to not interfere with staining quantifications. Quantifications were performed by multiplying the percent area of the arterial region of interest expressing the stain, by the average intensity of the stain. Endothelial regions were defined by hand using a Wacom graphic tablet (Wacom, Saitama, Japan).

**Statistics.** The data are reported as mean values  $\pm$  standard deviation. Two-way analysis of variance statistics were performed to assess significant differences between shear stress and cyclic stretch groups. A value of  $P < 0.05$  was considered significant.

## RESULTS

### Reduced stretch modulates smooth muscle contractile capacity

Smooth muscle contractile function, as assessed at the  $10^{-4}$  mol/l dosage of norepinephrine, was significantly modulated for both HSS and OSC groups by reduced stretch with a 20 and 24% decrease, respectively (Figure 1,  $P < 0.05$ ).

### Radial expression of superoxide anion is modulated by reduced cyclic stretch and oscillatory shear

Radial vascular ROS magnitude and distribution are modulated almost identically by both reduced stretch and oscillatory flow, which maintain a sustained level of ROS throughout the measured region. The combination of the two stimuli are additive, nearly doubling the amount of superoxide anion present in the vascular wall, which is sustained throughout the region of interest (Figure 2a,b).

### Radial expression of p22-phox correlates with superoxide ion production, but suggests it is not the only factor responsible for its measured increases

The radial magnitude of p22-phox expression is modulated by reduced stretch and oscillatory shear stress, the two effects

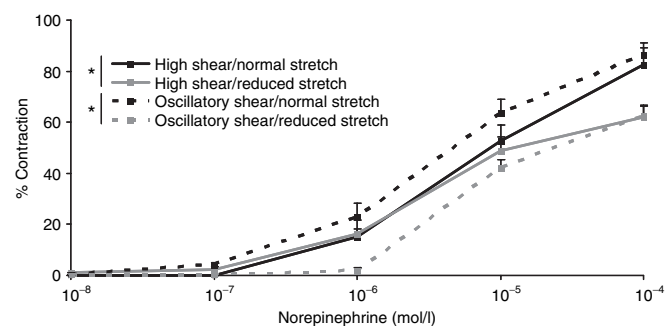
being additive and correlating well with the magnitude of DHE expression. In the first 130  $\mu$ m, reduced stretch and oscillatory shear stimulate nearly identical amounts of p22-phox, at which point they diverge, and oscillatory shear is shown to be the more prominent stimulus of p22-phox. The combination of both reduced stretch and oscillatory shear creates a sustained amount of vascular p22-phox (Figure 3a,b).

### MMP-2 is significantly modulated by shear stress, but not stretch

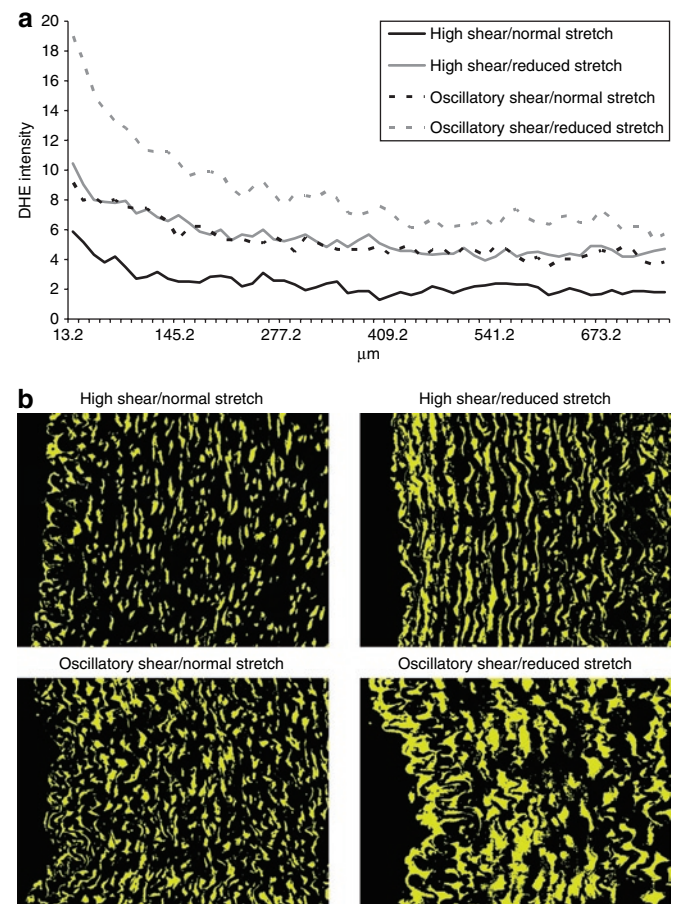
In arteries exposed to OSC shear stress, MMP-2 protein expression was significantly upregulated in both normal and reduced stretch groups (21 and 18%, respectively, Figure 4a,b,  $P < 0.05$ ).

### Reduced stretch activates gelatinase in the endothelial region, whereas oscillatory shear increases gelatinase activity throughout the vascular wall

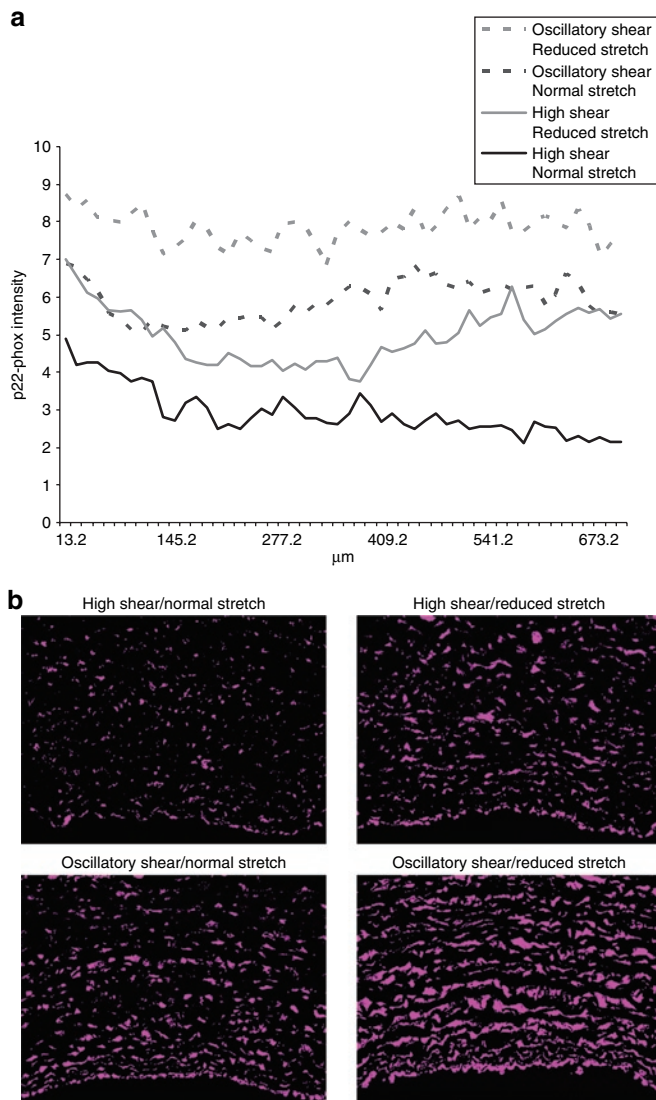
When focusing on the endothelial region, arteries exposed to a reduced stretch show significant increases in gelatinase activ-



**Figure 1** | Norepinephrine (NE) dose-dependent contraction capacity of porcine carotid arterial segments after 72 h of *ex vivo* perfusion. Data are represented as mean  $\pm$  s.e.m.,  $n = 6$ ,  $*P < 0.05$ .



**Figure 2** | Superoxide expression and distribution. (a) The effect of reduced cyclic stretch and oscillatory shear stress on dihydroethidium (DHE) staining vs. arterial wall radius. Curves represent the average of four images from each artery,  $n = 6$ . (b) DHE is represented in yellow; all images were taken at the same contrast and luminescence levels at  $\times 10$  magnification. Arterial lumen orientation is facing left.



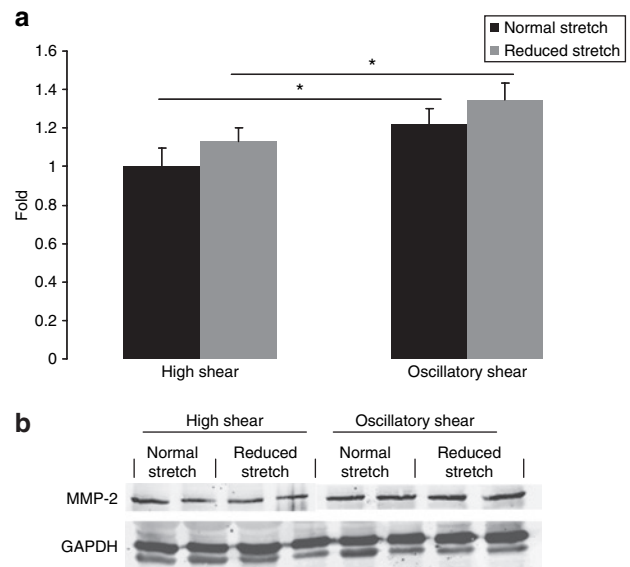
**Figure 3** | p22-phox expression and distribution. (a) The effect of reduced cyclic stretch and oscillatory shear stress on p22-phox expression vs. arterial wall radius. Curves represent the average of four images from each artery,  $n = 6$ . (b) p22-phox is represented in purple; all images were taken at the same contrast and luminescence levels at  $\times 10$  magnification. Arterial lumen orientation is facing down.

ity for both HSS and OSC groups (66 and 50%, respectively, **Figure 5a**,  $P < 0.05$ ). Representative gelatinase images used for analysis have been included (**Figure 5b**).

Analysis of total vascular gelatinase activation was significantly increased by OSC shear for both the normal and reduced stretch groups (67 and 32%, respectively, **Figure 5c**,  $P < 0.05$ ). Representative gelatinase images used for analysis have been included (**Figure 5d**).

#### Reduced cyclic stretch and oscillatory shear stress modulate gelatinase activity differently in the arterial wall

In the first 100  $\mu\text{m}$  of the arterial wall, we see that the magnitude of gelatinase is modulated by reduced stretch and OSC shear, and the shapes of the curves are similar. From



**Figure 4** | MMP-2 expression. (a) The effect of reduced cyclic stretch and oscillatory shear stress on MMP-2 expression as evaluated by immunoblot. Data are expressed as mean  $\pm$  s.e.m.,  $n = 6$ ,  $*P < 0.05$ . (b) Representative immunoblot. MMP-2, matrix metalloproteinase-2.

100 to 400  $\mu\text{m}$ , gelatinase activity for the HSS 5% group steadily declines and introduction of either reduced stretch or OSC shear reduces this decline. The combination of reduced stretch and OSC shear causes the activation of gelatinase to peak in this region. From 400 to 600  $\mu\text{m}$ , we see all four groups come together and begin to behave in a similar manner (**Figure 5e**).

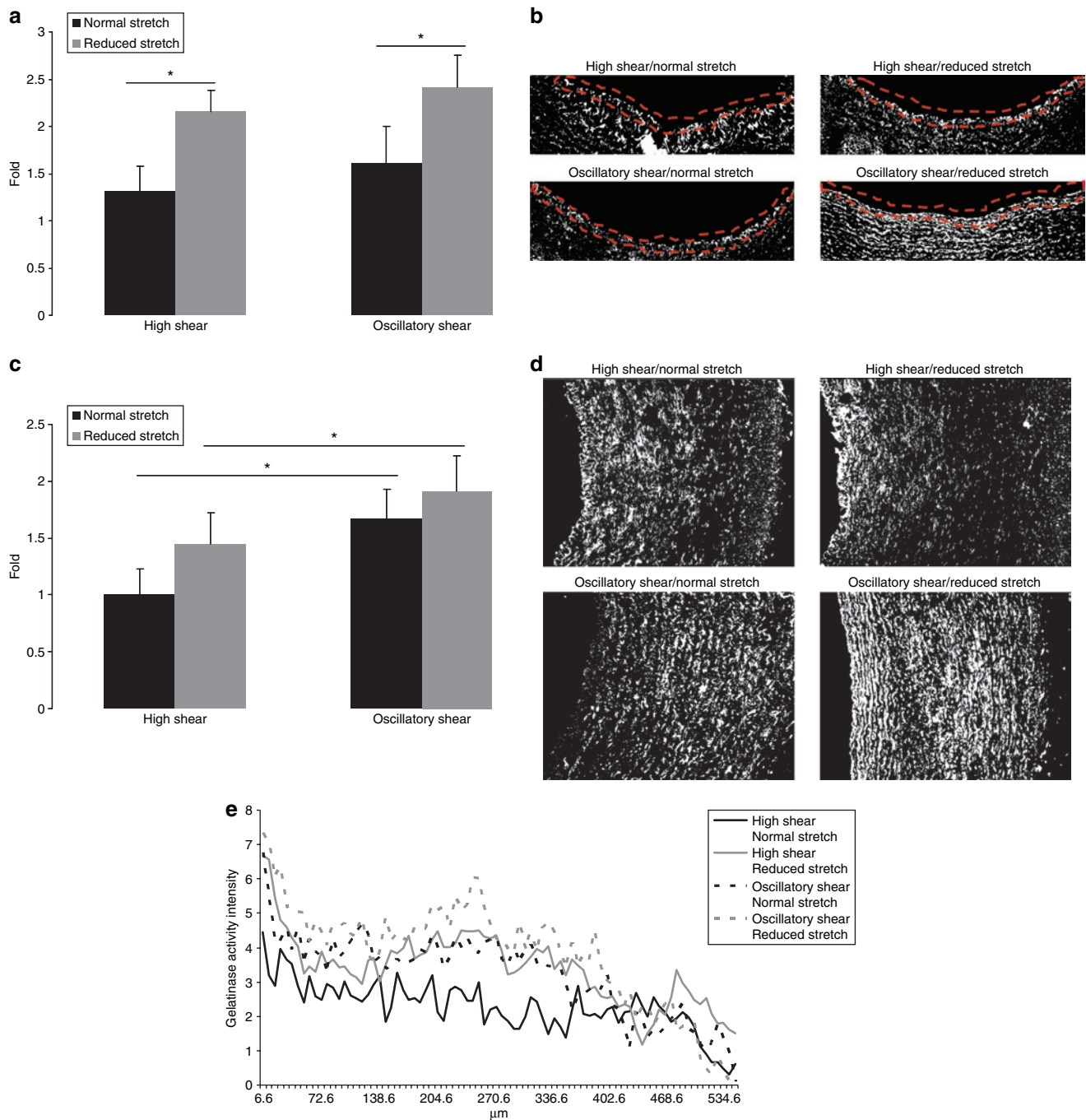
#### Reduced stretch modulates the expression of fibronectin in the endothelial region, whereas oscillatory shear increases gelatinase activity throughout the vascular wall

When focusing on the endothelial region, arteries exposed to reduced stretch show significant increases in fibronectin expression for both HSS and OSC groups (40 and 41%, respectively, **Figure 6a**,  $P < 0.05$ ). Representative fibronectin images used for analysis have been included (**Figure 6b**).

Total vascular fibronectin expression was significantly increased by OSC shear for both the normal and reduced stretch groups (68 and 74%, respectively, **Figure 6c**,  $P < 0.05$ ). Representative fibronectin images used for analysis have been included (**Figure 6d**).

#### Reduced cyclic stretch and oscillatory shear stress modulate fibronectin expression differently in the arterial wall

From the endothelium penetrating 0.05 mm into the arterial wall, we see that the magnitude of fibronectin expression is modulated by both reduced stretch and OSC shear, but the shapes of the curves fall into two distinct groups, modulated by shear. The expression of fibronectin in the HSS groups decreases exponentially with radius, whereas the OSC groups have a more sustained expression before diminishing. As the radius increases, we see that reduced stretch, OSC shear, and the combination of the two still modulate the magnitude of



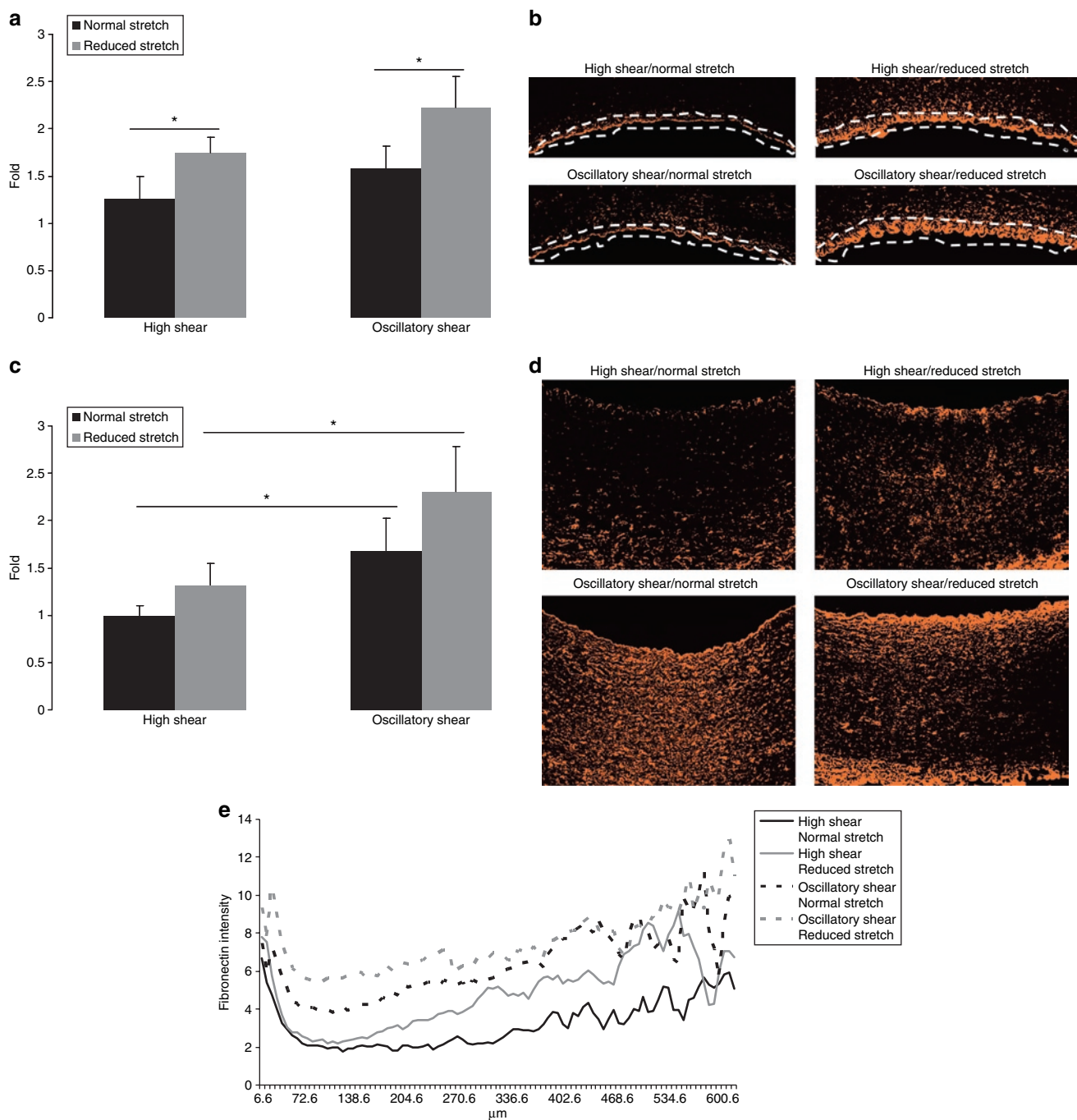
**Figure 5** | Gelatinase activity and distribution. **(a)** The effect of reduced cyclic stretch and oscillatory shear stress on endothelial gelatinase activity as evaluated by image analysis. Data are expressed as mean  $\pm$  s.e.m.,  $n = 6$ ,  $*P < 0.05$ . **(b)** Gelatinase activity is represented in white and was evaluated at the endothelium. All images were taken at the same contrast and luminescence levels at  $\times 10$  magnification. Arterial lumen orientation is facing up. **(c)** The effect of reduced cyclic stretch and oscillatory shear stress on total gelatinase activity as evaluated by image analysis. Data are expressed as mean  $\pm$  s.e.m.,  $n = 6$ ,  $*P < 0.05$ . **(d)** Gelatinase is represented in white and was quantified over the entire region. All images were taken at the same contrast and luminescence levels at  $\times 10$  magnification. Arterial lumen orientation is facing left. **(e)** The effect of reduced cyclic stretch and oscillatory shear stress on gelatinase activity vs. arterial wall radius. Curves represent the average of four images from each artery,  $n = 6$ .

fibronectin expression. The shapes of the curves for the four groups follow a similar trend, steadily increasing with radius.

## DISCUSSION

Previous works have shown that reduced arterial compliance<sup>12</sup> and oscillatory shear stress<sup>13</sup> stimulate the

production of vascular superoxide,<sup>14</sup> which in turn contributed to the processes of arterial remodeling.<sup>15</sup> However, the experimental conditions traditionally used to study shear stress and cyclic stretch have often left these two very important stimuli coupled, or exploited in such a way that is less physiologic, making it difficult to determine their



**Figure 6** | Fibronectin expression and distribution. **(a)** The effect of reduced cyclic stretch and oscillatory shear stress on endothelial fibronectin expression as evaluated by image analysis. Data are expressed as mean  $\pm$  s.e.m.,  $n = 6$ ,  $*P < 0.05$ . **(b)** Fibronectin is represented in orange and was evaluated at the endothelium. All images were taken at the same contrast and luminescence levels at  $\times 10$  magnification. Arterial lumen orientation is facing down. **(c)** The effect of reduced cyclic stretch and oscillatory shear stress on total fibronectin expression as evaluated by image analysis. Data are expressed as mean  $\pm$  s.e.m.,  $n = 6$ ,  $*P < 0.05$ . **(d)** Fibronectin is represented in orange and was quantified over the entire region. All images were taken at the same contrast and luminescence levels at  $\times 10$  magnification. Arterial lumen orientation is facing up. **(e)** The effect of reduced cyclic stretch and oscillatory shear stress on fibronectin expression vs. radius in the arterial wall. Curves represent the average of four images from each artery,  $n = 6$ .

individual contributions to vascular disease. In our study, we have performed experiments coupling high and oscillatory shear stress with normal and reduced stretch, and studied how shear stress and cyclic stretch, two different mechanical stimuli, modulate remodeling throughout the arterial wall.

Arterial stiffness is strongly affected by endothelial cell signaling<sup>16</sup> and smooth muscle contractile capacity.<sup>17</sup> By limiting the range of arterial adaptability in response to hemodynamic stimuli, the artery becomes more susceptible to vascular disease, thus provoking vascular remodeling.<sup>18</sup> To understand the effects of shear stress and cyclic stretch on arterial smooth

muscle contractile capacity, we performed vascular reactivity studies on tissue segments from our four experimental groups: HSS/5% stretch, HSS/1% stretch, OSC/5% stretch, and OSC/1% stretch. We noticed introducing an oscillatory shear had little effect, whereas reducing stretch caused significant decreases in smooth muscle contractile capacity for both the HSS and OSC groups. Indicating that on a functional level, reduction of cyclic stretch has a more dramatic effect on smooth muscle contractile capacity than oscillatory flow.

Fluctuations in superoxide ion production increasing vascular levels of ROS are understood to have dramatic effects on vascular health, especially those occurring in the endothelial region.<sup>19</sup> Basal levels of ROS are necessary for vascular cell physiologic function, increases of which have been shown to accelerate and contribute to the signaling required for vascular remodeling.<sup>20,21</sup> Dramatic increases of ROS have been shown to scavenge vasoprotective molecules such as NO, thus modulating vascular tone.<sup>22</sup> Although ROS production is mediated at many levels, the predominant system producing ROS in vascular cells is recognized as being the NADPH oxidase cascade,<sup>23</sup> which is mediated, in part, by the expression of p22-phox.<sup>24</sup> Thus, we found it interesting to see how shear stress and cyclic stretch modulate ROS production and the expression of p22-phox in the vascular wall. Upon staining with DHE, we found that oscillatory flow upregulated the total formation of superoxide anion as compared to reduced stretch.<sup>25</sup> Yet when we focused on just the endothelial region, it was found that reduced stretch is a more significant modulator of superoxide than oscillatory flow.<sup>25</sup> Upon measuring the intensity of DHE staining vs. radius, both reduced stretch and oscillatory shear have similar effects in the expression of DHE and when combined, they have a significant additive effect. This distribution suggests that increased superoxide is a process that is at least partly mediated by the variation of stress throughout the vascular wall due to change in radius. When looking at the expression of p22-phox vs. arterial wall radius, we see that its expression follows a similar trend, but the shape of the curves indicates that expression is nearly independent of radius. This feature could help explain the sustained increases of superoxide production as radius increases throughout the arterial wall.

Arterial wall remodeling in response to mechanical stimuli is a process that involves increased cellular proliferation and reorganization of the ECM.<sup>26</sup> MMP-2 is a very important ECM protein that is suspected to play a significant role in arterial matrix reorganization, a process governed by both its expression and activation. Also known to be actively involved in ECM remodeling, as well as cellular migration and proliferation, is fibronectin.<sup>27</sup> Our results showed that total MMP-2 expression was significantly upregulated by oscillatory shear stress for both the normal and reduced stretch groups. Comparing this to total MMP activation, as assessed by gelatinase activity, we observed a similar trend. Yet when we observed gelatinase activation at just the endothelial region, we saw it was significantly modulated by reduced stretch. To further investigate this regional distribution of gelatinase activity, we plotted gelatinase activity vs. arterial radius and

found a trend suggesting the distribution of gelatinase activation is strongly mediated by stress variations due to radius. When the same analysis was performed for fibronectin expression, we found that again, total arterial fibronectin expression was mediated by oscillatory shear stress for both normal and reduced stretch groups. Analysis of just the endothelial region showed that reduced stretch was the significant upregulator. When plotting fibronectin expression vs. radius, we observed that the endothelial region expression is strongly related to the stress distribution in this area, yet as radius increases, this relation is slightly inverted.

To conclude, this study demonstrates for the first time in an arterial tissue model the individual contributions of shear stress and cyclic stretch to arterial remodeling while emphasizing how these processes are mediated and distributed throughout the vascular wall. Our results showed that total superoxide production, fibronectin expression, and gelatinase activation are predominantly mediated by oscillatory shear stress, whereas their expression in the endothelial region is mediated by reduced cyclic stretch, correlating well with results from total MMP-2 expression. By plotting intensity vs. radius for these markers of vascular remodeling, we were able to see that superoxide production and gelatinase activity followed trends indicating their expression was in part mediated by stress distributions throughout the vascular wall due to changing radius, whereas fibronectin and p22-phox were much less or not at all. Most importantly, these findings, when coupled with our results from tissue reactive studies, suggest that the arterial remodeling process triggered in the endothelial region due to reduced stretch causes the most significant changes in arterial smooth muscle function. Because perturbed shear stress and reduced arterial compliance have both been implicated in the initiation and progression of vascular disease, this work provides a new perspective into how these stimuli are perceived throughout the vascular wall.

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