Effects of Prostacyclin Analogues on Human Endothelial Cell Tissue Factor Expression

David J. Crutchley, Lobella B. Conanan, Andy W. Toledo, Denis E. Solomon, and Benito G. Que

Prostacyclin analogues have been reported to inhibit the expression of tissue factor procoagulant activity in human monocytes, primarily by elevating intracellular levels of adenosine 3',5'-cyclic monophosphate (cAMP). The present studies have investigated whether prostacyclins can also inhibit tissue factor expression in endothelial cells. Iloprost, carbacyclin, and ciprostene had no effect on human umbilical vein endothelial cell tissue factor activity induced by lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), or interleukin-1β (IL-1β). Iloprost failed to elevate intracellular levels of cAMP, even when combined with a phosphodiesterase inhibitor. In contrast, forskolin increased endothelial cAMP and inhibited tissue factor expression. Conditioned medium from LPS-challenged monocytic THP-1 cells, which contained both TNF-α and IL-1β, induced endothelial cell procoagulant activity to levels 20-fold higher than those achieved in response to LPS alone. Iloprost abolished LPS-induced TNF-α secretion by THP-1 cells and inhibited IL-1β secretion by 45%. In keeping with this, iloprost reduced levels of TNF-α and IL-1β mRNA in LPS-challenged cells. Treatment of THP-1 cells with iloprost strongly inhibited the ability of conditioned medium to induce endothelial tissue factor expression, an effect that was mimicked by treating the medium with blocking antibodies to the cytokines. We conclude that although prostacyclin analogues do not directly suppress endothelial tissue factor expression due to their failure to elevate cAMP, they may do so indirectly by inhibiting the amplification produced by monocyte-derived cytokines.

KEY WORDS • iloprost • prostaglandin I₂ • interleukin-1β • thromboplastin • tumor necrosis factor-α • cyclic AMP

Tissue factor (thromboplastin, factor III) is a membrane-bound glycoprotein that plays a critical role in initiating blood coagulation. Tissue factor binds with high affinity to factor VII, leading to the rapid formation of factor VIIa; the resulting tissue factor-factor VIIa complex then activates factors IX and X, leading to thrombin generation and clot formation (for reviews, see References 1 and 2).

Under normal conditions, tissue factor is thought to be absent from the blood and vascular intima but present in subendothelial and perivascular tissues. This selective localization would minimize unwanted coagulation in healthy vessels while providing for effective hemostasis in the event of vascular damage. However, in vitro studies suggest that under appropriate conditions, tissue factor can be synthesized by two types of cells that are in contact with the blood at all times: monocytes and the cells of the vascular endothelium. For example, exposure to bacterial lipopolysaccharide (LPS) leads to transcription of the tissue factor gene and expression of procoagulant activity by both cell types. Endothelial tissue factor expression is also stimulated by exposure to interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Both of these cytokines are secreted by activated monocytes, suggesting that monocytes can induce or amplify the expression of procoagulant activity by endothelial cells.

We have recently shown that stable analogues of prostacyclin are potent inhibitors of tissue factor expression in human peripheral blood monocytes and a monocytic tumor cell line, THP-1. Since vascular endothelial cells are a primary source of native prostacyclin, it was of interest to determine whether prostacyclin analogues could inhibit tissue factor expression in these cells. Our results suggest that although the analogues do not directly affect endothelial tissue factor expression, they may do so indirectly by inhibiting monocyte cytokine secretion. These results suggest that a complex interplay may exist between prostacyclin, monocytes, and endothelial cells in the regulation of the tissue factor pathway.

Methods

Antibiotics and materials for the preparation of cell culture media were obtained from Gibco, Grand Island, NY. Fetal bovine serum was obtained from Hyclone, Logan, Utah. Heparin (from porcine intestinal mucosa), bovine fetuin, human thrombin, forskolin, isobutylmethylxanthine (IBMX), dibutyryl adenosine 3',5'-cyclic monophosphate (cAMP), dibutyryl guanosine 3',5'-cyclic monophosphate (cGMP), indomethacin, and polymyxin B were obtained from Sigma Chemical Co, St Louis, Mo. Type I collagenase was obtained from Worthington, Freehold, NJ. Bovine brains were obtained from Pelfreze, Rogers, Ark. Bacterial endotoxin (LPS B; Escherichia coli 0111:B4) was obtained from Difco, Detroit, Mich. Human recombinant TNF-α and IL-1β were obtained from Boehringer, Inc.
Indianapolis, Ind. Neutralizing polyclonal antibodies to human TNF-α and IL-1β were obtained from R&D Systems, Minneapolis, Minn. DNA polymerase from Thermus aquaticus (Tagg polymerase) and pAW109 RNA primers for TNF-α and IL-1β were obtained from Perkin-Elmer Cetus, Norwalk, Conn. Primers for glyceraldehyde-3-phosphate dehydrogenase were obtained from Clontech, Palo Alto, Calif. Recombinant Moloney murine leukemia virus (M-MLV) reverse transcriptase was obtained from Gibco BRL, Gaithersburg, Md. Rabbit brain thromboplastin standard was obtained from Ortho, Raritan, NJ. Human plasma deficient in factor VII was obtained from Helena Labs, Beaumont, Tex, and American Diagnostics, Greenwich, Conn. Iloprost was provided by Berlex, Cedar Knolls, NJ. Ciprostene and carbacyclin were provided by Upjohn Diagnostics, Kalamazoo, Mich. H-d-hexahydrotyrosyl-L-arginine p-nitroanilide (Spectrozyme-TH) was obtained from American Diagnostics. Primaria-coated 24-well dishes were obtained from Becton Dickinson, Lincoln Park, NJ, and Sarstedt, Newton, NC.

Cell Culture

Human endothelial cells were isolated from umbilical veins by perfusion with 0.2% collagenase and seeded onto 25-cm² gelatin-coated flasks. Cells were grown in medium 199 supplemented with 2 mmol/L L-glutamine, 20% heat-inactivated fetal bovine serum, and 10 mmol/L HEPES, pH 7.4.

For studies on the direct effects of prostacyclin analogues, endothelial monolayers were incubated in medium 199 containing 20% fetal bovine serum plus antibiotics and prostacyclins but lacking growth factors and heparin. Iloprost was added to the medium from a sterile saline stock solution. Carbacyclin and ciprostene were dissolved in ethanol and added to the medium so that the concentration of ethanol was 0.1% (vol/vol); at this concentration ethanol had no effect on cell tissue factor expression. After incubation for 30 minutes at 37°C, sterile saline containing LPS, IL-1β, or TNF-α was added and cells were incubated further for 4 hours. For coculture experiments, THP-1 cells (10⁶ cells/mL) were incubated for 30 minutes with RPMI 1640 containing 10% fetal bovine serum plus iloprost or saline vehicle and then incubated further for 4 hours with LPS. Cell-free conditioned medium was collected under aseptic conditions and stored at −70°C until it was either added to endothelial cells or analyzed for cytokine content. For immunoneutralization experiments, samples of conditioned medium were incubated for 60 minutes at 37°C with 2 µg/mL of blocking antibodies to TNF-α or IL-1β, both antibodies, or nonimmune goat immunoglobulin G (IgG). Endothelial and THP-1 cells treated by the aforementioned procedures remained more than 95% viable, as determined by exclusion of trypan blue.

After incubation, endothelial monolayers were washed with warm, serum-free medium 199. They were then studied as intact monolayers or scraped carefully with a rubber policeman, collected by centrifugation, and gently resuspended in tris(hydroxymethyl)aminomethane (Tris)–saline buffer by passage through a 12-gauge needle or plastic pipette tip. Phase-contrast microscopy in the presence of trypan blue showed that this approach produced a suspension of single cells of >90% viability.

Procoagulant Activity Assays

Procoagulant activity was assessed either on the surface of intact monolayers by using a chromogenic assay or in single-cell suspensions by using a clotting assay. For the former, endothelial monolayers were incubated with 1 mL of warm Tris-saline buffer containing 2.5% normal citrated human plasma and 8.3 mmol/L CaCl₂. Incubations were carried out at 37°C for 10 minutes. The reaction was stopped by removing 200-µL samples of supernatant, which were added to the wells of a 96-well plate containing 20 µL of a 5-mmol/L solution of a chromogenic substrate for thrombin, H-d-hexahydrotyrosyl-L-ala-lyl-L-arginine p-nitroanilide (Spectrozyme-TH). Absorbance at 405 nm was read by using an automatic plate reader (model 3550, Bio-Rad, Rockville Centre, NY) and compared with the absorbance generated by a standard curve of 0.01 to 5 U/mL human thrombin. For studies with single-cell suspensions, 50 µL of a suspension containing 0.3 to 1.8×10⁶ human cells was mixed with 50 µL of a 25-mmol/L solution of CaCl₂. Clotting was initiated by the addition of 50 µL normal human plasma, and the time for clotting to occur at 37°C was recorded by using a fibrometer (BBL, Cockeysville, Md). Procoagulant activity was quantified by reference to a rabbit brain thromboplastin standard; vials were reconstituted in 5 mL buffer and assigned a value of 100 000 U/mL; a 10-fold dilution of this preparation clotted plasma in approximately 28 seconds. A standard curve was constructed by plotting log units versus log clotting time. The activity of untreated endothelial cells was low and was subtracted from that of treated cells. None of the prostacyclin analogues affected the procoagulant ability of cell suspensions or standard thromboplastin when added at the assay stage.
Procoagulant activity was functionally characterized as tissue factor by establishing that cells failed to shorten the clotting time of plasma deficient in factor VII. Immunological characterization was achieved by demonstrating that HTF1-7B8, a blocking monoclonal antibody to human tissue factor (generously provided by Dr Steven Carson, University of Nebraska Medical Center, Omaha, Neb), specifically inhibited the ability of the cell suspensions to shorten the clotting times of normal plasma (50% inhibition, 500 ng/mL for each cell type).

Cytokine Assays

Total RNA was prepared from THP-1 cells by the method of Chomczynski and Sacchi. One microgram RNA from control and treated cells was reverse-transcribed with recombinant M-MLV reverse transcriptase in a reaction mixture containing 20 mmol/L Tris, pH 8.3, 2.5 mmol/L MgCl2, 50 mmol/L KCl, 100 μg/mL bovine serum albumin, 0.5 mmol/L deoxynucleotide triphosphates, and 10 U placental RNase inhibitor. Reaction mixtures were incubated for 90 minutes at 42°C, heated to 95°C for 5 minutes, and then quickly chilled on ice. One tenth of the reaction mixture was used for polymerase chain reaction (PCR) amplification. Specific primers for TNF-α (5'-end primer, 5'-CAGAGGGAAGGTCCCCCAG-3'; 3'-end primer, 5'-CCTGCTGGTAGGAGACG-3') and for IL-1β (5'-end primer, 5'-AAAGATGAGTGGCTCCCCAGG-3'; 3'-end primer, 5'-TGAGAAA-CACCAGTTGTCTCCA-3') were used as previously described. For control purposes, primers for the "housekeeping gene" glyceraldehyde-3-phosphate dehydrogenase (5'-end primer, 5'-TGAAGTGGGTGAGGACACG-3'; 3'-end primer, 5'-CATGTGGGCAATGAGGTTGCACCAC-3') were used in a side-by-side PCR amplification with the target genes for TNF-α and IL-1β. PCR was performed at a final concentration of 10 mmol/L Tris, pH 8.3, 50 mmol/L KCl, 0.1 mmol/L deoxynucleotide triphosphates, 0.1 mmol/L each of 5' and 3' primer, and 1 U Taq polymerase in a total volume of 50 μL. The mixture was overlaid with mineral oil and then amplified with the Perkin-Elmer Cetus Thermal cycler. The amplification profile consisted of denaturation at 95°C for 2 minutes, primer annealing at 60°C for 2 minutes, and primer extension at 72°C for 3 minutes in a 30- to 50-cycle reaction. Five to ten microliters of each PCR reaction mixture was electrophoresed in 1.5% agarose gel or 8% polyacrylamide gel in Tris/borate/EDTA buffer. Gels were stained with 0.5 μg/mL ethidium bromide and photographed.

Conditioned medium from THP-1 cells was assayed for secreted TNF-α and IL-1β by using commercial enzyme immunoassay kits (Cistron Biotechnology, Pine Brook, NJ), according to the manufacturer's instructions.

cAMP Assays

Endothelial cells were preincubated for 10 minutes with growth medium containing 1 mmol/L IBMX and then further incubated for 30 minutes with 100 mmol/L iloprost or 100 μmol/L forskolin. After incubation, cells were rapidly washed with cold phosphate-buffered saline and scraped into ethanol containing 0.01N HCl. Cells were then disrupted by brief sonication, and sonicates were incubated at room temperature for 5 minutes. Coagulated protein was removed by centrifugation and washed once with ethanol/water (2:1). Supernatants were combined and evaporated to dryness at 55°C under nitrogen, and residues were dissolved in assay buffer (50 mmol/L Tris and 4 mmol/L EDTA, pH 7.5). cAMP was measured by a commercial kit (Amer sham Corp, Arlington Heights, Ill) according to manufacturer's instructions.

Prostacyclin Assays

Prostacyclin in endothelial cell-conditioned medium was measured by radioimmunoassay of its hydrolysis product, 6-ketoprostaglandin F1α, as described previously.

Results

We first explored the ability of prostacyclin analogues to directly inhibit tissue factor expression by endothelial cells. Exposure of human umbilical vein endothelial cells to LPS (100 ng/mL), IL-1β (2 U/mL), or TNF-α (5 ng/mL) for 4 hours led to the expression of significant tissue factor procoagulant activity. Preliminary experiments established that these were submaximal concentrations of challenging agents. In marked contrast to results obtained with monocytic cells,18,19 iloprost over a wide concentration range (100 pmol/L to 10 μmol/L) failed to affect LPS-induced procoagulant activity as measured by thrombin generation on the surface of intact monolayers (Fig 1). In a similar manner, iloprost and two other analogues, ciprostene and carbacyclin, failed to modify LPS-, TNF-α-, or IL-1β-induced procoagulant activity. Prostacyclin, however, inhibited LPS-induced procoagulant activity in a concentration-dependent manner as depicted in Fig 1.
procoagulant activity as measured by clotting assays of single-cell suspensions (Table 1). The data shown were derived from four separate endothelial cell isolates that were passaged by mechanical means and tested in the first to fifth passages. Iloprost also had no effect on cells passaged with trypsin or in freshly isolated, nonpassaged cells (not shown).

We then explored the possibility that endothelial cells failed to respond to prostacyclin analogues because these cells were already producing optimal levels of the native prostaglandin. This is a potentially important point, since recent studies with porcine endothelial cells suggest that endogenous prostacyclin production may desensitize receptors for the exogenous ligand.33 Under basal conditions, prostacyclin synthesis by human umbilical vein endothelial cells was negligible, since 6-ketoprostaglandin F₁α was undetectable in medium collected after a 4-hour incubation. Conditioned medium from cells challenged with TNF-α contained low but measurable levels of 6-ketoprostaglandin F₁α (0.53±0.01 ng/mL; mean±SE; three incubations). Indomethacin (5 μmol/L) abolished TNF-α-induced prostacyclin synthesis but had no effect on tissue factor expression (Table 2). These results are consistent with previous reports that aspirin and indomethacin fail to modify endothelial tissue factor induction by IL-1 or LPS34,35 and indicate that endogenous prostacyclin does not modulate tissue factor expression. Furthermore, iloprost again failed to inhibit TNF-α-induced procoagulant activity in indomethacin-treated cells (Table 2), indicating that endogenous prostacyclin synthesis does not account for their unresponsiveness.

Previous studies have shown that iloprost inhibits tissue factor expression in THP-1 monocytic cells primarily via elevation of cAMP.19 The ability of iloprost to stimulate adenylate cyclase in endothelial cells and the relation of cAMP to endothelial tissue factor expression were therefore investigated. As shown in Table 3, 100 nmol/L iloprost had no effect on intracellular cAMP levels, either alone or in combination with the phosphodiesterase inhibitor IBMX. The failure of iloprost to inhibit endothelial tissue factor expression would therefore appear to be due to an inability to elevate cAMP in these cells. In keeping with this, elevation of cAMP in endothelial cells by other means did inhibit tissue factor expression. Thus, 100 μmol/L forskolin, a diterpene that is thought to stimulate the catalytic subunit of adenylate cyclase,36 produced a modest (1.8-fold) but significant increase in cAMP and inhibited LPS-induced tissue factor expression. Studies on human umbilical vein endothelial cells treated with 100 μmol/L forskolin produced a 4.2-fold increase in cAMP and a 64±3% inhibition of tissue factor expression. IBMX alone produced a 28±5% inhibition of tissue factor expression without detectable changes in cAMP. This apparent discrepancy may be a reflection of the different times at which measurement of cAMP and tissue factor were performed. Alternatively, these data suggest that even small changes in cAMP in cAMP are sufficient to inhibit endothelial tissue factor expression. Further evidence of a role for cAMP in regulating endothelial tissue factor expression was provided by experiments that showed that 1 mmol/L of a membrane-penetrable analogue, dibutyryl cAMP, produced a 39±7% inhibition of LPS-induced tissue factor expression; in contrast, 1 mmol/L dibutyryl cGMP produced a slight potentiation (8±6%, mean±SE; four determinations).

### Table 1. Failure of Prostacyclin Analogues to Suppress Tissue Factor Procoagulant Activity In Human Umbilical Vein Endothelial Cells

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Concentration (nmol/L)</th>
<th>Procoagulant activity (U/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LPS</td>
</tr>
<tr>
<td>None</td>
<td>...</td>
<td>2150±224</td>
</tr>
<tr>
<td>Iloprost</td>
<td>10</td>
<td>2379±361</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1749±160</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1947±189</td>
</tr>
<tr>
<td>Carbacyclin</td>
<td>10</td>
<td>1982±217</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1957±313</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1996±375</td>
</tr>
<tr>
<td>Ciprostene</td>
<td>10</td>
<td>1930±247</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1724±353</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1707±343</td>
</tr>
</tbody>
</table>

Endothelial monolayers were preincubated for 30 minutes with prostacyclin analogues and then incubated for 4 hours with 100 ng/mL lipopolysaccharide (LPS), 2 U/mL interleukin-1β (IL-1β), or 5 ng/mL tumor necrosis factor-α (TNF-α). Cells were harvested, and procoagulant activity of single-cell suspensions was determined. Values shown are mean±SE of three experiments performed on separate isolates. Basal activity of untreated cells was 76±20 U/10⁶ cells for nine experiments.

### Table 2. Failure of Iloprost to Suppress Tissue Factor Expression by Human Umbilical Vein Endothelial Cells Treated With Indomethacin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Procoagulant activity (U/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>60</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1280</td>
</tr>
<tr>
<td>Iloprost plus TNF-α</td>
<td>1094</td>
</tr>
</tbody>
</table>

Endothelial monolayers were incubated for 15 hours with or without 5 μmol/L indomethacin. Cells were then incubated with freshly added indomethacin plus 100 μmol/L iloprost for 30 minutes, followed by 5 ng/mL tumor necrosis factor-α (TNF-α) for 4 hours. Indomethacin was present throughout the challenge period. Procoagulant activity of single-cell suspensions was measured. Values are means of duplicate determinations.
These agents were similarly effective against tissue factor expression induced by 5 ng/mL TNF-α, with an =40% inhibition being produced by either 100 μmol/L forskolin or 0.5 mmol/L IBMX. We conclude, therefore, that although elevation of cAMP in endothelial cells does inhibit tissue factor expression, prostacyclin analogues do not affect endothelial cAMP levels and so have no appreciable effect.

We then explored whether products of activated THP-1 cells could influence endothelial tissue factor expression and, if so, whether iloprost could affect the phenomenon. Endothelial cells were incubated for 4 hours with conditioned medium obtained from THP-1 cells previously incubated with or without 1 μg/mL LPS. This relatively high concentration of LPS was employed to ensure that cytokine secretion would be readily measurable. For comparison, endothelial cells were also incubated with nonconditioned medium containing 1 μg/mL LPS.

No changes in endothelial cell attachment, morphology, or viability were noted during the 4-hour incubation with nonconditioned medium (RPMI 1640 containing 10% fetal bovine serum) compared with normal growth medium (medium 199 containing 20% fetal bovine serum). Procoagulant activity was also low or undetectable and did not change when endothelial cells were incubated with medium conditioned by THP-1 cells in the absence of LPS. In contrast, endothelial cells incubated with medium conditioned by LPS-challenged THP-1 cells generated high procoagulant activity, which reached levels 20-fold higher than those observed with cells incubated with LPS directly (Fig 2). The effects were concentration dependent with respect to the amount of conditioned medium added; as little as a 10-fold dilution was effective, representing a monocyte–endothelial cell ratio of ≈3:1. These effects were not due solely to residual LPS in conditioned medium, since 10 μg/mL polymyxin B abolished the direct effects of LPS but inhibited the effects of conditioned medium by only 20% to 30%.

Pretreatment of THP-1 cells with 50 nmol/L iloprost before the addition of LPS led to a marked inhibition of the ability of the conditioned medium to induce endothelial tissue factor expression (Fig 2). Inhibition ranged from 40±3% observed with undiluted medium, to 77±7% observed with 10-fold–diluted medium. It is noteworthy, however, that complete inhibition was not observed, despite the fact that this concentration of iloprost was maximal and produced 91±3% inhibition of tissue factor expression in the THP-1 cells from which the conditioned medium was obtained.

Levels of TNF-α and IL-1β were measured in the conditioned medium added to endothelial cultures. Both cytokines were readily detected in the medium from LPS-challenged THP-1 cells. Iloprost abolished TNF-α secretion and inhibited IL-1β secretion by 45±5% (Fig 3). Levels of a 325-bp fragment of TNF-α mRNA and a 391-bp fragment of IL-1β mRNA, obtained after reverse transcription and amplification with appropriate primers, were low or undetectable in control THP-1 cells but readily detected in cells treated with 1 μg/mL LPS (Fig 4). Iloprost (50 nmol/L) abolished the LPS-induced increase in TNF-α mRNA and reduced that in IL-1β mRNA, suggesting that iloprost

| TABLE 3. Effect of Iloprost and Forskolin on cAMP Levels and Tissue Factor Expression in Human Umbilical Vein Endothelial Cells |
|----------------|----------------|----------------|
| Agent          | cAMP (pmol/10⁶ cells) | Inhibition of PCA (%) |
|                | −IBMX            | +IBMX          | −IBMX | +IBMX |
| None           | 10.1±1.5         | 11.0±1.4       | ...   | 28±5  |
| Iloprost       | 11.1±1.7         | 13.4±1.6       | 0±0   | 25±3  |
| Forskolin      | 17.9±2.5*        | 42.9±6.3*      | 42±7  | 64±3  |

*Significantly different from "None" at P<.01.

For measurement of adenosine 3',5'-cyclic monophosphate (cAMP), cells were incubated for 10 minutes with 1 mmol/L isobutylmethylxanthine (IBMX) and then for 30 minutes with 100 mmol/L iloprost or 100 μmol/L forskolin. For measurement of procoagulant activity (PCA), 0.5 mmol/L IBMX was used, and cells were studied after a 4-hour challenge with lipopolysaccharide. Values are mean±SE of three to seven determinations.

Levels of TNF-α and IL-1β were measured in the conditioned medium added to endothelial cultures. Both cytokines were readily detected in the medium from LPS-challenged THP-1 cells. Iloprost abolished TNF-α secretion and inhibited IL-1β secretion by 45±5% (Fig 3). Levels of a 325-bp fragment of TNF-α mRNA and a 391-bp fragment of IL-1β mRNA, obtained after reverse transcription and amplification with appropriate primers, were low or undetectable in control THP-1 cells but readily detected in cells treated with 1 μg/mL LPS (Fig 4). Iloprost (50 nmol/L) abolished the LPS-induced increase in TNF-α mRNA and reduced that in IL-1β mRNA, suggesting that iloprost

![Fig 2. Bar graph showing amplification of endothelial tissue factor expression by THP-1 monocytic cell-conditioned medium. Endothelial monolayers were incubated for 4 hours with 1 mL nonconditioned medium containing 1 μg/mL lipopolysaccharide (LPS) (●), 0.1 to 1 mL medium conditioned by THP-1 cells previously incubated for 4 hours with 1 μg/mL LPS (○), or LPS plus 50 nmol/L iloprost (□). Conditioned medium was adjusted to 1 mL with nonconditioned medium when appropriate. After incubation, endothelial cell procoagulant activity (PCA) was measured, and the PCA of untreated cells (97±29 Ui10⁶ cells) was subtracted to give the net increase in PCA. Values are mean±SE of six experiments with five different endothelial isolates at the second through seventh passage. Iloprost plus LPS was significantly different from corresponding LPS alone at *P<.01 and **P<.005.](https://jab Group)
exerted its effects, at least in part, at the level of gene transcription. The effects were specific, since levels of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, were unchanged by treatment with either LPS or iloprost (Fig 4).

These results are consistent with the hypothesis that the procoagulant activity–inducing effects of THP-1 cell–conditioned medium were due to a combination of TNF-α and IL-1β and that iloprost blunted the effects by reducing the levels of both cytokines. The hypothesis would also explain why iloprost did not completely inhibit the procoagulant activity–inducing properties of conditioned medium; complete inhibition would not be expected, in view of the failure of iloprost to abolish the secretion of IL-1β.

Confirmation of the hypothesis was provided by experiments using polyclonal antibodies to specifically neutralize the biological activity of the cytokines. Incubation of conditioned medium from LPS-treated THP-1 cells with a nonimmune goat IgG had no effect on its ability to induce endothelial tissue factor expression (Fig 5). However, a blocking antibody to TNF-α produced 45±5% inhibition, a blocking antibody to IL-1β produced 48±5% inhibition, and both antibodies together produced 86±2% inhibition (mean±SE; six experiments with five endothelial isolates at the third through seventh passages).

Discussion

Monocytes and endothelial cells are currently thought to be the only cell types within a normal, intact vasculature capable of expressing tissue factor procoagulant activity. Although tissue factor expression in both cell types shows several similarities, including induction by common agents such as LPS and cytokines, the present studies show that important differences do exist. Prostacyclin analogues, which are potent inhibitors of tissue factor expression in human peripheral blood monocytes and monocytic tumor cells, 18,19 do not directly inhibit tissue factor expression in human endo-
thelial cells. This appears to be due primarily to an inability to elevate intracellular levels of cAMP. Thus, incubation of cells with 100 nmol/L iloprost, alone or in combination with a phosphodiesterase inhibitor, had no effect on cAMP levels measured at 30 minutes, the time at which challenging agents were added. In contrast, 5 nmol/L iloprost produced a 27-fold increase and 50 nmol/L iloprost produced a 95-fold increase in cAMP in THP-1 cells under similar conditions.19 However, cAMP levels could be elevated in endothelial cells by other agents, such as forskolin and dibutyryl cAMP, and under these conditions tissue factor expression was inhibited.

Previous studies on the effects of prostacyclin on endothelial cell cAMP have produced conflicting results. Native prostacyclin and another of its analogues, beraprost, have been reported to produce variable increases while iloprost had no effect.24-40 A recent study suggested that endothelial cells lack prostacyclin receptors.41 Our results showing that prostacyclin analogues are inactive in endothelial cells suggest that prostacyclin itself may have a paracrine rather than an endocrine function. Consistent with such a function, the present studies suggest that prostacyclin can indirectly modulate the expression of endothelial cell procoagulant activity by suppressing monocyte-associated cytokine release.

In summary, these findings suggest that a complex interplay exists between vascular cells in the regulation of tissue factor-initiated blood coagulation. Thus, endothelial cells can produce prostacyclin and may thereby downregulate monocyte tissue factor expression and cytokine production; in turn, monocytes may upregulate endothelial cell tissue factor expression via cytokine secretion. Under normal conditions, prostacyclin release from healthy endothelium presumably helps to maintain monocyte tissue factor expression and cytokine secretion at minimal levels. Under certain pathological conditions, however, including severe atherosclerosis, prostacyclin secretion may be impaired,42,43 allowing tissue factor expression by both monocytes and endothelium to be enhanced. If this is true, stable prostacyclin analogues may have therapeutic potential, not only for inhibiting the expression of tissue factor by monocytes but also for reducing the ability of monocytes to induce or amplify tissue factor expression by the endothelium. In addition, the ability of iloprost to inhibit the secretion of IL-1β and especially of TNF-α may itself have widespread clinical implications in view of the possible contributions of these potent agents to a variety of pathological states.14,15

Acknowledgments

This work was supported in part by a grant from the Walter G. Ross Foundation. We wish to thank Ms Ana Maria Muniz, Ms Viviana Barria, and Mr Franco Arias for their excellent technical assistance. We would also like to thank Dr Steven Carson of the University of Nebraska Medical Center, Omaha, Neb, for his generous gift of monoclonal antibody to human tissue factor and Dr Richard Hart of American Diagnostica, Greenwich, Conn, for his gift of Spectrozyme-TH. We wish to thank the nurses of the Obstetrics Department of Mt Sinai Medical Center, Miami Beach, Fla, for providing umbilical cords. Finally, we wish to acknowledge the many helpful discussions with Drs Berthold Baldus and Karl-Heinz Thierschrauf of Schering AG, Berlin, FRG.

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*Arterioscler Thromb Vasc Biol.* 1993;13:1082-1089
doi: 10.1161/01.ATV.13.7.1082

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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