Prostacyclin Analogues Inhibit Tissue Factor Expression in the Human Monocytic Cell Line THP-1 Via a Cyclic AMP-Dependent Mechanism

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Increased expression of tissue factor procoagulant by peripheral blood monocytes has been implicated in a number of thrombotic disorders. The present studies were undertaken to determine whether stable analogues of prostacyclin, a potent endothelium-derived platelet inhibitor and vasodilator, could inhibit tissue factor expression by human monocytic cells. Exposure of monocytic tumor THP-1 cells to 100 ng/ml endotoxin, 2 units/ml interleukin-1β, or 5 ng/ml tumor necrosis factor-α for 4 hours led to increased tissue factor procoagulant activity. Preincubation for 30 minutes with iloprost, ciprostene, and carbacyclin led to a dose-dependent inhibition of tissue factor expression induced by all three challenging agents. Iloprost was the most potent: 50% inhibition occurred at 5 nM, a concentration close to the reported dissociation constant for iloprost binding to the platelet prostacyclin receptor. An orally active analogue, cicaprost, was equally effective against endotoxin-induced tissue factor expression. Carbacyclin and ciprostene were 100 times less potent. Iloprost prevented the endotoxin-induced expression of tissue factor antigen on the surface of THP-1 cells, as determined by flow cytometry. Iloprost (500 pM–50 nM) increased intracellular levels of cyclic AMP. This effect was potentiated by isobutylmethylxanthine, an inhibitor of phosphodiesterase. The inhibitory effects of iloprost on tissue factor expression were also potentiated by isobutylmethylxanthine and mimicked by forskolin and dibutyryl cyclic AMP but not dibutyryl cyclic GMP. These results suggest that prostacyclin may play a role in downregulating tissue factor expression in monocytes, at least in part via elevation of intracellular levels of cyclic AMP.

Key Words • iloprost • interleukin-1β • tumor necrosis factor-α • cytokines • thromboplastin • cyclic AMP

Exposure of peripheral blood monocytes to a variety of agents leads to the expression of tissue factor, a membrane-bound glycoprotein that plays a critical role in blood coagulation as the cellular receptor and cofactor for factor VIIa. The agents include bacterial endotoxin; antigen–antibody complexes; activated complement fragments; and cytokines, such as the T-cell–derived monocyte procoagulant–inducing factor, interleukin-1 (IL-1), tumor necrosis factor (TNF), and interleukin-1 (IL-1). Although the efficacy of the latter two has been questioned. Monocyte tissue factor expression is thought to be a result of gene transcription and mRNA stabilization. At present, the physiological factors that downregulate these processes are not well identified.

We have previously shown that iloprost, a stable analogue of prostacyclin, is a potent inhibitor of endotoxin-induced tissue factor expression in human peripheral blood monocytes and a monocytic tumor cell line, THP-1. These preliminary studies suggest a novel role for prostacyclin in addition to its well-established role as a vasodilator and a platelet inhibitor (for review, see Reference 16). The present studies were undertaken to determine whether the effects of iloprost are 1) shared by other prostacyclin analogues and 2) expressed against tissue factor induction by agents other than endotoxin. In addition, because both iloprost and prostacyclin stimulate adenylate cyclase, we have explored the role of adenosine 3',5'-cyclic monophosphate (cAMP) in mediating the inhibitory effects of iloprost on monocytic cell tissue factor expression.

Methods

Monocytic tumor THP-1 cells were purchased from the American Type Culture Collection, Rockville Pike, Md. Antibiotics and materials for the preparation of cell-culture media were obtained from Gibco, Grand Island, N.Y. Fetal bovine serum was obtained from HyClone, Logan, Utah. Forskolin, 3-isobutyl-1-methylxanthine (IBMX), dibutyryl cAMP, and dibutyryl cGMP were obtained from Sigma Chemical Co., St. Louis, Mo. Bacterial endotoxin (lipopolysaccharide B; Escherichia coli 0111:B4) was obtained from DIFCO, Detroit, Mich. Human recombinant TNF-α and IL-1β were obtained from Boehringer, Indianapolis, Ind. Rabbit brain thromboplastin standard was obtained from Ortho, Raritan, N.J. Human plasma deficient in factor VII was obtained from Helena Labs, Beaumont, Tex.
and American Diagnostica, Greenwich, Conn. Iloprost was provided by Berlex, Cedar Knolls, N.J. Cicaprost was provided by Schering AG, Berlin, FRG. Ciprostene and carbacyclin were provided by Upjohn Diagnostics, Kalamazoo, Mich.

**Cell Culture and Incubations**

The cells were grown in RPMI-1640 medium containing 100 μg/ml streptomycin, 100 units/ml penicillin, 10% fetal bovine serum, and 10 mM N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid (HEPES), pH 7.4. Serum was heated at 56°C for 30 minutes to inactivate complement. For the experiments, cells were seeded into 24-well dishes at a density of 5×10⁵ cells/ml and incubated for 30 minutes at 37°C with growth medium containing varying concentrations of prostacyclin analogues or other inhibitors. Iloprost and cicaprost were supplied in sterile saline solution. Carbacyclin, ciprostene, and forskolin were dissolved in ethanol and added to the medium so that the concentration of ethanol was 0.1% (vol/vol). Ethanol at this concentration had no effect on cell tissue factor expression. Cyclic nucleotides and IBMX were dissolved directly in the medium. After a 30-minute incubation, endotoxin, IL-1β, or TNF-α in sterile saline was added, and the cells were incubated further for 4 hours. Cells treated with all of the aforementioned combinations remained more than 95% viable, as determined by exclusion of trypan blue. After incubation the cells were collected by centrifugation, washed with Puck’s saline A solution or serum-free RPMI-1640 medium, and resuspended in Tris-saline buffer (50 mM tris(hydroxymethyl)aminomethane [Tris] and 110 mM NaCl, pH 7.4).

**Procoagulant Activity Assays**

Procoagulant activity on the surface of THP-1 cells was determined by a single-stage clotting assay. Fifty microliters of cell suspension containing 0.3–1.8×10⁴ cells was mixed with 50 μl 25 mM 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 units/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) values. The procoagulant activity of standard thromboplastin and cell suspensions was not affected by prostacyclin analogues or other inhibitors when added at the assay stage.

Procoagulant activity was functionally characterized as tissue factor by establishing that the cells failed to shorten the clotting time of plasma deficient in factor VII. Immunological characterization was achieved by demonstrating that HTF3-7B8, a blocking monoclonal antibody to human tissue factor, specifically inhibited the ability of the cell suspensions to shorten the clotting times of normal plasma (50% inhibition, 500 ng/ml).

**cAMP Assays**

THP-1 cells were incubated for 30 minutes with growth medium containing 500 pM–50 nM iloprost, 200 μM IBMX, 100 μM forskolin, or combinations thereof. After incubation, the cells were rapidly centrifuged by using a microfuge, washed with cold phosphate-buffered saline, and resuspended in ethanol containing 0.01N HCl. The cells were disrupted by brief sonication, and supernatants were incubated at room temperature for 5 minutes. Coagulated protein was removed by centrifugation and washed once with ethanol/water (2:1, vol/vol). Supernatants were combined and evaporated to dryness at 55°C under nitrogen, and residues were dissolved in assay buffer (50 mM Tris and 4 mM EDTA, pH 7.5). cAMP was measured by a commercial kit (Amersham Corp., Arlington Heights, Ill.) according to the manufacturer’s instructions.

**Flow Cytometry**

Fluorescent flow cytometry was carried out essentially according to the method of Carson et al. Briefly, THP-1 cells were incubated with or without 100 nM iloprost for 30 minutes and then incubated for 4 hours longer with 100 ng/ml endotoxin. After washing, a sample of the cell suspension was tested for surface procoagulant activity. The remaining cells were incubated with normal human serum for 15 minutes to block the Fe binding sites. The serum contained 10 mM EDTA to block factor VII binding. Cells were then washed twice with Tris-saline buffer and incubated with 20 μg/ml of a fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody to human tissue factor (4508Cl, generously provided by Dr. Richard Hart, American Diagnostica) or FITC-conjugated nonimmune immunoglobulin G (IgG). Incubations were carried out at 4°C for 30 minutes. The cells were then washed twice, and the fluorescence intensity of 5–10×10⁴ cells was measured at 488 nm in a FACScan flow cytometer (Becton Dickinson, Rutherford, N.J.) equipped with an air-cooled argon ion laser. The instrument was gated to the appropriate cell size by comparing forward-angle versus 90° light scatter.

**Data Analysis**

Statistical analysis of data was performed by Student’s t test. Differences were considered significant at the 95% confidence level or higher. I₅₀ (the concentration causing 50% inhibition) values were calculated from a plot of percent inhibition versus log of inhibitor concentration. Percent inhibition was calculated as

\[
100 \times \left[ \frac{1}{\frac{1}{2} \text{(challenged-control)}+\text{inhibitor}}} \right]
\]

**Results**

Exposure of THP-1 cells for 4 hours to 5 ng/ml TNF-α produced significant (p<0.005) twofold increases in surface tissue factor activity. Similarly, exposure to 2 units/ml IL-1β produced three- to fivefold increases. Preliminary experiments established that these were submaximal concentrations for both agents. As shown in Figure 1, preincubation of the cells for 30 minutes with 100 pM iloprost slightly enhanced IL-1β- and TNF-α-induced activity, although only the latter
enhancement was statistically significant. Higher concentrations of iloprost strongly inhibited cytokine-induced tissue factor expression; at 100 nM the stimulatory effects of both cytokines were essentially abolished. An IC₅₀ of approximately 5 nM was obtained against each agent, identical to that observed with endotoxin-induced tissue factor expression.¹⁵

The ability of three other prostacyclin analogues to inhibit monocyte cell tissue factor induction was investigated. As shown in Figure 2, an orally active analogue, ciprostene, was equipotent with iloprost in inhibiting endotoxin-induced tissue factor expression. Carbacyclin and ciprostene were approximately two orders of magnitude less potent. Thus, ciprostene at concentrations of 100 nM or higher inhibited tissue factor activity in a concentration range over which cytokine activity have also been noted by Carson et al.²³

The role of cAMP in the inhibition of tissue factor expression by iloprost was then explored. Measurements of cAMP in THP-1 cells were made after a 30-minute incubation, the time point at which the challenging agents would routinely be added. Basal levels of cAMP were low (0.9±0.2 pmol/10⁶ cells) and increased modestly to 2.8±0.8 pmol/10⁶ cells after exposure to 500 pM iloprost (Figure 5). At this concentration iloprost had no observable effect on procoagulant activity. Higher concentrations induced dramatic increases in cAMP, 5 nM iloprost induced a 27-fold increase, and 50 nM induced a 95-fold increase, to 23.3±7.0 and 83.3±14.9 pmol/10⁶ cells, respectively. Iloprost therefore increased cAMP levels in THP-1 cells over the same concentration range at which it inhibited tissue factor expression.

As expected, the stimulation of cAMP by iloprost was significantly enhanced in the presence of 200 μM IBMX, an inhibitor of phosphodiesterase. Thus, 500 pM, 5 nM, and 50 nM iloprost induced ninefold, 69-fold, and 234-fold increases in cAMP, respectively, relative to untreated cells (Figure 5). Importantly,
IBMX also strongly potentiated the inhibitory effects of iloprost on monocytic cell tissue factor expression. Thus, although iloprost alone had no effect at 50 and 500 pM, it produced approximately 30% inhibition at 50 pM (not shown) and completely inhibited tissue factor expression at 500 pM when combined with IBMX (Figure 6). Indeed, iloprost combined with IBMX was more potent than would be predicted from its effects on cAMP levels. Thus, 500 pM iloprost plus IBMX raised cAMP levels to 8.0±2.5 pmol/10⁶ cells and abolished tissue factor expression, whereas 5 nM iloprost alone increased cAMP levels to 23.3±7.0 pmol/10⁶ cells and produced only 70% inhibition. This apparent discrep-
Further experiments showed that the effects of iloprost could be mimicked by agents that elevate intracellular cAMP by receptor-independent mechanisms. These included forskolin, which is thought to directly activate the catalytic subunit of adenylate cyclase, and dibutyryl cAMP, a membrane-penetrable analogue of cAMP. As shown in Figure 7, both endotoxin- and TNF-α-induced tissue factor expression in THP-1 cells was almost completely inhibited by 100 μM forskolin or 1 mM dibutyryl cAMP; in contrast, 1 mM dibutyryl cGMP had no effect. Sodium butyrate at 1 mM was similarly inactive (not shown).

Discussion

Our previous studies have shown that iloprost potently inhibits endotoxin-induced expression of tissue factor procoagulant activity by human peripheral blood monocytes and THP-1 monocytes. Iloprost also blunted further increases in procoagulant activity in cells already exposed to endotoxin, although higher concentrations were required under these conditions. Iloprost was approximately one order of magnitude more potent than prostaglandin E₂, which has also been shown to inhibit monocyte tissue factor expression.

We have now extended these observations to show that iloprost inhibits tissue factor induction by two cytokines, TNF-α and IL-1β, with the same potency observed with induction by endotoxin. Furthermore, the inhibitory properties were shared by three other prostacyclin analogues, cicaprost, ciprostene, and carbacyclin. Thus, inhibition of tissue factor expression by monocytic cells in response to various stimuli appears to be a general property of this class of drugs.

The I₅₀ values of 5 and 500 nM obtained for iloprost and carbacyclin, respectively, are very close to the reported dissociation constants (8 nM and 528 nM, respectively) for the binding of these agents to the prostacyclin receptor on platelet membranes. These observations suggest that prostacyclin itself is likely to...
be the endogenous ligand and may, therefore, be a physiological regulator of monocyte tissue factor expression. In view of the suggested relation between tissue factor and the receptors for several growth factors and cytokines, it is reasonable to ask whether prostacyclin may have other effects on monocyte function and whether the monocyte, like the platelet, may be a primary target of this agent.

The present studies also provide additional insight into the mechanism of action of iloprost. Three lines of evidence suggest that iloprost, like prostacyclin, exerts its effects at least in part via stimulation of adenylate cyclase. First, iloprost increased cAMP levels in THP-1 cells over the same concentration range at which it inhibited tissue factor expression. Second, the effects of iloprost on both cAMP and tissue factor were potentiated by an inhibitor of phosphodiesterase. Third, both endotoxin- and TNF-α-induced tissue factor expression were effectively inhibited by other agents known to elevate cAMP. The latter observation confirms earlier reports of variable inhibition by such agents on tissue factor activity in human peripheral blood monocytes. The ability of iloprost to block the expression of tissue factor antigen on the surface of THP-1 cells (Figure 4) would be consistent with a role of the inhibition of tissue factor synthesis, perhaps by interference with the process of gene transcription and/or stabilization of tissue factor mRNA. Further studies are required to clarify this point.

Increased tissue factor expression by monocytes may contribute to the disseminated intravascular coagulation associated with disorders such as allograft rejection, bacterial infection, carcinoma, and immune disease (for reviews, see References 1 and 30). More recently, it has been suggested that increased monocyte tissue factor activity may also contribute to the postoperative thrombotic state. Other investigators have shown that the development of fulminating hepatitis is accompanied by increased monocyte procoagulant activity in a virally infected mouse model. Both the hepatitis and the monocyte procoagulant activity are inhibited by the administration of prostaglandin E₂ and its 16,16-dimethyl analogue. The potency of iloprost and its effectiveness against multiple challenging agents suggest that it may also be valuable in the treatment of monocyte-driven coagulopathies, particularly if used in conjunction with a clinically effective phosphodiesterase inhibitor.

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