The Modulation of Tissue Factor by Endothelial Cells during Heat Shock*

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Tissue factor (TF) initiates the extrinsic coagulation cascade on the surface of macrophages and endothelial cells. In septic patients, the extrinsic coagulation cascade is activated. When septic patients are febrile, mortality is decreased. The purpose of this study was to investigate the role of elevated temperatures on TF expression by endothelial cells during a sepsis-like challenge. Human endothelial vein cells (HUVECs) were incubated with lipopolysaccharide (LPS) or interleukin-1β (IL-1β) for 0, 2, 4, 6, or 8 h. At the 0-h time point, some HUVECs were heat shocked at 43 °C for 2 h and then recovered at 37 °C for 0, 2, 4, or 6 h. Heat-shocked and non-heat-shocked LPS-stimulated HUVECs were analyzed for TF-specific mRNA expression by ribonuclease protection assay (RPA), surface TF expression by flow cytometry, and TF activity by a two-stage clotting assay. Heat shocked LPS-stimulated HUVECs expressed significantly reduced TF-specific mRNA, TF surface protein levels, and TF surface activity when compared with non-heat-shocked, LPS-stimulated HUVECs (p < 0.0125, p < 0.0125, and p < 0.0001, respectively; repeated measures analysis of variance, ANOVA). If heat shock models elevated core temperature, these results suggest that fever may protect the host during sepsis by reducing TF activity on the surface of endothelial cells.

Disseminated intravascular coagulation (DIC)1 is a pathological condition precipitated by sepsis, trauma, or certain cancers (1–4). In DIC the coagulation system activates, promoting fibrin deposition in the microvasculature leading to thrombosis, organ failure, depletion of coagulation factors, and uncontrolled bleeding (5). Tissue factor (TF) is involved in the development and progression of DIC during sepsis; however, its precise role is unknown (3, 6–9).

Heat shock inhibits cytokine- and endotoxin-mediated NF-κB nuclear translocation and IκB degradation in cultured cells (15, 16) and in vivo (17). Thus, the heat shock response may protect the host during infection by modulation of proinflammatory genes during sepsis. TF expression in response to LPS and cytokines is also partially regulated by NF-κB. We hypothesized, therefore, that heat shock modulates tissue factor expression by endothelial cells during LPS challenge. In this study we show that heat shock significantly reduces expression of TF-specific mRNA, surface protein, and activity by LPS-stimulated endothelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture, Heat Shock, and LPS—HUVECs were isolated as described previously (18, 19). HUVECs, which were provided by Dr. Gregory Vercellotti (Dept. of Medicine, University of Minnesota), were grown in modified Eagle’s media 199 containing 10% fetal bovine serum (Invitrogen), 4.7 mM t-glutamine, 1 mM sodium pyruvate, 100 μg/ml penicillin/streptomycin, 25 μg/ml ampicillin (Invitrogen), 5 units/ml heparin sulfate (Sigma), and 50 μg/ml ENDOGRO™ (VecTechnologies, Rensselaer, NY) at 37 °C in 5% CO2. HUVECs from passages 1 to 4 were used in all experiments. To induce heat shock, HUVEC-containing flasks were immersed in a water bath equilibrated in a 43, 41.5, or 40 °C incubator. Some HUVEC cultures were stimulated with E. coli LPS serotype 55:B5 (Sigma) or IL-1β (R & D Systems, St. Paul, MN).

RNA Preparation—Total RNA was isolated from HUVEC monolayers by the guanidinium-phenol extraction method with TriZol™ reagent (Invitrogen) according to the manufacturer’s protocol (20). Cell suspension/Trizol was stored at −20 °C until RNA purification. The total amount of RNA isolated from each sample was quantified by absorbance at 260 nm. Purified RNA was stored at −20 or −80 °C until analyzed by ribonuclease protection assay (RPA).

Generation of Radioactive Antisense cRNA Probes—Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), heat shock protein 72 (HSP72), and TF-purified polymerase chain reaction products were ligated into linearized plasmids that contained T3 and T7 RNA polymerase promoters (PCR-Script™; Amp Cloning Kit, Stratagene). The primers used to generate PCR products were as follows: GAPDH (5′-CGAGTCAACGAGTGGTGCTAT-3′, 5′-AGCCTTCCATGCTGAGAAGAC-3′; PCR product length 307 bp), GAPDH(5′-GACCCCTATGGGTACAG-3′), PCR product length 234 bp); GAPDH(5′-CGGGCTCGAAGAC-3′), PCR product length 222 bp); TF (5′-GAAATTCGAGTGGGAACCC-3′, 5′-CATTTTTGCTCCACCTG-3′; PCR product length 234 bp). The products were purified on agarose gel slabs, excised and purified on Microcon (Millipore) columns. The purity of each product was confirmed by nuclease sequencing. Using a combination of primers specific for each gene product and phage promoter flanking region, PCR products were generated containing...
LPS-stimulated HUVECs. The results are reported as mean ± S.E. (n = 7, n = 3, respectively). *, p < 0.0125, repeated measures ANOVA.

**Fig. 1.** Optimizing LPS and heat shock conditions for the expression of TF- and HSP72-specific mRNA by HUVECs. HUVECs were stimulated with 0.1 μg/ml LPS for 0, 1, 2, 4, or 6 h at 37 °C (A) or with 0, 0.01, 0.1, 1, or 10 μg/ml of LPS for 2 h at 37 °C (C) and then analyzed for TF- and HSP72-specific mRNAs by RPA. RNase-protected fragments of TF and GAPDH were separated according to size by gel electrophoresis (TF, 310 bp; GAPDHs, 222 bp) and analyzed from the phosphorimage. Ratios of TF/GAPDHs were calculated to determine relative levels of mRNA for TF- and HSP72-specific mRNAs by RPA. RNase-protected fragments of TF and GAPDH were separated according to size by gel electrophoresis (unprotected 310 bp; and HSP72 (unprotected 280 bp, protected 234 bp). TF (unprotected 396 bp, protected 222 bp); TF (unprotected 396 bp, protected 222 bp). To determine target probe specific activity, a liquid scintillation counter (LKB Wallac 1214 Rackbeta) was used to obtain counts per minute from aliquots of the transcription reaction mixture and target probe eluent. Probes were stored at −20 °C until use. Probe sizes were as follows: GAPDH (unprotected 395 bp, protected 307 bp); GAPDHs (unprotected 368 bp, protected 222 bp); TF (unprotected 396 bp, protected 310 bp); and HSP72 (unprotected 280 bp, protected 234 bp).

Ribonuclease Protection Assay—RPA was used for the following procedures. Ribonuclease Protection Assay—RPA was used for the following procedures (Ambion RPA II kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Total RNA was isolated from LPS-stimulated HUVECs at final concentrations of 300 μg/ml. RNA probe mixture was hybridized overnight at 42 °C to 45 °C and then incubated with 1:50 RNase solution (T1/A) for 30 min at 37 °C. Protected RNA products were precipitated and then separated by electrophoresis on 5% acrylamide gel containing 8 M urea (60 min at 200 V). Gels were exposed to a PhosphorImager (Amersham Biosciences) for 20–24 h.

Analysis of mRNA—To quantify mRNA, the phosphorimage of an exposed gel was scanned with a Storm System 840 scanner; ImageQuant analysis software (version 4.2a) was used to circumscribe the “protected” mRNA fragment and analyze the density of each pixel within each “box.” After correction for background, relative abundance of TF- and HSP72-specific mRNAs was quantified as the ratio to GAPDH or GAPDHs probes. The RNA/probe mixture was hybridized overnight at 42 °C to 45 °C and then incubated with 1:50 RNase solution (T1/A) for 30 min at 37 °C. Protected RNA products were precipitated and then separated by electrophoresis on 5% acrylamide gel containing 8 M urea (60 min at 200 V). Gels were exposed to a PhosphorImager (Amersham Biosciences) for 20–24 h.

Flow Cytometry—HUVECs were analyzed for surface TF expression by flow cytometry (BD Biosciences). Trypsin/EDTA solution was used to disrupt cell monolayers. Preliminary experiments were performed to assess the effect of trypsin on the detection of surface TF. Two monolayer disruption protocols were compared. The first used EDTA to disrupt HUVEC monolayers, and the second protocol used a trypsin/EDTA combination. The trypsin/EDTA protocol produced no detectable differences in TF surface expression compared with EDTA treatment when assessed by flow cytometry (data not shown). Because TF was unaffected and the protocol disrupted HUVEC monolayers more efficiently, the trypsin/EDTA protocol was used for the flow cytometry experiments. HUVEC monolayers were washed with PBS containing 0.5 mM EDTA for 2 min. The supernatant was discarded, and HUVEC monolayers were dispersed by incubation with 0.5 mM EDTA/0.017% trypsin for 1 min. Dispersed HUVECs were washed with cold PBS containing 10% (v/v) FBS, centrifuged at 400 rpm for 5 min at 4 °C, and counted in a hemocytometer after resuspension in 250 μl of PBS containing 2% (v/v) FBS. HUVECs (5 × 10⁵) were incubated with 8 μg/ml anti-TF or IgG isotype control antibodies for 30 min at 4 °C, washed with 25× the incubation volume with PBS containing 2% FBS, and incubated with an anti-isotype secondary antibody labeled with fluorescein isothiocyanate (FITC, Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min at 4 °C. To assess nonspecific binding, some HUVECs were washed and resuspended in 300 μl of PBS containing 2% FBS and incubated with the secondary antibody only. To assess HUVEC viability, propidium iodine (1 μg/ml final concentration) was added to each cell suspension prior to flow cytometry.

Two-stage TF Activity Assay—HUVEC monolayers were washed with PBS containing 0.5 mM EDTA for 2 min. Supernatants were removed, and the monolayers were disrupted by incubation with 0.5 mM EDTA/0.017% trypsin for 1 min. To inhibit trypsin activity, HUVEC suspensions were washed with cold Tris-HCl, pH 7.4, containing 10% (v/v) FBS. HUVECs (6 × 10⁵) were pelleted, washed with buffer, and then incubated with 15 μl factor Xa (Clinical Enzyme Laboratories, South Bend, IN) and S-2222 (Diagnostics 96-well microtiter plates. Factor X (Clinical Enzyme Laboratories, South Bend, IN) and S-2222 (DiaPharma Group Inc, West Chester, OH) were added to each cell suspension at final concentrations of 300 μM and 1.4 μg/ml, respectively. Cleavage of S-2222 by factor Xa was detected by a change in absorbance at λ = 405 nm (Bio-Rad microplate reader model 3550).

Statistical Methods—Repeated measures analysis of variance (ANOVA) was applied in which HUVECs were “subjects,” and the within-subject fixed effects were heat shock (present or absent), time (treated as categories), and ionomycin (present or absent). The one exception is the analysis supporting Fig. 3, which included the between-subject factors heat shock and temperature. All post hoc tests used the Bonferroni correction to maintain an alpha (type I error rate) of 0.05.

RESULTS

LPS-stimulated HUVECs Express Detectable TF-specific but not HSP72-specific mRNAs—Confluent HUVEC monolayers were incubated with 0.1 μg/ml LPS for various times. At each time point, cells were harvested, and total RNA was isolated and analyzed for TF-, HSP72-, and GAPDH-specific mRNA expression by an RPA. Fig. 1A is a representative phosphorimage from a time course experiment. The right panel shows
ribonuclease digestion of the unbound RNA target probe. In the left panel, TF-specific mRNA expression by HUVECs appeared to maximize at 2 h of LPS-stimulation and return to near baseline levels at 6 h. The increase in the TF-specific message was significant at 1, 2, and 4 h when compared with non-stimulated HUVECs and maximized at 2 h (Fig. 1B). To deter-

A

Heat Shock (hrs)

0 1 2 4 6

GAPDH

HSP72

200 bp

300 bp

400 bp

B

Heat Shock

LPS addition

43°C

Recovery phase at 37°C

Time (hrs)

0 2 4 6

C

Heat shock experiments. After LPS addition, HUVEC cultures were heat shocked for 2 h and then re-equilibrated at 37 °C for another 0, 2, 4, or 6 h. Some HUVEC monolayers were stimulated with LPS without heat shock or with heat shock without LPS. Untreated cells were compared and shown to be negative for TF- and HSP72-specific mRNA at each condition (data not shown). C, induction of TF-specific mRNA by heat-shocked and non-heat-shocked HUVECs at 2, 4, and 6 h of LPS stimulation. In a representative phosphorimage (left panel), TF- and GAPDHs-specific mRNA bands migrate at 310 bp and 222 bp, respectively. To confirm heat shock, HUVEC expression of HSP72 was analyzed after 2 h of heat shock (right panel). HUVECs expressed HSP72-specific mRNA (234-bp band). HUVECs treated with heat shock but without LPS did not express detectable levels of TF message (data not shown). D, relative abundance of TF-specific mRNA expressed by LPS-stimulated HUVECs. The results are reported as mean ± S.E. (n = 7), * p < 0.01 for heat shock versus no heat shock, repeated measures ANOVA).
**Fig. 3. Heat shock reduces iTF surface expression by stimulated HUVECs.**

A, a representative histogram from a flow cytometry experiment demonstrating the surface iTF-positive population of HUVECs after stimulation with LPS for 4 h (shaded curve). The unshaded curve represents the fluorescence of LPS-stimulated HUVECs stained with nonspecific isotype control primary antibodies. B, surface iTF positive HUVECs heat shocked for 2 h and stimulated with LPS for 4 h (shaded curve). C, the histogram generated by an unstimulated HUVEC population (shaded curve). Within the unstimulated HUVECs the unshaded curve represents the iTF positive HUVECs after incubation with a primary nonspecific IgG. Histograms are also shown for HUVECs incubated with isotype control antibody (unshaded curve) and anti-TF (shaded curve) after (D) heat shock (2 h), followed by 2 h at 37 °C, or (E) LPS-stimulation incubated with secondary antibody only. Immunoreactive TF expression by HUVECs incubated with LPS (F) or IL-1β (G) with or without heat shock. The results are expressed as mean ± S.E. (n = 7 and n = 3, respectively); statistically significant results are indicated with an asterisk (*, p < 0.01 for heat shock versus no heat shock, repeated measures ANOVA). H, viability of heat-shocked and non-heat-shocked LPS-stimulated HUVECs by propidium iodine exclusion staining. I, LPS-stimulated HUVECs were heat shocked for 2 h at 40, 41.5, or 43 °C and then returned to 37 °C for 0, 2, 4, or 6 h. In parallel, non-heat-shocked, LPS-stimulated HUVECs were harvested at the same time points. HUVECs were harvested at the times indicated and analyzed for iTF surface expression by flow cytometry. The graph represents the average percent reduction of surface iTF ± S.E. at each heat shock temperature over the time course.
mine the LPS dose that would maximally stimulate TF-specific mRNA expression, confluent HUVEC monolayers were incubated for 2 h with each LPS concentration. LPS-stimulated HUVECs did not express detectable quantities of HSP72-specific mRNA at any time or concentration tested (data not shown). LPS concentrations from 0.01 to 10 μg/ml induced TF-specific mRNA expression, which was maximal at 0.1 μg/ml (Fig. 1, C and D).

Heat-shocked HUVECs Induce Expression of HSP72 but Not TF-specific mRNAs—HUVEC cultures were heat shocked at 43 °C over time. At the times indicated, total RNA was isolated and analyzed for expression of HSP72-, TF-, and GAPDH-specific mRNA. HUVEC cells expressed HSP72-specific mRNA, which appeared to peak at 2 h (Fig. 2A). Heat-shocked HUVECs did not express detectable quantities of TF-specific mRNA (data not shown).

Heat-shocked, LPS-stimulated HUVECs Express Reduced TF-specific mRNA—Based upon the optimization experiments, a standard protocol was used unless noted (Fig. 2B). LPS (0.1 μg/ml) was added, and HUVEC cultures were heat shocked at 43 °C for 2 h and then re-equilibrated at 37 °C for up to 4 h (Fig. 2B). To determine the effect of heat shock on TF mRNA expression, LPS was incubated with HUVEC monolayers in the presence or absence of heat shock. LPS induced TF-specific mRNA in heat-shocked and non-heat-shocked HUVECs (Fig. 2C). Heat-shocked HUVECs express HSP72-specific mRNA (Fig. 2C, right panel). At 2 h, heat shock significantly reduced expression of TF-specific mRNA by LPS-stimulated HUVECs when compared with non-heat shocked, LPS-stimulated HUVECs (Fig. 2D). After 2 h of incubation with LPS, cells were allowed to re-equilibrate at 37 °C. The reduction in the expression of TF-specific mRNA caused by heat shock was not apparent (Fig. 2D).

Immunoreactive TF (iTF) Surface Protein Expression Reduced by Heat Shock—To determine whether the surface iTF expression of heat shocked, LPS-stimulated HUVECs paralleled the decrease in TF-specific mRNA expression, monolayers were stimulated with LPS or IL-1β and heat shocked. Monolayers were dispersed and analyzed by flow cytometry for iTF expression. Non-heat shocked, LPS-stimulated HUVECs were also analyzed for comparison. After 4 h of LPS stimulation, 31.7% of HUVECs were surface iTF-positive in a representative histogram (Fig. 3A, shaded area). After heat shock, only 22.2% of LPS-stimulated HUVECs were surface iTF positive.
Heat shock proportionally reduces the expression of TF-specific mRNA, iTF, and activity on HUVECs. Heat shock reduces the expression of TF-specific mRNA, iTF, and activity on LPS-stimulated HUVECs. The data from all experiments are expressed as the mean percent reduction ± S.E.

Fig. 5. Heat shock proportionally reduces the expression of TF-specific mRNA, iTF, and activity on HUVECs.

Heat shock significantly reduces surface iTF (40% of HUVECs were detected at 4 h of LPS-stimulation (Fig. 3F)). Heat shock significantly reduces surface iTF (+) HUVECs after stimulation with LPS for 2, 4, 6, or 8 h (Fig. 3F). Regulation of the expression of TF by heat shock may be under the control of the nuclear transcription factor NF-κB. NF-κB regulates cytokine- and LPS-mediated TF expression (21, 22). Heat shock attenuates NF-κB nuclear translocation and the induction of NF-κB-dependent nitric oxide synthase in murine epithelial cells (15). Future studies will determine whether NF-κB nuclear translocation is reduced in heat shocked, LPS-stimulated HUVECs.²

Heat shock regulation of TF expression was not specific to LPS. Flow cytometry experiments were repeated using IL-1β to stimulate TF expression. In response to IL-1β, heat shock significantly reduced surface iTF (+) HUVECs at all times (Fig. 3G). Although the magnitude of the heat shock inhibition of iTF expression was temperature dependent (Fig. 3F)), the extent of reduction appeared to be independent of the strength of the procoagulant signal. Interleukin-1β or LPS stimulation induced similar surface iTF expression, with peak expression appearing to occur at 4 h. At 4 h, however, IL-1β induced expression on more HUVECs (60%) compared with LPS (36%) (Fig. 3, F and G). Heat shock also caused a greater reduction in the iTF (+) HUVEC population when IL-1β was used as the stimulating agent. In contrast, heat shock of LPS-and IL-1β-stimulated HUVECs reduced iTF (+) to 19.5% of the population, respectively. Heat shock appeared to down-regulate TF expression to this minimum level despite differences in stimulus potency, which may be an important control of coagulation if the anti-coagulant effect of heat shock occurs in vivo.

TF surface expression was reduced on heat-shocked LPS- or IL-1β-stimulated HUVECs, but it was expressed. Therefore, we assessed surface TF procoagulant activity by a two-stage clotting assay. Heat shock significantly reduced surface TF activity by LPS-stimulated HUVECs (Fig. 4, A and C). Because TF-specific antibodies inhibited the two-stage clotting assay (Fig. 4B), the procoagulant activity expressed on HUVECs was produced by TF. The reduction in TF surface activity was propor-

² D. L. Basi, K. F. Ross, J. S. Hodges, and M. C. Herzberg, manuscript in preparation.
tional to decreases in TF protein and TF-specific mRNA expression (Fig. 5), which strongly suggests that heat shock mediated the modulation of transcription without detectable post-translational modification.

Encrypted TF is the non-functional portion of the total quantity of surface TF (23, 24). Heat shock may have altered the proportion of encrypted TF and, therefore, could account for the decreased TF activity. To assess the total functional TF activity, we added ionomycin to HUVECs prior to incubation with factor VIIa. Ionomycin treatment increased surface TF activity, suggesting that a fraction of the TF was encrypted. Heat-shocked, LPS-stimulated HUVECs treated with ionomycin, however, showed significantly reduced surface TF activity (Fig. 4C). Sixty-one percent of TF activity was encrypted on LPS-stimulated HUVECs compared with 83% on heat shocked cells. The data suggest that heat shock reduced the absolute quantity and functional activity of surface TF.

We considered several other explanations for the results. The reduction in TF expression may have reflected the loss of LPS or IL-1β activity due to heat denaturation during heat shock. Therefore, LPS and IL-1β were pre-heated at 43 °C for 2 h and then added to HUVEC cultures. The surface iTF (+) HUVEC population was virtually identical when stimulated with pre-heated LPS or IL-1β (data not shown), indicating that heat denaturation or degradation did not alter activity. Perhaps heat shock itself contributed to cell injury or death and thus reduced TF expression. To test this possibility, cell viability was compared in heat stressed and unstressed LPS-stimulated HUVECs. Although HSP72-specific mRNA was up-regulated by heat shock and served as a positive control, viability was unaffected by heat shock when assessed by propidium iodide exclusion staining (Figs. 2C and 3H). To rule out cellular injury as a cause of reduced TF expression, cellular respiration and gross morphological changes were compared in heat-stressed and -unstressed LPS-stimulated HUVECs. Cellular respiration and morphological changes were evaluated by AlamarBlue™ reduction and light microscopy, respectively. Heat shock did not produce detectable changes in HUVEC respiration or morphology (data not shown). Heat shock modulates the expression of certain genes without apparent effect on other cell functions. For example, heat shock reduced the expression of TNF-α mRNA and protein by LPS-stimulated macrophages, which retained the ability to ingest antibody-coated erythrocytes like non-heat-shocked macrophages (25). Collectively, these experiments suggest that heat shock can modulate TF gene expression without an effect on specific cell functions, injury, or death. Reduction of TF expression by heat shock in LPS-stimulated HUVECs was therefore not due to cellular injury or death.

Based on our study, we hypothesize that fever may protect septic hosts because of the reduced activation of proinflammatory cytokines. Attenuated expression of TF would be expected to decrease development of DIC and improve the clinical prognosis. Fever may also play a role in other disease processes such as atherosclerosis. Viral and bacterial infections have been suggested as being implicated in the pathogenesis of atherosclerosis (26–28). For example, specific pathogen-free chickens infected with Marek’s disease virus and fed cholesterol-supplemented diets develop arterial fatty-fibro lesions similar to atherosclerotic plaques (29). In humans, the presence of cytomegalovirus antibodies is an independent risk factor for the development of atherosclerosis (30). Chlamydia antigens are detected in ~80% of coronary atherectomy sites compared with 4% in non-diseased vessels (31). In view of our data, it is noteworthy that the host responds to chlamydia and cytomegalovirus infections with antibody production, but clinical symptoms such as fever are usually absent. We hypothesize that the absence of fever generation during acute chronic vascular infections may increase the risk of thrombosis, contributing to the development of atheromas and acute coronary events such as myocardial ischemia or infarction.

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