Tissue factor activity is increased in human endothelial cells cultured under elevated static pressure


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Silverman, M. D., C. R. Waters, G. T. Hayman, J. Wigboldus, M. M. Samet, and P. I. Lelkes. Tissue factor activity is increased in human endothelial cells cultured under elevated static pressure. Am. J. Physiol. 277 (Cell Physiol. 46): C233–C242, 1999.—We tested the hypothesis that elevated blood pressure, a known stimulus for vascular remodeling, and an independent risk factor for the development of atherosclerotic disease, can modulate basal and cytokine-induced tissue factor (TF; CD 142) expression in cultured human endothelial cells (EC). Using a chromogenic enzymatic assay, we measured basal and tumor necrosis factor-α (TNF-α; 10 ng/ml, 5 h)-induced TF activities in human aortic EC (HAEC) and vena cava EC (HVCEC) cultured at atmospheric pressure and at 170 mmHg imposed pressure for up to 48 h. Basal TF activities were 22 ± 10 U/mg protein for HAEC and 14 ± 9 U/mg protein for HVCEC and were upregulated in both cell types >10-fold by TNF-α. Exposure to pressure for 5 h induced additional elevation of basal TF activity by 47 ± 16% (P < 0.05, n = 6) for HAEC and 17 ± 5% (P < 0.05, n = 3) for HVCEC. Pressurization also enhanced TF activity in TNF-α-treated cells from 240 ± 28 to 319 ± 32 U/mg protein in HAEC (P < 0.05, n = 4) and from 148 ± 25 to 179 ± 0.8 U/mg protein (P < 0.05, n = 3) in HVCEC. Cytokine stimulation caused an ~100-fold increase in steady-state TF mRNA levels in HAEC, whereas pressurization did not alter either TF mRNA or cell surface antigen expression, as determined by quantitative RT-PCR methodology and ELISA. Elevated pressure, however, modulated the EC plasma membrane organization and/or permeability as inferred from the increased cellular uptake of the fluorescent amphipathic dye merocyanine 540 (33 ± 7% P < 0.05). Our data suggest that elevated static pressure modulates the hemostatic potential of vascular cells by modifying the molecular organization of the plasma membrane.

UNDER PHYSIOLOGICAL CONDITIONS, the endothelial cell (EC) monolayer lining the entire circulatory system acts as a nonthrombogenic blood-contacting surface. In disease states, upregulation of tissue factor (TF; CD 142) expression, an inducible transmembrane glycoprotein that functions as the primary physiological initiator of blood clotting (6), results in a procoagulant EC phenotype. Recent evidence suggests that TF expression by EC is likely an important mediator of in vivo pathologies, such as atherosclerosis (42). In cultured EC, TF expression is upregulated by a variety of agonists, including endotoxin and inflammatory cytokines (2, 7), as well as by environmental factors such as hypoxia (8).

In vivo, EC are constantly exposed to hemodynamic forces resulting from the flow of blood in the vasculature. These forces include shear stress, cyclic strain, and pressure. Numerous studies have established that EC are not just passively tolerant of such forces but, rather, are exquisitely responsive to these mechanical influences, which alter gene expression and, subsequently, a variety of morphological and functional characteristics (17, 39). To date, most studies of the EC response to mechanical stimuli have focused on evaluating the biological effects of fluid shear stress and cyclic strain. In relevance to the current study, it has been demonstrated that exposure of cultured human EC to either of these hemodynamic forces upregulates the expression and biological activity of TF (19, 37).

In addition to cyclic strain and shear stress, blood pressure is also an important mediator of EC function (14). Elevated blood pressure is an independent risk factor for a host of vascular diseases that are characterized by dysfunctional endothelium and that manifest in altered vasomotor function and atherosclerosis (9). Recent clinical findings also suggest that hypertensive patients are hypercoagulable (21) and are at risk for thrombotic complications, particularly in pulmonary hypertension (11). A number of in vitro studies have shown that exposure of cultured EC to static or pulsatile pressure resulted in altered EC morphology, proliferation, and ion channel activity and modified the elaboration of growth factors, metalloproteases, and nitric oxide (12, 16, 34, 41, 43). We hypothesized that elevated pressure modulates the expression and/or activity of molecules that determine the pro- and/or anticoagulant balance of the EC surface. In this study, we report that elevated static pressure alters the hemostatic potential of cultured human EC by increasing the levels of cell-associated TF activity.

MATERIALS AND METHODS

Materials. Recombinant human tumor necrosis factor-α (TNF-α; 10 ng/ml) was purchased from R & D Systems (Minneapolis, MN). Dulbecco’s PBS containing 0.9 mM CaCl₂ (PBS) was from Mediatech (Herndon, VA). FCS was obtained from Hydose Laboratories (Logan, UT). Sodium heparin was from Lyphomed (Deerfield, IL). The chromogenic substrate S2222 was purchased from Chromogenic (Molndal, Sweden). Proplex T, a heat-treated concentrate containing coagulation factors II/VII/X/X, was from Baxter Hyland (Glendale, CA). The protein assay kit was from Pierce (Rockford, IL). Recombinant human TF was from American Diagnostica (Greenwich, CT). Ultrapure electron microscopy grade formalde-
hyde was from Polysciences (Warrington, PA). Cell culture media MDCB 131 and M199, and all other reagents, were from Sigma Chemical (St. Louis, MO), and all tissue culture plastic ware was from Corning (Corning, NY), unless otherwise indicated.

Cell isolation and culture. Adult human aortic EC (HAEC) and vena cava EC (HVCEC) were collected from pieces of the respective blood vessels, obtained at autopsy within 24 h postmortem, according to an Institutional Review Board-approved protocol. The cells from both vessel types were harvested following 20 min collagenase type Ia treatment (0.15% in PBS). All EC cultures were grown in a culture medium composed of MDCB 131/M199 (50%-50% vol/vol, pH 7.4) supplemented with 10% FCS, 30 µg/ml EC growth supplement, 1 U/ml sodium heparin, 10 µg/ml gentamicin, and 125 ng/ml fungizone (complete medium) and maintained in a 5% CO2-95% air, 100% humidity, 37°C tissue culture incubator. After the primary isolates were manually weeded, the ensuing cultures were essentially free of contaminating fibroblasts and/or smooth muscle cells. The endothelial nature of the cultured cells was ascertained by their typical cobblestone morphology and contact-inhibited monolayer growth. The cultured cells were ascertained by their typical fibroblast morphology and contact-inhibited monolayer growth, as well as by the ability of >99% of the cells to endocytose di-I-acetylated low-density lipoprotein and to express von Willebrand factor, platelet-EC adhesion molecule-1. All EC types were used at passages 5–10. In preliminary experiments we verified that 1) there were no significant variations in the basal and cytokine-stimulated TF activities among the passages of each EC type used and 2) total cellular protein levels in pressurized and cytokine-stimulated cells were similar to those found in control cultures (data not shown).

Human vena cava smooth muscle (HVCSM) cells, obtained from explants of the medial layer of the vessel after EC isolation, were cultured in medium identical to that used for EC. HVCSM cells were characterized by morphology, absence of the above-mentioned EC-specific markers, and by positive immunostaining for smooth muscle actin. Human dermal fibroblasts (from neonatal foreskins) were a kind gift from Mishel Davis (Pathology Department, Sinai Samaritan Medical Center, Milwaukee, WI) and were cultured in medium M199 containing 10% FCS and antibiotics. Both of these cell types were used at passages 6–10.

Pressure application. Approximately 1 × 10^5 cells/cm² were plated in standard 24-well tissue culture plates. Under these conditions, cells routinely reached confluence within 24 h. At confluence, all media were replaced with fresh complete medium. Confluent EC monolayers were treated with cytokines, exposed to elevated static pressure of 170 mmHg, or both, for up to 48 h. In 5-h experiments, some wells were refed with medium containing recombinant human TNF-α (10 ng/ml). In 24- and 48-h experiments, medium was replaced again after 19 or 43 h, respectively, to yield a consistent 5-h cytokine exposure period. No evidence of cytotoxicity due to any of the above treatments was observed in this study, as evaluated by trypan blue exclusion.

To expose the cells to elevated pressure, standard tissue culture plates were placed in pressure chambers situated inside a 37°C tissue culture incubator (Fig. 1A). Three isolated-atmosphere pressure chambers were connected in parallel, via two step-down regulators and a humidifying chamber, to a high-pressure tank containing a mixture of 5% CO₂-95% air. The second, high-pressure regulator (Watts FluidAir, Kittery, ME) accurately delivers pressures in the range of 0–430 mmHg. The Plexiglas pressure chambers were fitted with quick-release lids and venting ports (Fig. 1B). In all experiments, identical cultures were maintained in the same incubator, exposed to 95% air-5% CO₂ at ambient atmospheric pressure, and served as controls. Tissue culture media pH, Pco₂, and P O₂ levels, in both pressurized and nonpressurized samples, were monitored with a blood gas analyzer (model ABL-3; Radiometer, Copenhagen, Denmark).

Measurement of TF activity. Confluent EC monolayers, grown in standard six-well plates, were treated as stated above. On termination of each experiment, the monolayers were washed with ice-cold PBS and disrupted by three consecutive freeze-thaw cycles (−80°C/20°C). Cell lysates were collected in 0.75 ml/well PBS for TF and total protein measurements. Whole cell TF activity was assayed by a modified two-stage amidolytic assay using the chromogenic substrate S2222, as previously described. In each well of a 96-well microtiter plate, 50-µl aliquots of whole cell lysates were added to 100 µl of PBS-containing Proplex T and 350 µM chromogenic substrate S2222 and were incubated at 37°C. The final concentrations of the coagulation factors in this assay were (according to information provided by the manufacturer) 0.08 U/ml factor II, 1 U/ml factor VII, 0.26 U/ml factor IX, and 0.15 U/ml factor X. Cell surface-associated TF activity was evaluated in some experiments by similar methods. Briefly, EC monolayers, cultured in 96-well plates, were subjected to the various experimental regimens, as above, with the exception that cells were not frozen and thawed. Immediately on termination of the experiments, the wells were washed with PBS and the intact cells were incubated at 37°C with 100 µl/well of the Proplex T/chromogenic cocktail solution. Substrate deavage was quantitated by measuring changes in optical density at 410 nm in a microplate reader (Dynatech, Alexandria, VA) for 30–240 min.

Cellular TF activity was quantified by comparison to a standard curve of rabbit brain thromboplastin (TF) with known TF activity, which was run in parallel for each experiment. One unit of TF activity was arbitrarily defined as the enzymatic equivalent of 1 µl of the thromboplastin standard solution. Assay specificity for TF was ascertained by the failure of cell lysates to hydrolyze chromogenic substrate S2222 in the absence of coagulation factors, by adding buffer rather than Proplex T to the assay. Furthermore, we verified that the chromogenic substrate S2222 is a highly specific substrate for factor Xa rather than for thrombin (selectivity >50:1 over the thrombin-selective substrate S2338 for the duration of our incubations). Finally, inclusion of the high-affinity thrombin inhibitor hirudin (1 U/ml) did not alter our results. Protein content of the wells was determined with a commercially available protein assay kit (Pierce). Results are presented as units of TF activity per milligram of total cellular protein (U/mg protein).

TF ELISA. HAEC were plated at an initial density of 7.5 × 10^4 cells/cm² in standard tissue culture 96-well plates precoated with bicarbonate washing buffer, pH 7.4 (WB9-74; 0.05 M sodium bicarbonate, and 0.9% sodium chloride) containing 15 µg/ml calf skin collagen type I. HAEC were seeded 48 h before the experimental conditioning and refed once with fresh complete medium. Before the experiments, medium was aspirated and HAEC monolayers were refed with fresh complete medium or fresh complete medium containing 10 ng/ml of recombinant human TNF-α (as positive controls). Recombinant human TF (rHu-TF) standards in WB9-74 were also placed, in all 96-well plates, before the start of each experiment. HAEC monolayers were maintained at ambient pressure or exposed to elevated static pressure at 170 mmHg for 6 h, as described above.

On termination of each experiment, rHu-TF standards were aspirated and the wells were washed three times with 200 µl of WB9-74. The medium in the wells containing
HAEC monolayers was aspirated, the cells were washed once with 200 µl of BWB-7.4, and all wells were fixed with 100 µl of 4% formaldehyde in BWB-7.4 for 30 min at room temperature. After fixation, all wells were washed once with 200 µl of BWB-7.4 and blocked for 1 h at room temperature with 200 µl of bicarbonate blocking buffer (BBB; BWB-7.4 containing 2% BSA, pH 7.4). BBB was aspirated, and all wells were incubated for 1 h at room temperature with 100 µl of primary antibody (1.4 µg/ml; monoclonal anti-TF, clone TF9–5B7 (23); a generous gift from Dr. J. H. Morrissey, Oklahoma Medical Research Foundation) in BBB, except for secondary antibody control wells, which received 100 µl of BBB. All wells were aspirated, washed three times with 200 µl of BWB-7.4, and incubated for 1 h at room temperature with 100 µl of secondary antibody (200 U/ml; anti-mouse, whole molecule, alkaline phosphatase conjugate) in BBB. After two washes with 200 µl of BWB-7.4 and one wash with 200 µl of bicarbonate washing buffer, pH 9.6 (BBW-9.6), all wells were incubated with 100 µl of a fluorogenic phosphatase substrate solution (50 µg/ml 4-methylumbelliferyl phosphate in BWB-9.6). Fluorescence was measured at 10, 30, and 60 min using a CytoFluor 2350 fluorescent miniplate reader (PerSeptive Biosystems, Framingham, MA) with excitation and emission filters set at 360 and 460 nm, respectively. After each ELISA, cell number was determined fluorometrically using the bisbenzimide method, as previously described (28).

Competitive quantitative RT-PCR. PCR primers were designed (Primer Designer 2.01; Scientific & Educational Software, Durham, NC) and synthesized (Operon Technologies, Alameda, CA) based on the human TF cDNA sequence (35). The primers, positioned within the second and third exons, have the following sequences: 5′-ACCGACGAGATTGAGAAGG-3′ (sense) and 5′-GCTGTCTGTACTCTTCCGGT-3′ (antisense). To quantitatively measure changes in TF mRNA expression, a human TF-specific internal standard was constructed for use in competitive quantitative RT-PCR (qRT-PCR) (36). qRT-PCR was carried out essentially as previously described (15). The PCR product generated using the TF

Fig. 1. A: schematics of pressurized cell culture system that precisely regulates the atmospheric composition and pressure under which cells are cultured. B: pressurized cell culture chamber fitted with a quick-release lid and venting ports. For details, see text.
primers and human EC cDNA, 438 bp in size, was cloned (pCRII; Invitrogen, San Diego, CA) and confirmed by sequencing using DyeDeoxy Terminator chemistry (Applied Biosciences, Foster City, CA). To construct the internal standard plasmid, a procedure similar to the primer ligation technique of Borriello and Lederer (5) was used. First, primers were designed using the primerB sequence from a PCR MIMIC kit (Clontech Laboratories, Palo Alto, CA). These primers, 5'-GTGAGCTGATTGCAGAGT-3' (antisense), which generate a product of 223 bp with primerB DNA as template, were first phosphorylated with T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The phosphorylated 5' and 3' primers were then ligated, using T4 RNA ligase (New England Biolabs), to the 5' and 3' TF primers, respectively. In the presence of excess TF 5' and 3' primer, a PCR reaction was performed with primerB DNA as template, using conditions previously described (29). The single product, of the expected size of 262 bp, was cloned into pCRII to generate plasmid pHT211 and was confirmed by sequencing. Tenfold serial dilutions of the internal standard were complemented with equal amounts of cDNA, for all controls and treated samples, to determine the approximate internal standard concentration that produced a product equal in amount to that of the cDNA. Subsequently, 2- to 2.5-fold dilutions of the internal standard spanning that concentration were used in secondary PCRs, which were repeated. After amplification, agarose gel electrophoresis, and ethidium bromide staining of the samples, gel images were digitized and band intensities measured and quantified, exactly as described previously (15).

Uptake of merocyanine 540. Incorporation of the amphiphatic fluorescent dye merocyanine 540 (MC540) is an established marker for assessing membrane fluidity (18, 38). Recently, cellular uptake of MC540 has been used to assess membrane reorganization in EC exposed to fluid shear stress (I). In the current study, confluent cell monolayers were pressurized for 5 and 24 h, refed with complete medium containing 5 µg/ml MC540, and repressurized for an additional 30 min. After three washes with PBS, MC540 uptake was determined by measuring cell-associated fluorescence in a fluorescent miniplate reader (CytoFluor), using the appropriate excitation and emission filters. Nonpressurized monolayers served as controls. Cells treated with vehicle only did not display fluorescence above background levels. The data are normalized to the fluorescence of MC540 incorporated into nonpressurized controls. To ascertain that the pressure-induced change in MC540 incorporation was not due to altered integrity of the plasma membrane barrier function, we determined the cellular uptake of fluorescein-conjugated dextran (100 µg/ml, mol wt = 10,000) and trypan blue, and we also evaluated the release of lactate dehydrogenase into the culture supernatant. Uptake of fluorescein-conjugated dextran was evaluated as described for MC540, with the filters set for fluorescein. For measuring trypan blue exclusion, pressurized and control cells were treated with 0.4% trypan blue in saline, pH 7.4, for 5 min, followed by PBS washing and visual counting of stained cells by light microscopy. Lactate dehydrogenase release was determined using a commercial chromogenic kit, according to the manufacturer’s instructions (TOX-7; Sigma).

Evaluation of cell surface phosphatidylserine expression. Pressure- or membrane reorganization in particular phospholipid flip-flop, was assessed in HAEC grown in collagen type I-coated tissue culture chamber slides (Nunc, Naperville, IL) using a commercial kit (ApoAlert kit; Clontech Laboratories). This assay is based on the specific and high-affinity binding of fluorescein-labeled annexin V to phosphatidylserine exposed at the cell surface (32). Briefly, after pressurization with or without concurrent TNF-α stimulation, EC monolayers were washed with PBS and incubated with 0.5 µg/ml of fluorescein-labeled annexin V for 15 min at 20°C in the dark. As positive controls, cells were serum starved for 48 h to induce apoptosis. Slides were washed, fixed in 1% formaldehyde-PBS, rinsed with water, mounted, and viewed on a Nikon Microphot-FX fluorescence microscope, using optics appropriate for fluorescein detection.

Statistical analysis. Results for each experimental group were expressed as means ± SD for the indicated number of experiments that were performed in triplicate or more wells. The significance of variability among the means of the experimental groups was determined by one-way ANOVA, using INSTAT version 1.12a software (GraphPAD Software, San Diego, CA). Differences among experimental groups were considered to be statistically significant when P < 0.05.

RESULTS

Basal and cytokine-induced TF activity in vascular cells. Basal surface expression of TF activity in both HAEC and HVCEC was low and approached the detection limits of this assay. Basal, total cellular TF activities in lysates of both cell types were low but well within the range of our assay. Basal TF activity in HAEC lysates (22 ± 10 U/mg protein, n = 13) tended to be greater than that in HVCEC lysates (14 ± 9 U/mg protein, n = 6); this difference, however, was not statistically significant (P > 0.08). Both HAEC and HVCEC responded to TNF-α (0.01–100 ng/ml, 5 h) with dose-dependent increases in total cellular TF activity (Fig. 2). Significant elevation of TF activity was obtained with cytokine concentrations as low as 10 pg/ml (220 fmol/l), and the EC50 value for TNF-α-induced TF was ~10 pM for both EC types. More than 95% of maximal TF induction occurred with 10 ng/ml TNF-α, the dose used in all subsequent experiments. At all TNF-α concentrations tested, HAEC consistently expressed significantly more TF activity than HVCEC. The maximal level of TNF-α-induced TF activity in HAEC (240 ± 28 U/mg protein) was significantly higher than in HVCEC (148 ± 25 U/mg protein, P <
Aortic EC were also more responsive than EC of venous origin to TF induction by other inflammatory agents, such as bacterial endotoxin and interleukin-1β. In both EC types, cytokine-induced increases in surface levels of TF mirrored the cytokine-induced total cellular TF activities and amounted to ~20–30% of the activity measured in cryodisrupted cell lysates (data not shown). In contrast to EC, vascular smooth muscle cells and fibroblasts constitutively express large amounts of TF (46). Basal TF activities in HVCSM cells and fibroblast lysates were 620 ± 139 and 270 ± 43 U/mg protein, respectively (n = 5).

Pressure alters TF activity in cultured vascular cells. Cultured HAEC and HVCEC responded to elevated static pressure with an increase in total cellular TF activity (Fig. 3A). In HAEC, cultured at 170 mmHg for 5 h, basal TF activity increased from 22 ± 10 to 32.2 ± 5.2 U/mg protein, i.e., it was 147 ± 16% of that seen in atmospheric pressure controls (P < 0.05, n = 6). This effect was maintained through 24 h (140 ± 14% of control levels, P < 0.05, n = 4), after which TF activity began to decline, returning to control levels (101 ± 3% of controls, P > 0.8, n = 3) by 48 h. In HVCEC, exposure to 170 mmHg for 5 h caused a more moderate, yet significant, elevation of basal TF activity from 14 ± 9 to 16.4 ± 0.8 U/mg protein, resulting in levels 117 ± 5% of controls (P < 0.05, n = 3), and this level was maintained through 24 h (123 ± 7% of controls, P < 0.05, n = 2) and returned to baseline by 48 h.

To test whether pressure induction of TF activity was an EC-specific effect, we also tested vascular smooth muscle cells and fibroblasts, which constitutively express TF (Fig. 3B). Surprisingly, pressurization of medial HVCSM cells at 170 mmHg for 5 h resulted in a decrease of total constitutive TF activity to 84 ± 6% of control values (P < 0.05, n = 3), which remained depressed after 24 h (76 ± 15% of controls, n = 2). Similarly, total constitutive TF activity in human dermal fibroblasts was also reduced by 170 mmHg pressure to levels 75 ± 14% and 52 ± 4% of control values, after 5 h (n = 5) and 24 h (n = 2), respectively (P < 0.01 in both cases).

When elevated pressure was imposed concurrently with TNF-α stimulation for 5 h, TF activity in HAEC lysates was significantly increased from 240 ± 28 to 319 ± 32 U/mg protein, i.e., to a level 133 ± 10% of that induced by the cytokine alone (P < 0.05; n = 4). As in the unstimulated cells, pressure-induced elevation in TF activity was maintained through 24 h (143 ± 15% of nonpressurized controls, P < 0.05, n = 4) and returned to baseline levels by 48 h (92 ± 5%, P > 0.08, n = 4). In HVCEC, pressurization for 5 h caused a lesser, but significant, elevation of cytokine-induced TF activity in cell lysates (121 ± 3% of controls, P < 0.01, n = 3), which was also maintained at this level through 24 h (123 ± 7% of controls, P < 0.05, n = 2) and returned to baseline levels by 48 h. In cytokine-stimulated EC, pressure increased surface expression of TF activity parallel with the increased activity measured in whole cell lysates. For example, pressurization for 5 h at 170 mmHg increased surface TF activity in TNF-α-stimulated HAEC by ~40%, from 78 ± 6 to 106 ± 18 U/mg protein (P < 0.05, n = 4). This elevated coagulability was maintained through 24 h.

We detected a slight elevation in the pH of the culture from 7.21 at atmospheric pressure to 7.30 at 170 mmHg. However, in experiments where HAEC were grown in media in which the pH varied between 6.9 and 7.4, no differences were detected in either basal or TNF-α-induced TF activity after 5 h, indicating that changes in endothelial TF activity with pressure are unrelated to these subtle shifts in media pH. Pressurization also resulted in modestly increased media Po2 levels (from 155 to 180 mmHg). However, given that hypoxia has previously been shown to increase TF expression in human EC (8), it is unlikely that this mild hypoxic effect of pressure was responsible for the increase in TF activity. Pressure caused a slight decrease in media PCO2 (from 46 to 38 mmHg). Because increasing media pH in the above control experiments caused similar slight decreases in media PCO2 levels without altering TF activity, these findings imply a negligible contribution of slightly altered dissolved gas...
levels to the induction of EC procoagulant activity in our system.

Elevated static pressure does not stimulate immunoreactive TF antigen expression or alter TF mRNA levels. In contrast to the increased TF enzymatic activity observed in pressurized EC, no significant change in the level of cell surface-associated immunoreactive TF antigen was detected by ELISA in HAEC monolayers after 6 h of pressure, both under basal conditions as well as on stimulation of the cells with TNF-α (Table 1). Similarly, using Western blot techniques, we failed to detect any appreciable change in pressure-induced TF protein expression or alterations in the level of serine/threonine phosphorylation, a possible indicator of a posttranslational, functional modification of TF (data not shown) (45).

The effect of pressure on steady-state TF mRNA expression in cultured HAEC was evaluated by competitive qRT-PCR. In agreement with earlier reports (30), stimulation with 10 ng/ml TNF-α for 1.5 and 3 h resulted in a marked elevation of the TF message, to levels ~150- and 80-fold, respectively, over nonstimulated controls. Pressurization at 170 mmHg for up to 3 h had no significant effect on steady-state TF mRNA levels in either unstimulated or TNF-α-treated cells (Fig. 4). Consistent with a lack of change in steady-state TF mRNA levels due to pressure, 170 mmHg pressurization of HAEC for 30–240 min did not yield any appreciable alteration in the nuclear levels of the cis-acting DNA-binding proteins (NF-κB, AP-1, and SP-1), as assessed by electrophoretic mobility shift assays (data not shown).

Elevated pressure alters MC540 uptake but does not affect plasma membrane barrier function. Taken together, the above data suggest that the significantly increased procoagulant activity in pressurized EC does not involve appreciable alterations in TF gene and protein expression. Because TF is a transmembrane protein whose biological activity is modulated by its specific interactions with the plasma membrane (24), we hypothesized that the pressure-induced upregulation of TF activity might be caused by a direct effect of elevated pressure on plasma membrane organization (and/or its permeability functions). To assess the effects of pressure on membrane organization, we used the amphipathic fluorescent dye MC540, which has been previously employed to assess membrane permeability and fluidity (1, 18, 38). Pressurization of HAEC at 170 mmHg did result in a significant increase in the uptake of MC540 (Fig. 5), suggesting that elevated pressure can

Table 1. Effect of elevated pressure on tissue factor expression

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<th>Elevated Pressure</th>
<th>Basal</th>
<th>TNF-α</th>
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Values are means ± SD for 3 separate experiments, each performed in at least 6 independent samples. Results were normalized to the number of cells/well and are expressed as picograms of tissue factor (TF) per 2.5 × 10⁴ endothelial cells. Immunoreactive TF expression in human aorta endothelial cells (HAEC) was determined by ELISA (for details see MATERIALS AND METHODS). HAEC were subjected to normal static pressure or elevated static pressure at 170 mmHg for 6 h. TNF-α, tumor necrosis factor-α.

Fig. 4. Pressure-induced TF activity in human endothelial cells is not associated with an increase in elevation of steady-state TF mRNA levels. HAEC were cultured at ambient atmospheric pressure (0 mmHg) or exposed to a static pressure of 170 mmHg for 3 h in absence or presence of TNF-α (10 ng/ml). Steady-state TF mRNA levels were determined using competitive quantitative RT-PCR, as detailed in MATERIALS AND METHODS. Cytokine stimulation resulted in ~100-fold increase in steady-state TF mRNA levels. No significant differences were observed between TF mRNA levels in pressurized and nonpressurized HAEC. Data are means ± SD (n = 6).

Fig. 5. Pressure-induced alterations in TF activity are associated with plasma membrane reorganization. Cellular uptake of amphipathic fluorescent dye merocyanine 540 (MC540) into pressurized (170 mmHg) HAEC was significantly increased at 5 and 24 h, compared with controls maintained at ambient pressure. This increased MC540 uptake paralleled the increase in TF activity (see Fig. 3). Conversely, in HVCSM cells, pressurization resulted in a significant decrease in MC540 uptake, concomitant with a parallel decrease in TF activity (see Fig. 3B). Data represent means ± SD from 3 experiments conducted in quadruplicate wells. Results are presented as percentage of MC540-associated fluorescence in pressurized samples compared with nonpressurized controls. *Significant difference (P < 0.05) between mean MC540 uptake in pressurized samples and controls for respective cell type.
alter the organization of the plasma membrane. Visualization by fluorescence microscopy suggested that, under our experimental conditions (30 min incubation with MC₅₄₀ at 37°C), localization of MC₅₄₀ was not restricted to the EC plasma membrane but that the dye was also to some extent internalized (data not shown). In contrast to EC, in HVCSM cells, where TF activity was decreased by pressure (see Fig. 3B), MC₅₄₀ uptake was coordinately reduced (Fig. 5), suggesting a cell type-specific effect of pressure on membrane organization and function.

Using a variety of independent approaches, we ascertained that pressure did not overtly compromise the integrity of the plasma membrane. We did not find any gross damage to the EC membranes: trypan blue exclusion and lactate dehydrogenase release by pressurized HAEC were no different from that in nonpressurized controls, through 24 h. Furthermore, pressurization for up to 24 h did not alter the endocytotic properties of the HAEC plasma membrane, as assessed by the invariant intracellular uptake of fluorescein-conjugated dextran into control and pressurized cells (data not shown). Finally, pressurization in the absence or presence of TF-α did not increase nonspecific phospholipid flip-flop, as inferred from lack of annexin V binding to intact, pressurized HAEC. As a positive control, induction of apoptosis by serum deprivation for 48 h (4) yielded significant annexin V binding to the cells (data not shown).

**DISCUSSION**

The main finding of this study is that exposure of cultured human EC to elevated ambient pressure results in transient upregulation of TF activity. This process does not seem to involve transcriptional or translational regulation, as inferred from the unchanged levels of TF mRNA and protein. Furthermore, we did not find evidence for pressure-induced posttranslational modification of TF, such as alterations in serine phosphorylation, which might have accounted for the enhanced TF activity. Rather, elevated static pressure affects the organization of the plasma membrane and hence modulates indirectly, presumably via altered lipid-protein interactions, the enzymatic activity of TF, without significantly altering its net expression at either the gene or protein level.

In vivo, EC are continuously exposed to the complex interplay of distinct hemodynamic forces, i.e., pressure, cyclic strain, and shear stress. By using in vitro cell culture systems, which mimic each of these forces independently, it is possible to evaluate their individual influences on EC biology. Using this approach, it has been demonstrated that mechanical stimuli cause profound changes in EC gene expression and modulate numerous cellular functions, including their procoagulant/fibrinolytic profiles. In the past, Grabowski and Lam (10) described increased abluminal accumulation of TF procoagulant activity in human umbilical vein EC (HUVEC) that were exposed to low levels of shear stress. More recently, Lin et al. (19) demonstrated shear stress-induced upregulation of both TF gene expression and protein activity in HUVEC. We previously reported elevated TF activity in human EC subjected to cyclic straining (37). The current study is the first demonstration that elevated static pressure can modulate TF activity and thus the thrombogenic potential of human endothelium.

The signaling pathways by which inflammatory cytokines such as TNF-α induce endothelial TF expression and activity are well characterized (3, 30). The human TF gene promoter region contains consensus binding sites for the nuclear transcription factors NF-κB, AP-1, SP-1, and EGR-1 (22, 30). In addition, cell type-specific negative transcriptional regulatory elements (silencing) mechanisms have been proposed to explain the low basal levels of TF expression in quiescent EC (27). Upregulation of EC TF expression by TNF-α appears to require cooperative interactions between the two AP-1 binding sites and the lone NF-κB site (30) and might be further mediated by the SP-1 and EGR-1 elements.

The molecular mechanisms through which mechanical forces are perceived by EC, transduced intracellularly, and finally translated into elevated endothelial TF expression and/or activity are less well elucidated. The promoter region of several vasomodulatory proteins (e.g., platelet-derived growth factor-B, intercellular adhesion molecule-1, and nitric oxide synthase) contains a cis-acting shear-stress-responsive element (SSRE) that acts as a regulatory element in the transcriptional activation of these genes by laminar shear stress (33). The sequence of this prototypic SSRE (GAGACC), however, is not present in the human TF promoter. Shear-stress-induced TF upregulation in human EC is apparently mediated by alternative mechanisms, such as the flow-induced induction of EGR-1 (13) and/or flow-induced increase in the serine phosphorylation of the nuclear transcription factor SP-1 (19). Similar signaling mechanisms have also been proposed to function in the activation of SSRE-containing genes by cyclic strain (33, 40). We, therefore, hypothesized that the TF promoter might also harbor an analogous pressure-responsive element. However, the present study does not support this hypothesis. We did not observe any significant effect of pressure on either steady-state TF mRNA expression in human EC or on the nuclear levels of relevant transcription factors that activate positive response elements in the TF gene. Thus we speculate that increased TF activity due to steady elevated pressure is mediated by mechanisms other than a putative nuclear pressure-responsive element.

Because pressure-induced changes in TF activity were not regulated at either the transcriptional or translational levels, we hypothesized that pressure might modulate TF activity indirectly by affecting protein-lipid interactions at the level of the plasma membrane. The specific phospholipid composition and organization of the membrane in which TF is embedded is pivotal in regulating the formation of TF complexes with circulating plasma coagulation cofactors and, as such, for its procoagulant activity (24). In testing our hypothesis, we evaluated the potential for elevated
pressure to induce membrane reorganization in EC by measuring the incorporation and/or cellular uptake of the amphipathic fluorescent dye MC540. This fluorescent molecule has been shown to preferentially insert itself into loosely packed phospholipid membranes, thus affording a relative measure of membrane fluidity (1, 18, 38). Prior in vitro studies suggested that EC plasma membrane organization and function are altered by mechanical perturbations and that exposure of these cells to physiological levels of shear stress resulted in increased cellular uptake of MC540, unrelated to elevated endocytic activity (1). In the current study, uptake and/or incorporation of MC540 was significantly elevated in EC cultured at elevated pressure for up to 24 h, concomitant with increases in TF activity. In contrast to the increased cellular uptake of MC540 after pressurization, no change was seen in the uptake of fluorescein-dextran and trypan blue or in the release of lactate dehydrogenase. Similarly, using fluorescein-conjugated annexin V, we failed to detect any evidence for nonspecific phospholipid flip-flop, as a sign for mechanical force-induced apoptosis and subsequent membrane rearrangement. Taken together, our data suggest that pressure might induce subtle alterations in the architecture and/or organization and function of the plasma membrane, without compromising its structural integrity. We propose that elevated steady pressure at (patho)physiological levels may change the physical properties of the EC plasma membrane, such as fluidity, hydration at the protein/lipid interface, heterogeneity of microdomains, interactions of boundary lipids, and/or associations with underlying cytoskeletal elements (38), and thus enhance the latent biological activity of TF already present at the cell surface. These membrane changes might also be related to alterations in the activity of specific plasma membrane ion channels that have been demonstrated to result from pressurization of EC (26). In support of a causal correlation between pressure, membrane alterations, and procoagulant TF activity, pressurization of smooth muscle cells resulted in a decrease in TF activity (Fig. 3B) concomitant with a significant reduction in MC540 uptake (Fig. 5). A possible explanation for the opposite effects of pressure on membrane reorganization, as reflected by MC540 uptake, might be found in the well-documented, distinct cytoskeletal organization in EC and smooth muscle cells (perimembranal web vs. elaborate network of criss-crossing stress fibers).

Another possible explanation of our results may be a hydrostatic pressure-induced modulation in the expression and/or secretion of TF pathway inhibitor (TFPI) (20). In an in vitro flow model, Grabowski and Lam (10) showed that shear stress elevated TF activity in both cultured human EC and in fibroblasts and that this response was magnified by inclusion of an anti-TFPI neutralizing antibody. Thus, although TFPI affected the magnitude of response to flow, it did not alter the basic observation that shear stress directly activated the TF pathway in these cells. More recent findings have corroborated these conclusions by providing direct evidence for flow-induced upregulation of TF gene expression (13, 19). In our study, we noticed an opposite effect of pressure on TF activity in these two cell types (Fig. 5), suggesting that pressure modulation of procoagulant activity likely involves additional, cell type-specific mechanisms beyond TFPI availability. This is certainly an intriguing issue; however, it is beyond the scope of the current investigation.

Insight into the regulation of endothelial TF by hemodynamic forces has important clinical relevance. Vascular cell expression of TF is known to mediate thrombotic events in a variety of disease states such as septic shock, disseminated intravascular coagulation, and atherosclerosis. In addition, in coronary bypass procedures, pressure-induced procoagulant activity in the traumatized EC of transplanted veins, which are unaccustomed to elevated hemodynamic loads, may play a role in graft failure. This notion is supported by a recent study in which TF activity was increased 2.5-fold on human saphenous vein segments that were perfused ex vivo under conditions that mimic arterial hemodynamics (25). Our study indicates that the pressure-induced upregulation of TF activity in cultured EC is abated after ~48 h. Thus, if the pressure-induced endothelial TF expression proves to be problematic, it is likely that preconditioning the endothelialized grafts in a mock circulation apparatus, in vitro, might help downregulate TF activity before implantation. In any case, a thorough understanding of the mechanisms by which EC procoagulant profiles are modified by hemodynamic influences will enable us to better assess and regulate EC involvement in thrombogenic sequelae associated with both naturally arising vascular pathologies and clinically generated scenarios using cardiovascular prostheses.

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