The Regulation of Tissue Factor mRNA in Human Endothelial Cells in Response to Endotoxin or Phorbol Ester*

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Tissue factor (TF) is the membrane-bound glycoprotein whose co-factor activity with factor VIIa causes activation of the extrinsic pathway of coagulation. The transition of endothelium to a procoagulant state by agents such as bacterial lipopolysaccharide (LPS) is the result of TF expression by these cells. The mechanism of TF induction in human umbilical vein endothelial cells (HUVEC) was investigated in response to LPS and phorbol 12-myristate 13-0-acetate (PMA). Northern blot analysis of total RNA from HUVEC showed a rapid rise in TF mRNA levels which was maximal at 2 h and had fallen to low levels by 6 h following both LPS (10 µg/ml) and PMA (10 ng/ml) stimulation. Nuclear run on experiments showed at most a 2-fold increase in transcription of the TF gene following LPS stimulation but a 10-fold increase following PMA stimulation. In addition 24-h pre-incubation with PMA desensitised HUVEC to further PMA exposure, but caused no alteration in the response to LPS. Cycloheximide (10 µg/ml) alone caused induction of TF mRNA. Treatment of cells previously exposed to LPS for 1 or 4 h with actinomycin D indicated a 12-fold difference in the TF mRNA half-life. Therefore the rapid accumulation of TF mRNA in HUVEC stimulated by PMA is largely a result of an increase in mRNA stability rather than an increased rate of transcription of the gene.

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†The abbreviations used are: TF, tissue factor; LPS, bacterial lipopolysaccharide; HUVEC, human umbilical vein endothelium; PMA, phorbol 12-myristate 13-0-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; kb, kilobase; SPARC, secreted protein acid rich in cysteine.

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Experimental Procedures

**Materials**

**Cell Culture**—Medium 199 (M199), L-glutamine, penicillin, and streptomycin were from Flow Laboratories, Irvine, United Kingdom. Fetal calf serum, newborn calf serum, and trypsin, were from Gibco, Paisley, UK. Endothelial cell growth supplement was prepared in-house from bovine brain by the method previously described (17). Heparin (sodium salt from porcine intestinal mucosa) was from Sigma, Poole, UK. Tissue culture flasks were from Falcon, Irvine, UK and were otherwise stated. Gelatin was from British Drug House (BDH) Poole, UK.

**Chemicals Used**—CaCl₂, dimethyl sulphoxide; formaldehyde solution (40%), glycerol from BDH, Poole, UK; actinomycin D, benzamidine, cycloheximide, dihydrothreitol, EDTA, heparin, HEPES, KC1, LiCl, MgCl₂, Nonidet P-40, phenylmethylsulfonyl fluoride, polyvinylpyrrolidone, sodium acetate, sodium citrate, sodium dodecyl sulfate (SDS), Na₂HPO₄, NaCl, and Tris-HCl were all from Sigma, Poole, UK and were Molecular Biology Reagent grade; ATP, CTP, and GTP and Ficoll400 were from Pharmacia, Milton Keynes, UK. RNase A was from Sigma, Poole, UK. Human placental RNase inhibitor was from Amersham International, Amersham, UK. HindIII, SacI, SP6 and T7 RNA polymerase, proteinase K, and yeast tRNA were from BRL; Lewes, UK; oligo(dT)-cellulose from Collaborative Research Inc, Bedford, UK; agarose, DNase Type I, phenol, and urea from Bethesda Research Laboratories, Gibco; chloroform, May & Baker Ltd., Dagenham, UK; ethanol, J. Burrows Ltd., Paisley, UK and were otherwise stated. Gelatin was from BDH, Poole, UK.

**Methods**

**Cell Culture**—HUVEC were isolated and cultured as described previously (18). All cultures were grown in medium 199 with 10%
newborn calf serum, 10% fetal calf serum, 4 mM L-glutamine, penicillin, and streptomycin. Between passage 1 and 3 the medium was supplemented with heparin (90 µg/ml) and endothelial cell growth supplement (20 µg/ml). Confluent primary cultures were removed from flasks by brief exposure to 0.1% trypsin, 0.02% EDTA, pooled with other cultures, and seeded at approximately one-half confluent density. mRNA extraction experiments were performed on passage 3 HUVEC (passage 3) at confluence at passage 3 in 175 cm² flasks (NUNC). Briefly, cells were detached by rinsing with ice-cold phosphate-buffered saline, 7 ml of 10 s saliva, 3 M LiCl at 4 °C was added to the cell surface of a flask, which was left on ice for 5 min. This was then recovered and stored overnight at 4 °C to precipitate the RNA which was then collected by centrifugation and redissolved in water. RNA from human placenta was extracted in essentially the same way, except the tissue was disrupted in 6 M urea, 3 M LiCl by homogenization for 1 min. Polyadenylated RNA was prepared by affinity chromatography using an oligo(dT)-cellulose column.

RNA samples were electrophoresed in denaturing 1% agarose/formaldehyde gels (20). 10 µg of total RNA was loaded per lane from HUVEC samples. The gel was washed in 20 × SSC and the RNA transferred overnight to GeneScreen (Du Pont-New England Nuclear) membrane using 20 × SSC. RNA was coupled to the membrane by UV cross-linking (120,000 J, Stratagene TM-1800) followed by baking at 80 °C for 2 h. Filters were prehybridized for 2 h at 65 °C and then hybridized overnight at 68 °C in 60% formamide, 50 mM sodium phosphate, 5 × SSC, 1% SDS, 5 × Denhardt’s, 200 µg/ml denatured sheared salmon sperm DNA, and 100 µg/ml yeast tRNA. Blots were then washed twice for 30 min with each in 0.1 × SSC, 0.5% SDS at 75 °C, and exposed to Kodak XAR film at −70 °C using an image intensifying screen. Where appropriate images were quantified by densitometry (Chromoscan 3, Joyce Loebl-Vickers).

Where described filters were stripped prior to rehybridization in 70% formamide, 1% SDS for 50 min at 80 °C. Some filters were treated with RNase A to demonstrate the specificity of the hybridization. For this the membrane was soaked in 2 × SSC with 20 µg/ml RNAse A at room temperature for 30 min. The filters were then washed twice, each for 30 min in 2 × SSC, 0.5% SDS at 50 °C.

3P-Labeled RNA Probes—1360 base pairs of the human TF cDNA (21) was obtained in pBR347 from Genentech Inc, San Francisco, CA. This was linearized with SfuI, and single-stranded, antisense RNA was synthesized with [3P]UTP (800 Ci/mm, Amer sham International, Amersham, England) by transcription using SP6 polymerase. The antisense parental (mRNA) strand of a murine α-actin DNA clone, pAM 91 (22), was recloned at the PstI site of pGEM3 (Promega). This was linearized with HindIII, and 3P-labeled antisense RNA was made using T7 RNA polymerase.

Dot Blots of Serial Dilutions of HUVEC mRNA—5 µg of total RNA from HUVEC stimulated with either LPS (10 µg/ml) or PMA (10 ng/ml) for 1 h, was made up to 200 µl at a final concentration of 5 × SSC. These were applied to GeneScreen membrane (Du Pont-New England Nuclear) in a dot blot apparatus, washed with 100 µl of 5 × SSC and fixed by UV cross-linking and baking as above. This was hybridized with 3P-labeled antisense RNA probes for TF following the manufacture’s instructions. The dot blots were exposed to Kodak XAR film at −70 °C with an image intensifying screen.

Nuclear Run-On Experiments—Nuclei were extracted from 175 cm² flask (NUNC, Rakilde, Denmark) of HUVEC (passage 3) at confluence. The cells were washed with ice-cold phosphate buffered saline and removed by brief exposure to 1.5 ml of 0.1% trypsin, 0.02% EDTA. The cells were resuspended in 10 ml of resuspension buffer (RNAse free Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and benzamidine) and pelleted at 500 x g for 5 min and washed twice with 10 ml of RSB. The pellet was resuspended in 7 ml of lysis buffer (100 mM HEPES buffer (pH 7.9), 10 mM NaCl, 3 mM MgCl₂, and 0.05% Nonidet P-40). Nuclei were then washed with 10 strokes of a pestle of a Dounce homogenizer. The nuclei were pelleted at 500 x g for 5 min, washed twice with RSB, resuspended to a final volume of 210 µl in 50 mM HEPES buffer (pH 7.9), 40% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA, and frozen at −70 °C until required. Run on reactions were performed using the nuclei obtained from one 175 cm² flask for each time point = 3.0 x 10⁶ cells). The nuclei were added to 5 x run on buffer (25 mM Tris-HCl (pH 8.0), 12.5 mM MgCl₂, 750 mM KCl, 1.25 mM ATP, 1.25 mM CTP, 1.25 mM GTP, and 1000 units/ml placental RNAse inhibitor), and 330 µCi of [32P]UTP (3000 Ci/mmol). This was incubated for 40 min at 28 °C. Following the run on reaction the sample was incubated with DNase I type 1 at 30 °C for 5 min. This was completed by the addition of 0.2 volume 5 × SET (5% SDS, 25 mM EDTA, 50 nM Tris-HCl (pH 7.4)). The sample was then treated with proteinase K (200 µg/ml) for 45 min at 37 °C. The synthesized 3P-labeled RNA was extracted with phenol/chloroform (1:1) and precipitated with 2.5 M ammonium acetate and an equal volume of isopropanol. The RNA was collected by centrifugation, rinsed in isopropanol, and dissolved in 10 µl of 1 × SSC. The DNA was then UV cross-linked to the filter as described above. An 1100-base pair cDNA clone for von Willebrand factor in pUC18 (a generous gift of Dr. Pannekoek, Central Laboratory of the Netherlands Red Cross, Amsterdam) corresponding to the 1147-base pair fragment of the 5' region of the SPARC (secreted protein acidic rich in cysteine) cDNA in pGEMI (23), the α-actin and TF cDNAs described above, and control plasmids without inserts for the α-actin and TF cDNA were all applied to the membrane. Following prehybridization at 65 °C as above, the filters were hybridized for 36 h at 45 °C with the run-on RNAs and were washed twice for 15 min each in 2 × SSC, 0.1% SDS at room temperature and once at 55 °C in 0.1 × SSC, 0.1% SDS for 30 min. The filters were then exposed to Kodak XAR film at −70 °C with an image intensifying screen.

RNA Studies—One flask of cells was used for each time point with an agonist. LPS (10 µg/ml) and PMA (10 ng/ml) were added to the flasks in serum free culture medium and the 6 ml urea, 3 M LiCl extraction started at the specific time of exposure designated for that flask. HUVEC grow much more slowly and stop growing at early passage compared with bovine or porcine aortic endothelium. The number of cells used in these studies for HUVEC are considerable and for this reason the same passages were done in three stages for both LPS (10 µg/ml) and PMA (10 ng/ml) short 0, 10, 30, and 60 min, intermediate 0, 1, 2, 4 h, and long 0, 3, 6, and 12 h. Each stage for each agonist, therefore, represents the same batch of cells from a pooled population of umbilical cords.

TF Activity Studies—HUVEC at passage 2 were removed by trypsinization, as above, and subcultured into 1% gelatin coated 24-well trays (NUNC). Cells were used at confluence. Agonists used in TF induction experiments were added in M199 + 5% fetal calf serum, to a final volume of 1 ml/well. After removal of the medium, the cells were washed twice with phosphate-buffered saline at 37 °C and then lysed by repeated (3 times) freeze/thawing using a methanol/cardice bath and warming to 37 °C. TF activity was measured in the resulting cell lysate by adding 50 µl of normal human cultured platelet-poor plasma to each well. TF activity was detected as the time to clot formation at 37 °C after the addition of CaCl₂, to a final concentration of 25 mM. This was standardized against serial dilutions of rabbit brain thromboplastin. 1 unit of TF is taken to be equivalent to the amount of thromboplastin required to clot normal citrated plasma in 20 s.

Protein Kinase C Depletion Studies—HUVEC at passage 3 in 24-well trays were exposed to 10 ng/ml PMA in M199 + 5% fetal calf serum for 2 h or medium alone. This was removed and each LPS (10 ng/ml) or PMA (10 µg/ml) was added to the cells for 6 h and the resulting TF activity measured as described above.

RESULTS

TF mRNA and Nuclear Run-On Studies—The 1.35-kb human antisense RNA probe for TF hybridized with a major transcript of 2.4 kb as well as a characteristic doublet of two minor transcripts at 3.5 and 3.1 kb (21). These species were...
in both polyadenylated placental RNA (data not shown) and total RNA from HUVEC following 2-h stimulation with LPS (10 μg/ml). Furthermore, the minor 2.4-kb species, were found to be resistant to RNase A digestion.

TF mRNA was seldom detectable in unstimulated HUVEC and when observed was present at very low levels. Confluent cultures of cells at passage 3 were stimulated with maximally active concentrations of LPS (10 μg/ml) or PMA (10 ng/ml) (see below). Fig. 1 shows Northern blots of RNA from HUVEC stimulated for 0, 1, 2, and 4 h with either PMA or LPS. TF mRNA shows rapid induction to achieve peak levels by 2 h following stimulation with either agent. Substantial depression of the TF mRNA has occurred by 4 h which appears to be more rapid in the case of PMA.

Other experiments performed to examine the initial part (0, 10, 30, and 60 min) and the late part of the time course (0, 3, 6, 12, and 24 h) of TF mRNA response to LPS and PMA confirm this rapid inductive effect. For both agents TF mRNA is easily detectable by 30 min following stimulation. In the late time course studies TF mRNA levels were found to have fallen to very low levels by 12 h and fall to barely detectable amounts at 24 h. As is shown in Fig. 1, all three species of TF mRNA responded in a parallel manner.

Total RNA from HUVEC stimulated for 1 h with either LPS (10 μg/ml) or PMA (10 ng/ml) was serially diluted and applied to a dot blot filter for comparison of the change in steady state level of TF mRNA in each sample at that time point. Hybridization of the filter for TF mRNA showed that the stimulation produced by PMA caused a 30-fold greater increase in steady state mRNA levels compared to elevations achieved with LPS.

The effects of LPS and PMA upon the rate of transcription were examined in nuclear extracts from HUVEC. Fig. 2 shows the results from a nuclear run-on experiment where cells of the same batch had been exposed to either serum-free medium alone, LPS (10 μg/ml) or PMA (10 ng/ml) for 1 h. A low level of transcription of the TF gene is seen in the control nuclei, and densitometric analysis, correcting to the actin signal, indicates only a 2-fold increase with LPS, in the experiment shown. A second run-on experiment (data not shown) confirmed an identifiable basal rate of transcription of the TF gene under control conditions, which was not altered by LPS. In contrast PMA caused a marked, 10-fold, increase in the level of transcription of the TF gene under control conditions, which was not altered by LPS.

The level of transcription of the actin and von Willebrand’s factor genes are also shown for comparison. SPARC mRNA hybridization is negative, indicating that there is no transcription of this gene by these cells under these conditions. SPARC is secreted by bovine aortic endothelial cells when injured in culture, or plated at nonconfluent density, and has been interpreted as a marker of culture induced damage (24). The absence of transcription of the SPARC gene suggests that the TF response seen was not a consequence of nonspecific cellular damage.

The effect of cycloheximide (10 μg/ml) was examined upon the basal and stimulated level of TF mRNA in HUVEC. Fig. 3 shows a Northern blot of RNA isolated from cells exposed to serum-free medium alone (cont), 10 μg/ml cycloheximide (cyclo), 10 μg/ml LPS (LPS), or 10 μg/ml cycloheximide and 10 μg/ml LPS (cyclo + LPS) each for 1 h. Total RNA was extracted and 10 μg added to each lane. The filter was hybridized with antisense RNA probes for TF and α-actin. The actin bands represent hybridization to the 2100-base pair mRNA species.
The half-life of TF mRNA was examined by inhibiting transcription with actinomycin D (10 μg/ml) after stimulation of HUVEC with LPS (10 μg/ml) for either 1 h when the level of TF mRNA is increasing or 4 h when it is decreasing. Fig. 4 shows that TF mRNA was induced by LPS at both times compared with control, however, the message was substantially more stable following a 1-h stimulation with LPS compared with 4 h. Densitometric analysis of autoradiographs exposed so that the zero time point for the 1- and 4-h stimulated cells were quantitatively similar in density indicates half-lives for TF mRNA of 2 h following LPS stimulation for 1 h and 10 min following LPS stimulation for 4 h. Fig. 4 shows stability of the actin mRNA throughout both of these periods.

**Tissue Factor Activity**—TF activity was not detectable in unstimulated HUVEC. PMA and LPS each caused dose-dependent increases in TF activity in HUVEC that were maximal with 10 ng/ml PMA and 10 μg/ml LPS. The maximal TF activity achieved with PMA was 10-fold greater than that with LPS. TF activity was not detectable until 2 h after stimulation with either agent, peaked at 6 h, and declined to low levels at 24 h (Fig. 5). Agonist-induced changes were consistently greater for both agents when incubation was performed in 5% serum but were still detectable when serum-free medium was used (data not shown). Exposure to serum-free medium alone for up to 6 h was without effect upon tissue factor expression.

When either cycloheximide (10 μg/ml) or actinomycin D (10 μg/ml) was co-incubated with either PMA (10 ng/ml) or LPS (1 μg/ml) there was complete abolition of the TF response to these agents.

**Protein Kinase C Desensitization Studies**—Long-term exposure of endothelial cells to PMA down-regulates protein kinase C activity to almost undetectable levels (28). In other cell types this phenomenon has been shown to have a half-life of about 6 h and leads to near complete abolition of protein kinase C activity by 24 h (28). HUVEC when treated for 24 h with PMA (10 ng/ml) expressed little TF activity: medium alone 24 h, 5.2 ± 0.9 milliunits of TF/10⁶ cells; medium + PMA (10 ng/ml) for 24 h, 39.7 ± 2.9 milliunits of TF/10⁶ cells (mean ± S.E., n = 12, two experiments). When cells were then further challenged for 6 h with PMA (10 ng/ml) TF activity did not rise in those previously exposed to PMA (49.0 ± 3.2 milliunits of TF/10⁶ cells), whereas the expected response was obtained from cells preincubated for 24 h with medium alone (485 ± 31.0 milliunits of TF/10⁶ cells (mean ± S.E., n = 12, two experiments)). In contrast, LPS (1 μg/ml) for 6 h induced equal amounts of TF activity in cells pretreated for 24 h with medium alone (111.0 ± 7.7 milliunits of TF/10⁶ cells), or with PMA (10 ng/ml) (120.8 ± 26.7 milliunits of TF/10⁶ cells (mean ± S.E., n = 12, two experiments)).

**DISCUSSION**

The TF mRNA found in HUVEC upon stimulation with either LPS or PMA is composed of one major transcript (2.4 kb) and two larger, less abundant transcripts (3.1 and 3.5 kb). This is in accordance with the original description of this cDNA clone (21). The origin of these minor transcripts is not clear, but induction of these species parallels the change in level of the major transcript upon stimulation, and furthermore, the hybridization is resistant to RNase A digestion and is, therefore, specific. The presence of the larger transcripts in the polyadenylated placental RNA, and the demonstration in other cell types this phenomenon has been shown to have a half-life of about 6 h and leads to near complete abolition of protein kinase C activity by 24 h (28). The TF mRNA response of HUVEC to tumor necrosis factor has been reported to show maximal levels at 4 h following stimulation, but in this study earlier time points were not included (13). The rapid TF mRNA response to LPS described here is similar to the mRNA time courses in HUVEC of the early response gene KC induced by LPS, which...
was reported to be mediated by protein kinase C activation (32) and c-fos mRNA induced by interleukin 1 (33).

The response to PMA was similarly brisk and decay of TF mRNA was, if anything, quicker. The latter may be the result of desensitization of protein kinase C upon prolonged exposure to PMA (see below). The steady state levels of TF mRNA achieved with PMA were some 30-fold greater than with LPS when compared at 1 h of exposure, and this is reflected in the observation that PMA was consistently able to produce greater maximal TF activity in these cells.

Exposure of the cells to cycloheximide, at a concentration that blocked protein synthesis, induced TF mRNA to a level greater than that achieved with LPS alone. It is clear, therefore, that the induction of TF mRNA under these circumstances does not require protein synthesis, and this is in accordance with the rapidity of the LPS response.

The nuclear run-on experiments indicate that HUVEC in culture had a low basal rate of transcription which was only slightly increased or unaltered, by LPS, but substantially stimulated by PMA. The latter finding is in keeping with the presence of a consensus sequence for a phorbol response element in the 5'-flanking region of the TF gene (28). The absence of a clear stimulatory effect of LPS upon transcription is, however, at variance with the increase in TF transcription produced by LPS in the human monocyte (12). This may represent a significant cell type-specific difference in the regulation of the TF gene.

The predicted presence of AU sequences (known to be important in rendering mRNA unstable) (34) in the 3'-untranslated region of the TF mRNA, and the rapid induction of TF mRNA by cycloheximide without effect on the rate of transcription suggests that message stability is a potential control point regulating the level of TF mRNA. In addition, the lack of clear effects of LPS upon transcription, despite the demonstration of increases in transcription with PMA, suggested that LPS is acting by increasing stability of TF mRNA. The actinomycin D experiments reported here show the demonstration of increases in transcription with PMA, and the rapid induction of the translated region of the TF mRNA, and the rapid induction of TF mRNA.
Tissue Factor mRNA Changes in Human Endothelium

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