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# Cyclic Strain Induces Redox Changes in Endothelial Cells

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## Abstract

**Our previous studies have shown that cyclic strain to endothelial cells (ECs) increases reactive oxygen species (ROS) that act as second messengers. The potential impact of these enhanced ROS levels on ECs was examined by studying the antioxidant activities and heme oxygenase-1 (HO-1) expression in strained ECs. Cyclic strain to ECs increased lipid peroxidation and augmented oxidation of low-density lipoproteins. ECs subjected to strain increased their superoxide dismutase activities. Concomitantly, glutathione peroxidase activities increased in 3 to 6 hr and returned to basal level 24 hr after continuous cyclic strain treatment. A decrease of glutathione (GSH) was accompanied with an increase of oxidized glutathione (GSSH) level in ECs 3 to 6 hr after strain treatment. This was followed with a return of both GSH and GSSH to basal levels in 24 hr. Consistently, $H_2O_2$ treatment of ECs decreased the GSH/GSSG ratio. ECs pretreated with catalase abolished the strain-induced change in GSH/GSSG. Strain treatment, similar to $H_2O_2$ exposure, induced HO-1 expression in a time-dependent manner. This induction was inhibited after treating ECs with catalase or free radical scavenger. ECs treated with N-acetyl-cysteine abolished HO-1 gene induction. Our results suggest that cyclic strain-induced ROS cause a transient increase of glutathione peroxidase activity that results in a decrease of GSH level in ECs and that this decrease is crucial to HO-1 induction.**

**Key Words: hemodynamic forces, reactive oxygen species, glutathione**

## Introduction

Endothelial cells (ECs) lining vascular walls are constantly under the influence of hemodynamic forces including cyclic strain due to pulsatile pressure and wall compliance that can have profound effects on endothelial function (7, 14, 20). Although the molecular mechanisms that transmit the applied cyclic strain to intracellular signals remain unclear, studies including ours indicate that cyclic strain modulates vascular gene expression including endothelin-1 (Et-1) (38), monocyte chemotactic protein-1 (MCP-1) (39) and intercellular adhesion molecule-1 (ICAM-1) (9).

Cells constantly produce reactive oxygen species (ROS) including superoxide, hydrogen peroxide and hydroxyl radicals during electron transport. Enhanced ROS production is common in cells when they are activated. Evidence suggests that these intracellular ROS can act as second messengers in cells exposed to various stimuli. ROS have been shown to be involved in the activation of transcription factors such as AP-1 and NFκB (4, 21, 27, 36). Our previous studies indicate that intracellular ROS levels are induced in ECs exposed to hemodynamic forces (8, 11, 18, 41, 42). Evidence that these elevated ROS were directly involved in shear- or strain- induced gene expression was based on observations that antioxidant pretreatment of cells inhibited hemodynamic forces-induced gene expression of MCP-1, ICAM-1, Egr-1

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and c-*fos* as well as plasminogen activator inhibitor-1 (PAI-1) release (8, 9, 11, 18, 41, 42). Therefore, we conclude that intracellular ROS play an important role in mediating hemodynamic forces-induced gene expression.

While intracellular ROS levels are elevated, the potential damage of ROS has to be carefully monitored in ECs. To prevent the oxidative stress caused by excess production of ROS, cells have evolved elaborate antioxidant defense mechanisms that include the utilization of non-enzymatic antioxidants (vitamin C, vitamin E and glutathione) and invigorated enzymatic activities such as superoxide dismutase (SOD), catalase, glutathione peroxidase and reductase (13, 23, 28, 29). We previously demonstrated (9, 18) that rapid induction of catalase activities was detected in strain- and shear- treated ECs. In these earlier studies, the increase of catalase activities was apparently hemodynamic forces-dependent because they returned to control basal levels after the removal of forces.

In addition to the induction of antioxidant activities, many cells respond to oxidative stress by synthesizing antioxidant proteins. Among those proteins, the heme degrading enzyme heme oxygenase (HO-1) is consistently activated by oxidative stress (28). The magnitude of HO-1 induction after oxidative stress and the wide distribution of this enzyme in systemic tissues coupled with the intriguing biological activities of the catalytic byproducts, carbon monoxide, iron and bilirubin, make HO-1 a highly attractive candidate for serving as the stress-response protein. Since the induction of the HO-1 gene leads to the degradation of pro-oxidant heme and production of antioxidant biliverdin, it represents a potentially potent cellular defense mechanism. Shear stress and cyclic strain induce the expression of HO-1 in smooth muscle cells (37). Whether intracellular ROS levels contribute to HO-1 induction by the oxidative agents or hemodynamic forces remain to be confirmed. The change of ROS levels in cells may influence the intracellular glutathione concentration and consequently affect redox status (2, 13, 23, 29). Intracellular glutathione (GSH) is a major thiol reservoir that serves to maintain proper redox state and protects cells from potential oxidative damage (2). The imbalance of intracellular redox status may participate in triggering various cellular responses during cardiovascular disorders (5, 12, 30, 40). GSH also modulates redox-sensitive transcription factors (40) that consequently alter gene expression.

We have previously indicated that ROS are involved in the hemodynamic forces-induced expression of various atherosclerosis-associated genes (10, 11, 18, 41, 42). The present study further demonstrates that intracellular redox changes are involved in the cyclic strain-induced HO-1 expression in ECs. Our study thus illustrates the importance of intracellular redox status in modulating endothelial responses to hemodynamic forces. The imbalance of intracellular redox status may trigger gene expression and consequently progress into various vascular disorders including atherosclerosis.

## Materials and Methods

### *Materials*

Media and chemicals used for cell cultures were purchased from Gibco (Grand Island, NY, USA). Sodium arsenite, a trivalent arsenic compound $NaAsO_2$, was obtained from E. Merck (Darmstradt, Germany). DCFH was purchased from SERVA, Germany. Other chemicals of reagent grade were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

### *EC Cultures*

Human umbilical vein ECs were isolated from human umbilical cord as previously described (16). ECs were grown in petri dishes and seeded onto the silicone membrane base of a cell plate (Flexcell, Mekeesport, PA) by 1 % trypsin solution (Gibco) until the monolayer became confluent. The culture medium was changed to medium 199 containing only 2% fetal bovine serum (FBS), and the cells were incubated overnight prior to the experiment.

### *In vitro Cyclic Strain on Cultured ECs*

The strain unit Flexcell FX-2000 (Flexcell, McKeesport, PA) consisted of a vacuum unit linked to a valve controlled by a computer program as previously described (10). ECs cultured on the flexible membrane base were subjected to cyclic strain produced by this computer-controlled application of sinusoidal negative pressure. The flexible membranes supporting the cultured cells were deformed by a sinusoidal negative pressure with a peak level of ~20 kPa which produced an average strain of 12% at a frequency of 1 Hz (60 cycles/min) for various periods of time. After the strain treatment, the cells were lysed for further analysis.

### *Measurement of Intracellular ROS*

ROS levels were estimated by a method previously described (19). Strained ECs were scraped and re-suspended in PBS followed by incubation in 50 μM non-polar 2, 7 -dichlorofluorescein diacetate (DCFDA) for 30 min at 37°C in the dark, and stopped

by centrifugation at 200 Xg for 5 min. The cell pellets were re-suspended in 1 ml of PBS. The relative fluorescence intensity of DCF, which is formed by peroxide oxidation of its non-fluorescent precursor, was detected at an emission wavelength of 525 nm by using an excitation wavelength of 475 nm with a Hitachi 4010 fluorescent spectrophotometer. DCFH with fresh medium was used as a blank control.

*Measurement of Lipid Peroxidation*

The extent of oxidation of the total cell lipoprotein was measured by thiobarbituric acid reaction assay (TBARS) as equivalents of MDA (malondialdehyde) per mg protein (43) in a microtiterplate. In brief, conditioned ECs were scraped and counted by trypan blue exclusion. ECs ($10^6$ cells) were centrifuged, re-suspended in PBS and subjected to sonication (Sonicator, Heat System, Ultrasonics Inc. NY) Tetramethoypropane (TMP) was used as a standard for MDA. In some experiments, TBARS were analyzed for the oxidation of low-density lipoproteins (LDL, 50 μg/ml) in medium from strained cells.

*RNA Isolation and Northern Blot Analysis*

Total RNA was isolated from ECs by the guanidium isothiocyanate/phenol-chloroform method as described (39). RNA (10 μg/lane) was separated by electrophoresis on a 1.2% agarose formaldehyde gel and transferred onto a nylon membrane (Nytran, Schleicher & Schuell Inc.) by a vacuum blotting system (VacuGene XL, Pharmacia, Piscataway, NJ). This membrane was hybridized with a $^{32}$P-labeled HO-1 cDNA probe. This membrane was then washed with 1x SSC containing 1% SDS at room temperature for 30 min and exposed to X-ray film (Kodak X-Omat-AR) at -7°C. Autoradiographic results were scanned and analyzed with a densitometer (Computing densitometer 300S, Molecular Dynamics, Sunnyvale, CA).

*Immunoblot Analysis*

Proteins were extracted in SDS buffer and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of cell protein was determined using a BCA protein assay kit (PIERCE, USA). The HO-1 was transferred onto a nitrocellulose membrane and detected immunologically using rabbit anti-human HO-1 antibody (StressGen Biotechnologies Corp., Victoria, BC, Canada). Antigen-antibody complexes were detected using horseradish peroxide-conjugated goat anti-rabbit IgG and an ECL detection system (Amersham International plc, Buckinghamshire, England).

*Assay of Glutathione Content*

Total glutathione levels including the reduced (GSH) and oxidized glutathione (GSSG) were assayed by incubating 100 μl of cell lysate with 800 μl of reaction buffer containing 0.3 mM NADPH, 125 mM sodium phosphate, 6.3 mM EDTA and 100 μl of 6 mM DTNB (17). After addition of 25 U/ml GSH reductase, the change in optical density at 412 nm was measured as total glutathione level. To measure GSSG, GSH in samples was modified by adding 2 μl of 20 mM 4-vinylpyridine per 125 μl of sample solution and mixing vigorously for 1 min. The concentration of GSH was calculated as the difference between total glutathione levels and GSSG. The results were expressed as the amount of glutathione (μg) per mg protein.

*Superoxide Dismutase (SOD) and Glutathione Peroxidase (GP) Assay*

Strained ECs were washed, suspended in potassium-phosphate-buffer (120 mM, pH 7.0) and then sonicated. Superoxide dismutase (SOD) was measured as previously described (31). Briefly, ECs were scraped from plates and suspended in ice-cold water. After vortexing, ECs were extracted by adding ice-cold ethanol/chloroform (62.5/37.5, v/v). After vortexing and centrifugation, the upper aqueous layer was collected for assay. SOD activity was measured by using a SOD-525 assay kit (Bioxytech, Marne cedex, France). Glutathione peroxidase activity was assayed according to the method of Lawrence and Burk (26). This assay is based on monitoring the oxidation of NADPH to NADP at 340 nm. An enzyme unit was defined as the amount of enzyme causing oxidation of 1 μmol of NADPH per minute.

*Statistical Analysis*

Statistical analysis was carried out using the Student's t-test. Data are expressed as mean±SEM. Statistical significance was defined as $P<0.05$.

## Results

*Cyclic Strain Induces ROS Generation and Lipid Peroxidation*

Intracellular ROS levels were determined by the fluorescent intensity of the peroxidative product of DCFH. ECs exposed to 12% cyclic strain increased 2.9-fold in fluorescent intensity compared to static

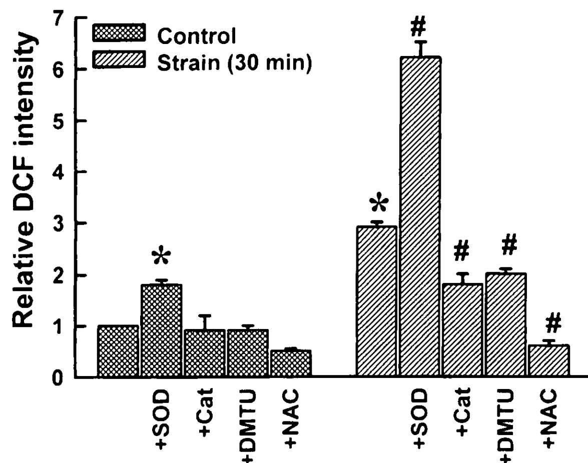
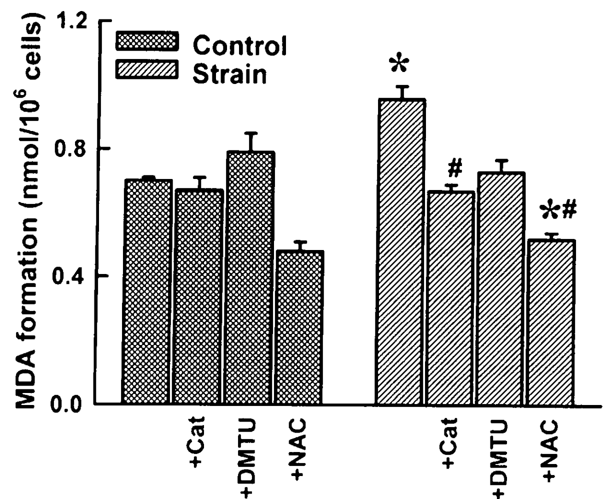



Fig. 1. Cyclic strain increased intracellular ROS in ECs. ECs under static or cyclic strain treatment were scraped and suspended in phosphate buffer saline containing 50 μM DCFH as described in methods. In some experiments, ECs were preincubated with superoxide dismutase (SOD), catalase (Cat, 300 U/ml) or dimethyl-thiourea (DMTU, 5 mM) for 0.5 hr before strain treatment. ROS were detected by the fluorescence intensity of the oxidative product of DCFH. Data are shown as mean±SE in terms of relative intensity compared to control static cells from at least three separate experiments. *P<0.05 vs. unstrained control cells. #P<0.05 vs. strained cells.



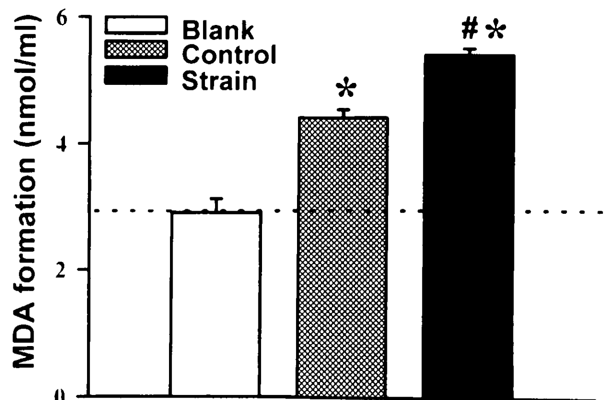




Fig. 2. Cyclic strain induces lipid peroxidation in ECs. A. ECs were in static condition or subjected to cyclic strain for 48 hr. In some experiments, ECs were pretreated with catalase (Cat, 300 U/ml), dimethyl-thiourea (DMTU, 5 mM) or N-acetyl-cysteine (NAC, 20 mM) for 0.5 hr before strain treatment. Total cell lysate was measured for the oxidation of lipoprotein by thiobarbituric acid reaction assay (TBARS) as equivalents of MDA (malondialdehyde) per mg protein in a microtiter plate. *P<0.05 vs. unstrained control cells. #P<0.05 vs. strained cells. B. Low density lipoprotein (LDL) was co-incubated with control or strained ECs for 24 hr. LDL in cell-free medium was used as blank. LDL in the media was analyzed for MDA formation. The values are shown as mean±SE expressed in equivalents of MDA per mg protein.

controls (Fig. 1). This result agrees with our previous study that indicated that cyclic strain increases intracellular superoxide levels according to analysis using the lucigenin method (10). The increased superoxide was apparently quickly converted into $H_2O_2$ via superoxide dismutase (SOD) because the addition of SOD to the cellular lysate rapidly increased DCF levels in both control and strained groups. However, these increases did not affect the induction ratio of strained vs control cells. These strain-induced ROS were inhibited after pretreating ECs with free radical scavengers; i.e., catalase, dimethyl-thiourea (DMTU, a hydroxyl radical scavenger) or N-acetyl-cysteine (NAC, a glutathione precursor) (Fig. 1). The inhibition of ROS induction by free radical scavengers indicates that strain-induced ROS are mainly derived from hydrogen peroxide. Due to the increase of intracellular ROS, we further analyzed the lipid peroxidation by MDA formation in strained cells. In ECs subjected to strain for 48 hr, an augmentation of lipid peroxidation was indicated by a 33.3% increase in MDA formation. The increase of lipid peroxidation was reduced after treating cells with an antioxidant, i. e., catalase, DMTU or NAC (Fig. 2A). ECs are known to be able to oxidize low-density lipoprotein (LDL). To further demonstrate the effect of cyclic strain on lipid peroxidation of LDL, LDL was added and co-incubated with ECs during cyclic strain. As shown in Fig. 2B, LDL added in cell-free medium as a blank control resulted in a MDA value of 2.92±0.22 after 24 h of incubation. In LDL co-incubated with control static cells, MDA formation increased to 4.4±0.13. However, co-incubation of LDL with strained ECs further elevated the MDA value to 5.4±0.1 – a 23% increase compared to static control cells (Fig. 2B). These results indicate that cyclic strain treatment of ECs increases ROS levels that subsequently increase intracellular lipid peroxidation and accelerate LDL oxidation.

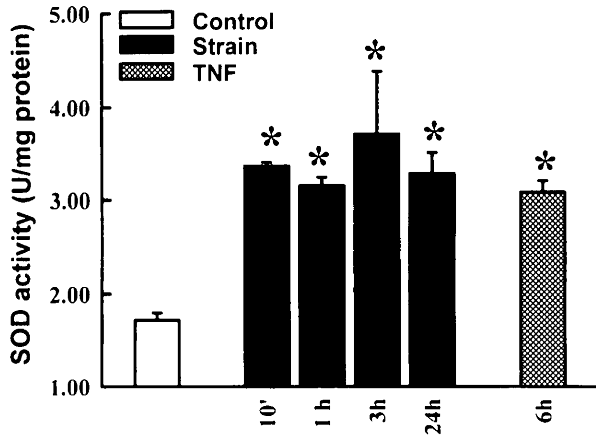



Fig. 3. Cyclic strain induces superoxide dismutase activity in ECs. ECs were in static condition (C) or subjected to cyclic strain (S) for 0.5, 1, 3 or 24 hr. ECs were removed and treated with ethanol/ chloroform. The aqueous layer was collected and the superoxide dismutase (SOD) activity was measured. ECs treated with tumor necrosis factor (TNF) for 6 h were used as positive controls.

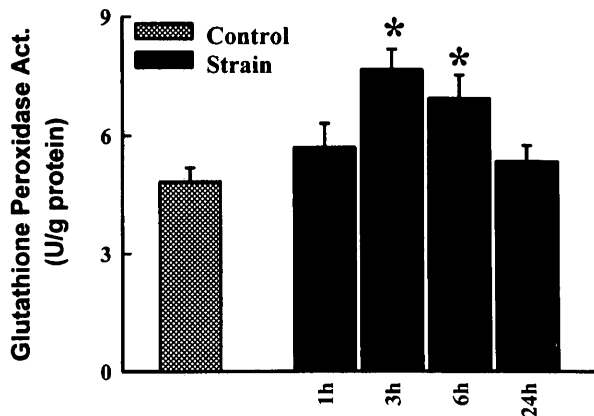



Fig. 4. Cyclic strain increases glutathione peroxidase activity in ECs. ECs were in static condition or subjected to cyclic strain for 1, 3, 6 or 24 h. Glutathione peroxidase (GP) activity was monitored by oxidation of NADPH to NADP. Enzyme units were defined as the amount of enzyme causing oxidation of 1 μmol of NADPH per min. *P<0.05 vs. unstrained control cells.

*Cyclic Strain Induces Endothelial Superoxide Dismutase and Glutathione Peroxidase Activities*

Strained ECs rapidly increased intracellular ROS levels via superoxide generation. Because superoxide is converted to hydrogen peroxide via superoxide dismutase, we next investigated the superoxide dismutase (SOD) activity in strained cells. As shown in Fig. 3, SOD activity in ECs after strain treatment was rapidly induced by about 2-fold vs. control static cells. The increase of SOD activity was maintained at an elevated level as strain continued. It is known from previous study that the induction of SOD activity results in an increase of $H_2O_2$. To prevent oxidative stress, catalase and glutathione peroxidase may be activated or upregulated to reduce intracellular $H_2O_2$ levels. We previously demonstrated that intracellular catalase activity was activated and remained in an elevated level as cyclic strain continued (10). In the present study, we analyzed the glutathione peroxidase activity in the strained ECs. As shown in Fig. 4, cyclic strain to ECs stimulated glutathione peroxidase activity within 6 hr after strain treatment. However, this enzyme activity returned to basal level 24 hr after continuous strain treatment. This result indicates that ECs exposed to cyclic strain transiently increase glutathione peroxidase activities whose functions are to remove the excess ROS generated in strained cells.

**Table 1. Effect of Cyclic Strain on the Levels of Total Glutathione (GSH+GSSG), Reduced Glutathione (GSH), Oxidized Glutathione (GSSG) and GSH/GSSG Ratio in Cultured ECs**

| | GSH+GSSG | GSH | GSSG | GSH/GSSG |
|---|---|---|---|---|
| C | 40.0±1.6 | 35.4±1.4 | 4.6±0.5 | 7.7±0.2 |
| S1 | 38.0±4.1 | 32.9±2.8 | 5.1±0.5 | 6.3±0.6 |
| S3 | 31.8±2.3* | 24.9±2.5* | 6.9±0.5* | 3.7±0.7* |
| S6 | 34.2±2.1* | 27.2±2.2* | 6.8±0.4* | 4.0±0.7* |
| S24 | 43.8±3.4 | 38.1±4.5 | 5.7±0.7 | 6.7±0.3 |
| C+Cat | 41.4±2.7 | 37.0±3.6 | 4.4±0.3 | 7.1±0.3 |
| S3+Cat | 39.1±3.3# | 34.5±2.9# | 5.1±0.4# | 6.7±0.3# |

*P<0.05 vs. control; #P<0.05 vs. S3. All data are presented as glutathione level (μg) per mg of cellular protein from at least 3 separate experiments.

*Cyclic Strain Affects Intracellular GSH and GSSG Levels*

ECs after strain treatment changed their intracellular glutathione (GSH) and oxidized glutathione (GSSG) concentrations as well as their GSH/GSSG ratio (Table 1). ECs after 3 and 6 hr of strain treatment decreased GSH concentration by approximately 30% compared to that of static controls. Similar to the change of glutathione peroxidase, this decrease was transient because the GSH level in ECs returned to normal basal level 24 hr after continuous cyclic strain treatment. While GSH concentration decreased within 6 hr after strain, GSSG content increased about 48%. The GSH/GSSG ratio was thus reduced. Pretreatment of ECs with catalase abolished the change of strain-induced GSH/GSSG ratio. Similarly, ECs exposed to $H_2O_2$ also decreased the GSH/GSSG ratio (Table 2). This result indicates that ECs subjected to cyclic strain, similar to $H_2O_2$ treatment, cause a transient decrease of GSH content in ECs.

**Table 2. GSH/GSSG Ratio in Cultured ECs after $H_2O_2$ Exposure**

| $H_2O_2$ | GSH/GSSG |
|---|---|
| C | 8.3±0.2 |
| 1h | 3.1±0.5* |
| 3h | 3.4±0.4* |
| 6h | 2.3±0.6* |
| 4h | 7.1±0.1 |

*P<0.05 vs. control static ECs from 3 to 6 separate experiments

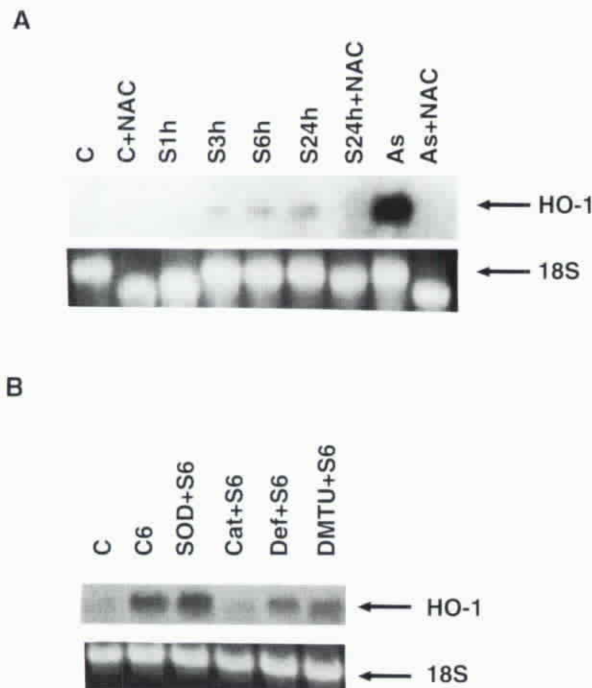



Fig. 5. ROS mediate strain-induced HO-1 expression. A) Time course of strain- induced HO-1 gene expression. The confluent monolayer of ECs were in static condition (C) or subjected to cyclic strain (S) for 1, 3, 6 or 24 hr. In some experiments, ECs were pretreated with N-acetyl-cysteine (NAC, 20 mM) for 0.5 hr before strain treatment. ECs incubated with sodium arsenite (As) for 3 h were used as positive controls. B) ROS mediate strain-induced HO-1 gene expression. ECs were strained for 6 hr (S6) after treating with superoxide dismutase (SOD), catalase (Cat, 300 U/ml), deferoxamine (Def, 1 mM) or 1,3-dimethyl-2-thiourea (DMTU, 5mM). Total RNA was analyzed for HO-1 levels. Equal amounts of RNA(10 µg) applied to each lane are demonstrated by the 18S RNA shown for each lane. Data are representative of triplicate experiments.

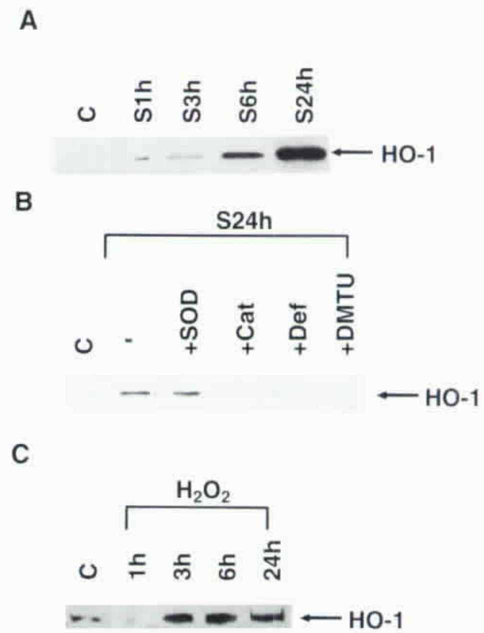



Fig. 6. Cyclic strain induced HO-1 protein levels. A) ECs were in static condition (C) or subjected to cyclic strain (S) for 1, 3, 6 or 24 h. In some experiments, ECs were pretreated with N-acetyl-cysteine (NAC, 20 mM) for 0.5 hr before strain treatment. ECs treated with sodium arsenite (As) for 3 hr were used as positive controls. B) Antioxidant inhibits HO-1 protein expression. ECs pretreated with superoxide dismutase (SOD), catalase (Cat, 300 U/ml), deferoxamine (Def, 1 mM) or 1, 3-dimethyl-2-thiourea (DMTU, 5 mM) followed with cyclic strain for 24 hr. Protein levels were analyzed by Western blot on total cell lysate. Data are representative of triplicate experiments. C) $H_2O_2$ induces HO-1 protein expression in ECs. ECs exposed to 100 mM $H_2O_2$ for 1 (H1), 3 (H3), 6 (H6) or 24 hr (H24).

*Cyclic Strain Induced Heme Oxygenase-1 Gene Expression*

ECs subjected to cyclic strain increased HO-1 gene expression (Fig. 5A). This increase was clearly observed 3 h after strain treatment. This increase was sustained and remained in an elevated level up to 24 hr. This strain-induced HO-1 expression was significantly inhibited after treating ECs with an antioxidant, i.e., NAC, catalase, deferoxamine (an iron chelator) or DMTU. In contrast, SOD treatment of ECs had no effect on strain-induced HO-1 induction (Fig. 5B). Similar induction in the levels of HO-1 protein was also shown from results of Western blotting (Fig. 6A). Cyclic strain induced a time-dependent increase in HO-1 protein levels, with a maximal level shown at 24 hr after cyclic strain treatment. Similarly, this induction was strongly inhibited after treating ECs with NAC, catalase, deferoxamine or DMTU (Fig. 6B). In parallel experiments, addition of 100 µM $H_2O_2$ to ECs resulted in a similar induction of HO-1 expression (Fig. 6C). These results indicate that cyclic strain, similar to $H_2O_2$ treatment, alters redox state and results in HO-1 induction.

## Discussion

The increase of intracellular ROS levels

associated with redox changes has been linked to cellular activation (5, 12, 30, 40). Although the mechanisms of ROS effects on cellular metabolism are still unclear, substantial evidence suggests that ROS may act as a modulator in the signaling pathway (4, 21, 27, 36) followed by transcriptional activation that contributes to endothelial gene induction. We have shown that cyclic strain induces a sustained increase of ROS according to analyses using an indicator lucigenin or DCF which measure mainly superoxide anion or $H_2O_2$, respectively (10, 42). In this study, we demonstrated that cyclic strain-induced ROS levels indicated by DCF intensity was inhibited after treating ECs with catalase, DMTU or NAC. In contrast, SOD treatment of cellular lysate enhanced these ROS levels, thus confirming an increase of superoxide levels by cyclic strain. This SOD treatment, however, did not alter the ROS ratio of strained vs. static ECs. We previously demonstrated that cyclic strain-induced ROS activate the signaling pathway Ras/Raf/ERK (41) and result in an increase of gene expression of *c-fos* (18), early growth response-1 (41), MCP-1 (42) and ICAM-1 (10, 11). We further showed that AP-1 binding activity in the MCP-1 promoter was increased in strained ECs, a phenomenon similar to that in ECs treated with $H_2O_2$ (42). Together, these results indicate that cyclic strain to ECs increases intracellular superoxide followed by $H_2O_2$ formation and that these increased ROS modulate endothelial responses.

Due to their constant interaction with blood, ECs are potential targets of activated compounds derived from stimulated white blood cells and blood components such as oxidized LDL or cytokines. These disturbed ECs generate ROS that may trigger initial responses including immediate early gene expression in ECs. Sustained ROS levels resulting in altered intracellular redox status may progress into chronic injury of the vascular wall. The alteration of redox status in the vascular wall results in the recruitment of monocytes into the vascular wall and is the underlying mechanism for atherogenesis (33, 44). In the present study, cyclic strain, a component of pulsatile pressure, induced ROS levels that were followed by an increase of lipid peroxidation in the cell membranes. This increased lipid peroxidation could be attenuated after treating ECs with an antioxidant. Increased ROS levels also enhanced LDL oxidation when LDL was co-incubated with strained cells. Thus, the change of redox status caused by increased ROS during cyclic strain to ECs may provide some insights into how hypertension accelerates atherogenesis.

It is becoming clear that oxidant-induced genes and/or their resulting proteins play vital roles in the adaptive and/or protective response in cells subjected to oxidant stress. HO-1 is one such responsive protein. HO-1 induced by a variety of oxidative agents decreases the concentration of intracellular heme and concomitantly increases the antioxidant bilirubin. Bilirubin, an efficient scavenger of singlet oxygen, is capable of reacting with superoxide anion and peroxyl radical (34). HO-1 induction, as part of a protective response, also modulates vessel tone and blood pressure by virtue of its product, carbon monoxide, to increase cGMP via activating intracellular guanylate cyclase (35). Studies of the promotor region of HO-1 revealed several transcriptional responsive elements including AP-1, NFκB and IL-6 that are known to be sensitive to oxidative stress (1, 6, 25). HO-1 induction in artery walls inhibits the migration of monocytes triggered by oxidized LDL (22). In our study, catalase but not SOD, inhibits cyclic strain-induced HO-1 expression. Both catalase and SOD are large molecules and a half-hour preincubation with ECs is not likely for them to entry into cells. However, catalase is able to remove the intracellular produced $H_2O_2$ because $H_2O_2$ is relatively stable and permeable to cell membrane. In contrast, the superoxide has short half-life and not accessible to extracellular SOD. In addition to catalase, Def and DMTU treatment of ECs showed partial inhibition of HO-1 induction, indicating hydroxyl radical plays a role. Although the relatively stable $H_2O_2$ may be the main contributing species, our results indicate that its related species, superoxide and hydroxyl radical, are also involved in the strain induced HO-1 expression. Thus, cyclic strain exerts its effects on ECs by HO-1 induction in a manner similar to that of $H_2O_2$ treatment. Similarly, HO-1 is induced after depleting GSH in cells (24, 32). Thus, treating ECs with a GSH precursor, NAC, abolishes strain-induced HO-1 expression. Hemodynamic forces to smooth muscle cells also induce HO-1 gene expression and carbon monoxide production (37). Our results are in agreement with the notion that HO-1 induction is an adaptive mechanism that protects cells from cyclic strain-induced oxidative injury.

Cyclic strain induces a moderate increase in ROS levels. This ROS increase apparently does not harm ECs since strained ECs appeared not only morphologically intact but also did not increase the cellular leakage according to measurement of the lactate dehydrogenase released into culture medium (data not shown). This may be due to the concomitant induction of antioxidant activities in strained cells under oxidative damage. One of our previous studies showed a rapid increase of catalase activity in ECs after the onset of cyclic strain (10). The present study further demonstrates the increased activities of SOD and glutathione peroxidase in strained ECs. SOD activity remained at an elevated level when cyclic strain continued, a phenomenon consistent with the

behavior of increasing superoxide levels. Since that cyclic strain induces a sustained elevation of intracellular ROS, the sustained increase of SOD and catalase activities may help to prevent the cells from oxidative damage. In contrast, glutathione peroxidase activity reached its maximal activity at 3 hr but returned to basal level 24 hr after continuous cyclic strain treatment. Interestingly, this maximal activity coincides with the GSH/GSSG ratio that reached a minimal level 3 hr after strain treatment. $H_2O_2$ appeared to be responsible for this GSH reduction since $H_2O_2$ treatment to cells caused a similar decrease in the GSH/GSSG ratio. Catalase treatment, however, reversed this ratio change. These results together with the abolishment of HO-1 induction by NAC and catalase treatment indicate that a rapid and transient reduction of intracellular GSH level in strained ECs may be crucial for HO-1 induction. This is consistent with previous reports that HO-1 levels are augmented in conditions of decreased intracellular glutathione (24, 32). An increase of SOD activity with a consequent increase of $H_2O_2$ and a reduction of GSH may trigger HO-1 induction. However, the HO-1 expression in strained cells was sustained up to 24 hr. The mechanism that contributes to this long-term effect on HO-1 induction is not clear. It may be due to the post-transcriptional regulation of HO-1. Alternatively, the intracellular metabolites such as nitric oxide (NO) in strained cells may also influence HO-1 induction. Cyclic strain inducing NO production in ECs has been reported (3) and the increased NO may in turn modulate HO-1 expression (15).

In summary, our data strongly indicate that cyclic strain to ECs causes an increase of ROS and a transient induction of glutathione peroxidase activity that results in a decrease of GSH levels. A decrease of GSH level may trigger signaling mechanisms that result in HO-1 expression. Thus, the intracellular redox status plays an important role in modulating gene expression in ECs constantly under the influence of hemodynamic forces. Any imbalance of intracellular redox status coupled with insufficient antioxidant activities may trigger endothelial activation and contribute to vascular disorders.

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