Upregulation of Glutathione Peroxidase Offsets Stretch-Induced Proatherogenic Gene Expression in Human Endothelial Cells

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Objective—Localization of atherosclerotic plaques typically correlates with areas of biomechanical strain where shear stress is decreased while stretch, thought to promote atherogenesis through enhanced oxidative stress, is increased.

Methods and Results—In human cultured endothelial cells, nitric oxide synthase expression was exclusively shear stress–dependent whereas expression of glutathione peroxidase-1 (GPx-1), but not of Cu^{2+}/Zn^{2+}-superoxide dismutase or Mn^{2+}-superoxide dismutase, was upregulated solely in response to cyclic stretch. GPx-1 expression was also enhanced in isolated mouse arteries perfused at high pressure. Combined pharmacological and decoy oligodeoxynucleotide blockade revealed that activation of p38 MAP kinase followed by nuclear translocation of CCAAT/enhancer binding protein plays a pivotal role in stretch-induced GPx-1 expression in human endothelial cells. Antisense oligodeoxynucleotide knockdown of GPx-1 reinforced both their capacity to generate hydrogen peroxide and the transient stretch-induced expression of CD40, monocyte chemoattractant protein-1, and vascular cell adhesion molecule-1. Consequently, THP-1 monocyte adhesion to the GPx-1–depleted cells was augmented.

Conclusions—Stretch-induced proatherosclerotic gene expression in human endothelial cells seems to be hydrogen peroxide-mediated. The concomitant rise in GPx-1 expression, but not that of other antioxidant enzymes, may comprise an adaptive mechanism through which the cells maintain their antiatherosclerotic properties in spite of a decreased bioavailability of nitric oxide. (Arterioscler Thromb Vasc Biol. 2009;29:1894-1901.)

Key Words: glutathione peroxidase ■ oxidative stress ■ cyclic stretch ■ endothelial cells ■ atherosclerosis

Atherosclerotic plaques are often located at arterial bifurcations and curvatures. Although the primary risk factors for atherosclerosis (ie, diabetes, dyslipidemia, hypertension, and cigarette smoking) contribute to pathophysiological mechanisms which affect the endothelium in general, they do not explain the focal nature of the disease. Otherwise, it is well known that biomechanical forces exerted on the vessel wall by the flowing blood tend to vary at these predilection sites due to oscillations in blood flow. As a result, laminar shear stress is reduced, whereas volume (pressure)-dependent deformation of the vessel wall is enhanced. Whereas laminar shear stress protects arteries from developing atherosclerosis by maintaining endothelial cell nitric oxide (NO) synthesis, cyclic deformation promotes atherosclerosis through an increased formation of reactive oxygen species (ROS), namely superoxide anions (O_2^-). Both radicals rapidly neutralize each other, hence reducing the level of biologically active NO even further.

Whereas cyclic stretch in endothelial cells, presumably through activation of protein kinase C and subsequent assembly of NOX-2, initially triggers the formation of O_2^- , this is rapidly converted to hydrogen peroxide (H_2O_2) by the various superoxide dismutases (SOD) present in these cells. There is accumulating evidence that it may not be O_2^- per se or its neutralization of NO but the SOD-derived H_2O_2 which either directly or through activation of distinct protein kinases modulates the activity of transcription factors which are collectively referred to as redox-sensitive. Activation of these transcription factors can alter the endothelial cells phenotype from antiinflammatory to proinflammatory and hence promote atherosclerosis through increased expression of proinflammatory gene products such as monocyte chemoattractant protein-1 (MCP-1) or vascular cell adhesion molecule-1 (VCAM-1). Subsequently, H_2O_2 is degraded to water and oxygen by catalase or glutathione peroxidase-1 (GPx-1). GPx-1 is a selenium-dependent enzyme that inactivates H_2O_2 as well as various lipid hydroperoxides. The cellular form of GPx-1 is dispersed throughout the cytoplasm, but also found in mitochondria. Its Michaelis constant (Km value) for H_2O_2 is significantly lower than that of catalase which appears to become more important under conditions of excessive H_2O_2 formation.

Thus, GPx-1 is considered as the rate-limiting H_2O_2-degrading enzyme under physiological conditions.
conditions in eukaryotic cells (for a comprehensive review see reference10).

Given the association between biomechanical and oxidative stress and its relation to atherosclerosis, studying the regulation of this antioxidant system might provide important insights into the mechanisms of early lesion formation at arterial branches or bifurcations. In the present study, we first analyzed the effects of cyclic stretch on the expression pattern of the major redox enzymes in human cultured endothelial cells. As there was a robust upregulation of GPx-1 expression in response to cyclic stretch, the potential of this putative compensatory mechanism for maintaining the antiinflammatory properties of these cells was investigated further by using an antisense oligodeoxynucleotide (ODN) based loss of function approach.

Methods

A full description of the methods can be found in the supplemental materials (available online at http://atvb.ahajournals.org). In brief, human umbilical vein endothelial cells (HUVECs) were exposed as primary culture to fluid shear stress (cone-and-plate viscometer) or cyclic stretch (Flexercell strain unit FX-3000). Antisense oligodeoxynucleotides were efficiently transfected into these cells by using Metafectene. Hydrogen peroxide formation in cells seeded onto gelatin-coated glass coverslips or BioFlex culture plates was monitored through changes in dichlorofluorescein fluorescence. Human premonocytic THP-1 cells were maintained under standard conditions. Murine smooth muscle cells were isolated from branches of the mouse mesenteric artery and murine endothelial cells from mouse lungs with the aid of a magnetic cell separation method. Cell–cell interactions were monitored with a color CCD camera and a parallel plate flow chamber mounted on the stage of a high-end inverted research-grade microscope.

Protein detection by Western blot or electrophoretic mobility shift analyses was done according to standard protocols. Total RNA from the blood vessels or cells was isolated by solid-phase extraction followed by reverse transcription and semiquantitative or quantitative real-time PCR analyses.

Statistical calculations were done by exact 2-sided Wilcoxon rank-sum tests to compare 2 samples and exact 2-sided Kruskal-Wallis tests to compare 3 or more groups with resulting probability values corrected for multiple testing using appropriate permutation procedures.

Results

Stretch-Induced ROS Formation and \( \text{H}_2\text{O}_2^{-} \)-Mediated Proinflammatory Gene Expression

On exposure to cyclic stretch there was a transient increase in intracellular ROS in the cultured HUVECs, reaching a maximum at approximately 7 hours and returning to control levels after 24 hours (Figure 1A). This increase in intracellular ROS, mainly reflecting \( \text{H}_2\text{O}_2 \) formation as demonstrated by the addition of PEG-catalase (cf Figure 1B and 1C), after 3 hours of stretching was associated with an increased expression of proinflammatory gene products such as eg, CD40 (185±10% of static control; n=11, adjusted \( P<0.01 \)). This rise in CD40 protein was completely abrogated in the presence of the ROS scavenger Tiron (5 mmol/L, 108±19% of static control; n=4, adjusted \( P<0.01 \)). To further demonstrate that the increase in \( \text{H}_2\text{O}_2 \) formation was in fact responsible for boosting proinflammatory gene expression, the cultured cells were exposed to glucose oxidase which results in a moderate but steady production of \( \text{H}_2\text{O}_2 \).14 Intracellular \( \text{H}_2\text{O}_2 \) levels rose approximately 4-fold and triggered a transient (maximum at 2 hours, not shown) and significant increase in the expression of CD40 (213±17% of the static controle; n=6, adjusted \( P<0.01 \)), MCP-1 (198±16%; n=4, adjusted \( P=0.01 \)), and VCAM-1 mRNA (291±32%; n=6; adjusted \( P<0.01 \)). This \( \text{H}_2\text{O}_2 \)-induced increase in VCAM-1 mRNA, for example, was inhibited by the glutathione peroxidase mimetic ebselen15 (range 1 to 15 \( \mu \text{mol/L} \); cf supplemental Figure II), further corroborating

![Figure 1](http://atvb.ahajournals.org)
the notion that the transient stretch-induced expression of these gene products was mediated by H$_2$O$_2$.

**Cyclic Stretch Modulation of Redox Enzyme Expression**

Next the effects of cyclic stretch on the expression of various prooxidant or antioxidant enzymes was investigated. Stretching of the cells for 6 hours did not affect Cu$^{2+}$-Zn$^{2+}$-SOD, NOS-3, and NADPH oxidase (NOX-2) subunit expression whereas mRNA levels of catalase, GPx-1, and Mn$^{2+}$-SOD were clearly upregulated (Figure 1D), possibly attributable to the increased intracellular ROS formation. However, this observation did not reach statistical significance for GPx-1 and catalase. In contrast, exposure to shear stress for 6 hours resulted in a decreased GPx-1 mRNA expression (65±9% of the static control; n=4, adjusted P=0.03) while mRNA levels of catalase were not, those of Cu$^{2+}$-Zn$^{2+}$-SOD slightly and those of Mn$^{2+}$-SOD and NOS-3 significantly elevated (not shown). The stretch-induced increase in GPx-1 mRNA was confirmed on the protein level (Figure 2A), whereas catalase abundance had a tendency to be decreased (Figure 2A, nonsignificant). In contrast to the transient rise in GPx-1 mRNA with a maximum at 6 hours (not shown), GPx-1 protein levels steadily increased, reaching a plateau after 16-hour exposure to cyclic stretch (Figure 2A). Cyclic stretch also caused a rise in Mn$^{2+}$-SOD and heme oxygenase-1 (HO-1) abundance with Mn$^{2+}$-SOD protein levels returning to baseline at 24 hours, whereas those of HO-1 had risen sharply at this point (Figure 2B). On the protein level, 12-hour exposure to shear stress also significantly downregulated GPx-1 expression (77±6% of the static control; n=20, global P<0.001).

To verify the transferability of these data to the in vivo situation, isolated mouse mesenteric arterial segments were perfused at different levels of intraluminal pressure. GPx-1 mRNA levels were elevated only on raising the perfusion pressure from 90 to 160 mm Hg (supraphysiological level) for 6 hours (Figure 2C). CD31 immunohistochemistry confirmed that the segments remained endothelium-intact throughout the 6-hour perfusion period (data not shown). Because of a lack of a suitable antibody, changes in GPx-1 protein in these segments could not be determined. Therefore, mouse cultured arterial smooth muscle cells and pulmonary endothelial cells were exposed to cyclic stretch for the same period revealing a marked increase in GPx-1 expression only in the latter cell type (Figure 2D and 2E), even though this effect did not reach statistical significance because of the small number of replicates (n=3).

Because both exposure to the O$_2^-$ generator menadione (Figure 3A) and blockade of endogenous NO formation with nitro-$\varepsilon$-arginine (not shown) apparently increased GPx-1 protein abundance in the cultured HUVECs, cyclic stretch–induced ROS formation seems to be the pivotal driving force therein. In fact, stretch-induced GPx-1 expression was completely blocked by scavenging H$_2$O$_2$ with ebselen (Figure 3B, supplemental Figure II).

**Effects of Cyclic Stretch on Effector Kinases and Transcription Factor Activation**

With the aid of appropriate pharmacological and nucleic acid–based inhibitors we next screened for potential effector kinases and downstream transcription factors involved in stretch-induced H$_2$O$_2$–mediated GPx-1 expression. Both c-Jun N-terminal kinase (JNK) and Rho kinase blockade significantly augmented the stretch-induced increase in GPx-1 protein abundance, whereas this was abrogated after inhibition of p38 mitogen–activated protein kinase (p38 MAPK; Figure 3C). Time course experiments revealed a rather transient activation of JNK as compared to that of p38 MAPK (Figure 3D). Interestingly, stretch-induced JNK activation was virtually absent in GPx-1 antisense ODN-transfected cells, whereas that of p38 MAPK was only minimally affected (Figure 3E).

Cyclic stretch elicited, albeit with different kinetics, a distinct rise in DNA-binding activity of both AP-1 and C/EBP (presumably C/EBPβ and δ according to our earlier work$^{16}$) in nuclear extracts of the cultured HUVECs (Figure 4A). Decoy ODN neutralization of AP-1 tended to augment...
the stretch-induced rise in GPx-1 protein, whereas this was abolished by the C/EBP decoy ODN (Figure 4B) even though the latter effect did not quite reach statistical significance (adjusted $P=0.08$). The corresponding control oligodeoxynucleotides exerted no significant effects (adjusted $P=0.81$).

**GPx-1 Antisense ODN Effects**

To delineate functional consequences of the stretch-induced rise in GPx-1 expression, an antisense ODN-based loss of function approach was used. The effective antisense ODN (AS2) reduced GPx-1 protein levels in the cultured HUVECs to approximately 40% of control (Figure 5A). As a consequence, their capacity to generate H$_2$O$_2$ was markedly increased (Figure 5B) along with a significant rise in CD40, MCP-1, and VCAM-1 expression both on the mRNA (Figure 5C) and protein level. For example, CD40 protein abundance increased to 201% of control in antisense ODN-treated cells as compared to 109% of control in scrambled control ODN-treated cells ($n=4$, adjusted $P=0.04$). One consequence of this enhanced proinflammatory gene expression was an increased adherence of THP-1 monocytes to the antisense ODN-treated HUVECs (Figure 5D).

Because of the elevated H$_2$O$_2$ formation observed under static conditions, antisense ODN downregulation of GPx-1 protein levels also significantly augmented the transient (maximum after 3 hours) stretch-induced expression of CD40, MCP-1, and VCAM-1 in the cultured HUVECs (Figure 6A) but, as exemplified for VCAM-1 (Figure 6B), abrogated the fall in gene expression in cells stretched for longer periods of time (24 hours). This decline in VCAM-1 mRNA below baseline was confirmed on the protein level (Figure 6C) and may have occurred independently of a
putative change in the bioavailability of NO because it was not affected by blockade with the NOS inhibitor nitro-L-arginine.

Discussion

That atherosclerosis predominantly develops at arterial bifurcations or curvatures has been known for some 150 years, but only during the past 20 years or so has it become apparent that biomechanical forces altering endothelial cell (and also smooth muscle cell) function may ultimately be responsible for its initiation. Today atherosclerosis is considered as a chronic inflammatory disease of the vessel wall, and biomechanical forces, namely oscillatory shear stress and cyclic stretch, have been shown to alter the endothelial cell phenotype from antiinflammatory to proinflammatory. Moreover, the pulsatile distension of the vessel wall together with the disturbed blood flow yields low flow zones at, eg, arterial bifurcations, which facilitate the contact between circulating leukocytes and the endothelium.

Laminar shear stress is the physiologically most important stimulus for maintaining NO synthesis as well as NOS-3 expression in endothelial cells, so that a shift from laminar to oscillatory shear stress diminishes the local concentration of NO in the vessel wall. Cyclic stretch, on the other hand, increases the formation of $\text{O}_2^{-}$ in endothelial cells (and in smooth muscle cells) through increased activity of the corresponding NADPH oxidase (NOX-2 or NOX-4). This can be observed in cultured endothelial cells derived from various species, as well as in native endothelial cells. Because NO and $\text{O}_2^{-}$ rapidly react with each other to yield peroxynitrite, both oscillatory shear stress and cyclic stretch will synergize in reducing the bioavailability of NO to a level where it can no longer control proinflammatory gene expression in the endothelium.

In addition to loosening this “NO brake,” cyclic stretch through protein kinase C–dependent assembly hence activation of NOX-2 and subsequent (enzymatic or nonenzymatic) dismutation of $\text{O}_2^{-}$ to $\text{H}_2\text{O}_2$ may also directly affect transactivation of these genes. Thus, expression of the chemokine MCP-1 as well as that of the adhesion molecule VCAM-1 in endothelial cells has been shown to be $\text{H}_2\text{O}_2$-sensitive. Our data confirm an induction by $\text{H}_2\text{O}_2$ of MCP-1 and VCAM-1 expression in human primary cultured endothelial cells and extend this observation to the T-cell costimulatory molecule CD40.

Figure 5. GPx-1 knockdown effects on ROS formation, proatherosclerotic gene expression, and endothelial cell–leukocyte interaction in HUVECs cultured under static conditions. A, Basal GPX-1 protein levels (relative to vehicle-treated cells) in cells pretreated with the GPX-1 antisense ODN (AS2) or the scrambled (SCR) control ODN. Statistical summary (n=3; global $P=0.055$) and representative Western blot. B, Phorbol 12,13-dibutyrate (PDB, 1 $\mu$mol/L) stimulated ROS formation in GPx-1–depleted cells (n=6 to 12 with 6 different batches of cells; a, adjusted $P=0.02$ vs vehicle; b, adjusted $P=0.02$ vs SCR-ODN). C, Changes in CD40, MCP-1, and VCAM-1 mRNA expression in cells over 24 hours posttransfection (n=12 to 16 with 8 different batches of cells; a, adjusted $P=0.01$ vs vehicle or SCR-ODN; b, adjusted $P<0.01$ vs vehicle; c, adjusted $P=0.02$ vs SCR-ODN). D, Effects of GPX-1 knockdown on THP-1 cell adhesion (n=4 to 8 with 4 individual batches of cells; a, adjusted $P<0.01$ vs vehicle; b, adjusted $P<0.01$ vs SCR-ODN).
overexpression facilitates intracellular H$_2$O$_2$ accumulationabolished, as exemplified for VCAM-1. It is known that SOD
detects only H$_2$O$_2$, lipid hydroperoxides, and peroxynitrite, but
rofluorescein (DCF) fluorescence, which is considered to
tackle). C, Lack of effect of the NO synthesis inhibitor NG-nitro-L-
SCR-ODN; d, adjusted
vs cyclic stretch; c, adjusted
/ H$_{11005}$
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cells maintained under static conditions) in nontransfected and
H$_2$O$_2$-induced proinflammatory gene expression could be
similar effects were observed with the
culture medium, resulting in a steady but moderate increase in
intracellular H$_2$O$_2$.14 Similar effects were observed with the
O$_2^-$-generating redox cycler menadione.

Stretch-induced ROS formation was monitored by dichlo-
rofluorescein (DCF) fluorescence, which is considered to
detect only H$_2$O$_2$, lipid hydroperoxides, and peroxy nitrite, but
not O$_2^-$ or hydroxyl radicals.28 Even though some caution is
warranted with respect to interpreting results obtained with this
fluorescent dye (ie, there are reports suggesting that it
generates redox cycler menadione, whereas expression of the other antioxidant
enzymes either decreased (catalase) or remained unaffected
(NOS-3, both superoxide dismutases).

In line with these results, GPx-1 knockout mice have been
reported to present with endothelial dysfunction and in-
creased oxidative stress in the arterial vessel wall.32 More-
over, GPx-1/apolipoprotein E–double-knockout mice, with
or without streptozotocin-induced diabetes, develop significa-
tively more atherosclerotic lesions than apolipoprotein E–
deficient animals, an effect that is accompanied by an
increased expression of proinflammatory marker molecules
such as VCAM-1.33 Likewise in humans, decreased or absent
GPx-1 activity in atherosclerotic plaques appears to be
associated with the development of more severe lesions.34
Furthermore, the German AtheroGene Study and others have
proposed that increasing GPx-1 activity or expression might
rescue endothelial dysfunction and thus lower the risk of
cardiovascular events.35

To assess the functional implications of the stretch-induced
rise in GPx-1 expression for the cultured endothelial cells, the
classical antisense ODN approach was chosen for the follow-
ing reasons: (1) there is no specific GPx-1 inhibitor available
to date, and (2) at least in our hands transfection of small
interfering RNAs into human primary cultured endothelial
cells did not prove satisfactory. In fact, one of the antisense
ODNs designed, but not the corresponding scrambled control
ODN, effectively reduced GPx-1 protein levels. As a conse-
quence, the endothelial cell capacity to generate ROS in-
creased more than 4-fold within 24 hours posttransfection,
and this was paralleled by a comparable rise in expression of the
3 proinflammatory gene products monitored. Presumably as
a result of the increase in VCAM-1 expression, monocyte
adhesion to the antisense ODN-treated endothelial cells was
significantly augmented. GPx-1 thus seems to effectively re-
move the accumulating H$_2$O$_2$ even under static conditions hence
maintaining proinflammatory gene expression.30 In this context, our data imply that GPx-1 may
play a more important role than the 2 superoxide dismutases in preventing proinflammatoty gene expression at atheroscle-
rosis predilection sites.

The aforementioned data suggest that cyclic stretch
through activation of NOX-2 elicits an increase in O$_2^-$ which
is effectively converted to H$_2$O$_2$ by Cu$^{2+}$/Zn$^{2+}$-SOD or
Mn$^{2+}$-SOD and in turn, either directly or through an inter-
mediate, triggers the observed increase in proinflammatory
gene expression. To prevent this, endothelial cells must
rapidly degrade the accumulating H$_2$O$_2$. Rate-limiting for this
is the activity of GPx-1 which, because of the characteristics of
the enzyme,10 most likely hinges on its level of expression.
In this context, it was noteworthy that expression of both
GPx-1 and the normally shear stress–responsive antioxidant
enzyme heme oxygenase-131 increased in the stretched endo-
thelial cells, whereas expression of the other antioxidant
enzymes either decreased (catalase) or remained unaffected
(PEG catalase and this fluorescent dye (ie, there are reports suggesting that it
}

## Figure 6. Changes in proinflammatory gene expression in GPx-
1–depleted HUVECs exposed to cyclic stretch. A, Changes in
CD40, MCP-1, and VCAM-1 mRNA expression (relative to cells
transfected with the SCR-ODN) in cells transfected with the GPx-
1 antisense (AS2) or the scrambled (SCR) control ODN after 3
hours of cyclic stretch (n=4 to 5; a, adjusted \( P<0.01 \) vs SCR-
ODN, B, Changes in VCAM-1 mRNA abundance (relative to
cells maintained under static conditions) in nontransfected and
transfected cells in response to 24 hours of cyclic stretch
(n=4 to 7; a, adjusted \( P<0.01 \) vs static control; b, adjusted \( P<0.01 \)
vs cyclic stretch; c, adjusted \( P<0.01 \) vs cyclic stretch plus
SCR-ODN; d, adjusted \( P=0.05 \) vs SCR-ODN-treated static control).
C, Lack of effect of the NO synthesis inhibitor N$^\circ$-nitro-L-
arginine (N-Arg, 1 mmol/L, 1 hour preincubation) on the stretch-
induced decline in VCAM-1 protein levels. Summary (n=6 to 11
out of 4 batches of cells; a, adjusted \( P=0.01 \) vs static control) and
representative Western blot.
stretch-induced ROS formation may be transient but also degradation of H$_2$O$_2$ by GPx-1 may be accelerated later on, so that stretch-induced ROS formation is fully compensated at that time. This hypothesis is further supported by the finding that the decrease in proinflammatory gene expression after 24-hour exposure to cyclic stretch was abolished in cells treated with the GPx-1 antisense ODN. It was remarkable though that 24-hour cyclic stretch plus knockdown of GPx-1 did not augment proinflammatory gene expression further, as one might have expected from the short term stretch results. In fact, VCAM-1 protein abundance at this point decreased well below the basal level in static controls, and this effect was not influenced by blocking NO synthesis. However, other antioxidative enzymes such as heme oxygenase-1, the expression of which was upregulated more than 5-fold after 24 hours cyclic stretch, may account for this effect.

We$^{16,37}$ and others$^{38}$ have previously shown that stretch-induced changes in gene expression in vascular cells proceed through activating several intracellular signaling pathways. Our screen with pharmacological inhibitors of some of the candidate effector kinases confirmed a role for p38 MAPK in stretch-induced H$_2$O$_2$-mediated GPx-1 expression in the cultured endothelial cells. Rho kinase or JNK activation, on the other hand, appeared to limit GPx-1 expression. Interestingly, GPx-1 knockdown barely affected p38 MAPK activation, whereas that of JNK was virtually abolished. Possibly the function of an upstream effector kinase, JNK phosphatase or JNK itself is redox-sensitive in that the cysteine oxidation state determines their activity.$^{39}$

Of the two downstream transcription factors likely to account for the stretch-induced rise in GPx-1 expression,$^{37,40}$ a C/EBP family member, presumably C/EBP$\beta$ or $\delta$, was identified by using the so-called decoy ODN technique. Although the corresponding effect of an AP-1 decoy ODN did not achieve statistical significance, it seemed to corroborate the notion that the JNK–AP-1 pathway inhibits rather than boosts stretch-induced GPx-1 expression. MatInspector analysis$^{41}$ in fact revealed one C/EBP$\beta$ binding site at position $-814$ and one AP-1 binding site at position $-290$ in the promoter of the human GPx-1 gene (GenBank accession No. AF029317) Previously, redox-sensitive transcription factors such as nuclear factor erythroid 1-related factor 1 and 2 (Nrf1/Nrf2) as well as the Ku antigen have been shown to transactivate the GPx-1 gene through antioxidant response elements (ARE) located at positions $-267$ and $-1209$, respectively.$^{12-44}$ Activation of these transcription factors is dependent on protein kinase C, MAP kinase (eg, ERK1/2, p38 MAPK), and phosphotyridinositol 3-kinase activities.$^{45}$ In addition to the single binding site at position $-814$, C/EBP$\beta$ may also bind to these AREs in the GPx-1 promoter, as previously demonstrated in murine vascular smooth muscle cells.$^{46}$

Hitherto, shear stress but not cyclic stretch has been reported to upregulate GPx-1 mRNA expression in bovine aortic endothelial cells (passages 6 to 12).$^{47}$ In human umbilical vein endothelial cells (passages 3 to 8) shear stress causes a decrease in both GSH peroxidase and catalase activity,$^{48}$ whereas exposure of the same cells (passages 4 to 6) to cyclic stretch not only raises the level of H$_2$O$_2$ but (consequently) also the activity of GSH peroxidase.$^{49}$ In our primary human umbilical vein endothelial cells exposure to shear stress (30 dyn/cm$^2$, 12 hours exposure) caused a moderate (23% to 35%) decrease in GPx-1 expression both on the mRNA and protein level. The reason for these somewhat discrepant findings between bovine and human endothelial cells is unclear at present but may not simply be related to a species difference. On the other hand, it is well known that working with endothelial cells at higher passages may be artifact-prone because they hardly produce any NO.$^{50}$ In addition, it is important to distinguish between shear stress and (cyclic) stretch which per se are very different biomechanical forces both in terms of direction (unidirectional versus bidirectional deformation) and relative strength (typically stretch is 3 to 4 orders of magnitude greater than shear stress).

Thus, besides the documented stretch-induced rise in GSH peroxidase activity in human cultured endothelial cells,$^{40}$ the present findings demonstrate for the first time by using an antisense ODN approach the pivotal role of GPx-1 expression in the protection against oxidative stress and a dedifferentiation of these cells toward a proinflammatory phenotype both under static conditions and in response to cyclic stretch. They further reveal that this biomechanical force upregulates GPx-1 expression in human cultured endothelial cells through the generation of ROS, more precisely H$_2$O$_2$,$^{51}$ followed by activation of the transcription factor C/EBP via the p38 MAPK pathway.$^{40}$ Because H$_2$O$_2$-mediated control of proinflammatory gene expression in endothelial cells may be especially relevant at atherosclerosis predilection sites, our findings indicate that boosting O$_2^-$ dismutation to H$_2$O$_2$ may be therapeutically less meaningful than accelerating the degradation of H$_2$O$_2$. 

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Disclosures
None.

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Methods

Cell culture

Human endothelial cells (HUVEC) were isolated from umbilical cord veins as described in detail elsewhere, and employed as a primary culture throughout. Murine arterial smooth muscle cells (MASMC) were isolated from branches of the mouse mesenteric artery and cultured in D-MEM (Invitrogen) supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin and 15% fetal bovine serum. The phenotype of the cells was confirmed by anti-mouse smooth muscle α-actin immunofluorescence staining. Pulmonary endothelial cells (MPEC) were isolated from mouse lungs using a magnetic cell separation method, as previously described. In brief, magnetic beads (Dynabeads; Dynal) were coated with anti-CD31 antibodies (Mec13.3; BD Biosciences). The lungs of two mice were minced, and digested with collagenase A solution (Roche). Cells were incubated with anti-CD31–coated Dynabeads and separated in a magnetic field. After washing away unbound cells, bound cells were released from the beads by trypsin/EDTA treatment. To determine the percentage of endothelial cells in the cultures, immunofluorescence staining with a monoclonal rat anti-mouse CD31 antibody (BD Pharmingen) or uptake of Dil-labeled Ac-LDL particles (Cellsystems) was performed, revealing a purity of more than 95%. The human pre-monocytic cell line THP-1 (American Type Culture Collection) was cultured in RPMI 1640 medium (Life Technologies) containing 10% FBS and antibiotics. In some experiments, 12 mU/mL glucose oxidase (Sigma-Aldrich) was added to the medium to continuously generate H₂O₂ (up to 95 μmol/L of H₂O₂ in
1 hour, cf. reference 5). The cultured HUVECs were exposed to laminar shear stress (30 dyn/cm²) with the aid of a cone-and-plate viscometer.¹ For application of cyclic stretch (0.5 Hz, 16-18% elongation) they were grown on BioFlex™ Collagen type I six-well plates (FlexCell International) that had additionally been coated with gelatine.⁶ Cell viability was assessed colorimetrically with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-etrazolium-bromide.² In some experiments, the cell-permeable glutathione peroxidase mimetic ebselen (range 1-15 µmol/L) was added 48 hours prior to the start of the experiment. None of the different treatments exerted any cytotoxic effect (not shown).

**Perfusion of isolated mouse arteries**

Animals were euthanized, and the intestine was dissected. Second order branches of the mesenteric artery were extracted from fat tissue and mounted in a specially designed 4-position perfusion chamber where they were stretched back to their in situ length by the aid of moveable cannulas onto which the segments were tied; they were subsequently perfused as described previously.⁷ After an equilibration period of 30 minutes, the segments were perfused at 0, 45, 90 or 160 mmHg for 6 hours with the aid of an adjustable afterload device system (Hugo Sachs Elektronik). The functional and cellular integrity of the segments was routinely checked by immunohistochemical staining for the endothelial cell marker CD31. At the end of the perfusion, the segments were snap-frozen in liquid nitrogen and stored at -80°C.

**GPx-1 antisense ODN transfection**

Efficient transfection of the cultured HUVECs was achieved with Metafectene (Biontex Laboratories). In brief, 10 µl Metafectene was mixed with 100 µl of medium M199 free of serum and antibiotics. Five microgram antisense-ODN and 100 µl medium M199 free of serum and antibiotics were mixed in a separate vial.
Subsequently, both solutions were combined and incubated at ambient temperature for 20 minutes to form the lipid-DNA complexes. The lipid-DNA complexes were added to the cells when they were about 70% confluent and incubated for 3.5 hours at 37°C. Thereafter, the medium was replaced and the cells were cultured for another 24 hours. Under these conditions, a transfection efficiency of 80% was achieved (cf. Suppl. Fig. I); this was verified by employing an atto590-labeled ODN. The following antisense ODN sequences were employed (* denotes phosphorothioate-bonded bases): GPx-1 antisense (AS2) ODN 5’-T*G*T*CC*A*A*G*A*A*GCC*A*G-3’ and scrambled (SCR) control ODN 5’-A*G*T*C*A*CC*A*GG*A*C*A*G*T-3’ (IBA).

**Detection of H₂O₂ formation**

H₂O₂ formation in HUVECs seeded onto gelatine-coated glass coverslips or BioFlex™ 6-well culture plates was monitored through changes in dichlorofluorescein (DCF) fluorescence. The specificity of the DCF signal for detection of H₂O₂ was confirmed by pre-incubating the cells on coverslips with polyethylene glycol (PEG) catalase (100 U/mL, Sigma-Aldrich) for 40 minutes prior to adding 12 mU/mL glucose oxidase (Sigma-Aldrich) for another 40 minutes or by exposing the cells on flexible membranes to cyclic stretch for 3 hours. To visualize stretch-induced H₂O₂ formation, the BioFlex elastomers with the attached HUVECs were excised and placed face down onto glass coverslips for fluorescence microscopy analysis.

**Endothelial cell-leukocyte interaction**

Cell-cell interactions were monitored with a SPOT RT color CCD camera (Diagnostic Instruments) and a parallel plate flow chamber (Warner Instruments) placed on the stage of an Axiovert S100 TV microscope (Zeiss). The cultured HUVECs were superfused with 1.5×10⁶ THP-1 cells (5×10⁵ THP-1 cells/mL) at a wall shear stress of 5 dyn/cm² and a shear rate of 10 s⁻¹ with the aid of a precision pump (Ismatec). Three
images from different regions were recorded for subsequent automatic counting of adherent cells with the MetaMorph 6 software package (Universal Imaging).

**Decoy oligodeoxynucleotide (dODN) technique**

Double-stranded activator protein-1 (AP-1) and CCAAT/enhancer binding protein (C/EBP) dODNs were prepared from complementary single-stranded phosphorothioate-bonded ODNs obtained from Eurogentec as described earlier.8 The sequences of the single-stranded ODNs were as follows (* denotes phosphorothioate-bonded bases): AP-1, 5’-C*G*C*TTGATGACTCAGCCG*G*A*A-3’; AP-1mut, 5’-C*G*C*TTGATTACTTAGCCG*G*A*A-3’; C/EBP, 5’-T*G*C*AGATT-GCGCAATCT*G*C*A-3’; C/EBPmut, 5’-T*G*C*AGAGACTAGTCTCT*G*C*A-3’.

Nucleic acid uptake was achieved without using any cationic lipid or liposomal complex.

**Electrophoretic mobility shift analysis (EMSA)**

Preparation of nuclear extracts from the cultured HUVECs and subsequent non-denaturing 4% polyacrylamide gel electrophoresis were carried out as described previously.8 The double-stranded gel shift ODNs (Santa Cruz Biotechnology) for activator protein-1 (AP-1, 5’-CGCTTGATGACTCAGCCGAA-3’) and CCAAT/enhancer binding protein (C/EBP, 5’-TGCAGATTGCGCAATCTGCA-3’) were end-labellled with \([\gamma^{32}P]ATP\) by using the 5’-end labelling kit from Amersham GE Healthcare. To ascertain the binding specificity, 1.0-2.0 µL of the appropriate gel supershift antibody (2 mg mL\(^{-1}\), Santa Cruz Biotechnology) per 6.0-7.0 µL of nuclear extract (3-10 µg protein) were pre-incubated overnight at 4°C or at room temperature for 60 minutes before the EMSA was performed.9
RT-PCR analysis and real-time PCR

Total RNA was isolated from cultured cells by solid-phase extraction with the RNeasy kit (Qiagen). Reverse transcription and polymerase chain reaction for CD40, monocyte chemoattractant protein-1 (MCP-1) and VCAM-1 was performed as described previously. Amplification of 60S ribosomal protein L32 (RPL32) cDNA served as an internal standard (house-keeping gene). Details of the PCR conditions and primer sequences are given in Table I. In addition, real-time PCR for VCAM-1 (81-bp PCR fragment, GenBank accession No. NM_001078) was performed in a LightCycler (Roche Diagnostics) using the QuantiTect SYBR Green RT-PCR kit (Qiagen) and the primer pair 5'-CATGGAATTCGAACCAAACA-3' (sense) and 5'-GACCAAGACGTTGTATCTCTGG-3' (antisense) as described previously. Total RNA from mouse mesenteric arteries was isolated using the RNeasy mini kit from Qiagen (Hilden). Copy DNA synthesis was carried out using the Sensiscript RT kit (Qiagen). The following gene specific primers for quantitative real-time PCR were used: Mouse GPx-1 forward, 5'-CCACCGTGATGCCTTCTCC-3'; mouse GPx-1 reverse, 5'-GATCGTGGTGCCTCAGAGAG-3' (GenBank accession number NM_008160); mouse RPL32 forward, 5'-GGGAGCAACAAAGAAACCAA-3'; mouse RPL32 reverse, 5'-ATTGTGGACCAGAACTTG-3' (GenBank accession number XR_031977).

Western blot analysis

Protein extracts were separated by denaturing 10% polyacrylamid gel electrophoresis in the presence of SDS according to standard protocols. Subsequent transfer to a BioTrace™ polyvinylidene fluoride transfer membrane (Pall Corporation) and standard detection of the target protein with Amersham ECL Plus (GE Healthcare) was performed as described elsewhere. The following antibodies were used:
polyclonal anti-CD40 and anti-VCAM-1 (1:1000, Santa Cruz Biotechnology), monoclonal anti-GPx-1 (1:500, MBL International), polyclonal GPx-1 (1:2000, Lab Frontier Life Science Institute), polyclonal anti-catalase (1:8000, Calbiochem EMD Biosciences), monoclonal anti-manganese containing superoxide dismutase (Mn-SOD; 1:2000, Lab Frontier Life Science Institute) and monoclonal anti-heme oxygenase-1 (HO-1; 1:250, BD Transduction Laboratories). Loading and transfer of equal amounts of protein was verified by reprobing the membrane with a monoclonal anti-β-actin antibody (1:3000, Sigma-Aldrich) followed by densitometry. The VCAM-1, GPx-1 and β-actin antibodies were also used for detection of the mouse proteins.

A phospho-MAPK antibody sampler kit (Cell Signaling Technology via NEB) containing the primary antibody for phospho-p38 MAPK (Thr180/Tyr182) and phospho-SAPK/JNK (Thr183/Tyr185) and a MAPK antibody sampler kit (Cell Signaling) were used for verifying activation of the kinases in response to cyclic stretch. A casein-based blocking reagent (I-Block, Applied Biosystems) was used for immunodetection of the activated kinases.

**Data analysis**

Unless indicated otherwise, results are expressed as means ± SEM of $n$ observations. Stress effects, for example the relationship between cyclic stretch and ROS formation, were investigated by rank-based analysis of variance (ANOVA) procedures. Exact, two-sided Kruskal-Wallis tests were used to compare three or more groups, and exact, two-sided Wilcoxon rank-sum tests were used to compare two samples. Exact probability values were estimated by Monte Carlo simulations based on 100,000 samples using the procedure NPAR1WAY from SAS version 9. Probability values from exact tests are reported with three significant digits and referred to as global probability values (global P). If heterogeneity among three or
more groups was found (global P smaller than 0.05), subsequent post-hoc analyses based on exact, two-sided two-sample Wilcoxon rank-sum tests were used to compare group pairs. P-values from the post-hoc analyses were corrected for multiple testing using a permutation approach: the P-value was calculated based on the actual data set, 10,000 pseudo data sets were created by permutation of data into artificial groups and pseudo-P-values were calculated based on permuted data. Comparison of the actual P-value with the distribution of pseudo P-values resulted in the adjusted P-value.\textsuperscript{10} This correction for multiple testing by permutation was implemented in SAS version 9. Adjusted P-values are reported with two significant digits.
References


5. Salazar JJ, Van Houten B. Preferential mitochondrial DNA injury caused by glucose oxidase as a steady generator of hydrogen peroxide in human fibroblasts. Mutat Res. 1997;385:139-149.


Legends to supplemental figures

**Figure I**: Transfection efficiency in cultured primary human endothelial cells after metafectene transfection with an atto590-labeled ODN (cf. Methods section). Transfection efficiency was estimated by visual counting. Approximately 80% of HUVECs were transfected at the end of the transfection process (3.5 hours). About 18% of the cells were penetrated by the labelled ODN without using any transfection reagent. (global P<0.001, n=9 out of 2 independent experiments).

**Figure II**: Effects of H$_2$O$_2$ on pro-inflammatory gene expression. Inhibition by the peroxide mimetic ebselen (range 1–15 µmol/L, 48 hours pre-incubation) of glucose oxidase (GlcOx, 12 U/mL, 2 hours) induced VCAM-1 mRNA expression as judged by quantitative real-time PCR analysis (n=4; a, adjusted P=0.01 vs. GlcOx stimulation).

**Figure III**: Representative Western blot analyses of (A) time-dependent effects of cyclic stretch on GPx-1 and catalase protein expression in the cultured HUVECs and (B) GPx-1 and VCAM-1 protein abundance in mouse pulmonary endothelial cells (MPEC) after 16 hours exposure to cyclic stretch (15% elongation at 0.5 Hz).
**Suppl. Table I:** Primers and PCR conditions.

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Suppl. Fig. I

The bar graph shows the percentage of total cell numbers for non-transfected and transfected conditions. The transfected group has a significantly higher percentage compared to the non-transfected group.
Suppl. Fig. II

VCAM-1 mRNA (% of GlcOx)

GlcOx

− − 1 3 6 15 ebselen (µmol/L)
Suppl. Fig. III

A  HUVEC

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B  MPEC

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