Influence of Oscillatory and Unidirectional Flow Environments on the Expression of Endothelin and Nitric Oxide Synthase in Cultured Endothelial Cells

Thierry Ziegler, Karima Bouzourène, Vanessa J. Harrison, Hans R. Brunner, Daniel Hayoz

Abstract—In vivo, endothelial cells (ECs) are subjected to a complex mechanical environment composed of shear stress, pressure, and circumferential stretch. The aim of this study was to subject bovine aortic ECs to a pulsatile pressure oscillating from 70 to 130 mm Hg (mean of 100 mm Hg) in combination with pulsatile shear stresses from 0.1 to 6 dyne/cm² (1 dyne/cm² = 0.1 N/m²) with or without a cyclic circumferential stretch of 4% for 1, 4, and 24 hours. The effect of highly reversing oscillatory shear stress (range −3 to +3 dyne/cm², mean of 0.3 dyne/cm²) typical of regions prone to the development of atherosclerotic plaques was also studied at 4 and 24 hours. Endothelin-1 (ET-1) and endothelial constitutive nitric oxide synthase (ecNOS) mRNA expression was time and mechanical force dependent. ET-1 mRNA was maximal at 4 hours and decreased to less than static culture expression at 24 hours, whereas ecNOS mRNA increased over time. Pressure combined with low shear stress upregulated ET-1 and ecNOS mRNA compared with static control. Additional increase in expression for both genes was observed under a combination of higher shear stress and pressure. A cyclic circumferential stretch of 4% did not induce a further increase in ET-1 and ecNOS mRNA at either low or high shear stress. Oscillatory shear stress with pressure induced a higher expression of ET-1 mRNA but lower expression of ecNOS mRNA compared with unidirectional shear stress and pressure. We have shown that the combination of pressure and oscillatory shear stress can downregulate ecNOS levels, as well as upregulate transient expression of ET-1, compared with unidirectional shear stress. These results provide a new insight into the exact role of mechanical forces in endothelial dysfunction in regions prone to the development of atherosclerosis. (Arterioscler Thromb Vasc Biol. 1998;18:686-692.)

Key Words: mechanical stress ■ hemodynamics ■ vascular endothelium ■ nitric oxide synthase ■ endothelin

The fluid mechanics of blood are known to transiently regulate vascular tone. In addition to short-term action, fluid mechanics have also been shown to regulate vascular remodeling in the case of long-term changes in pressure (hypertension) and flow rate (pregnancy, arteriovenous shunts, and exercise).1 More importantly, it has been demonstrated that the localization of the atherosclerotic plaque is correlated with regions characterized by oscillatory shear stress environment.2 This regulation has been demonstrated in part to occur via two molecules secreted by the ECs and which have been identified as ET-1 and NO.

ET-1 is a 21-amino acid peptide acting as a powerful vasoconstrictor and an SMC mitogen.3 ET-1 is thought to play an active role in SMC proliferation during remodeling of arteries. ET-1 mRNA expression has been shown to be transiently stimulated at 1 to 4 hours in cultured porcine aortic ECs exposed to a shear stress of 5 dyne/cm² (1 dyne/cm² = 0.1 N/m²) and 30 minutes in BAECs exposed to 15 dyne/cm².4,5 Longer time exposure to 15 dyne/cm² resulted in a decrease in ET-1 mRNA in BAECs.6 This decrease was not observed when ECs were exposed to an oscillatory shear stress ranging from −21 to 21 dyne/cm².5 Another study using venous ECs suggested that there exists a threshold value of steady shear stress around 5 to 6 dyne/cm² above which ET-1 mRNA is downregulated and below which it is upregulated.6,7 It should be noted, however, that the perfusion system used in the latter study subjects ECs to both shear stress and unspecified pressure. A recent study has demonstrated an increased ET-1 release by hydrostatic pressure as low as 40 mm Hg.8 No change in ET-1 mRNA levels was shown after the cells were exposed to a 20% stretch for 2 to 10 hours.8 Other studies, however, found an upregulation of ET-1 mRNA, as well as an increased ET-1 secretion into the medium, by cyclic stretch of 5% to 20% by a transcriptional regulation mechanism.9

NO is the product of conversion of L-arginine into L-citrulline by NOS and was found to play an important role in the regulation of vascular tone.10 NO was shown to inhibit SMC growth through a cyclic GMP–dependent mechanism.11 NO is released in bigger amounts by ECs subjected to both steady and pulsatile shear stresses.12,13 Both mRNA and protein expressions of ecNOS and NO release are enhanced.
selected Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>BAEC</td>
<td>bovine aortic EC</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>ecNOS</td>
<td>endothelial constitutive NOS</td>
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<tr>
<td>EMEM</td>
<td>eagle modified essential medium</td>
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<tr>
<td>ET-1</td>
<td>endothelin-1</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>NO synthase</td>
</tr>
<tr>
<td>SI</td>
<td>shape index</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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as early as 3 hours after exposure to 15 or 25 dyne/cm². Although a lower shear stress of 4 dyne/cm² has been shown to induce mRNA expression, this level was not sufficient to induce higher protein content than in static culture. Using a vacuum-powered stretching device, it was demonstrated that ecNOS mRNA and protein were increased in BAECs exposed to 1 Hz cyclic stretch of 6% to 10%. The level of ecNOS protein.

Methods

Cell Culture

BAECs were isolated from aortae obtained from the local slaughterhouse. The aorta was cannulated using plastic syringes and all collaterals were clamped. The aorta was then filled with a solution of 1% collagenase CLS4 (Worthington) in modified EMEM (GIBCO) and incubated 15 minutes at 37°C. After this time period, the collagenase solution was centrifuged 5 minutes at 1000 rpm and the supernatant discarded. The pellet was resuspended in culture medium composed of EMEM supplemented with 10% FBS (Seromed), 100 U/mL penicilin-streptomycin (GIBCO), 2 mmol/L glutamine (GIBCO), and nonessential amino acids (Seromed) and seeded into several T-75 (Falcon, Inotech). All experiments were carried out using cells at passage 4 from the same aorta.

After extensive cleaning, five silicone tubes (see following section) were treated with 70% sulfuric acid (Merck) for 2 minutes. After several rinses with deionized water, each tube was mounted onto an individual fitting and longitudinally stretched by 10%. This amount of stretching in the longitudinal direction prevented buckling of the tube under pressure. All fittings were sterilized by autoclaving. After cooling, the tubes were incubated overnight with a solution of PBS containing 10 μg/mL bovine fibronectin (Sigma). The following day, the tubes were seeded with a suspension of BAECs at a density of 150 000/cm². The cells were allowed to attach under rotation for 4 hours in a tissue-culture incubator, at which time confluence was checked under the microscope.

Experimental System

The system has been described in more detail elsewhere (Reference 17 and Fig 1A). Briefly, it is composed of compliant tubes made of Sylgard 184 (Dow Corning Europe) of 6 mm diameter and 76 mm length. The tubes are mounted onto specially designed fittings, which in turn are inserted in parallel into a perfusion loop composed of a reservoir and a gear pump (Ismatec). The pump is controlled by a function generator (Hewlett Packard) to produce a 1-Hz sinusoidal flow rate. Medium in the reservoir is composed of culture medium with 2% dextran, M₆=70,000 (Sigma), to increase the viscosity to 1.07×10⁻³ N · m⁻² · s⁻¹. The reservoir is constantly gassed by a 5% CO₂/95% air mixture to keep a constant pH of 7.2. Both reservoir and fittings were kept at a constant temperature of 37°C in a water bath.

Flow rate was measured with ultrasonic flowmeters (Transonic System, Inc) with a precision of ±2%.

Cell Harvesting and Northern Blot Analysis

Each tube was exposed to a different mechanical environment (Fig 1A). Tubes 1 and 4 were exposed to the combination of shear stress, circumferential stretch and pressure. Tubes 2 and 3 were exposed to high pulsatility, oscillatory shear stress of 0.3 dyne/cm² with a high pulsatility, and unidirectional shear stress of 6 dyne/cm² with a high pulsatility. In addition, we evaluated the effect of a 4% cyclic circumferential stretch in combination with the stresses described above. Flow experiments were run for 1, 4, and 24 hours.

Cell Harvesting and Northern Blot Analysis

At the end of the experiment, the fittings were quickly dismounted. The tubes were then rinsed once with PBS and filled with 3 mL of a trypsin-EDTA solution (GIBCO). After 3 minutes, the cells were completely detached by gentle tapping of the tubes and the cell suspension obtained was centrifuged for 10 minutes at 800g and 4°C. The cell pellet was lysed in a buffer containing guanidinium isothiocyanate and total RNA was isolated using a kit (RNasy, Quiagen). Total RNA (5 μg) was loaded into wells and separated according to its size in a 1% agarose/6% formaldehyde gel. RNA was transferred overnight by capillarity to a
nylon membrane (Hybond-N, Amersham). The membranes were prehybridized at 42°C for a minimum of 1 hour in 50% formamide, 0.2% polyvinylpyrrolidone, 0.2% BSA, 0.2% Ficoll, 0.05 mol/L Tris (pH 7.5), 1.0 mol/L NaCl, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate, and 100 mg/mL denatured salmon sperm DNA (Boehringer Mannheim). Hybridization was carried out in the same solution containing 32 P-random-primed–labeled cDNA (Boehringer Mannheim) specific for bovine preproendothelin-1 (gift of Dr Quetermous), bovine ecNOS (gift of Dr D. Harrison, Atlanta, Ga), rat b-actin or human GAPDH. After overnight incubation, the membranes were washed in 2 x SSC/0.1% SDS at room temperature for 30 minutes and in 0.1 x SSC/0.1% SDS (for ET-1 and ecNOS) or 0.5% SSC/0.1% SDS (b-actin and GAPDH) at 60°C for 1 hour and then exposed to X-ray film (Kodak X-Omat) at −80°C. Transcript levels were quantified using an electronic autoradiography apparatus (Instant Imager, Packard) or by scanning the x-ray film (Apple OneScanner) followed by densitometric analysis (NIH Image).

Protein Harvesting and Western Blot Analysis

Total cellular protein was solubilized in lysis buffer (50 mmol/L Tris, pH 6.8, 2% SDS) containing 1 mmol/L PMSF (Sigma) and 10 μmol/L leupeptin (Sigma). Samples were boiled for 5 minutes and protein concentration was measured using a protein assay (BCA, Pierce). Samples of protein (10 μg) were separated on a 7% SDS-polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes (Hybond-ECL, Amersham) using a semidry transfer cell (Bio-Rad). The membrane was blocked in PBS containing 3% nonfat milk and incubated overnight at 4°C with a monoclonal antibody directed against ecNOS (Transduction Laboratories). The following day, the membrane was washed in PBS with Tween 20 (Sigma) and incubated 1 hour at room temperature with a secondary horseradish peroxidase conjugate (Amersham). After washing, the proteins were visualized by chemiluminescence using the ECL revealing kit (Amersham). Transcript levels were quantified as for Northern blot analysis.

Cell Staining and Measurement of Cell Elongation

The borders of the cells were silver stained as previously described.17 The perimeter, area, and major axis of single cells were measured from images taken with a confocal microscope (MRC 500, Bio-Rad) using a dedicated software (SOM, Bio-Rad). The SI was then computed as 4π(area/2)2. Shear stress was calculated from a measurement of the flow rate using a Womersley approximation.18 The duration of a cycle is 1 second (1 Hz). Typical patterns representative of oscillatory (B) and unidirectional (C) shear stresses are depicted.

Figure 1. A, Diagram of the perfusion system containing four tubes seeded with BAECs and perfused with culture medium at a pressure oscillating from 70 to 130 mm Hg. The flow environments generated in tubes 1 through 4 are noted alongside the diagram. A fifth tube (static control) was kept in a tissue-culture incubator. Pump 1 is used to produce a 1-Hz sinusoidal unidirectional flow. To make an oscillatory flow, a second pump (pump 2, in the dashed box) is added to the system to reduce the mean flow rate created by pump 1 to almost zero. PP indicates pulsatile pressure; SS shear stress, and HS, hoop stretch. B and C. Shear stress was calculated from a measurement of the flow rate using a Womersley approximation. The duration of a cycle is 1 second (1 Hz). Typical patterns representative of oscillatory (B) and unidirectional (C) shear stresses are depicted.

Figure 2. Time- and force-dependent effect on the expression of mRNA for ET-1 in BAECs by unidirectional flow environment characterized by a shear stress of 6 (range of 3 to 9) dyne/cm2, a pressure oscillating from 70 to 130 mm Hg, and a cyclic stretch of 4%. A, Northern blot analysis of total mRNA obtained from BAECs exposed to different combinations of mechanical stresses (see Fig 1 for the definition of each tube) for 1, 4, and 24 hours hybridized with bovine cDNA for preproendothelin-1 (top) and GAPDH. B, Expression of normalized levels of ET-1 mRNA in BAECs exposed to unidirectional flow environment for 1, 4, and 24 hours. Normalized ET-1 mRNA is the ratio of transcript levels of ET-1 mRNA quantified using electronic autoradiography apparatus to corresponding levels of GAPDH mRNA in tubes 1 through 5 divided by the value obtained for the static control (tube 5). Results for the 4- and 24-hour time points are shown as mean±SEM of four experiments. Tubes 1 through 4 are statistically different from tube 5 (P<.01).
Results

Effect of Mechanical Forces on ET-1 mRNA Expression

Fig 2A and 2B show a time course of ET-1 mRNA induction by mechanical forces. After 1 hour of exposure of BAECs to a unidirectional flow environment composed of a shear stress of $6\pm 3$ dyne/cm$^2$, 4% stretch, and pressure, we observed a slight induction of ET-1 mRNA by mechanical forces (tubes 1 through 4) compared with static control (tube 5). At 4 hours, ET-1 mRNA induction was maximally induced in all tubes compared with static control values. The effect of pressure, shear stress, and cyclic stretch on ET-1 mRNA expression in BAECs seen at the 1-hour and 4-hour time points was no longer observed at 24 hours. Furthermore, the expression levels showed a return to those of the static culture.

A dose response of the effect of shear stress in the presence of pressure on BAEC ET-1 mRNA at 4 hours is shown in Fig 3A. Very low steady shear stresses of 0.08 to 0.3 dyne/cm$^2$ were sufficient to induce a 2.5-fold to 3-fold increase in ET-1 mRNA. A pulsatile shear stress of 6 (range 3 to 9) dyne/cm$^2$ further upregulated ET-1 mRNA expression (3.7-fold). Interestingly, oscillatory shear stress (mean of 0.3, range from −3 to +3 dyne/cm$^2$) maximally induced ET-1 mRNA, with an approximate 6-fold increase compared with static control. The presence of a 4% cyclic stretch did not significantly affect ET-1 mRNA expression at both low and high shear stress values and in the presence of oscillatory shear stresses (Fig 3A).

Effect of Mechanical Forces on ecNOS mRNA Expression

The pattern of ecNOS mRNA expression in BAECs exposed to the same unidirectional environment described in the previous paragraphs for 1, 4, and 24 hours is shown in Fig 4A and 4B. At 1 hour and 4 hours, there was no effect of any combination of mechanical environment on ecNOS mRNA expression. However, by 24 hours, there was a dramatic
increase in ecNOS mRNA expression in all tubes exposed to mechanical forces compared with static control.

Very low shear stress values of 0.08 dyne/cm² in combination with pressure did not induce ecNOS mRNA at all time points studied (Fig 3B). A 6-fold induction of ecNOS mRNA was observed in ECs cultured in the presence of a steady shear stress of 0.3 dyne/cm². Increasing both the mean and the pulsatility of shear stress (mean of 6 dyne/cm², range from 3 to 9) resulted in a further induction of ecNOS mRNA expression (13-fold compared with static). At 24 hours and in the presence of pressure, oscillatory shear stress induced a lower normalized level of ecNOS mRNA compared with unidirectional shear stresses of 0.3 and 6 dyne/cm² (Fig 3B). Similarly to ET-1, cyclic stretch had no additional effect on the expression of ecNOS mRNA in the presence of low, high, and oscillatory shear stresses (Fig 3B).

These results were confirmed at the protein level by Western blot analysis of total cell lysate of BAECs exposed to unidirectional flow environment for 24 hours, as depicted in Fig 5. In comparison to increasing mRNA expression, cyclic stretch did not increase ecNOS protein level in the presence of either low or high shear stress. Oscillatory shear stress in the presence or in the absence of a 4% circumferential stretch only slightly induced ecNOS protein expression in BAECs compared with static culture (Fig 5).

In the light of the different results obtained with oscillatory shear stress, we exposed BAECs to oscillatory shear stress to assess their shape change and compare it with that seen under unidirectional shear stress. Elongation and alignment of BAECs has been well documented in the presence of shear stress only, but less when shear stress is combined with pressure and/or cyclic circumferential stretch. 17 In this study, we found again that BAECs exposed to a high unidirectional shear stress of 6 (range 3 to 9) dyne/cm² for 24 hours were elongated and aligned with the direction of flow (Fig 6). In the presence of oscillatory shear stress of 0.3 dyne/cm² (range −3 to +3), the SI of BAECs did not change compared with cells exposed to a low shear stress of 0.3 dyne/cm² (range 0.2 to 0.4), implying that the cells did not elongate. Furthermore, no particular alignment of the cells was observed in both low and oscillatory shear stresses. When a cyclic stretch of 4% was combined with the oscillatory shear stress, the SI decreased, demonstrating cell elongation. However, these morphological changes were less pronounced than those with the combination of high unidirectional shear stress and stretch.

**Discussion**

Previous studies of how mechanical forces affect EC gene expression have used a variety of different apparatuses that look at individual force effects (for review see Reference 16). Our study relies on a perfusion system in which ECs are exposed to a more complex mechanical environment. In addition, we can clarify more fully the contribution of an individual force to EC morphology and function in a complex environment. Using this system, we have previously shown a synergistic effect of pressure, shear stress, and cyclic stretch on EC elongation and alignment. 17

The main findings of this study are that (1) pressure in combination with a shear stress lower than 0.1 dyne/cm² induced ET-1 mRNA expression at 4 hours but failed to change ecNOS mRNA expression at 4 to 24 hours; (2) higher shear stress magnitudes increased ecNOS mRNA and protein at 24 hours but not at 4 hours; (3) the presence of a 4% cyclic circumferential stretch did not alter ET-1 and ecNOS mRNA expression induced by shear stresses ranging from 0.08 to 6 dyne/cm²; and (4) oscillatory shear stress upregulated ET-1 mRNA at 4 hours and downregulated ecNOS mRNA at 24 hours compared with unidirectional shear stress.

The effect of shear stress on ET-1 mRNA expression in cultured ECs is controversial, with some studies showing a downregulation of ET-1 at levels similar to the ones used in our study.6 However, it has been demonstrated that a nonpulsatile shear stress of 5 dyne/cm² at atmospheric pressure
increased ET-1 mRNA expression by ECs in a time-dependent manner.4,22 Our study has shown that very low unidirectional shear stresses in combination with a pulsatile pressure ranging from 70 to 130 mm Hg induced ET-1 mRNA in BAECs in a similar manner. As this level of expression was further upregulated by a higher pulsatile shear stress, we have demonstrated that a pulsatile shear stress can individually affect EC expression in a pressurized system.

In the presence of a pulsatile pressure, lowering shear stress to less than 0.1 dyne/cm² had no effect on expression levels of ET-1 mRNA. This observation could suggest that the effect observed in tube 3 may be mainly due to pressure and not shear stress. However, because we are unable to show the effect of shear stress alone in our current system, we have recently designed a new perfusion apparatus that will allow us to further investigate the effect of pressure on ET-1 expression. This new generation of flow device will also allow for measurement of secreted ET-1.

Although small increases in mRNA expression were observed at 1 hour, ecNOS mRNA was not significantly upregulated by mechanical forces at 4 hours. A more dramatic change was observed at 24 hours, with a 7-fold to 13-fold increase in mRNA expression compared with the static control. The combination of pressure and a shear stress of 0.1 dyne/cm², a level previously shown not to induce any increase in ecNOS content and NO release,23 slightly upregulated ecNOS mRNA (1.5-fold) at 24 hours. However, our results show that a slightly higher shear stress magnitude to 0.3 dyne/cm² resulted in a 7-fold increase in ecNOS mRNA at 24 hours. This observation suggests that the ecNOS expression level is not affected by the presence of a pulsatile pressure but may be mostly due to the small magnitude of shear stress.

At all levels of shear stress studied here, the presence of a 4% cyclic circumferential stretch did not significantly alter either ET-1 or the ecNOS mRNA response. Other studies have shown that ECs cultured on flexible membranes and stretched up to 20% release ET-1 after 4 hours due to induced gene expression.9,24,25 Similarly, NO release and ecNOS mRNA expression were increased after exposure of BAECs to 20% stretch.14,15 The main differences between these stretch studies and our experiments are the presence of pressure and shear stress and the lower level of stretch used here. Further experiments to show whether stretch levels greater than 4% can affect expression of ET-1 mRNA in the presence of pressure and low and high shear stresses are being carried out at present in our laboratory. It may be that the stretch effect on ecNOS and ET-1 mRNA and protein levels is blunted by the shear stress effect. This result shows that the only way to evaluate the relative importance of pressure, shear stress, and cyclic stretch is by combining mechanical forces in a single experiment.

Oscillatory flow, characterized by a low mean component and an important amount of flow in the reverse direction, has been widely studied because it was demonstrated to be correlated with the localization of the atherosclerotic plaque.2 In these regions, the phenotype of the endothelium is characterized by a rounded shape with an increased proliferation rate and increased permeability.20 In this study, we found that a 24-hour exposure of BAECs to an oscillatory shear stress ranging from -3 to +3 dyne/cm² did not induce elongation and alignment of the cells with the direction of flow. This result is consistent with in vivo data and in vitro works which show that an environment composed of a highly reversing shear stress with a low mean component did not affect cell shape and extracellular matrix composition.21,26 Moreover, pressure more than anything else was the factor inducing changes in the quantity and organization of fibronectin.26 Unlike unidirectional shear stress, which acted synergistically to enhance elongation and alignment in response to cyclic stretch,27 the presence of an oscillatory shear stress did not change the morphological response of BAECs to cyclic stretch.

We have found that BAECs exposed to a combination of pressure and oscillatory shear stress produced more ET-1 mRNA at 4 hours and less ecNOS mRNA at 24 hours than BAECs exposed to pressure and unidirectional shear stress. This enhanced ET-1 mRNA production and lower ecNOS expression in cells exposed to oscillatory shear stress is consistent with data showing increased release of ET-1 at the level of the atherosclerotic plaque,27 as well as higher levels of free radicals. It should be emphasized that these in vitro results cannot be directly extrapolated to in vivo conditions because both the biochemical environment and time of exposure to mechanical forces are different. However, these results imply that oscillatory shear stress may contribute to the endothelial dysfunction by inducing increased ET-1 and decreased NO production, thus leading to cellular toxicity, SMC proliferation, and atherogenesis, as hypothesized by Loscalzo and Welch.10 This difference in cell behavior due to the highly reversing nature of flow (which is the only difference between tube 1 and tube 3, in which ECs were exposed to a low amplitude of shear stress) is worth studying in greater details.

Using a novel perfusion apparatus, we have shown that both ET-1 and ecNOS mRNA were upregulated by the combination of pressure and shear stress but at different time points. Oscillatory shear stress, characteristic of regions prone to the development of atherosclerotic plaque and characterized by an important amount of stress in the reverse direction, was shown to induce more ET-1 mRNA at 4 hours and less ecNOS mRNA and protein at 24 hours than unidirectional shear stress. Overall, these results emphasize the importance of using a quasiphysiological mechanical environment combining all stresses to assess endothelial function in vitro.

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