Fluid Shear Stress Attenuates Hydrogen Peroxide–Induced c-Jun NH₂-Terminal Kinase Activation via a Glutathione Reductase–Mediated Mechanism

Yukihiro Hojo, Yuji Saito, Tatsuo Tanimoto, Ryan J. Hoefen, Christopher P. Baines, Kei Yamamoto, Judith Haendeler, Reto Asmis, Bradford C. Berk

Abstract—c-Jun NH₂-terminal kinase (JNK) is activated by a number of cellular stimuli including reactive oxygen species (ROS). Previous studies have demonstrated that fluid shear stress (flow) inhibits cytokine-induced JNK activation in endothelial cells (ECs). In the present study, we show JNK activation by ROS in ECs and hypothesized that flow inhibits ROS-induced JNK activation in ECs via modulation of cellular protection systems against ROS. JNK was activated by 300 μmol/L hydrogen peroxide (H₂O₂) in bovine lung microvascular ECs (BLMVECs) with a peak at 60 minutes after stimulation (6.3 ± 1.2-fold increase). Preexposure of BLMVECs to physiological steady laminar flow (shear stress = 12 dyne/cm²) for 10 minutes significantly decreased H₂O₂-induced JNK activation. Thioredoxin and glutathione are cellular antioxidants that protect cells against ROS. Flow induced a significant increase in the ratio of reduced glutathione to oxidized glutathione consistent with a 1.6-fold increase in glutathione reductase (GR) activity. Preincubation of BLMVECs with the GR inhibitor, 1,3 bis-(2 chloroethyl)-1-nitrosourea, abolished the inhibitory effect of flow. In contrast, preincubation of BLMVECs with azelaic acid, a specific inhibitor for thioredoxin reductase, did not alter the effect of flow on H₂O₂-induced JNK activation. Overexpression of GR mimicked the effect of flow to inhibit JNK activation. These results suggest that flow activates GR, an important regulator of the intracellular redox state of glutathione, and exerts a protective mechanism against oxidative stress in endothelial cells. (Circ Res. 2002;91:712-718.)

Key Words: oxidative stress ■ endothelial cell ■ signal transduction

A growing body of evidence indicates that reactive oxygen species (ROS) are involved in cardiovascular diseases such as atherosclerosis, reperfusion injury, heart failure, and hypertension. Previous studies have clarified that ROS are important regulators of signaling events in pathological cardiovascular condition. Among cellular signals, members of mitogen-activated protein (MAP) kinases play critical roles in cellular proliferation, inflammation, and apoptosis. We have demonstrated that hydrogen peroxide (H₂O₂) activates MAP kinases in cultured cells derived from vascular tissue.

c-Jun NH₂-terminal kinase (JNK) is activated by a number of cellular stimuli including proinflammatory cytokines and ROS. It is speculated that JNK plays an important role in proatherogenic signal events through phosphorylation of c-Jun, activation of AP-1, and stimulation of proinflammatory gene expression such as ICAM-1. Our previous studies have demonstrated that laminar fluid shear stress (flow) inhibits cytokine-induced JNK activation in vascular endothelial cells. These results are consistent with the concept that flow exerts an atheroprotective effect against inflammatory cytokines.

Cardiovascular risk factors such as smoking, hypercholesterolemia, diabetes, and hypertension have been shown to share a common pathogenic mechanism in that they increase ROS and are associated with endothelial dysfunction. Because laminar flow is atheroprotective, it is possible that flow inhibits ROS-induced signaling events related to endothelial dysfunction. Two major intracellular antioxidant systems are the thioredoxin and glutathione systems. In the present study, we hypothesized that flow inhibits ROS-induced JNK activation in endothelial cells via modulation of these cellular antioxidant systems.

Materials and Methods

Reagents

Cell culture media was purchased from GIBCO-BRL. Azelaic acid, 1,3 bis-(2 chloroethyl)-1-nitrosourea (BCNU), mercapto succinic acid, 5,5′-dithiobis (2-nitrobenzolic acid) (DTNB), NADPH, glutathione (oxidized and reduced form), and metaphosphoric acid were...
from Sigma. Anti-JNK2 antibody was from Cell Signaling. Antibody against green fluorescent protein (GFP) was purchased from Clontech.

**Cell Culture**

Bovine lung microvascular ECs (BLMVECs) were purchased from VEC technologies (Rensselaer, NY), maintained in MCDB 131 medium supplemented with 10% fetal bovine serum, 0.09 mg/mL heparin, 10 ng/mL EGF, and endothelial growth factors. BLMVECs at passage between 5 to 8 were used for experiments.

**Shear Stress Protocol**

BLMVECs were grown on 60-mm dishes. Before the experiment, cells were rinsed free of culture medium with Hanks’ balanced saline solution (in mmol/L: 130 NaCl, 5 KCl, 1.5 CaCl₂, 1.0 MgCl₂, and 20 HEPES, pH 7.4) supplemented with 10 mmol/L glucose and either maintained at static condition or subjected to shear stress. For inhibitor experiments, cells were treated with 10 mmol/L glucose and either maintained at static condition or subjected to shear stress. Shear stress protocol was performed as previously described.

**Measurement of GSH/GSSG Ratio**

GSH/GSSG ratio was measured with commercially available kit (OxisResearch) according to the manufacturer’s instruction. Briefly, BLMVECs grown on 60-mm dishes were harvested with 100 μL of ice-cold cell lysis buffer (in mmol/L: 20 Tris-HCL, pH 7.4, 1 EDTA, 1 NaVO₃, and 50 NaF) and protease inhibitors. Cell lysates were centrifuged at 10 000 g for 10 minutes, and supernatant was collected. Lysates were mixed with 100 μL of ice-cold cell lysis buffer same as GR assay for total glutathione and 100 μL of cell lysate for protein quantification with 50 μg/mL hydrocortisone, 10 ng/mL EGF, and endothelial growth factors. BLMVECs at passage between 5 to 8 were used for experiments.

**Activity of glutathione reductase (GR) was calculated by the linear regression line generated by GR (from Baker’s yeast) purchased from Sigma. There was a linear relationship between rate of increase in the absorbance at 405 nm and GR activity from 2 to 40 mU/mL of GR in the reaction buffer.**

**Glutathione Peroxidase Activity Assay**

Cell lysates were prepared with the same protocol for GR activity measurement. The reaction mixture contained 100 mmol/L sodium phosphate buffer, pH 7.0, 0.5 mmol/L EDTA, 1 mmol/L NaN₃, 0.2 mmol/L NADPH, 1 U of GR, and 2 mmol/L reduced form of glutathione (GSH). After addition of 5 μL of cell lysate, 990 μL of reaction mixture was incubated at 25°C for 2 minutes in 1.5 mL semimicro cuvette. Enzymatic reaction was initiated by addition of 10 μL of 1.5 mmol/L H₂O₂. The conversion of NADPH to NADP⁺ was monitored by continuous recording of changes in absorbance at 340 nm between 2 and 4 minutes after initiation of the reaction with a 1-cm light path. The relationship between rate of decrease in the absorbance at 340 nm and glutathione peroxidase (GPX) activity was linear from 2.5 to 20 mU/mL of GPX in the reaction buffer.

**Catalase Activity Assay**

Briefly, BLMVECs grown on 60-mm dishes were harvested with 250 μL of ice-cold cell lysis buffer (150 mmol/L NaCl, 5 mmol/L EDTA, 0.01% digitonin, 0.25% deoxycholic acid). Cell lysates were centrifuged at 14,000 g for 10 minutes, and supernatant was collected. The following reagents were prepared for enzyme reaction (in mmol/L): 50 Tris-HCl, pH 8.0, 0.25 EDTA, and 9 H₂O₂. The reaction was initiated by the addition of 2 μL of cell lysates to 998 μL of reaction buffer. Absorbance at 240 nm was measured continuously at 25°C. Catalase activity was expressed by the rate constant of a first-order reaction (k). For a time interval of 15 seconds, the following equation was used for calculation of k: k = 0.153(log A₁/A₂)/(sec⁻¹), where A₁ is absorbance at 240 nm at t=0, A₂ is absorbance at 240 nm at t=15 seconds. Activity was standardized by the amount of protein in the samples.

**Measurement of GSH/GSSG Ratio**

GSH/GSSG ratio was measured with commercially available kit (OxisResearch) according to the manufacturer’s instruction. Briefly, BLMVECs grown on 60-mm dishes were harvested with 100 μL of ice-cold cell lysis buffer same as GR assay for total glutathione and with the buffer containing 3 mmol/L 1-methyl-2-vinylpyridium trifluoromethane sulfonate for GSSG measurement. Cell lysates were centrifuged at 14,000 g for 10 minutes, and supernatant was collected. Lysates was mixed with 100 μL of 5% metaphosphoric acid and centrifuged again. Supernatant was mixed with assay buffer with 10-fold dilution. After addition of 200 μL of diluted sample, 200 μL of DTNB reagent and 200 μL of enzyme were added and incubated at 25°C for 5 minutes in 1.5 mL semimicro cuvette. Enzymatic reaction was initiated by addition of 200 μL of NADPH reagent. Changes in absorbance at 412 nm were monitored for 3 minutes after initiation of the reaction with a 1-cm light path.

**Cell Transfection**

The mammalian cell expression vector encoding GR was kindly provided by Dr Asmis. Mouse GR was cloned into a pcDNA3 vector.
in-frame with the expression cassette for enhanced green fluorescence protein (EGFP) and is expressed as a C-terminal EGFP fusion protein. BLMVECs were grown to 80% confluence in 60-mm dishes and transfected with 4 μg plasmid DNA using LipofectAMINE Plus reagent (Life Technologies Inc) according to the manufacturer’s instruction. Assays of GR activity and JNK kinase activity were performed 2 days after transfection.

Statistical Analysis
Data are expressed as mean±SEM. Differences were analyzed by 1-way analysis of variance followed by Tukey’s post hoc test. A value of P<0.05 was considered significant.

Results
Effect of Flow on H₂O₂-Induced JNK Activation
As shown in Figure 1A, 300 μmol/L H₂O₂ activated JNK (6.3±1.2-fold increase) with a peak at 60 minutes after stimulation in BLMVECs. As shown in Figure 1B, JNK was activated by H₂O₂ in a dose-dependent manner with maximum at 1000 μmol/L H₂O₂ (6.2±0.3-fold increase).

The effect of flow on JNK activation by H₂O₂ was studied (Figure 2). To investigate the effect of flow, we used the following protocol. BLMVECs were preexposed to flow (12 dyne/cm² of shear stress) for 10 minutes or maintained in medium for 10 minutes under static conditions. Then, cells were kept under static conditions for an additional 60 minutes with or without stimulation by 300 μmol/L H₂O₂. Flow alone did not have any effect on JNK activity (Figure 2, lane 4). H₂O₂ induced a significant increase in JNK activity and preexposure of BLMVECs to flow decreased H₂O₂-induced JNK activation significantly (43% inhibition, Figure 2, lanes 2 and 3).

Role of Thioredoxin System in H₂O₂-Induced JNK Activation
To determine the mechanism responsible for flow-mediated increases in GSH/GSSG and inhibition of JNK activation, we studied enzymes responsible for regulation of thioredoxin and glutathione, the major intracellular antioxidants. Figure 3 shows the effect of a specific inhibitor for thioredoxin reductase (TrxR), azelaic acid, on changes in H₂O₂-induced JNK activation. Inhibition of TrxR did not affect the inhibitory effect of flow on H₂O₂-induced JNK activation (inhibition of 50% before treatment and 45% after treatment). A similar result was obtained using another inhibitor of TrxR, 13-cis-retinoic acid (data not shown).

Role of Glutathione System in H₂O₂-Induced JNK Activation
Because inhibition of TrxR did not prevent flow-mediated effects, we examined the involvement of the glutathione system in H₂O₂-induced JNK activation. As shown in Figure 4A, pretreatment of BLMVECs with 25 μmol/L BCNU, which lowers GSH, abolished the inhibitory effect of flow, suggesting an important role for glutathione. The two major regulators of GSH concentration are glutathione peroxidase (GPX) and glutathione reductase (GR). GPX catalyzes H₂O₂ to H₂O by using GSH as an electron donor. We investigated the effect of the GPX inhibitor, mercaptosuccinic acid on H₂O₂-induced JNK activation. As shown in Figure 4B, inhibition of GPX by 100 μmol/L mercaptosuccinic acid did not have a significant effect on flow-mediated inhibition of JNK activation. There are no well-characterized GR pharmacological inhibitors. Therefore, we evaluated changes in the
enzyme activity of GR induced by flow. As shown in Figure 5A, flow increased GR activity by 1.6-fold (peak at 5 minutes). In contrast, no significant changes were observed in GPX activity or catalase activity induced by flow (Figures 5B and 5C). Consistent with the data in Figure 4A, treatment of BLMVECs with 25 μmol/L BCNU decreased GR activity, both during incubation and after exposure to flow (below the detection limit). Treatment of BLMVECs with 300 μmol/L H₂O₂ did not change GR activity within 60 minutes (data not shown). TrxR activity was also below the detection limit in BLMVECs under basal condition and after flow. These results suggest that GR activity is the major determinant of GSH concentration in response to flow.

Changes in Cellular Redox State Induced by Flow

We next examined the effect of flow on cellular redox state by measuring reduced glutathione (GSH) and oxidized glutathione (GSSG) levels in BLMVECs. There was no significant change in total glutathione level in BLMVECs before and after exposure of flow (Figure 6A). However, the relative amount of GSSG to total glutathione decreased significantly after flow exposure (Figure 6B). As a consequence, flow induced significant increases in the GSH/GSSG ratio, a marker of a less-oxidized cellular redox state (Figure 6C).

Figure 5. Changes in activity of glutathione reductase, glutathione peroxidase, and catalase by flow. BLMVECs were subjected to flow as indicated times. Activity of each enzyme was measured by the protocol described in Materials and Methods. GR activity increased significantly after flow exposure (A). There were no significant changes in GPX (B) or catalase (C) activity by flow. Data are expressed as mean±SEM (n=5). *P<0.05 vs static condition (time=0).

Effect of GR Overexpression on H₂O₂-Induced JNK Activation

To confirm the role of GR in mediating inhibition of H₂O₂-induced JNK activation, we overexpressed GR, by adenoviral-mediated transfection of BLMVECs with a green fluorescence protein (GFP)–tagged GR construct. We found that transfection efficiency was approximately 50% by immunohistochemical staining using anti-GFP antibody. As shown in Figure 7A, GR activity in BLMVECs overexpressing GFP-GR was significantly higher than that in control or BLMVECs transfected with plasmid DNA encoding GFP alone. H₂O₂-induced JNK activation in BLMVECs overexpressing GFP-GR was significantly inhibited compared with control or BLMVECs overexpressing GFP alone (Figure 7B).

Discussion

The major finding of the present study is that flow inhibits H₂O₂-induced JNK activation in endothelial cells via a mechanism dependent on glutathione reductase. Recently, apoptosis signal–regulating kinase 1 (ASK1) was identified as an upstream activating kinase for JNK, which plays an important role for JNK activation by tumor necrosis factor (TNF) and H₂O₂. Saitoh et al. found that the reduced form of Trx binds to ASK1 and acts as a direct inhibitor of ASK1.
Initially, we hypothesized that steady laminar flow activates TrxR and that reduced Trx binds to ASK1, leading to the inhibition of JNK. However, our results indicate that TrxR is not the enzyme primarily responsible for JNK inhibition, because TrxR inhibition did not prevent the inhibitory effect of flow. Instead, we found a key role for GR, because on one hand, flow induced rapid GR activation and increased the GSH/GSSG ratio; and on the other hand, inhibition of GR abolished the inhibitory effect of flow, and overexpression of GR inhibited activation of JNK.

Flow induced a significant increase in GR activity but did not change GPX or catalase activity. Because catalase is mainly localized in peroxisomes, it is unlikely to be involved in cytosolic signaling events. Activation of GR increases

Figure 6. Changes in redox state of glutathione by flow. BLMVECs were subjected to flow as indicated times. Levels of GSH and GSSG were measured by the protocol described in Materials and Methods. Total glutathione level was not changed by flow (A). Flow induced significant decreases in relative amount of GSSG to total glutathione (B) and increases in GSH/GSSG ratio (C). Data are expressed as mean±SEM (n=3). *P<0.05, †P<0.01 vs static condition (time=0).

Figure 7. Effect of GR overexpression on JNK activation by H2O2. A, BLMVECs were transfected with plasmid DNA encoding GR. Mock transfection was used for control. GR activity in BLMVECs that overexpress GFP tagged GR (GFP-GR) was significantly higher than in control or GFP-transfected BLMVECs. Data are expressed as mean±SEM (n=6). †P<0.01. B, Effect of GR overexpression on JNK activation was examined in BLMVECs. For stimulation, BLMVECs were incubated with 300 μmol/L H2O2 for 60 minutes. JNK activity in GFP-GR expressing BLMVECs was significantly less than in control or GFP-expressing BLMVECs. Equal expression of JNK2 and GFP were confirmed by Western blotting. Data are expressed as mean±SEM (n=3). *P<0.05.
GR activation might play some role in modulation of 14-3-3/ASK1 interaction. Future study is necessary to clarify the role of the glutathione system for JNK signaling. The results of the present study suggest that flow augments intracellular protection against ROS via a GR-dependent mechanism. Our findings are in agreement with the results of Hermann et al.\(^\text{35}\) who showed that NO and glutathione play important roles for protection of endothelial cells from \(\text{H}_2\text{O}_2\)-induced apoptosis in the long-term flow model. In their model, combined inhibition of NO and \(\gamma\)-glutamylcysteine synthase (a rate limiting enzyme for glutathione synthesis) was required to reverse the protective effect of flow. Interestingly, Moeller et al.\(^\text{24}\) reported that NO induces glutathione synthesis through activation of \(\gamma\)-glutamylcysteine synthase. In summary, we found that GR plays an important role in flow-mediated inhibition of JNK signaling via inducing a reduced intracellular redox state. These findings suggest that GR and Trx represent important molecules to study for therapies that improve endothelial dysfunction and limit atherosclerosis.

**Acknowledgments**

This research was supported by grants from Banyu Fellowship in Lipid Metabolism and Atherosclerosis to Y.H., and NIH HL1B1 grants (HL68409 and 49192) to B.C.B.

**References**


Fluid Shear Stress Attenuates Hydrogen Peroxide–Induced c-Jun NH₂-Terminal Kinase Activation via a Glutathione Reductase–Mediated Mechanism
Yukihiro Hojo, Yuji Saito, Tatsuo Tanimoto, Ryan J. Hoefen, Christopher P. Baines, Kei Yamamoto, Judith Haendeler, Reto Asmis and Bradford C. Berk

Circ Res. 2002;91:712-718; originally published online September 19, 2002; doi: 10.1161/01.RES.0000037981.97541.25
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/91/8/712

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/