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Shear Stress Enhances Glutathione Peroxidase Expression in Endothelial Cells

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Hemodynamic forces have profound effects on vasculature. Laminar shear stress upregulates superoxide dismutase (SOD) expression in endothelial cells. SOD converts superoxide anion to H_2O_2, which, however, promotes atherosclerosis. Therefore, defense against H_2O_2 may be crucial in reducing oxidative stress. Since glutathione peroxidase (GPx-1) reduces H_2O_2 to H_2O, the regulation of GPx-1 expression by mechanical stress was examined. Cultured bovine aortic endothelial cells (BAECs) were subjected to laminar shear stress and stretch force. Shear stress upregulated GPx-1 mRNA expression in a time- and force-dependent manner in BAECs, whereas stretch force was without effect. Furthermore, shear stress increased GPx activity. L-NAME, an inhibitor of nitric oxide synthase, did not affect shear stress-induced GPx-1 mRNA expression. The ability of laminar shear stress to induce GPx-1 expression in endothelial cells may be an important mechanism whereby shear stress protects vascular cells against oxidative stress.

Key Words: **hemodynamic force; shear stress; stretch force; glutathione peroxidase; antioxidant; oxidative stress; atherosclerosis; gene expression; endothelial cells.**

Mechanical forces such as shear stress and stretch force play important roles in maintaining the homeostasis of the vessel wall. Depending on the circumstances, however, they act as pathophysiological factors in cardiovascular diseases such as atherosclerosis and hypertension. Atherosclerosis occurs predominantly at low shear stress areas or in regions exposed to turbulent flow (1, 2). Shear stress modulates the process of atherosclerosis through its effects on endothelial-mediated alterations in vascular relaxation, coagulation, leukocyte and monocyte migration, smooth muscle growth, lipoprotein uptake and metabolism, and endothelial cell survival (3). These observations suggest that laminar high shear stress may have antiatherogenic properties. In contrast, stretch force stimulates cell proliferation, modulates cell phenotype (4), enhances the oxidative modification of LDL (5), and stimulates superoxide production (5, 6) on vascular smooth muscle cells. These cellular responses may promote the process of atherosclerosis. Since the degree of stretch force on vessel walls depends on blood pressure, the alteration of stretch force might be one of the pathological factors in the hypertensive state.

Imbalance between prooxidants and antioxidants like superoxide dismutase (SOD) causes oxidative stress, which, according to accumulating evidence, may play a pivotal role in atherogenesis. Laminar shear stress upregulates the expressions of Cu/Zn SOD (7) and Mn SOD (8) in endothelial cells; and this enhancement may be one of the mechanisms whereby high laminar shear stress protects vascular cells against oxidative stress. SOD potently converts superoxide anion to H_2O_2; however, H_2O_2 so formed has atherogenic properties such as growth promoting effects of smooth muscle cells (9) and enhancement of endothelial adhesiveness (10). Therefore, defense against H_2O_2 may be crucial in reducing oxidative stress. H_2O_2 is reduced by glutathione peroxidase (GPx) and catalase to H_2O. GPx, a soluble selenoprotein, is expressed in most tissues and plays an important role in cellular antioxidant defenses against H_2O_2 and organic hydroperoxides. It reduces H_2O_2 and organic hydroperoxides to H_2O and corresponding alcohols, respectively, as shown in the following formula:

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG \text{ or}$$

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O.$$

So far, five GPx isoenzymes have been identified: cytosolic and mitochondrial GPx (GPx-1) and phospholipid

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hydroperoxide GPx (GPx-4 or PHGPx) are found in most tissues; gastrointestinal GPx (GPx-2) and extracellular GPx (GPx-3) are localized in the gastrointestinal tract and kidney, respectively; and GPx-5, a selenium-independent GPx specifically expressed in mouse epididymis. GPx-1 is expressed ubiquitously and plays a central role in cellular defense against H_2O_2 and organic hydroperoxides.

Given the importance of mechanical and oxidative stress, investigation of the regulation of the antioxidant system might provide new insights into in the understanding of atherogenesis. In the present study, the effects of mechanical shear stress and stretch force on GPx-1 expression in bovine aortic endothelial cells were examined.

MATERIALS AND METHODS

Materials. GSH reductase, reduced GSH, NADPH and L-NAME were purchased from Sigma Chemical, Hepes from DOJINDO, sodium selenite and restriction enzymes from Wako Pure Chemical Industries, Ltd., and DMEM and fetal calf serum from GIBCO BRL.

Cell culture. Bovine aortic endothelial cells (BAECs) were isolated by scraping the internal surface of the thoracic aorta excised from a freshly slaughtered cow as previously described (11). BAECs were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated fetal calf serum. Confluent BAECs were seeded onto rectangular plastic tissue culture plates for shear stress experiments, ordinary tissue culture dishes for static controls, and laminin-coated deformable elastic dishes made of silicone for stretch force experiments and non-stretch controls. After reaching confluence, the cells were washed with and incubated in serum-free DMEM with 50 nM sodium selenite for 18 h at 37°C before being subjected to mechanical stress. Cells between passages 6 and 12 were used for the experiments.

Shear stress and stretch force experiments. BAECs were exposed to steady laminar shear stress as described by Levesque *et al.* with some modifications (12). Cells were grown on rectangular plates and placed in a parallel-plate flow chamber (9 cm long, 5.5 cm wide, 0.02 cm deep) that was part of a closed-loop arrangement where tissue culture media passed from a reservoir to the flow chamber through a roller pump and was then recirculated to the reservoir connected with tubing. The mean laminar shear stress (τ_w) to which the cells were exposed was calculated by the following formula: $\tau_w = (6\mu/h^2b)Q$, where μ is the dynamic viscosity measured by Ostwald viscosimeter, b, the flow chamber width, h, the flow chamber height, and Q, the flow rate. Shearing experiments were carried out in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes, 10 mM) buffered DMEM (pH 7.4) containing 50 nM sodium selenite, 100 μg/ml lilacillin, 15 μg/ml gentamicin and 2 μg/ml amphotericin B in a 100% air incubator at 37°C. Culture dishes were used as static controls under the same condition as those of the shearing experiments. BAECs subjected to laminar shear stress at 20 dynes/cm^2 (physiological artery level) over 12 h became elongated and aligned in the direction of flow, whereas cells at the venous level (5 dynes/cm^2) did not change morphologically.

To subject BAECs to stretch force, elastic dishes made of silicone rubber were used as described previously (5). In stretch experiments, the dishes were stretched by 10 or 20% in length along a single axis and incubated in DMEM containing 50 nM sodium selenite, 100 μg/ml lilacillin, 15 μg/ml gentamicin and 2 μg/ml amphotericin B in a humidified 5% CO_2 and 95% air incubator at 37°C.

Northern blot analysis. Total RNA was isolated from BAECs by guanidine thiocyanate-phenol extraction. To make a probe of GPx-1, the region of bovine GPx-1 cDNA (from 82 to 635) was amplified with forward primer (5′-CACAGTGTACGCCTTCTCCG) and reverse primer (5′-CCTGGGACAGCAGGGTTTCA) based on published sequences (13) from total RNA of BAECs by reverse transcription–polymerase chain reaction (RT–PCR). The amplified cDNA was inserted into the pCR 2.1 plasmid vector (TA Cloning Kit, Invitrogen). Orientation of the cDNA insert was confirmed by sequencing by the dideoxy termination method (Sequenase Version II DNA sequencing kit, USB). The cDNA insert was digested by *Eco*RI, and used as a probe for Northern blot analysis. Total RNA (15 μg per lane) of BAECs was electrophoresed on a 1% agarose-5% formaldehyde gel containing 0.5 μg/ml ethidium bromide and transferred to a nylon membrane (Hybond N+, Amersham) in 10× standard sodium citrate (SSC) solution. The membrane was baked for 1 h at 80°C and prehybridized in 50% formamide, 5× SSC, 0.5% SDS, 5× Denhardt's solution, and 40 μg/ml denatured herring sperm DNA for 1 h at 42°C. Bovine GPx-1 cDNA was random-primed and labeled using [α-^{32}P]dCTP as a probe. The denatured probe was added to the prehybridization solution and hybridized for 16 h at 42°C. After hybridization, the membrane was washed twice in 1× SSC and 0.1% SDS for 15 min at 55°C, then exposed to an imaging plate and analyzed on a Bio-Imaging Analyzer BAS 2000 (FUJI XEROX). Correction for loading variations was carried out by scanning the ethidium bromide staining of 18S ribosomal RNA using the NIH image.

Glutathione peroxidase activity. GPx enzymatic activity was assayed by the indirect, coupled test procedure as described by Paglia and Valentine (14). The oxidized glutathione (GSSG) produced during GPx enzyme reaction was immediately reduced by NADPH and glutathione reductase. Therefore, the rate of NADPH consumption was regarded as the rate of GSSG formation during the GPx reaction. BAECs were washed three times with ice-cold phosphate-buffered saline and gently scraped with lysis buffer containing protease inhibitors [50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 500 μM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 2 μM pepstatin A]. The cell suspension was homogenized with an ultrasonicator (4 × 5 s) on ice. Protein concentrations were determined by the method of Bradford with bovine serum albumin as the standard protein. The enzyme reaction was assayed in a buffer containing 100 mM sodium phosphate (pH 7.0), 2 mM sodium azide, 2 mM GSH, 200 μM NADPH, 1 unit/ml of glutathione reductase and 250 μM H_2O_2 at 25°C, and the rate of decrease in absorption of NADPH was followed at 340 nm. The activity was calculated using an extinction coefficient of 6.22 for NADPH and expressed as milliunits (mU) per milligram of protein, 1 mU representing 1 nmol GSH oxidized per minute.

Statistical analysis. Values in the figures are means ± SE, and statistical analysis was carried out using unpaired *t* test. *P* values <0.05 were considered significant.

RESULTS

Effect of Laminar Shear Stress on GPx-1 mRNA Expression in BAECs

Selenium is required for GPx expression since it is incorporated into selenoproteins by recognizing stop codon UGA as a codon for selenocystein (15). Our examination of the effect of selenium revealed that 50 nM sodium selenite was sufficient for enhancing GPx-1 mRNA expression in BAECs (data not shown); this quantity was therefore added to the media in the following all experiments.

As shown in Fig. 1A, exposure of BAECs to steady laminar shear stress at 20 dynes/cm^2 resulted in a time-dependent increase in GPx-1 mRNA levels; the

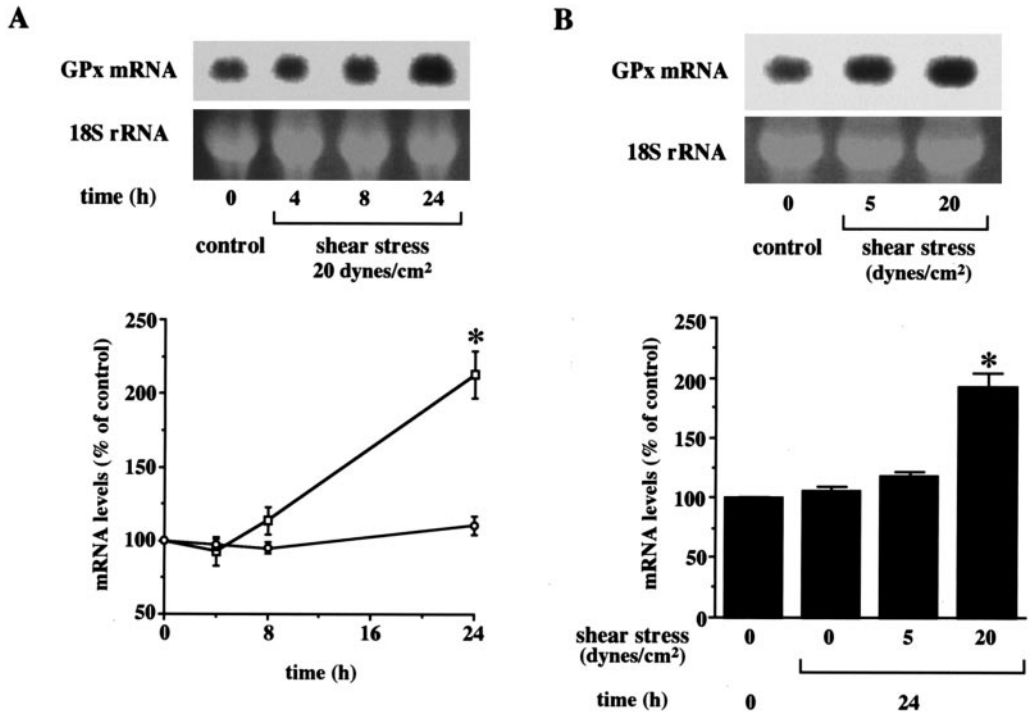

FIG. 1. Time course and dependence on the degrees of GPx-1 mRNA expression by laminar shear stress in bovine aortic endothelial cells (BAECs). BAECs were exposed to laminar shear stress at 20 dynes/cm^2 for the indicated time periods, and Northern blot analysis was carried out (A). □ indicates laminar shear stress, ○; static control. BAECs were exposed to laminar shear stress at 0, 5, or 20 dynes/cm^2 for 24 h (B). Representative blot and relative levels of mRNA are shown. Correction for loading variations was carried out by scanning the ethidium bromide staining of 18S ribosomal RNA in each experiment. Data represent the mean values ± SE of three independent experiments. *$P <$ 0.01 vs control.

increase appeared 8 h after the application of shear stress and reached a peak level at 24 h (2.1 ± 0.16 fold vs static control, $n = 6$, $P < 0.01$). Figure 1B shows the effect of shear force on the levels of GPx-1 mRNA expression in BAECs exposed to low (5 dynes/cm^2) and high (20 dynes/cm^2) laminar shear stress for 24 h; the levels increased significantly at high shear stress, at the physiological artery level, but not significantly at low shear stress.

Effect of Laminar Shear Stress on GPx Enzymatic Activity in BAECs

As shown in Fig. 2, exposure of BAECs to steady laminar shear stress at 20 dynes/cm^2 for 24 h increased the enzymatic activity of GPx from 3.2 to 4.9 mU/mg protein (1.52- ± 0.16-fold vs static control, $n = 5$, $P < 0.05$).

Effect of Stretch Force on GPx-1 mRNA Expression in BAECs

As shown in Fig. 3, application of stretch force to BAECs in stretching dishes by 10 and 20% for 24 h did not enhance GPx-1 mRNA expression, rather it tended to decrease it.

Effect of Nitric Oxide on Shear Stress-Induced GPx-1 mRNA Expression in BAECs

Shear stress enhances the expression of endothelial constitutive NO synthase (ecNOS), increase NO pro-

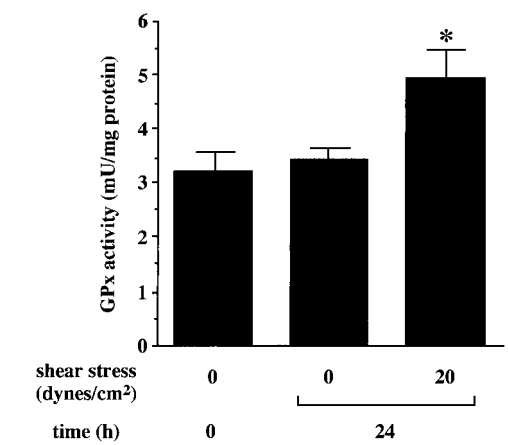

FIG. 2. Effect of laminar shear stress on GPx enzymatic activity in BAECs. BAECs were exposed to laminar shear stress at 20 dynes/cm^2 for 24 h, and GPx enzymatic activity was measured as described under Materials and Methods. Data represent the mean values ± SE of five independent experiments. *$P < 0.05$ vs control.

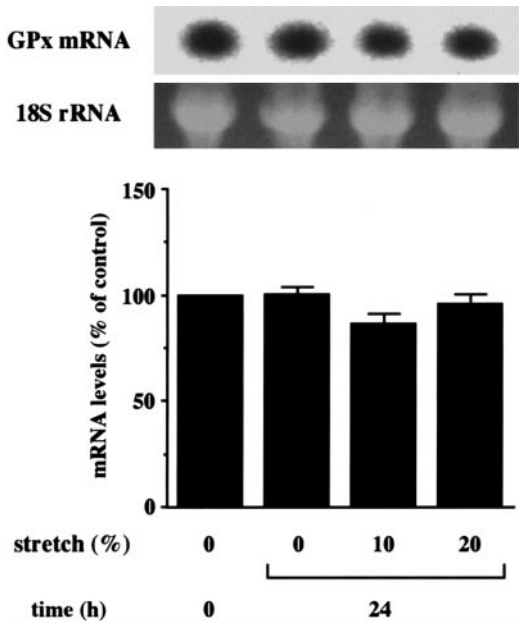

FIG. 3. Effect of stretch force on GPx-1 mRNA expression in BAECs. BAECs were subjected to stretch force of 10 or 20% for 24 h, and Northern blot analysis was carried out. Representative blot and relative levels of mRNA are shown. Correction for loading variations was carried out by scanning the ethidium bromide staining of 18S ribosomal RNA in each experiment. Data represent the mean values ± SE of four independent experiments.

duction (16) and is one of the principal stimuli for release of NO. Our examination of the involvement of NO in shear stress-induced GPx-1 mRNA expression with 1 mM NOS inhibitor, L-NAME, revealed that exposure of BAECs to steady laminar shear stress at 20 dynes/cm^2 for 24 h had no affect on the expression (Fig. 4).

DISCUSSION

The present study demonstrated that a physiological level of laminar shear stress induced sustained upregulation of GPx-1 mRNA expression and GPx enzymatic activity in endothelial cells, whereas stretch force did not have any significant effect.

In developing atherosclerotic lesions, enhanced production of reactive oxygen species such as O_2^-, H_2O_2, and hydroxyl radicals results in oxidative stress within the vascular wall and further promotes atherosclerosis (17, 18). H_2O_2 itself has atherogenic properties and is reduced to hydroxyl radicals in the presence of transitional metals through the Fenton reaction. Hydroxyl radicals also are strong prooxidants. GPx is essential for removing H_2O_2 and subsequent products, hydroxyl radicals *in vivo* (19). Indeed, GPx has been demonstrated to be much more effective than both SOD and catalase in protecting human cells exposed to O_2 (20). Furthermore, recent studies have demonstrated that the GPx enzymatic activity is markedly reduced in human atherosclerotic plaques (21) and that GPx is inactivated by some oxidant species such as myeloperoxidase-derived hypochlorous (22) and 4-hydroxynonenal (23). Therefore, GPx might be a key enzyme in protecting vessels against oxidative stress and atherogenesis. On the other hand, the distribution of hemodynamic forces is thought to contribute to the development of atherosclerosis. Pathological observations have demonstrated that the regions exposed to low shear stress are more prone to developing atherosclerosis than are high shear regions (1). In endothelial cells, laminar shear stress modulates various kinds of gene expression such as those of ecNOS (16), endothelin-1 (24), cyclooxygenase-2 (8), Cu/Zn and Mn SOD (7, 8), C-type natriuretic peptide (CNP) (25), platelet-derived growth factor (PDGF) (26, 27), angiotensin converting enzyme (ACE) (28), tissue factor (29), monocyte chemotactic protein-1 (MCP-1) (30), intercellular adhesion molecule-1 (ICAM-1) (31), and vascular cell adhesion molecule-1 (VCAM-1) (32). These genes have various patterns of gene expression in response to shear stress. Laminar high shear stress induces sustained upregulation (greater over 24 h) of the ecNOS, CNP and SOD genes, the products of which have antiatherogenic or antioxidative properties. The upregulation of the ICAM-1, MCP-1, and tissue factor gene expression is transient, whereas the expression of the

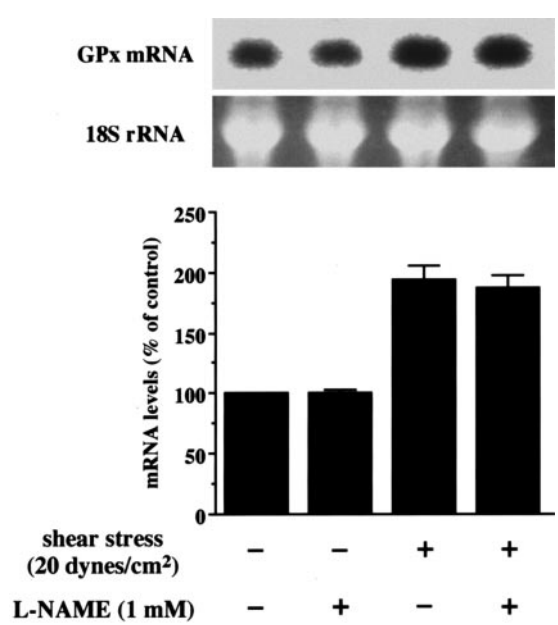

FIG. 4. Effect of NO on shear stress-induced GPx-1 mRNA expression in BAECs. BAECs were exposed to laminar shear stress at 20 dynes/cm^2 for 24 h with or without 1 mM L-NAME, and Northern blot analysis was carried out. Representative blot and relative levels of mRNA are shown. Correction for loading variations was carried out by scanning the ethidium bromide staining of 18S ribosomal RNA in each experiment. Data represent the mean values ± SE of three independent experiments.

ACE, endothelin-1 and VCAM-1 genes is downregulated by laminar shear stress. The present data demonstrated that laminar shear stress at the physiological artery level induced sustained upregulation of GPx-1 gene expression, but that low shear stress did not. Laminar high shear stress may be requisite to maintaining the antioxidant state in vessel walls; therefore, reduced expression of GPx-1 might be one of the reasons for the susceptibility of the low shear regions to developing atherosclerosis. In contrast, stretch force failed to upregulate GPx-1 gene expression in endothelial cells. Since stretch force enhances superoxide generation in vascular cells, it could cause the imbalance between antioxidants and prooxidants and contribute to the pathogenesis of vascular diseases.

Several mechanisms are involved in the regulation of gene expression in response to shear stress. The nucleotide sequence GAGACC, termed shear stress responsive element (SSRE), has been shown to enhance promoter activity of PDGF-B chain (27). NFκ-B binds to SSRE and promotes PDGF-B chain gene expression in response to shear stress (33). In addition, activation of AP-1 has been shown to be responsible for shear stress-induced MCP-1 gene expression (34). Both SSRE (GAGACC, −432) and AP-1 consensus sequence (TGACTCA, +488) exist in the 5′ flanking region and the intron of the GPx-1 gene, respectively (35). The regulatory mechanisms of shear stress-induced GPx-1 gene expression were unclear in the present study; however, the kinds of intracellular signal pathways responsible for the upregulation of GPx-1 gene expression need to be elucidated in future studies.

Laminar shear stress upregulates GPx-1 gene expression and GPx enzymatic activity in endothelial cells. Given the importance of oxidant stress in atherogenesis and the antioxidative effects of GPx, these findings may signify an important mechanism whereby shear stress protects vascular cells against atherosclerosis.

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