Glutaredoxin Mediates Akt and eNOS Activation by Flow in a Glutathione Reductase-Dependent Manner

Jing Wang, Shi Pan, Bradford C. Berk

Objective—The glutathione (GSH)/glutaredoxin (Grx) system regulates activities of many redox sensitive enzymes. This system has been shown to protect cells from hydrogen peroxide–induced apoptosis by regulating the redox state of Akt. Grx can be regulated by redox state; the oxidized Grx is selectively recycled to the reduced form by GSH. Flow can maintain endothelial cells in a reduced state by activating glutathione reductase (GR) and increasing the GSH/GSSG ratio. Because steady laminar flow exerts an antioxidant effect, we hypothesized that Grx mediates flow induced Akt and eNOS phosphorylation in a GR dependent manner.

Methods and Results—Exposure of endothelial cells (ECs) to physiological steady laminar flow (shear stress = 12 dyn/cm²) for 5 minutes significantly increased Grx activity (1.9±0.2-fold), and also increased Akt and eNOS phosphorylation. Overexpression of GFP-GR in ECs significantly increased Grx activity by 1.6±0.1-fold. Pretreatment with the GR inhibitor 1,3-bis[2-chloroethyl]-1-nitrosourea (BCNU) for 30 minutes dramatically reduced Grx activity and inhibited the increase in Akt and eNOS phosphorylation induced by flow. Overexpression of wild-type Grx in ECs increased both Akt and eNOS phosphorylation. In contrast, a mutated Grx (C22S/C25S), which lacks thioltransferase activity, had no effect. Therefore, flow-induced Akt and eNOS phosphorylation depend on Grx thioltransferase activity. Downregulation of Grx by small interfering RNA decreased flow induced Akt and eNOS phosphorylation.

Conclusions—These data suggest that Grx is an important mediator for flow-induced Akt and eNOS activation, and Grx activity depends on GR-mediated changes in EC redox state. (Arterioscler Thromb Vasc Biol. 2007;27:1283-1288.)

Key Words: Grx • Akt • eNOS • GR • endothelial cells

Fluid shear stress (flow) is the frictional force exerted by blood flow acting on vascular endothelial cells (ECs). Flow modulates endothelial structure and function, and it is a major determinant factor of vascular remodeling, arterial tone, and atherosclerosis.1–3 Physiological levels of steady laminar shear stress exert potent antiapoptotic and antiatherosclerotic effects. In contrast, flow that has low mean shear stress and turbulence is strongly correlated with EC dysfunction, EC apoptosis, and atherosclerosis.3–6

The mechanisms by which flow prevents atherosclerosis are not well known. It has been reported that flow is an important stimulus for the continuous formation of nitric oxide (NO) via endothelial nitric-oxide synthase (eNOS) both in cultured ECs and in intact vessels. And endothelial-derived NO plays lots of essential roles on the local regulation of vascular homeostasis including vessel relaxation, inhibition of apoptosis and platelet coagulation, and antiinflammation. A decrease in the bioavailability of NO is a characteristic feature in patients with coronary artery disease and promotes the development of atherosclerotic lesions.

We previously reported that flow-stimulated phosphorylation of eNOS at Ser1179, via the PI3K–Akt–eNOS signaling pathway.7 Akt is a serine/threonine kinase, and phosphorylation at Thr-308 and Ser-473 increases its enzyme activity. Akt is involved in many important signaling pathways that regulate survival and apoptosis.8 Increasing evidence suggests that Akt is a redox-regulated protein. Grx is a small (12-kDa) dithiol protein involved in many cellular events by regulating the redox status of cellular proteins via de-glutathionylation.9,10 The Grx redox system includes NADPH, glutathione (GSH), glutathione reductase, and Grx. Grx catalyzes the reduction of S-glutathionylated proteins via a disulfide exchange reaction in its catalytic center.11 Oxidized Grx is reduced by glutathione via consuming NADPH. Previous studies have demonstrated that protein glutathionylation can occur in cells,12–14 and it plays an important role to stabilize extracellular protein, and protect proteins against irreversible cysteine oxidation under oxidative stress.15–17

Grx has been shown to protect cell from hydrogen peroxide (H₂O₂)-induced death by regulating the redox state of Akt.13 Flow can activate Akt and show atheroprotective effect via modulation of cellular redox systems against ROS.18 We hypothesized that Grx participated in the atheroprotective effect of flow by regulating Akt–eNOS–NO signaling pathway in a GR dependent manner.
Methods

DNA Constructs and Reagents
Grx expression construct was a generous gift from Dr Y.J. Lee (University of Pittsburgh, Pa). C22/25S mutated Grx construct was generated by mutagenesis as described.19 Grx siRNA was described previously19 and ordered from Dharmacon. Control siRNA was purchased from Qiagen. GFP-GR adenovirus was a generous gift from Dr Reto Asmis (University of Kentucky).

Cell Culture and Flow Experiments
Bovine aortic endothelial cells (BAECs) were isolated according to previously published protocol,14 and maintained in medium 199 (M-199) (Gibco) supplemented with 100 U/mL of penicillin and 100 mg/mL of streptomycin (Gibco), 1% MEM amino acids (Gibco), 1% MEM vitamins (Cellgro), 10% fetal clone III (bovine serum product, HyClone), in a 5% CO2/95% O2 incubator at 37°C. Cells were used at passages 1 to 4. Flow experiments were performed with confluent cells grown in 60-mm dishes (growth-arrested for 1 day by serum deprivation) to decrease basal kinase activity. Cells were exposed to laminar flow (shear stress of 5 to 8 dyn/cm²) in a cone and plate viscometer.

Transient Transfection With DNA Constructs
BAECs were seeded onto 60-mm dishes 24 hours before transfection, and transiently transfected with 2 µg DNA (pcDNA3 vector was used as mock) per dish at 90% confluence with Lipofectamine 2000 reagent (Invitrogen) in OptiMEM medium (Gibco). After 4 hours, the medium was changed back to 10% serum BAEC medium.

Transient Transfection With siRNA
HUVECs were seeded onto 60-mm dishes 24 hours before transfection, and transiently transfected with 100 nmol/L siRNA per dish at 90% confluence with Lipofectamine 2000 reagent in OptiMEM medium. After 2 hours, 5% serum medium was added.

Cell Lysate Preparation
Cells were rinsed with ice-cold phosphate-buffered saline (PBS: 150 mmol/L NaCl, 20 mmol/L Na,PO4, pH 7.4) on ice and harvested in lysis buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 5 mmol/L NaF, 1 mmol/L Na3VO4 plus 1:1000 protein inhibitor cocktail (PIC, Sigma) and clarified by centrifugation. The protein concentration was determined by the Bradford assay (Bio-Rad).

Western Analysis
Total cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, and the membranes were incubated with appropriate primary antibodies: Grx (American Diagnostica), Actin (Santa Cruz), phospho-eNOS (Cell Signaling), eNOS (BD Bioscience), phospho-Akt (Cell signaling), and Akt antibodies (Cell Signaling). After washing and incubating with secondary antibodies, immunoreactive proteins were visualized by the Odyssey infrared imaging system (LI-COR Biotechnology). Densitometric analyses of immunoblots were performed with NIH Image software.

Grx Activity Assay
BAEC cell lysates were used to measure Grx activity by monitoring the decrease in absorbance of NADPH at 340 nm using Beckman DU 640 Spectrophotometer (Beckman).20,21 All reagents for the assay were purchased from Sigma.

NO Production Measurement
BAECs were seeded onto 60-mm dishes and transiently transfected with WT Grx or vector control. After 48 hours, medium was replaced with PBS, incubated for 30 minutes, and NO production was measured by chemiluminescent NO analyzer (model 270B, Sievers).22

Results

Flow Increases Grx Activity
Flow activates many oxidoreductases and protects against oxidative stress. We previously showed that flow regulates EC redox state by activating GR and increasing the GSH/GSSG ratio.18 Grx is regulated by redox state, because only the reduced form of Grx is active, and it functions via a disulfide exchange reaction by utilizing the active site Cys-Pro-Tyr-Cys. Oxidized Grx is selectively recycled to the reduced form by GSH.23 Therefore, we hypothesized that flow increases Grx activity by maintaining Grx in the reduced form. To test this hypothesis, BAECs were exposed to flow for 0 to 30 minutes. Grx activity was assayed using whole cell lysates. As shown in Figure 1, flow significantly increased Grx activity with a peak at 5 minutes (1.9±0.2-fold, P<0.01) compared with control.

Glutathione Reductase (GR) Is a Mediator for Grx Activity Induced by Flow
A previous paper from our laboratory showed that flow significantly increased the GSH/GSSG ratio and maintained cellular redox state by increasing glutathione reductase (GR) activity.18 To examine whether GR plays a role in flow induced Grx activation, BAECs were treated with the GR inhibitor 1,3-bis[2-chloroethyl]-1-nitrosourea (BCNU). As shown in Figure 2, preincubation of BAECs with 25 µmol/L BCNU for 30 minutes dramatically reduced Grx activity induced by flow at both 5 and 10 minutes (n=3, P<0.05). To confirm the role of GR in mediating Grx activity, we overexpressed GR by GFP-tagged GR adenovirus (Ad.GR) in BAECs. The infection efficiency was 80% by immunohistochemical staining using anti-GFP antibody (data not shown). Grx activity in BAECs infected with Ad.GR was significantly higher than in control (1.6±0.1-fold increase, n=3, P<0.01, data not shown).

Figures

Figure 1. Flow increases Grx activity. BAECs were exposed to flow for the indicated time. Grx activity was measured by Grx activity assay. One unit of Grx activity was defined as 1 µmol of NADPH oxidized per min under the standard assay conditions (data were expressed as mean±SEM, n=3, #P<0.01 vs control).
GR Mediates Flow-Induced Akt and eNOS Activation by Increasing Grx Activity

We next examined the effect of GR inhibitor on Akt and eNOS activation induced by flow. After pretreatment with BCNU, BAECs were exposed to flow for different time. Phosphorylation of Akt at Ser473 and eNOS at Ser1177 were analyzed by immunoblot with phosphospecific antibody.7 As shown in Figure 3A, phosphorylation of both Akt and eNOS was significantly stimulated by flow in untreated cells. BCNU treatment significantly inhibited phosphorylation of these two proteins as shown in Figure 3B and 3C. To confirm the role of GR, we next tested the effect of GR overexpression on basal Akt and eNOS activation. As shown in Figure 4, infection with Ad.GR increased Akt and eNOS basal phosphorylation relative to cells infected with LacZ. GR overexpression had no significant effect on expression level of Akt and eNOS. Thus GR plays an important role in Akt and eNOS activation induced by flow.

Grx Overexpression Increases Akt and eNOS Phosphorylation

It has been shown that Grx can regulate Akt redox state and increase its activity.24 To test whether Grx activity increases Akt activation in ECs, we overexpressed wild-type Grx (WT) in BAECs. The Grx protein expression level was confirmed by Western blot analysis. Grx activity assay confirmed that the overexpressed WT Grx was enzymatically active (Figure 5A). Basal Akt and eNOS phosphorylation were greatly enhanced in cells transiently transfected with WT Grx compared with empty vector pcDNA3. Grx overexpression had no significant effect on total protein levels of Akt and eNOS (Figure 5B).

Because Grx thioltransferase activity is dependent on the 2 cysteines in the catalytic domain, we next tested the effect of inhibiting thioltransferase function on activation of Akt and eNOS. The catalytic center of Grx contains a conserved CPYC sequence in which the 2 cysteines (cysteine 22 and cysteine 25) have been demonstrated to be necessary for Grx thioltransferase activity. Therefore, a mutated Grx (C22/25S) lacks thioltransferase activity. We overexpressed the mutated Grx in BAECs, and confirmed that the mutated Grx had no enzymatic activity (Figure 5A). The mutated Grx (C22/25S) protein expression level was similar to WT Grx expression (Figure 5B). Cells transfected with Grx (C22/25S) did not increase Akt and eNOS phosphorylation in the contrast to overexpression of WT Grx. Grx overexpression had no significant effect on total protein level of Akt and eNOS. Thus, the Grx increase in Akt and eNOS phosphorylation is dependent on thioltransferase activity.

To demonstrate that eNOS phosphorylation induced by Grx is functional, we measured NO production. Transient transfection with WT Grx increased NO production relative to cells transfected with empty vector (1.4 ± 0.1-fold increase, n = 3, P < 0.05, data not shown). These results show that Grx activity increases Akt and eNOS activation.

Flow Activation of Akt and eNOS Requires Grx

Flow activates Akt and eNOS in ECs by a VEGF receptor–VE-cadherin–PI3K signaling pathway.7 To evaluate the role
of Grx, we designed Grx siRNA to study the specific role of Grx in flow-induced Akt-eNOS pathway. After transfection with Grx siRNA for 48 hours, Grx expression was significantly reduced, whereas control siRNA had no effect (Figure 6A). Grx siRNA had no significant effect on the expression levels of Akt and eNOS (Figure 6B). Treatment with Grx siRNA and control siRNA did not increase cell death or cause apparent changes in cell morphology (data not shown). After siRNA transfection, HUVECs were exposed to flow for different time, and phosphorylation of Akt and eNOS was analyzed by immunoblot. Phosphorylation of Akt and eNOS was significantly stimulated by flow in cells treated with control siRNA (Figure 6C and 6D, 2.6±0.2- and 2.8±0.4-fold at time 10 minutes, respectively). In contrast, phosphorylation of Akt and eNOS was significantly inhibited in Grx siRNA-treated cells (Figure 6C and 6D, 1.0±0.2- and 1.1±0.2-fold at time 10 minutes, respectively). These results indicate that Grx is required for flow induced activation of Akt and eNOS.

Discussion

The major finding of this study is that flow stimulation of the Akt–eNOS signaling pathway in endothelial cells is regulated by the thioltransferase, Grx, via a mechanism dependent on GR. Specifically, we define a novel role for Grx to mediate eNOS activation by regulating Akt activation (supplemental Figure I, available online at http://atvb.ahajournals.org). Grx has been shown to protect Akt from disulfide bond formation under oxidative stress.13 We propose that flow increases GR activity, which increases GSH/GSSG ratio18 and activates Grx (supplemental Figure I). Grx now maintains Akt in the reduced form, which enables its activation and stimulation of the eNOS-NO signaling pathway. Evidence to support this concept includes: (1) Flow increases Grx activity (Figure 1); (2) GR mediates Grx activity, Akt and eNOS phosphorylation induced by flow (Figures 2 and 4); (3) Inhibiting GR with BCNU blocks Akt and eNOS phosphorylation induced by flow (Figure 3); (4) Grx overexpression significantly increased Akt and eNOS activation basally (Figure 5); and (5) Grx knockdown inhibits Akt-eNOS activation induced by flow (Figure 6).

We have recently shown that unidirectional laminar shear increases the intracellular levels of GSH, which is the major intracellular antioxidant. Thus, over the long term, unidirectional laminar shear stress has a predominant antioxidant effect.25 We previously showed that flow significantly increased GR activity, but did not change GPX or catalase activity.18 Activation of GR increases the ratio of GSH/GSSG and inhibits H2O2-induced JNK activation.18 Here we show that flow increases Grx activity via a GR-dependent mechanism (Figure 2). However, it is still not clear how flow activates GR in ECs. It is possible that flow causes a posttranslational modification of GR that enhances its activity.

Flow may regulate other proteins besides GR, such as the multidrug resistance protein-1 (MRP1), to increase Grx.
activity. It has been recently reported that human endothelial cells express MRP1 and use this as their major exporter of GSSG.26 MRP1 inhibition reduced apoptosis caused by oscillatory shear by increasing the intracellular GSH/GSSG ratio. Although decreasing MRP1 expression with siRNA had minimal effect on GR activity induced by flow,26 it is possible that MRP1 affects Grx activity by changing the GSH/GSSG ratio.

It has been reported that Grx and GSH play important roles in regulating Akt phosphorylation.24 There are multiple mechanisms by which Grx may affect Akt activation. First, many papers have suggested that Grx controls cell function by regulating Akt redox state.13,27 This is supported by data of Murata et al who showed that overexpression of Grx protected Akt from H2O2-induced oxidation.13 Second, Grx may regulate Akt phosphorylation by upregulating Akt activators, such as PI3K and VEGF receptor, or downregulating Akt inhibitors, such as apoptosis signal-regulating kinase1 (ASK1). It has been shown that Grx can bind to ASK1 and suppress its activation.28 Third, Grx may decrease recruitment of PP2A to Akt, resulting in a sustained phosphorylation of Akt and inhibition of apoptosis.13 Thus, Grx may affect Akt activation by inhibiting PP2A dephosphorylation of Akt and modifying function of proteins that activate or inhibit Akt.

In addition to effects on Akt, Grx may regulate other kinases, such as PKA, which can also increase eNOS phosphorylation. It has been demonstrated that PKA can be glutathionylated at cysteines 199 and 343.29 This modification inhibits PKA activity. Grx may de glutathionylate PKA and restore its activity.29 It has been shown that flow activates eNOS by phosphorylation at Ser1179 through a mechanism dependent on PKA activity.30 So it is possible that Grx may also regulate a flow–PKA–eNOS signaling pathway that results in eNOS activation.

In summary, we found that Grx plays an important role in flow-mediated Akt–eNOS–NO signaling pathway in a GR-dependent manner. These findings suggest that Grx may be an important molecule to study for therapies that can improve EC function and limit vascular disease.

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**Disclosures**

None.

**References**


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Supplemental data.

Fig. I. Model for Grx regulation of flow stimulated NO production.

Flow increases Grx activity by regulating GR activity. Grx enhances activation of Akt leading to phosphorylation of eNOS and NO production.
Figure I

Flow → GR → Grx → p-Akt → p-eNOS → NO