Cyclic Strain Delays the Expression of Tissue Factor Induced by Thrombin in Human Umbilical Vein Endothelial Cells

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ABSTRACT

Most studies of tissue factor (TF) expression in endothelial cells (EC) are performed under stationary culture conditions. The purpose of this study was to determine the influence of mechanical stimuli such as cyclic strain (CS) on the expression of TF in EC exposed to thrombin (Thr). Human umbilical vein endothelial cells (HUVEC) were exposed to $4 \text{ U} \cdot \text{mL}^{-1}$ Thr in the presence or absence of 10% average CS at $60 \text{ cycles} \cdot \text{min}^{-1}$ and then TF expression was measured. TF messenger RNA (mRNA) expression peaked at 2 hours in HUVEC exposed to Thr, but at 4 hours in HUVEC exposed to both Thr + CS. TF expression was inhibited by p38 and extracellular signal-regulated protein kinase (ERK) inhibitors. For both Thr or Thr + CS stimuli, p38 and ERK activity peaked at 5 minutes ($p < 0.05$). Nuclear factor-kappa B levels remained high in the Thr group but not in the Thr + CS group, while Egr-1 levels were elevated in the Thr + CS group. We demonstrated CS-delayed, Thr-induced TF mRNA expression in HUVEC, which may be modulated by p38 and ERK inhibitors.

KEYWORDS: Hemodynamic forces, thrombin, endothelium, tissue factor, atherosclerosis

Atherosclerosis is a chronic disease with systemic risk factors such as smoking, hypertension, hypercholesterolemia, diabetes, and male gender.¹ Despite these risk factors, the localization of atherosclerosis is mainly observed in areas of complex arterial geometry, such as bifurcations, branching points, and curvatures.² In these areas, the arterial wall receives complex physical forces generated by the pulsatile blood flow. The effects of hemodynamic forces in these areas have been postulated as a critical factor in atherogenesis.³

Numerous studies have focused on the response of endothelial cells (EC) to hemodynamic forces such as shear stress, pressure, and cyclic stretch and it is clear that these stimuli regulate protein synthesis, morphology, migration, apoptosis, proliferation, and survival in EC.⁴ Our laboratory has focused on the role of cyclic strain (CS), the repetitive, circumferential pulsatile pressure distention conferred to the vessel wall, and we have previously reported an increase in EC proliferation and expression of adhesion molecules, which can be involved in atherogenesis.⁵

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The endothelium can be activated not only by mechanical forces but also by chemical stimuli, such as nicotine, lipopolysaccharide (LPS), tumor necrosis factor (TNF), and thrombin (Thr).^{6–8} Thr is a multifunctional serine protease that is not only involved in mediating the cleavage of fibrinogen to fibrin in the coagulation cascade but can also activate a variety of cell types, including platelets and EC. Thr signaling in the endothelium results in a multitude of phenotypic changes, including alterations in cell shape, permeability, vasomotor tone, leukocyte trafficking, migration, DNA synthesis, angiogenesis, and hemostasis.⁹ Thr has been demonstrated to increase the expression on EC of tissue factor (TF), a membrane-bound glycoprotein with a key role in the regulation of hemostasis.¹⁰ Under physiological conditions TF is virtually undetectable in EC but is present in abundant quantities in subendothelial cells such as smooth muscle cells and fibroblasts. Several studies have now shown a substantial increase in TF levels in advanced atherosclerotic lesions.^{11,12} For example, in the tissue factor pathway inhibitor (TFPI) knockout mice, significantly more atherosclerotic lesions were observed in areas of disturbed flow such as the carotid and iliac artery bifurcations, indicating that inhibition of TF activity by TFPI may be an important atheroprotective mechanism.¹³ Furthermore, analysis of atherosclerotic plaques has demonstrated TF in leukocytes, smooth muscle cells, and EC overlying the lesion.¹¹

In vivo, EC are continuously exposed to mechanical forces and intermittently receive chemical stimuli. However, there are only few studies examining the effect of combined chemical and mechanical stimulation. The purpose of this study was to investigate the effect of CS, as a mechanical force, and Thr, as a chemical stress, on human umbilical vein endothelial cells (HUVEC) expression of TF and to elucidate the underlying mechanochemical signaling pathway.

MATERIALS AND METHODS

Cell Culture

We obtained primary cultures of HUVEC from Dr. Jordan Pober (Yale School of Medicine). Cells were cultured in T 75 flasks with M-199 culture medium enriched with 20% fetal bovine serum (FBS), 10 $\mu\text{g}\cdot\text{mL}^{-1}$ heparin, 5 $\mu\text{g}\cdot\text{mL}^{-1}$ EC growth supplement (BD Biosciences, Palo Alto, CA), penicillin–streptomycin antibiotic combination (100 $\text{U}\cdot\text{mL}^{-1}$ and 100 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively) in an incubator at 37°C and 95% humidity. Passage 3 to 6 HUVEC were seeded on 0.1% gelatin-coated flexible membrane (Flex I plate; Flexcell, McKeesport, PA) and when they reached confluence, they were incubated for 6 hours in 1% FBS medium for synchronization. The medium was then changed to FBS-free medium before experimentation.

Application of Mechanical and Chemical Stimuli

HUVEC were subjected to repetitive mechanical deformation in the presence or absence of 4 $\text{U}\cdot\text{mL}^{-1}$ Thr (Sigma-Aldrich, St. Louis, MO) with a Flexcell Strain Unit (FX-4000; Flexcell) as previously described.^{14,15} When a vacuum of 20 kPa was applied to the membrane the average strain was 10%. EC were subjected to this repetitive strain at 60 $\text{cycles}\cdot\text{min}^{-1}$ for up to 6 hours.

RNA Isolation and Estimation of TF mRNA Levels

HUVEC were exposed to CS in the presence or absence of Thr for 2, 4, and 6 hours and then RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's specifications. Reverse transcription of RNA was done using the iScript reverse transcription kit (Bio-Rad Laboratories, Hercules, CA) and polymerase chain reaction (PCR) performed in a C1000 Thermal Cycler (Bio-Rad). TF RNA levels were estimated with quantitative real-time PCR (qRT-PCR) with β -actin serving as the housekeeping gene. The iQ SYBR Green Supermix (Bio-Rad Laboratories) was used and all reverse transcription-PCRs were performed in a CFX96 Thermal Cycler machine (Bio-Rad).¹⁶ Primer sequences for TF were: TF forward 5'-GCCAGGA GAAAGGGGAAT-3'; TF reverse 5'-CAGTGCA ATATAGCATTTCAGTAGC-3'. Sequences for β -actin were: β -actin forward 5'-TTGGAGCTGTG GTATTTGTGGTCATCATCC-3'; β -actin reverse 5'-TCACCCACACTGTGCCCATCTACG-3'. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The Pfaffl method was used to calculate fold changes in TF mRNA expression levels.¹⁷

Inhibitor Experiments

We used 10 μM SB203580 as a selective inhibitor for p38, 2.5 μM LY294002 as a selective inhibitor of Akt, and 10 μM PD98059 as an inhibitor of extracellular signal-regulated protein kinase (ERK). All inhibitors were obtained from Promega (Madison, WI). HUVEC were exposed to inhibitors with CS, Thr alone or Thr + CS for 2 and 4 hours. Inhibitors were diluted in dimethyl sulfoxide (DMSO) according to manufacturer's specifications and therefore DMSO in equal concentration was used as a control (Ctrl) in each exposure. RNA was then isolated and TF mRNA level was estimated as described above.

Electrophoretic Mobility Shift Assay

HUVEC were exposed to CS in the presence or absence of Thr for 30, 60, and 120 minutes. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA) were then performed as described previously.¹⁸

Nuclear protein was extracted from HUVEC using NE-PER Nuclear Extraction Reagents added with Halt protease Inhibitor Cocktail (Pierce Biotechnology, Rockford, IL) according manufacturer's instructions. Samples were stocked at -80°C . Double-stranded oligonucleotides of SP-1 (5'-ATTTCGATCGGGGCGGGGCGAGC-3'), activator protein-1, AP-1 (5'-CGCTTGATGAGTCAGCCGGAA-3'), nuclear factor-kappa B, NF κ B (5'-CAGAGGGACTTTCGAGA-3'), and early growth response-1, Egr-1 (5'-CCCGGCCGCGGGGCGATTTCGAGTCA-3') were obtained from Integrated DNA Technologies. The oligonucleotides were end labeled with [^{32}P]ATP by T4 kinase.¹⁹ Nuclear extracts were incubated in a buffer containing poly (dI-dC) at room temperature for 10 minutes and then incubated with the radioactive oligonucleotide at room temperature for 20 minutes. The mixture was separated by electrophoresis on a 5% (wt/vol) nondenaturing polyacrylamide gel, and the dried gels were exposed to a film. For testing the specificity of the gel shift assay, unlabeled oligonucleotide was used as a competitor. Data analysis was performed for nuclear factor induction by determination of the ratio of densitometric integrated intensity compared with Ctrl.

Immunoblotting

Activation of the p38 mitogen-activated protein kinase (MAPK), Akt and ERK was assessed by determining their phosphorylation with immunoblotting using phospho-specific antibodies (Cell Signaling, Danvers, MA) as described previously.²⁰ HUVEC were exposed to CS in the presence or absence of Thr for 5, 15, and 30 minutes. EC were then lysed in 50 mM tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.25% deoxycholic acid, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4 , 1% sodium dodecyl sulfate (SDS), and protease/phosphatase inhibitor cocktail. The extracts were heat-denatured in Laemmli sample buffer (Bio-Rad) with 5% β -mercaptoethanol (American Bioanalytical, Natick, MA) size-fractionated in a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane (Bio-Rad). After blocking for 45 minute with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% nonfat dry milk, the blotted membrane was exposed to polyclonal antibodies, which were anti-p38, anti-Akt, anti-ERK, anti-phospho-p38, ant-phospho-Akt and ant-ERK (Cell Signaling Technology). After rinsing three times for 15 minutes in TBST, the blot was incubated with a 1:3000 dilution of anti-rabbit immunoglobulin G for 45 minutes. After three additional 30-minute rinses in TBST, the resulting protein-antibody complexes were detected by enhanced chemiluminescence (Perkin Elmer, Waltham, MA). All

analyses of scanned images were performed using densitometry software (ImageJ, NIH, Bethesda, MD).

Statistical Analysis

All results are expressed as mean $\pm$ SE. Two-way repeated measures analysis of variance (ANOVA) for between groups analysis, one-way ANOVA for within group analysis, and Bonferroni test as post hoc test were employed as indicated. A p value of <0.05 was considered significant.

RESULTS

CS Delays the TF mRNA Expression Induced by Thr

Fig. 1A summarizes the pattern of TF mRNA expression in HUVEC exposed to CS, Thr, or both for 6 hours. CS did not induce expression of TF even up to 24 hours of stimulation (data not shown). TF expression peaked at 2 hours when exposed to Thr only (6.60 ± 1.33 , $p < 0.05$ vs Ctrl) but at 4 hours with combined stimulation (Thr + CS) (6.16 ± 0.94 , $p < 0.05$ vs Ctrl). To confirm the delay of peak TF expression in Thr + CS exposure we compared the ratio of TF expression at 4 hours to that at 2 hours between the Thr and Thr + CS groups (Fig. 1B). The ratio in the Thr + CS group was significantly higher than in the Thr group (1.16 ± 0.07 vs 0.69 ± 0.05 , respectively, $p < 0.01$) confirming that TF expression in HUVEC exposed to both Thr + CS was delayed compared with exposure to Thr alone.

p38 Inhibitor and ERK Inhibitor Decreased TF mRNA Expression

Fig. 2 depicts the ratio of TF expression with inhibitor compared with no inhibitor at 2 hours and 4 hours exposure to mechanical and chemical stimuli. Although the Akt inhibitor, LY294002, seemed to have a tendency to increase TF expression in each exposure, it was not statistically significant but it was consistent with previous data.¹⁶ In contrast, the ERK inhibitor (PD98059) and the p38 inhibitor (SB203580) significantly inhibited TF mRNA expression in both groups and there was no difference in inhibition pattern between the Thr group and Thr + CS group.

Activation of p38 and ERK but Not Akt When HUVEC Exposed to CS and Thr

The activities of Akt, p38, and ERK can be determined based on the ratio of phosphorylated to total protein immunoblot intensity. As values of those activities were not distributed normally, we subjected them to normalized $\log_{10}$ transformation before statistical analysis

Figure 1 Tissue factor (TF) expression on human umbilical vein endothelial cells (HUVEC) exposed to cyclic strain (CS), thrombin (Thr), or Thr+CS. (A) HUVEC were exposed to 10% average CS at 60 cycles·min⁻¹, 4 U·mL⁻¹ Thr, or 4 U·mL⁻¹ Thr with 10% average CS at 60 cycles·min⁻¹ for 2, 4, and 6 hours. TF expression in CS group had minimal change during time course while in Thr+CS group it peaked at 4 hours, and the Thr only group clearly peaked at 2 hours. (B) Ratio of TF expression at 4 hours compared with that at 2 hours in Thr group and Thr+CS group (0.69 ± 0.05 vs 1.16 ± 0.07 , respectively, $p < 0.01$). The delay of Thr-induced TF expression by CS was demonstrated by (A) and (B). Results are mean $\pm$ SE from six different experiments. $\star p < 0.05$ compared with time 0; $\ast p < 0.05$ compared with 4-hour time point; $\dagger p < 0.05$ compared with 6-hour time point by post hoc test in analysis of variance; $\ddagger p < 0.01$ vs Thr group by paired *t*-test.

(Fig. 3, bottom figure).²¹ As shown in Fig. 3A, Akt activity was significantly reduced at 5 minute exposure in each stimulation ($p < 0.01$ vs Ctrl). The attenuation in the CS group showed significant difference compared with the Thr group at 15 and 30 minutes and to the Thr+CS group at 30 minute ($p < 0.05$, respectively). Although there was no difference in Akt activity between the Thr and Thr+CS group, the activity in the Thr+CS group continued to decline during exposure time, while in the Thr group it was sustained between 15 and 30 minutes.

Fig. 3B shows that p38 activity in the CS group seemed to be increased at 5 minutes and then was attenuated during the time course, but this did not attain significance. The activity of p38 in the Thr group was significantly increased at 5 minutes (0.76 ± 0.15 ,

$p < 0.05$ vs Ctrl) and decreased at 15 minutes compared with the 5-minute time point (0.40 ± 0.13 , $p < 0.05$ vs 5-minute time point), without significant increase compared with Ctrl. In Thr+CS group, the activity was increased at 5 and 15 minutes (0.73 ± 0.13 , 0.44 ± 0.14 , $p < 0.05$ vs Ctrl, respectively) and there was no significant difference between these two time points. Although there was no difference in activity magnitude between the Thr and Thr+CS group, the activity within each group exhibited a different temporal pattern during the time course. These results suggest that p38 activity in the Thr+CS group was sustained compared with the Thr group.

Fig. 3C shows that ERK activity was significantly increased at 5 minutes as a peak in the Thr and the Thr+CS group (0.84 ± 0.20 , 0.85 ± 0.18 , $p < 0.05$ vs Ctrl, respectively), while it had minimal change in the CS group. The activity in the Thr+CS group showed the same time course pattern as in the Thr group.

NF κ B Level Was Elevated in HUVEC Exposed to Thr with or without CS and Egr-1 Was Elevated in the Thr+CS Group

Fig. 4A shows typical patterns of EMSA and Fig. 4B shows the summary densitometric data. SP-1 level did not change through this time course for all experimental groups. Although AP-1 levels appear to be increased in the groups, these were not significant. NF κ B levels had only a slight rise with CS exposure but was only significantly increased in the Thr with or without CS groups at 30 minutes ($p < 0.05$ vs Ctrl). The levels remained high through the time course in the Thr group. In the Thr+CS group, NF κ B levels at 120 minutes were increased compared with that at 60 minutes ($p < 0.05$) after it lost significant difference at 60 minutes compared with Ctrl.

Conversely, Egr-1 levels in the Thr+CS group were significantly elevated at the 30- and 60-minute time points ($p < 0.05$ vs Ctrl), while in the CS group and the Thr group no significant increases were observed compared with Ctrl.

Although comparison of transcription factor levels between groups showed no significant differences in each EMSA, the levels of NF κ B, and Egr-1 within each group seemed to have different temporal patterns.

DISCUSSION

TF expression in ECs can be induced by various chemical stimuli such as LPS, TNF- α , and Thr.^{16,22-24} However, most of these reports about the effect of chemical stimuli on EC have been performed under static culture conditions. In vivo, ECs are continuously subjected to the pulsatile mechanical forces induced by the beating heart. In the present study, we exposed HUVEC to

Figure 2 TF expression in the presence of inhibitors. 10 μ M SB203580 was used as a selective inhibitor of p38, 2.5 μ M LY294002 as a selective inhibitor of Akt, and 10 μ M PD98059 as an inhibitor of ERK. HUVEC were subjected to inhibitors with CS, Thr, or Thr + CS for 2 and 4 hours. Inhibitors were diluted in dimethyl sulfoxide (DMSO) according to manufacturer's specifications. DMSO in equal concentration was used as a control in each exposure. Bar graph shows the ratio of TF expression with inhibitor compared with no inhibitor. Although LY294002 did not significantly increase TF expression, PD 98059 and SB 203580 significantly decreased TF expression. There was no difference in inhibition pattern between the Thr group and Thr + CS group. Results are mean $\pm$ SE from three separate experiments with LY 294002 ($\square$) or SB 203580 ($\blacksquare$), or from four separate experiments with PD 98059 ($\square$). * p < 0.05 vs no inhibitor, by paired Student's t -test.

combined stimulation with Thr and CS, and measured TF expression. Our results demonstrate that HUVEC exposed to CS had minimal effect on TF expression. The addition of Thr to HUVEC exposed to CS had a similar magnitude of TF expression compared with the addition of Thr to static HUVEC. The most significant observation of this study was that there was a significant delay in the peak of TF expression when Thr was added to HUVEC exposed to stretch. To our knowledge this finding has not been previously reported in the literature. In an effort to understand the underlying mechanism of this delay, we investigated the signaling pathways that regulate TF expression. In the present study selective inhibitors of p38 and ERK significantly inhibited TF mRNA expression in HUVEC exposed to CS, Thr or Thr + CS but did not affect the temporal delay seen in the latter groups.

TF expression can be regulated by both mechanical forces and chemical stimuli but the reports are inconsistent. Mazzolai et al previously reported that human EC exposed for 24 hours to oscillatory shear stress, significantly increased TF mRNA and TF protein expression; EC exposed to unidirectional shear showed a decrease in TF activity with a significant increase in TFPI mRNA and protein expression.²⁵ Lin et al demonstrated that $1.2 \cdot 10^{-4}$ μ N $\cdot$ cm $^{-2}$ laminar shear stress can induce the TF gene in both HUVEC and bovine aortic endothelial cells (BAEC).²⁶ Silverman et al used a chromogenic assay to measure TF activity in human microvessel and aortic ECs.²⁷ Basal TF activity

was low but exposure to CS for 5 hours induced significant elevation of TF activity. No assessment of TF expression was done. Our preliminary data indicate that the magnitude of TF mRNA expression was elevated transiently by laminar flow, was sustained by to-and-fro shear stress, but not elevated by orbital shear (data not shown). These findings suggest that different types of mechanical stress induce different responses on TF expression through different signaling pathways.

Endothelial TF can also be induced by several cytokines such as TNF- α or interleukin-1 β , by biogenic amines such as serotonin or histamine, and by mediators such as Thr, oxidized low density lipoprotein or vascular endothelial growth factor (VEGF). It is interesting that despite their diversity, most of these mediators share similar but not identical signal transduction pathways regulating TF induction.²⁸ For example, the MAPKs p38, ERK, and c-Jun terminal NH $_2$ -kinase (JNK) are involved in TNF- α -induced, histamine-induced, and Thr-induced TF expression, whereas the effect of VEGF is only mediated by p38 and ERK.

In addition, there is a synergistic effect of hemodynamic forces and chemical stimulation on TF activity. Silverman et al showed that concomitant application of CS and TNF- α (10 ng $\cdot$ mL $^{-1}$) resulted in synergistic elevation of TF expression which appeared to be dependent on cyclic AMP and protein kinase C.²⁷ No assessment of TF expression or direct measurement of intracellular signal protein levels was performed. Our preliminary data indicate a synergistic increase in TF

Figure 3 Akt, p38, and ERK activities in HUVEC exposed to CS, Thr, or CS + Thr for 5, 15, and 30 minutes. The activities are indicated by the ratio of phosphorylated/total protein band intensity in western blot. A representative result of western blot is shown in top portion and time course of densitometric data are shown in middle portion. As values of those activities were not distributed normally, we subjected them to normalized $\log_{10}$ transformation before statistical analysis (showing lower portion). (A) Akt activity decreased over the time course in each group. The activity in the Thr + CS group decreased with increasing exposure time, while in the Thr group it was sustained from 15 to 30 minutes (bottom). (B) p38 activities showed significant increase compared with control (Ctrl) only at 5 minutes in Thr group but significant increase was shown at 5 and 15 minutes in Thr + CS group. The activity in CS group had no change during time course. Results in (A) and (B) are mean $\pm$ SE from five separate experiments. (C) ERK activity showed transient increase in Thr group and Thr + CS group while in CS group had no change. Results are mean $\pm$ SE from three different experiments. $\ast p < 0.05$ vs Ctrl (exposure time = 0), $\ast p < 0.05$ vs 5 minutes, $\dagger p < 0.05$ vs 15 minutes.

Figure 4 Electrophoretic mobility shift assay (EMSA) for SP-1, activator protein-1 (AP-1), nuclear factor-kappa B (NFκB), and Egr-1 in HUVEC exposed to CS, Thr, or Thr + CS. (A) Representative results for each transcription factor are shown. C indicates control and arrow indicates cold competitor was added before incubating with radioactive oligonucleotide. Each lane includes 7.5 μg nuclear extraction protein. (B) Densitometric data for each transcription factor. (a) SP-1 binding activity had no change during time course; (b) AP-1 binding activity had no significant change for all groups; (c) NFκB binding activity had minimal change in the CS group. The activity was significantly increased and sustained in Thr group, but in Thr + CS group it showed different temporal pattern and seemed to increase later; (d) Egr-1 binding activity showed significant increase at 30 and 60 minutes in Thr + CS group only. The activity had an increasing trend in CS and Thr group but it was not significant change. Results are mean ± SE from five (a and b), nine (c), and seven (d) separate experiments. ☆ $p < 0.05$ vs Ctrl (exposure time = 0), * $p < 0.05$ vs 60 minutes.

expression when Thr is added to ECs exposed to pulsatile shear (data not shown) but not to ECs exposed to CS (Fig. 1A).

The Akt inhibitor did not have any significant effect on TF expression in any of the group. Akt is known as a protein which regulates multiple biological processes, including cell survival, proliferation, growth, and metabolism.^{29,30} Akt has also been reported to negatively regulate p38 MAPK.³¹ Surprisingly in this study, Akt activity was strongly decreased by all stimuli we applied. This may explain why TF mRNA expression was not increased significantly by the Akt inhibitor, LY294002.

In contrast, we have previously reported that CS inhibited apoptosis and enhanced cell survival in BAEC through increasing Akt activity.^{15,32,33} One reason of this discrepancy may be the use of serum-free medium to avoid the attenuation of Thr activity. Silverman et al reported TF expression was differentially modulated by CS in human EC derived from umbilical veins, aortae, and dermal microvessels.²⁷ The difference of cell origin and species may have an effect on cell responses. Nonetheless, the decrease of Akt activity by Thr stimulation in this study was consistent with a previous report in HUVEC.¹⁶

We observed a significant increase in p38 activity in the Thr group and the Thr + CS group. Although the magnitudes of p38 activity between both groups at each time point were similar, there was a small but significant difference in the temporal pattern of p38 activity (Fig. 3B bottom). The activity of p38 activity at 15 minutes was significantly higher than Ctrl in the Thr + CS group but not in the Thr group, and it was significantly decreased compared with that at 5 minutes in Thr group. This finding may provide an explanation for the delayed peak of the TF expression in Thr + CS group. Our data also confirm previous observations³¹ on the inverse correlation of Akt activity with p38 activity in TF regulation.

We have reported that p38 and ERK activities are upregulated in BAEC and bovine pulmonary arterial EC exposed to CS.^{14,34} However, ERK and p38 activity in HUVEC exposed to CS alone was not upregulated significantly in this study. This different response to CS may be due to different sensitivity to CS between bovine ECs and HUVEC as seen with the Akt results. In the Thr group and the Thr + CS group, ERK activity had a transient and significant increase but there was no difference in magnitude or timing between either group. Therefore the delay of Thr-induced TF mRNA expression by CS could not be explained by ERK stimulation.

We have previously demonstrated that AP-1 was increased and peaked at 4 hours in HUVEC and bovine pulmonary arterial EC exposed to CS^{18,34} and SP-1 levels and SP-1 phosphorylation was not altered in rat microvascular ECs exposed to CS.³⁵ However, in the

present study, CS did not induce significant activation of either AP-1 or SP-1. The reason for this discrepancy is likely due to the use of serum-free medium during experimentation. The Thr or Thr + CS stimulation groups also did not influence SP-1 and AP-1 levels. These data suggest that SP-1 and AP-1 do not play a central role in TF expression induced by Thr with or without CS in HUVEC.

In contrast, the temporal pattern of NF κ B in the Thr + CS group was increased later compared with that in the Thr group. Egr-1 levels in the Thr + CS group was also increased while in the Thr group no significant increases were noted. Taken together, the different temporal patterns in Akt and p38 activation and the levels of NF κ B and Egr-1 are consistent with the delay of Thr-induced TF expression in HUVEC exposed to CS. Although the ERK and p38 selective inhibitors did not affect the delay of Thr-induced TF mRNA expression with CS, it cannot be excluded that the timing of treatment with the inhibitors abrogates the p38 and ERK response to stimulation early and prevents analysis of the delayed response to the combination stimulation. In addition, we observed that PD or SB did not inhibit Egr-1 or NF κ B levels in the various groups (data not shown). This may suggest that p38 or ERK activate another transcription factor that regulates TF expression or another second messenger activates Egr-1 or NF κ B.

In this study we have not investigated other potential signaling pathways. For instance, other investigators have shown mechanical stress such as CS or shear stress-induced reactive oxygen species (ROS)^{36–39} and ROS upregulated NF κ B or Egr-1.^{36,40} ROS is also produced by agonists of protease-activated receptor (PAR-1), the primary mediator of Thr effects.^{16,24,40} Therefore, the ROS-mediated pathway to NF κ B or Egr-1 may be involved in TF expression induced by Thr with or without CS as nicotinamide adenine dinucleotide phosphate oxidase system. It has also been reported previously that protein kinase A (PKA) is involved in PAR-1 mediated pathway to NF κ B in glioblastoma cells,⁴¹ and that the pertussis toxin-sensitive G protein and PKA activation is participated in ROS generation in HUVEC.³⁴ These signal pathways may be involved in the delayed peak of TF expression confirmed with mechanochemical signaling.

Platelet endothelial cell adhesion molecule 1 (PECAM-1), which is also a mechanosensor, mediates Thr-induced TF expression on ECs.¹⁶ Previously we reported that PECAM-1 does not function as a major mechanoreceptor for activation of MAPK and Akt in ECs exposed to fluid shear stress.⁴² However, the mechanosensory complex composed of vascular ECs cadherin, VEGF receptor 2 and PECAM-1,⁴³ and its signaling pathway has not been elucidated completely. Therefore the signaling pathway induced by combined stimulation, may be mediated through a variety of

different routes such as PAR-1 and the mechanosensory complex pathway including PECAM-1. Further investigation of these pathways, including ROS, PKA, PAR-1, and mechanosensory complex pathway is needed to elucidate the mechanism of delay of Thr-induced TF mRNA expression by CS.

In vivo, vascular EC are exposed to many kinds of chemical stimuli with various intensities and durations which induce diverse phenotypic expressions in EC. It is important to elucidate the mechanism of EC responses to each stimulation in static culture condition to control these responses in the future. However, EC are constantly exposed to mechanical forces generated by blood flow and pressure which also induce gene expression. As we showed in this study, mechanical stimulation such as CS affects chemical stress-induced gene expression such as TF. Therefore, in vitro, the combination of chemical and mechanical stimuli may be a better mimic of in vivo situation where circulating chemicals interact with a pulsatile endothelium.

Our finding demonstrates the effect of CS in TF expression induced by Thr in HUVEC, which is different from those of laminar, oscillatory, to-and-fro or orbital shear stress. This implies that different types of mechanical stresses have different effects on phenotype expression in ECs.

CONCLUSION

In conclusion, we demonstrated that Thr-induced TF mRNA expression was delayed in HUVEC exposed to CS. It may be modulated by second messengers such as Akt, p38, and ERK and by transcription factors such as Egr-1 and NF κ B. However, we could not directly elucidate the mechanism of the delay by CS and further studies on other signaling pathways may be needed.

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