Cyclic Strain-Induced Thrombomodulin Expression in Endothelial Cells is Mediated by Nitric Oxide, but Not Hydrogen Peroxide

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Vascular endothelial cells (ECs) are constantly subjected to rhythmic distension because of pulsatile flow in the circulation. The rhythmic distension-induced cyclic strain plays an important role in modulating endothelial physiology. In the present study, we investigated the effects of cyclic strain on the expression of thrombomodulin (TM), an anti-coagulant protein that possesses anti-inflammation properties. Exposure of ECs to cyclic strain increased NADH/NADPH oxidase and endothelial nitric oxide synthase (eNOS) activities, as well as the nitric oxide (NO) production. When ECs were subjected to a greater cyclic strain (21%), TM expression was increased. The increased TM expression was not observed when ECs were subjected to a lower cyclic strain (15%). ECs treated with an NO donor (NOC18), induced TM expression, whereas hydrogen peroxide (H2O2) treatment did not have significant effects on TM expression. Pretreatment of ECs with an eNOS inhibitor (L-NAME) abolished the cyclic strain-induced TM expression in ECs. These results suggest that cyclic strain to ECs induces NO production which consequently results in an increase of TM expression in ECs. Our findings provide insights into the mechanisms by which cyclic strain induces TM expression in ECs, therefore playing important roles in anticoagulation and atheroprotection in the vascular system.

Key Words: Cyclic strain • Endothelial cell • Nitric oxide • Reactive oxygen species • Thrombomodulin

INTRODUCTION

ECs are constantly under the influence of hemodynamic forces, including blood flow-induced shear stress and rhythmic distension-induced cyclic strain. This cyclic strain to vessel walls plays an important role in the modulation of signaling pathways and gene expression in the vascular system. Any changes of cyclic strain may ultimately contribute to vascular complications, including hypertension-related vascular disorders. It is well documented that ECs subjected to cyclic strain transmit this mechanical force into intracellular signals and induce cellular responses. Recent evidence suggests that reactive oxygen species (ROS) play a pivotal role in growth factor- and hemodynamic force-induced endothelial responses. Increasing intracellular ROS levels in ECs induce redox-sensitive genes that may be involved in vascular dysfunction, including atherosclerosis and hypertension-related complications. It has been shown that ECs subjected to cyclic strain increase intracellular ROS levels, which subsequently induce the expression of various redox-sensitive genes, including monocyte chemotactic protein-1 (MCP-1), a protein that is shown to be involved in the recruitment of monocytes into the subendothelial space during athero-
Removal of ROS production by antioxidant treatment alleviated this cyclic strain-induced MCP-1 expression in ECs. The origin of ROS produced in activated ECs has not been fully clarified. NADPH oxidase has been suggested to be one of the major sources of ROS production in ECs in response to hemodynamic forces. Recent evidence indicates that a gp91phox-containing NADPH oxidase is selectively expressed in ECs.

Nitric oxide (NO), an important mediator that plays multiple roles in vascular biology, regulates blood pressure and regional blood flow as well as inhibiting vascular smooth muscle cell proliferation, platelet aggregation, and leukocyte adhesion. ECs constantly release NO via the activation of endothelial NO synthase (eNOS). NO has been shown to attenuate cytokine-induced expressions of MCP-1 and vascular adhesion molecules in ECs. A decrease in the levels of NO released from ECs aggravates vascular dysfunction. Moreover, it has been shown that local gene transfer of eNOS inhibits atherosclerotic lesions. There is increasing evidence suggesting that NO released from ECs enhances vascular resistance to ROS-induced damage.

Despite the well-recognized protective effects of NO on inflammatory responses of ECs, the detailed mechanisms of the effects of NO on ECs remain unclear. Endothelial NO appears to serve as a negative regulator to modulate redox-sensitive gene expression. Our earlier data have indicated that ECs exposed to hemodynamic forces increase their intracellular ROS levels that alter the Ras/Raf/extracellular signal-regulated kinase (ERK) signaling pathway and gene expression. Furthermore, NO negatively regulates the ERK signaling pathway and inhibits shear-induced early growth response-1 (Egr-1) expression. All these results support the notion that endothelial NO inhibits redox-sensitive gene expression, inflammation, and thrombosis, which are currently recognized as interrelated biological processes.

Thrombomodulin (TM), a vascular endothelial cell receptor and cofactor in the protein C anti-coagulant system, is potentially suppressed by inflammatory cytokines such as TNF-α and IL-1β. Furthermore, TM also plays a role in dampening inflammatory responses. While TM has been shown to exert atheroprotective effects, the effects of cyclic strain on the TM expression in ECs remain unclear. In the present study, we demonstrated that ECs exposed to a greater cyclic strain significantly enhanced the activities of NADPH/NADH oxidase and eNOS, as well as the expression of TM. The increase in TM expression by cyclic strain at least partially contributed to the higher NO production in strained ECs. Our results provide insight into the mechanisms by which cyclic strain plays atheroprotective roles in vascular biology by increasing NO production and thereby inducing TM expression in ECs.

**MATERIALS AND METHODS**

**EC cultures**

Human umbilical vein endothelial cells (HUVEC) were isolated as previously described. HUVECs were seeded on the flexible membrane base of a culture well and grown for 3 days until confluence. Cultured medium M199 containing 20% fetal bovine serum (FBS) was replaced with the same medium containing only 2% FBS, and cells were incubated overnight prior to use in the experiments. For gene transfection study, bovine aortic ECs (BAECs) were cultured in DMEM medium supplemented with 10% FBS, penicillin, and streptomycin.

**In vitro cyclic strain**

The strain unit Flexcell FX-2000 (Flexcell), described in detail elsewhere, consists of a vacuum unit linked to a valve controlled by a computer program. ECs cultured on a flexible membrane base were deformed by a sinusoidal negative pressure with a peak level of 20 kPa, which produced a strain on cells ranging from minimal strain at the center of the membrane to a peak value of 25% at the periphery (maximal strain is 25%, average strain is 12%) at a frequency of 1 Hz (60 cycles/min) for various intervals.

**Immunoblotting**

ECs were lysed with a buffer solution containing 0.1% SDS and 2-mercaptoethanol, and then subjected to SDS-PAGE. Antigens were analyzed using designated monoclonal antibodies (Calbiochem). Antigen-antibody complexes were detected using horseradish peroxide-labeled rabbit anti-mouse IgG and the results were analyzed using an enhanced chemiluminescence system (Pierce). The blots were then exposed to Kodak XAR-5 films to obtain fluorographic images.
NAD(P)H oxidase activity assay

EC lysate was washed with ice-cold PBS and homogenized in cold lysis buffer (20 mmol/L KH₂PO₄, pH 7.0, 1 mmol/L EGTA, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.5 mmol/L PMSF). The homogenate was centrifuged at 1000 g for 10 min at 4 °C. The pellet was resuspended in a lysis buffer containing protease inhibitors and manually homogenized on ice. NADPH oxidase activity was measured by a luminescence assay in a 50 mmol/L phosphate buffer, pH 7.0, containing 1 mmol/L EGTA, 150 mmol/L sucrose, 5 μmol/L dark-adapted lucigenin as the electron acceptor, and 100 μmol/L NADPH as the substrate in a final volume of 900 μL. The reaction was started by the addition of 100 μL of homogenate, and luminescence measurements were obtained every 15 sec for 5 min. Protein content was determined in an aliquot of the homogenate, and the result was standardized to the corresponding luminescence measurement.

Assay of eNOS activity

NOS activity was determined by measuring the conversion of [³H]-L-arginine to [³H]-L-citrulline as previously described.²³

Assay of NOx concentration

To measure the level of NO, the colorimetric Griess reaction for nitrite was applied. In brief, the conditioned medium was exposed to nitrate reductase (250 mU/ml) and NADPH (100 μM) for 30 min at 37 °C to reduce nitrate to nitrite. The nitrite-containing samples were treated with L-glutamine dehydrogenase (670 mU/ml), 2-oxoglutaric acid (4 mM), and NH₄Cl (100 mM) for 10 min at 37 °C to consume any residual NADPH. Finally, the samples were mixed with an equal volume of freshly prepared Griess reagent (0.05% N-(1-naphthyl) ethylenediamine dihydrochloride and 0.5% sulfanilamide in 2.5% ortho-phosphoric acid) for 5 min at 37 °C. The absorbance of each colored sample was measured at 540 nm using a spectrophotometer SPEKOL 221 (Carl Zeiss, Jena, Germany). Concentrations of NO in the samples were determined using a calibration curve generated from standard KNO₃ solutions.

Statistical analysis

Statistical analyses were performed using Student’s t-test. Data are presented as mean ± SEM. Statistical significance was defined as p < 0.05.

RESULTS

Cyclic strain induced NADPH & NADH oxidase activities in ECs

ECs cultured on flexible membrane bases were subjected to cyclic strain-induced deformation. ECs exposed to cyclic strain for 24 h remained morphologically intact. The activities of NADH and NADPH oxidase in strained ECs were examined. As shown in Figure 1, exposure of ECs to cyclic strain induced a rapid increase in the activity of NADH or NADPH oxidases. The increase in NADH oxidase or NADPH oxidase activities remained sustained over the 24-h period. These results suggest that cyclic strain to ECs may result in an increase in the intracellular ROS levels.

Cyclic strain induced eNOS and NO release

To investigate whether cyclic strain to ECs could induce intracellular NO levels, ECs were subjected to cyclic strain and the eNOS activity and the NO release were examined. As shown in Figure 2A, the activity of eNOS in cyclic strain-treated ECs was rapidly induced, reaching maximal levels within 3 to 6 hrs, and then declined but still remained at an elevated level after continuous cyclic strain for 24 hrs. Consistently, the levels of

![Figure 1](image-url)  
**Figure 1.** Cyclic strain stimulated NADPH and NADH oxidase activities in ECs. ECs were subjected to cyclic strain at various time points and their NADPH and NADH oxidase activities were examined. Data are expressed as mean ± SEM (n = 6).
NOx (nitrate+nitrite) in conditioned medium of strained ECs were also increased, reached its plateau at 3 hrs, and remained sustained levels after cyclic strain for 24 hrs (Figure 2B). These results suggest that ECs exposed to cyclic strain enhances eNOS activity and results in an increase of NO production.

**Cyclic strain induced the TM expression in a time- and strain force-dependent manner**

To investigate the effects of cyclic strain on the TM expression in ECs, ECs were subjected to 21% or 15% of cyclic strain for various time intervals, and their TM protein expression was examined by immunoblotting. As shown in Figure 3A, exposure of ECs to 21% of cyclic strain for 4 hrs induced TM expression. The increased TM expression was shown to be sustained after cyclic strain for 8 hrs. ECs exposed to a lower cyclic strain (15%) for 2 to 8 hrs did not induce TM expression (Figure 3B). This strain force-dependent expression of TM in ECs was further substantiated by subjecting ECs to a higher (21%) cyclic strain as compared to those under lower (15%) cyclic strain for 8 or 18 hrs. Exposure of ECs to 21% cyclic strain for 8 or 18 hrs increased their TM expression, whereas ECs under 15% cyclic strain for 18 hrs did not alter the TM expression, as compared with static controls (Figure 3C). This cyclic strain-induced TM expression was not a transcriptional event, as TM

![Figure 2](image-url)  
*Figure 2. Cyclic strain induces eNOS activity NO production in ECs. ECs were kept as control or subjected to cyclic strain at various time points, and their eNOS activity (A) and NO production (B) were examined. The activity of eNOS was monitored by the biochemical conversion of radioactive substrate $^3$H-arginine into L-citrulline (A). NOx (nitrate+nitrite) production was measured by reaction with Griess reagents (B). Results are expressed as mean ± SEM from 3 to 6 separate experiments. *P < 0.05 vs. unstrained control cells.*

![Figure 3](image-url)  
*Figure 3. Cyclic strain induced endothelial TM expression. ECs were subjected to different magnitude of cyclic strain [21% (A) vs. 15 % (B)] for 2, 4, 6 or 8 hrs (A and B) or 18 hrs (C), and their TM expression was determined using immunoblotting. Data are expressed as mean ± SEM from 3 to 6 separate experiments. *P < 0.05 HS-treated ECs vs. unstrained control cells.*
promoter activity was not induced by cyclic strain to ECs (data not shown). These results suggest that cyclic strain induces the TM expression in ECs in a time- and strain force- dependent manner.

**NO, but not hydrogen peroxide (H$_2$O$_2$), mediates cyclic strain-induced TM expression in ECs**

We have demonstrated that cyclic strain to ECs induces the activities of NADH and NADPH oxidase and eNOS, as well as the production of NO. We further investigated whether the intracellular NO or ROS level involved in cyclic strain-induced TM expression in ECs. As shown in Figure 4A, ECs treated with an NO donor (NOC18) at a concentration of 500 μM for 4 hrs significantly increased the TM expression in ECs. This NO-induced TM expression returned to the basal level 8 or 16 hrs after treatment. In contrast, ECs treated with H$_2$O$_2$ did not alter the TM expression, as compared to those control cells. These results suggest that the oxidative stress or increased ROS levels may not induce TM expression in ECs. To investigate whether NO mediates the cyclic strain-induced TM expression in ECs, ECs were pre-treated with an inhibitor to eNOS (L-NAME) and then subjected to cyclic strain for 6 hrs in the presence of L-NAME. As shown in Figure 4C, ECs treated with L-NAME remarkably inhibited the cyclic strain-induced TM expression. These results suggest that the cyclic strain-induced TM expression is mediated by the NO-dependent mechanisms in ECs.

**DISCUSSION**

ECs lining the vascular wall are constantly subjected to hemodynamic forces, including blood pressure-generated cyclic strain. How the ECs sense physical forces and transmit into intracellular signals remains an interesting issue. Our previous studies have shown that cyclic strain of physiological range (~15%) to ECs increases intracellular ROS levels that may act as secondary messengers for signaling modulation.$^{24,25}$ In addition, ECs subjected to hemodynamic forces enhance NO production, which has been shown to serve as a negative regulator by modulating redox-sensitive gene expression.$^5$ In the present study, we observed that ECs exposed to a greater cyclic strain induced NADH/NADPH oxidase activities, the major sources of ROS production in ECs. ECs under higher cyclic strain (24%), in contrast to those ECs under physiological 10%

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**Figure 4.** Cyclic strain-induced TM expression was mediated by NO. (A) ECs were treated with an NO donor (NOC18, 500 μM) for 4, 8, and 16 hrs, and TM protein expression was determined by using immunoblot analysis. Data are expressed as mean ± SEM from 3 separate experiments. *P < 0.05 vs. control cells. (B) To investigate the effects of ROS on endothelial TM expression, ECs were treated with H$_2$O$_2$ at concentrations of 100 or 500 μM for 4 or 8 hrs. (C) In some experiments, ECs were pre-treated with an eNOS inhibitor (L-NAME, 500 μM) for 30 min and then exposed to cyclic strain for 6 hrs in the presence of L-NAME.
strain, increased eNOS activity. These strain-dependent responses are consistent with our current finding that higher strain induces TM expression. All these results suggest that ECs subjected to cyclic strain increase intracellular ROS and NO levels, which may play important roles in modulating redox-based responses in ECs toward various stimuli.

TM forms a 1:1 stoichiometric complex with thrombin, which becomes less available to act on its substrates, notably platelets and fibrinogen. TM also accelerates protein C activation by thrombin. Activated protein C inactivates activated factors V and VIII, thereby exerting a potent negative feedback control on the generation of thrombin. Thus, the membrane glycoprotein TM is an important anti-coagulant component of ECs. In the present study, we demonstrated that a greater cyclic strain (21%) induced a sustained increase in TM expression in ECs, whereas normal range of cyclic strain (15%) did not show significant effects on TM expression. An earlier study demonstrated that fluid shear stress to bovine ECs resulted in a mild transient increase followed by a significant decrease in TM mRNA level. In a recent study, saphenous vein endothelium exposed to arterial flow downregulated TM. These results are different from our observations that TM was upregulated by cyclic strain. Indeed, independent roles for shear stress and cyclic strain as mechanical stimuli do occur in cells. The detailed molecular mechanisms responsible for the different downstream responses are not clear and need to be further investigated. Nevertheless, our results indicate that TM expression in ECs can be upregulated by the higher magnitude of cyclic strain. Because ECs are constantly under rhythmic distension-induced cyclic strain due to pulsatile flow, endothelial TM expression may be upregulated by higher blood pressure as circulating TM levels were reported to be elevated in hypertensive patients as compared to normotensive subjects. These are consistent with our finding that higher strain may upregulate TM expression in ECs. An increase of TM levels in hypertensive patients may support our notion that cyclic strain plays a role in regulating TM expression. Our results suggest that an increased TM expression in ECs exposed to cyclic strain may play important roles in anticoagulation and atheroprotection in vessel walls.

As cyclic strain to ECs induces intracellular levels of ROS and NO, our present results demonstrated that the increases in intracellular levels of NO, but not ROS, contributed to the cyclic strain-induced TM expression in ECs. ECs treated with an NO donor, NOC18, induced the TM protein expression in a time- and dose-dependent manner. However, treatment of ECs with H2O2 did not alter the TM expression as compared to static control cells. In concert with the increased TM expression in NO-treated ECs, pretreatment of ECs with an eNOS inhibitor (L-NAME) abolished the cyclic strain-induced TM expression. These results suggest that cyclic strain-induced NO production is involved in TM expression in ECs. However, TM promoter activity was not induced by cyclic strain to ECs. The stability of TM expression may be responsible for upregulation of TM expression in strain-ECs. How the increased intracellular NO activity affects TM expression in ECs remains unclear. NO treatment may attenuate growth factor-stimulated effects via the decrease of signaling responses and thus affect protein stability. NO has been shown to increase protein stability such as HIF-1. Alternatively, NO-mediated S-nitrosylation of protein may affect the protein stability. Despite the detailed mechanisms remain to be defined, our findings support the notion that NO plays anti-inflammatory and anti-thrombogenic roles in modulating gene expression and cellular function in ECs. The present results demonstrate the importance of intracellular NO in modulating cyclic strain-induced expression in ECs.

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REFERENCES


