Oscillatory and Steady Laminar Shear Stress Differentially Affect Human Endothelial Redox State
Role of a Superoxide-Producing NADH Oxidase

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Abstract—Atherosclerotic lesions are found opposite vascular flow dividers at sites of low shear stress and oscillatory flow. Since endothelial proinflammatory genes prominent in lesions are regulated by oxidation-sensitive transcriptional control mechanisms, we examined the redox state of cultured human umbilical vein endothelial cells after either oscillatory or steady laminar fluid shear stress. Endothelial oxidative stress was assessed by measuring activity of the superoxide (O$_2^-$)–producing NADH oxidase (a major source of reactive oxygen species in vascular cells), intracellular O$_2^-$ levels, induction of the redox-sensitive gene heme oxygenase-1 (HO-1), and abundance of Cu/Zn superoxide dismutase (Cu/Zn SOD), an antioxidant defense enzyme whose level of expression adapts to changes in oxidative stress. When cells were exposed to oscillatory shear (±5 dyn/cm$^2$, 1 Hz) for 1, 5, and 24 hours, NADH oxidase activity and the amount of HO-1 progressively increased up to 174±16% (P<0.05) and 505±111% (P<0.05) versus static conditions, respectively, whereas levels of Cu/Zn SOD remained unchanged. This upregulation of HO-1 was completely blocked by the antioxidant N-acetylcysteine (NAC, 20 mmol/L). In contrast, steady laminar shear (5 dyn/cm$^2$) induced NADH oxidase activity and NAC-sensitive HO-1 mRNA expression only at 1 and 5 hours, a transient response that returned toward baseline at 24 hours. Levels of Cu/Zn SOD mRNA and protein were increased after 24 hours of steady laminar shear. Furthermore, intracellular O$_2^-$, as measured by dihydroethidium fluorescence, was higher in cells exposed to oscillatory than to laminar shear. These data are consistent with the hypothesis that continuous oscillatory shear causes a sustained activation of pro-oxidant processes resulting in redox-sensitive gene expression in human endothelial cells. Steady laminar shear stress initially activates these processes but appears to induce compensatory antioxidant defenses. We speculate that differences in endothelial redox state, orchestrated by different regimens of shear stress, may contribute to the focal nature of atherosclerosis. (Circ Res. 1998;82:1094-1101.)

Key Words: shear stress • endothelium • NADH/NADPH oxidase • oxygen-derived free radical • heme oxygenase-1

Since the pathologists Rokitansky and Virchow described the focal nature of atherosclerotic plaque formation in the vascular tree, involvement of hemodynamic forces in the pathogenesis of atherosclerosis has been postulated. In the early 1980s, comparisons between the pattern of blood flow and the localization of atherosclerotic lesions showed that low rather than high mean shear was associated with atherogenesis. In addition, marked oscillations of flow (between −7 and +4 dyne/cm$^2$), but not turbulent flow, were observed at sites of atherosclerotic lesions. From these observations, it was postulated that oscillatory shear stress exerted a stimulus to the vascular wall that induced lesion formation and that high unidirectional shear stress might be protective.

Meanwhile, crucial discoveries in vascular physiology, morphology, and molecular biology have demonstrated that vascular endothelial integrity is obligatory for normal cardiovascular functioning and that endothelial dysfunction is a key initial event during atherogenesis. Endothelial dysfunction is responsible for impaired vessel relaxation and vasospasm and participates in endothelial-monocyte adhesion, oxidation of LDL, and increased susceptibility to thrombosis (for review, see Reference 5). Numerous studies have shown that shear stress both activates and inactivates many cellular processes, including the synthesis and release of vasoactive and coagulation factors (for review, see Reference 6) and the expression of inflammatory molecules, such as adhesion molecules. Recently, it was shown that steady unidirectional shear stress decreases the expression of VCAM-1 and almost completely prevents postshear cytokine-induced expression of VCAM-1 in human endothelial cells. Prolonged oscillatory shear, in contrast, robustly upregulates VCAM-1 expression.
These observations provide initial evidence that human endothelial cells can distinguish between different types of shear stress and suggest that steady laminar shear might induce protective anti-inflammatory responses.

As a first approach to clarify these observations, we examined the effect of oscillatory and unidirectional shear stress on endothelial oxidative mechanisms. Cellular redox state is an important functional parameter that modulates gene expression, activity of signaling pathways and para-crine factors, and cell growth. Cellular redox state reflects a balance between processes that promote either oxidative or reductive pathways in the cell. Thus, we examined the effect of steady laminar and oscillatory shear stress on the function of the superoxide radical (O$_2^-$)–producing NADH oxidase (a major source of reactive oxygen species in vascular cells) and on the expression of the cytosolic O$_2^-$–scavenging Cu/Zn SOD (an antioxidant defense enzyme whose level of expression adapts to changes in oxidative stress). In addition, the influence of oscillatory and steady laminar shear on the expression of the redox-sensitive gene HO-1 was assessed. The results of the present study suggest that steady and oscillatory shear stress differentially affect the endothelial redox state.

Materials and Methods

Cell Culture

Cryopreserved primary HUVECs were obtained from Clonetics Corp. HUVECs were grown in medium 199 supplemented with 20% FCS, 16 U/mL heparin, 50 μg/mL endothelial growth supplement, 2 mM/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and cultured at 37°C in dishes coated with 0.1% (wt/vol) gelatin to assist cell adhesion. For experiments, cells between passages 2 and 6 were seeded onto gelatin-coated plastic plates, fed every other day, and used at confluence. Cells for use as statically maintained samples were plated similarly. None of these conditions had any effect on cell viability.

Flow System

The steady laminar flow system has been described in detail elsewhere. Briefly, the cell culture slide containing the endothelial monolayer was inserted into a parallel-plate flow chamber, which was installed between upper and lower reservoirs containing culture medium. The chamber had a length of 11 cm, width of 6 cm, and height of 0.025 cm. After passage through the flow chamber, medium collects in the lower reservoir and is recirculated to the upper reservoir by means of a peristaltic pump. The height between the 2 reservoirs determines the steady flow rate. The mean shear stress ($\tau_r$) to which the cells were exposed was calculated using the following formula: $\tau_r = (6 \mu h/b)Q$, where $\mu$ refers to the dynamic viscosity, $b$ to the flow chamber width, $h$ to the flow chamber height, and $Q$ to the flow rate.

The oscillatory flow system has also been previously documented. A motor-driven syringe pump was inserted sidearm into the laminar flow system so that longitudinal oscillatory motion could be generated. This oscillatory component allowed pulsatility to be superimposed on the steady flow via a displacement of the medium back and forth above the endothelial cell surface. In order to dampen the motion so as to ensure a sinusoidal wave form, a small reservoir was placed between the syringe and the flow chamber. The frequency and amplitude of the oscillatory flow were controlled by adjusting the speed or the amplitude of the syringe motion, respectively. The pulsatile flow rate was monitored using an electromagnetic flowmeter probe that was calibrated with an in-line flowmeter. All shear stress experiments were conducted in a warm (37°C) room, and the flow medium was exposed to a 95% air/5% CO$_2$ mixture to maintain a pH of 7.2.

The monolayers were subjected either to a steady laminar shear stress of 5 dyne/cm$^2$ or to a very low mean shear stress with an instantaneous oscillatory shear stress between kn and $\sim$ 5 dyne/cm$^2$ at a frequency of 1 Hz, in an attempt to approximate physiological conditions. These experimental conditions were imposed for a range of time periods. For some experiments, the monolayers were incubated with the antioxidant NAC (20 mmol/L) either for 30 minutes, followed by shear stress in the presence of the antioxidant, or, in the case of the unshocked control samples, for the duration of the experiment.

Because of the requirements for securing the plate within the chamber, installation of the monolayer into the flow chamber necessarily destroys cells at the edges of the plate. In order to discount any effect of substances released by dead cells in the experimental homogenates, all control monolayers were secured within flow chambers for the length of time used in the shear experiments.

NADH Oxidase Assay

NADH oxidase activity was measured using a lucigenin assay as previously described. Briefly, control cultures and cultures that had been exposed to shear were washed and lysed in a buffer containing proteinase inhibitors (20 mmol/L monobasic potassium phosphate [pH 7.0], 1 mmol/L EGTA, 10 μmol/L aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.5 mmol/L phenylmethylsulfonyl fluoride). The cell suspension was then dounce-homogenized 100 times on ice, and the homogenate was stored on ice until use. Protein content was measured in an aliquot of the homogenate by the method of Lowry et al. NADH oxidase activity was measured using lucigenin, which is specific for O$_2^-$. The assay was performed in a 50 mmol/L phosphate buffer (pH 7.0) containing 1 mmol/L EGTA, 150 mmol/L sucrose, 500 mmol/L lucigenin as the electron acceptor, and NADH as the substrate (final volume, 0.9 mL). NADH was used at a final concentration of 100 μmol/L. The reaction was started by the addition of 100 μL of homogenate (50 to 150 μg protein). Photon emission was measured every 15 seconds for 10 minutes in a luminometer. No activity could be measured in the absence of NADH. A buffer blank containing lucigenin and NADH was subtracted (<5% of the cell signal) from each reading before transformation of the data by comparison with a standard curve generated with xanthine/xanthine oxidase. The chemiluminescence signal observed during the present study is unlikely to be related to lucigenin-induced O$_2^-$ formation in the cell homogenate, because no chemiluminescence signal was detected when lucigenin was assayed with cell lysate in the absence of NADH, and the validity of the assay was confirmed in separate experiments using cytochrome c as the electron acceptor.

In some experiments, inhibitors (diphenylene iodonium, KCN, 4,5-dihydroxy-1,3-benzene disulfonic acid [Tiron], allopurinol, and L-NAME) were added to samples 10 minutes before readings. As previously described, NADH oxidase activity was insensitive to KCN (100 μmol/L), L-NAME (100 μmol/L) and allopurinol (100 mmol/L), indicating that O$_2^-$ production was not derived from mitochondrial electron transport, nitric oxide synthase, or xanthine oxidase. NADH oxidase activity was, however, reduced by the flavin protein inhibitor diphenylene iodonium and by 10 mmol/L Tiron, verifying that the reactive oxygen species is O$_2^-$ and confirming that the endothelial oxidase is a flavin-containing enzyme.
Measurement of Intracellular \( O_2^- \) in Intact Cells

To measure intracellular \( O_2^- \) in intact HUVECs, we used dihydroethidium, a cell-permeant dye that is oxidized by \( O_2^- \) to yield the fluorescent ethidium cation.\(^{25}\) This dye has been shown to faithfully measure \( O_2^- \) in endothelial cells.\(^{27}\) Shear-preconditioned and static monolayers were rinsed 3 times with ice-cold PBS, treated with versene (0.2 g/L EDTA in PBS), and scraped from the plate. Cell suspensions were centrifuged at 500 g for 10 minutes at 4°C, and the supernatant was aspirated. Pellets were resuspended in 25 \( \mu \)mol/L dihydroethidium in serum-free culture medium and incubated in a light-protected environment for 30 minutes at 37°C. After centrifugation (500g for 10 minutes at 4°C), cells were washed and fixed with paraformaldehyde in PBS at a final concentration of 1% (wt/vol). The relative fluorescence intensities were quantified using flow cytometry in a fluorescence-activated cell sorter (FACScan IV, Becton Dickinson) with absorption set at 518 nm and the detector set at 605 nm. Untreated fixed HUVECs were used as a reference sample.

Northern Blot Analysis

Total RNA was isolated using TRI reagent. RNA (12 \( \mu \)g) was separated on 1% denaturing formaldehyde agarose gels, transferred to Nytran membranes (Schleicher & Schuell) by overnight upward capillary blotting with 10\( \times \) SSC, and immobilized by UV cross-linking as described previously.\(^{26}\) Consistency of total RNA loading between samples was controlled by densitometric analysis of 28S RNA ultraviolet fluorescence in the presence of ethidium bromide. Double-stranded full-length human Cu/Zn SOD cDNA and the full-length human HO-1 cDNA were labeled using a random primer labeling kit (Prime It II) and [\( ^{32}P \)]dCTP. Blots were prehybridized at least 2 hours and hybridized overnight at 42°C in the following solution: 1 mol/L NaCl, 50 mmol/L Tris HCl (pH 7.4), 5\( \times \) Denhardt’s solution, 50% formamide, 0.5% SDS, and 100 \( \mu \)g/mL sheared and denatured salmon sperm DNA. Denhardt’s solution was omitted during hybridization. After hybridization, the blots were washed 3 times in 1\( \times \) SSC and 1% SDS at 50°C. The blots were autoradiographed using Hyperfilm-MP at \(-80^\circ\)C, and the relative density of each band was determined using laser densitometry. Staining of the 28S band by ethidium bromide after transfer to the membrane was used for normalization.

Western Blot Analysis

Cells were lysed at 1 mL per dish with lysis buffer (mmol/L: HEPES 50, EDTA 5, NaCl 50, and \( \eta \)-octylglucoside 60, along with 1\( \% \) Triton X-100, [pH 7.5]) containing protease inhibitors (10 \( \mu \)g/mL aprotinin, 1 \( \mu \)mol/L phenylmethylsulfonyl fluoride, and 10 \( \mu \)g/mL leupeptin) and phosphatase inhibitors (50 \( \mu \)mol/L sodium fluoride, 1 \( \mu \)mol/L sodium orthovanadate, and 10 \( \mu \)mol/L sodium pyrophosphate). Extracted protein was quantified by the Lowry assay.\(^{25}\) Equal amounts of proteins were separated on 12% polyacrylamide gels using SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked with PBS containing 5% nonfat dry milk, 2\% BSA, and 0.2\% Tween 20. Antibodies against HO-1, Cu/Zn SOD, and the full-length human Cu/Zn SOD cDNA were used at 1:500 and 1:200 dilutions, respectively. The enhanced chemiluminescence Western blotting system was used for detection.

Calculations and Statistical Analysis

Comparison between groups was performed by unpaired \( t \) test. NADH oxidase enzyme activity was quantified by calculating the mean amount \( O_2^- \) produced per milligram protein per minute over a 10-minute period. Enzyme activity was linear over a wide range of protein content (<10 to 200 \( \mu \)g) within one sample.

Materials

All chemicals were of analytical grade or better. BSA and phenylmethylsulfonyl fluoride were from Boehringer Mannheim. Soybean trypsin inhibitor, glutamine, penicillin, streptomycin, calf serum, and trypsin/EDTA were purchased from GIBCO. FCS was purchased from Hyclone Laboratories. Endothelial growth supplement was purchased from Biomedical Technology. Common buffer salts and medium 199 were obtained from Fisher. Dihydroethidium was from Molecular Probes. Diphenylene iodonium was purchased from Toronto Research Chemicals. [\( ^{32}P \)]dCTP was purchased from DuPont NEN. Nytran membranes were purchased from Schleicher & Schuell, and Biospin columns were from Bio-Rad. Prime-It II probe labeling kits were purchased from Amersham Life Sciences. Cu/Zn SOD antibody was from Biodesign, Intl, and HO-1 antibody was purchased from StressGen Biotechnologies. All other chemicals and reagents were from Sigma Chemical Co. The full-length Cu/Zn SOD cDNA was a kind gift from Dr. David G. Harrison (Emory University, Atlanta, Ga.), and the human HO-1 cDNA was a kind gift from Dr Shigeaki Shibahara (Tohoku University, Japan).

Results

Effect of Oscillatory and Steady Laminar Shear Stress on NADH Oxidase Activity and \( O_2^- \) Levels

The effect of oscillatory shear stress (±5 dyne/cm\(^2\), 1 Hz) on NADH oxidase activity is shown in Figure 1. NADH oxidase was time-dependently activated by exposure to oscillatory shear. Activation was observed only when exposure was >1
hour and progressively increased in magnitude up to 24 hours after the onset of oscillatory shear (from $2342\pm 333$ nmol O$_2^\cdot$/mg protein per minute in static cells to $3940\pm 505$ nmol O$_2^\cdot$/mg protein per minute in cells after 24-hour shear, $P<0.05$).

Initiation of steady, but transient, laminar shear at 5 dyne/cm$^2$ also resulted in a significant time-dependent increase in oxidase activity (Figure 2). Enzyme activity was increased as early as 1 hour after exposure to steady laminar shear. However, when steady laminar shear stress was prolonged for 24 hours, oxidase activity in sheared cells was not different from activity in static cells ($1568\pm 194$ versus $1972\pm 295$ nmol O$_2^\cdot$/mg protein per minute in cells after 24-hour shear, $P<0.05$).

Increased NADH oxidase activity most likely resulted from activation of the existing enzyme, since neither type of shear stress altered the expression of p22phox, the only cloned subunit of the vascular NADH oxidase (data not shown).

This difference in oxidase activity between cells exposed to oscillatory and laminar shear stress for 24 hours was reflected in the O$_2^\cdot$ levels in intact cells. As measured by ethidium fluorescence, O$_2^\cdot$ levels in cells exposed to oscillatory shear were significantly higher (mean fluorescence intensity, $357\pm 29$; n = 2) than those in HUVECs exposed to laminar shear (mean fluorescence intensity, $102\pm 14$; n = 2) (Figure 3). Both were lower than in static cells (data not shown).

**Effect of Oscillatory and Steady Laminar Shear Stress on Cu/Zn SOD Expression**

The discrepancy in intracellular O$_2^\cdot$ levels between HUVECs exposed to oscillatory and laminar shear suggests a possible differential regulation of scavenging systems. Application of 24 hours of steady laminar shear (5 dyne/cm$^2$) increased the abundance of Cu/Zn SOD mRNA ($138\pm 5\%$ versus static conditions, $n=3$, $P<0.05$) and protein ($142\pm 18\%$, $n=3$) (Figure 4). In contrast, when cells were exposed to 24 hours of oscillatory shear (± 5 dyne/cm$^2$, 1 Hz), Cu/Zn SOD mRNA and protein levels were not altered ($99.6\pm 14\%$ [n=5] for mRNA and $110\pm 8\%$ for protein [n=3] versus static conditions, $P=NS$) (Figure 4). Identical results were obtained after 5 hours of oscillatory shear stress (data not shown). Thus, oscillatory shear apparently causes a
sustained increase in pro-oxidative mechanisms, whereas steady laminar shear induces only a transient oxidative insult.

**Effect of Oscillatory and Steady Laminar Shear Stress on HO-1 Expression**

HO-1 has been shown to be redox-sensitive in other non-endothelial cell types. In experiments anticipating studies on shear stress, we investigated the effects of oxidant stress on HO-1 mRNA expression in endothelial cells. As shown in Figure 5, extracellular reactive oxygen species (generated by xanthine oxidase [20 mU/mL] in the presence of xanthine [0.4 mmol/L] or by hydrogen peroxide [10 to 100 μmol/L]) markedly increased HO-1 mRNA expression. Under both conditions, this increase could be prevented, or at least attenuated, by previous administration of Tiron (an O₂ scavenger) or catalase (an enzymatic hydrogen peroxide scavenger), respectively. These data confirm that HO-1 mRNA expression is sensitive to changes in the intracellular redox state in endothelial cells.

Thus, we used HO-1 mRNA expression as an additional readout of net oxidative state of HUVECs exposed to shear stress. Figure 6 shows the time-dependent effect of oscillatory and steady laminar shear stress on HO-1 mRNA expression. When cells were exposed to oscillatory shear stress, a sustained increase in HO-1 mRNA was observed. HO-1 mRNA levels were increased by 4.5-fold above baseline (n=3, P<0.05) after 5 hours and remained upregulated by 5.1-fold above baseline (n=3, P<0.05) after 24 hours. This increase was reflected in protein levels, which were increased by 1.3-fold at 5 hours and by 2.0-fold at 24 hours (n=2–4). To demonstrate that HO-1 mRNA upregulation was redox-sensitive, in some experiments HUVECs were preincubated with the antioxidant thiol compound NAC (20 mmol/L) for 30 minutes before the application of oscillatory or steady laminar shear stress. As shown in Figure 7, for both types of shear, induction of HO-1 mRNA expression was completely inhibited. Incubation with NAC also de-
increased HO-1 protein expression for both types of shear (79% inhibition).

Discussion

The present study is focused on the differential effects of oscillatory and steady laminar shear stress on endothelial oxidative mechanisms. The data show that oscillatory shear, used at an amplitude modeled in vivo at vascular bifurcations (±5 dyne/cm²), progressively enhanced the activity of the O₂⁻-producing NADH oxidase and the expression of the redox-sensitive gene HO-1, whereas levels of the O₂⁻⁻⁻ scavenger enzyme Cu/Zn SOD remained unchanged. Steady laminar shear, used at 5 dyne/cm² to match mean peak levels of shear, initially increased NADH oxidase activity and HO-1 mRNA expression, but these responses were transient and returned toward baseline after 24 hours. Moreover, Cu/Zn SOD mRNA and protein expression were increased after 24 hours of steady laminar shear, suggesting that in cells exposed to this type of shear stress, mechanisms are induced to compensate for the oxidative stress. This was confirmed by measurement of intracellular O₂⁻⁻⁻, which showed that the overall O₂⁻⁻ levels in cells exposed to oscillatory shear for 24 hours were 3-fold higher than levels in cells exposed to laminar shear. Together, these data indicate that fluid shear forces may have important effects on endothelial oxidative mechanisms and that different types of shear stress may have different effects. Since fluid shear forces and endothelial redox state have been proposed to be involved in the pathogenesis of atherosclerosis, these observations may have mechanistic importance.

Growing experimental evidence suggests that the generation of reactive oxygen species participates in cellular activation and intracellular signal transduction. For example, both O₂⁻⁻ and H₂O₂ have been implicated in the activation of phospholipase D, p42/p44 mitogen-activated protein kinases, and p38 mitogen-activated protein kinase. Laurindo et al demonstrated that shear stress induced by increased flow through intact isolated rabbit aortas was accompanied by endothelium-dependent production of free radicals. Our data suggest that increased production of reactive oxygen species induces HO-1 mRNA expression. The biochemical pathways responsible for generation of reactive oxygen species during cell activation, however, remain unclear. Recently, several reports have shown that vascular smooth muscle and endothelial cells express an NADH/NADPH-dependent oxidase that is considered to be one of the most potent sources of O₂⁻⁻ in the vascular cell. We recently reported that enzyme activity of the vascular p22phox-based NADH oxidase is induced during cell activation by the cytokine tumor necrosis factor-α in vitro and by angiotensin II in vitro and in vivo. After induction with both stimuli, enzyme activity remained elevated for prolonged periods, as long as the stimulus was present (experiments were performed up to 24 hours). The major role of this oxidase may be as an inducible enzyme, since baseline NADH oxidase activity is unaffected by expression of antisense p22phox. This might explain why NADH oxidase activity levels are induced by shear stimuli, even though basal O₂⁻⁻ levels are high in endothelial cells.

In the present study, oscillatory shear--induced oxidase activity was sustained, similar to that observed after angiotensin II or tumor necrosis factor-α. The transient activation by steady laminar shear, however, is somewhat unusual. Numerous other cellular processes have indeed been demonstrated to be only transiently increased by acute in vitro application of steady laminar shear stress, such as ion channel activity, surface adenine nucleotide concentrations, and intracellular calcium (for review, see Reference 6). This phenomenon, known as feedback inhibition of mechanotransduction, is explained by adaptation or signal filtering. Feedback inhibition of mechanotransduction has been postulated to be necessary to prevent overstimulation by the normal mechanical environment. In the case of shear--induced activation of O₂⁻⁻ production, feedback inhibition seems crucial to avoid overproduction and adverse effects of reactive oxygen species. The inability of endothelial cells to adapt in the oscillatory environment may be a key feature leading to continuous activation of pro-oxidant processes under these conditions, whereas the lack of sustained NADH oxidase activity in cells exposed to steady laminar shear stress may contribute to the antiatherogenic effects of this type of shear stress.

The biological action of reactive oxygen species is counterbalanced by enzymatic and nonenzymatic scavenger systems. One of the major determinants of intracellular O₂⁻⁻ is the cytosolic copper/zinc-containing enzyme Cu/Zn SOD, which accelerates the conversion of O₂⁻⁻ to H₂O₂. In the present study, we observed that application of steady laminar shear during 24 hours increases Cu/Zn SOD mRNA and protein expression in HUVECs. This observation is consistent with the observations by Inoue et al in human arterial endothelial cells. Interesting is the somewhat smaller increase observed at 5 dyne/cm² in the present study (∼140% versus ∼200% by Inoue et al), a discrepancy that may point to differences between venous and arterial endothelial cells. The present finding that levels of Cu/Zn SOD mRNA were unchanged after exposure to oscillatory shear stress, however, is novel and intriguing. Differences in gene expression between various types of shear stress are only beginning to be observed. Although the 5' flanking promoter region of the Cu/Zn SOD gene contains 3 copies of the shear-responsive nucleotide sequence GAGACC and 2 copies of the activator protein-1 binding site, it is not known whether steady laminar shear--induced Cu/Zn SOD mRNA expression is modulated through these sequences. Whether oscillatory and steady laminar shear differentially affect Cu/Zn SOD mRNA expression by a differential effect on the activation of these promoter regions requires further investigation.

HO-1 is a member of the heat shock protein family, which catalyzes the initial rate-limiting step of heme degradation to CO and biliverdin. The physiological function of HO-1 is not yet fully understood, but CO-mediated changes in intracellular cGMP may be involved in the regulation of vascular tone and in the expression of endothelial mitogens. In addition, HO-1 may contribute to the control of oxidative stress in the vessel wall, since it decreases the cellular pro-oxidant heme and indirectly (via the action of biliverdin reductase on biliverdin) increases the antioxidant bilirubin. Induction of
HO-1 is also accompanied by increased expression of ferritin, a chelator of free iron. Previous experiments in fibroblasts and vascular smooth muscle cells have demonstrated that HO-1 is exquisitely sensitive to oxidative stress, including UV irradiation, \( \text{O}_2^\cdot \) and \( \text{H}_2\text{O}_2 \). Promoter studies of the human heme oxygenase gene have identified binding sites for nuclear factor-κB and activator protein-1 (both redox-sensitive transcription factors) and have shown that elements within 121 bp immediately upstream from the mRNA cap site respond to various forms of oxidative stress. Although we did not directly assess the involvement of these redox-sensitive DNA binding sites in shear-induced upregulation of HO-1, the ability of NAC to block HO-1 expression supports this hypothesis, especially since there is no evidence for the shear-responsive element GAGACC sequence in the HO-1 promoter. Whatever the mechanism, the potential importance of increased HO-1 expression in shear stress, which has also recently been reported in vascular smooth muscle cells, resides in endothelial cells as far as its potent antioxidant properties are concerned. Indeed, the antioxidant effects of HO-1 may be partly responsible for the lower intracellular \( \text{O}_2^\cdot \) in sheared cells compared with static cells.

In the present study, after confirming the redox sensitivity of HO-1 in HUVECs, HO-1 mRNA expression was used as a parameter of functional intracellular oxidative stress in order to better appreciate the overall balance between the observed increase in NADH oxidase activity and the expression of Cu/Zn SOD mRNA. Strikingly, results for HO-1 mRNA expression were consistent with results for NADH oxidase, Cu/Zn SOD expression, and intracellular \( \text{O}_2^\cdot \) levels. First, as a novel finding, both oscillatory and steady laminar shear upregulated HO-1 mRNA expression in endothelial cells. Second, experiments with NAC confirmed that redox pathways are involved in shear-induced HO-1 mRNA expression. Third, the time dependence of shear-induced HO-1 mRNA expression was consistent with the data observed for the NADH oxidase activation. Our attempts to further analyze the specific contribution of NADH oxidase activity in inducing HO-1 mRNA expression were restricted by a marked toxicity (at concentrations as low as 2 μmol/L) of the flavin enzyme inhibitor diphenyle iodonium on HUVECs. Therefore, although the vascular NADH oxidase is among the most powerful \( \text{O}_2^\cdot \)-regulating systems in the endothelial cell, we cannot completely exclude the possibility that other oxidases participate in the induction of HO-1 mRNA. Finally, the sustained induction of HO-1 may contribute to the antioxidant defenses summoned by the cell to combat the oxidative stress resulting from NADH oxidase activation.

The distribution of hemodynamic forces is thought to contribute to the focal nature of atherosclerosis. Comparisons between the pattern of blood flow and the localization of atherosclerotic lesions showed that low rather than high mean shear was associated with atherogenesis. Moreover, marked intimal thickening was most extensive when instantaneous shear oscillated between −7 and +4 dyne/cm². Elevated steady laminar shear stress tends to protect against intimal thickening. The present study, showing different effects of oscillatory and steady laminar shear on endothelial oxidative mechanisms, may help to explain these observations. The endothelial redox state may contribute to the initiation and progression of atherosclerosis by influencing the oxidative state of LDL, modulating the half-life of nitric oxide, and regulating inflammatory gene expression and cell growth. Thus, although both types of shear stress are initially pro-oxidant, the induction of SOD and the lowering of intracellular \( \text{O}_2^\cdot \) (ie, the net antioxidant effect) once laminar shear stress has been established may be protective against atherogenic stimuli, whereas oscillatory shear, by virtue of its inability to increase SOD expression and its higher intracellular \( \text{O}_2^\cdot \) levels, does not exert that protection and, thus, may contribute to atherogenesis.

In summary, the present study shows experimental evidence that continuous exposure of endothelial cells to oscillatory and steady laminar shear stress differentially affects endothelial oxidative mechanisms. This difference includes at least (1) the absence of feedback inhibition of oscillatory shear compared with steady laminar shear on the activity of the \( \text{O}_2^\cdot \)-producing enzyme, NADH oxidase, and (2) the differential regulation of the mRNA expression of the antioxidant enzymes Cu/Zn SOD and HO-1. Further studies are necessary to understand the molecular signaling pathways that allow endothelial cells to discriminate between various types of shear stress.

Acknowledgments

This study was supported by National Institutes of Health grants HL-38206 and HL-58863 and by a fellowship from the Belgian American Educational Foundation (to Dr De Keulenaer). The authors are grateful to Drs Bob Taylor and Stanislas Sys for helpful discussions.

References

2. Virchow R. Cellular Pathology as Based Upon Physiological and Pathological Histology. London, UK: Churchill; 1860.


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Circ Res. 1998;82:1094-1101
doi: 10.1161/01.RES.82.10.1094

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