Prostacyclin Production by Perturbed Bovine Aortic Endothelial Cells in Culture

By Peter P. Nawroth, David M. Stern, Karen L. Kaplan, and Hymie L. Nossel

This study reports that endotoxin (Escherichia coli serotype 026:B6) and 12-0-tetradecanoyl-phorbol-13-acetate stimulate cultured bovine aortic endothelial cells to generate prostacyclin. The prostacyclin concentration of the culture medium was measured indirectly by radioimmunoassay for 6-keto-PGF₁α. The amount of prostacyclin generated depended on the concentration of endotoxin or phorbol diester. Prostacyclin generation was not immediate, but occurred slowly after a six-hour lag period. The perturbed cells contracted and showed marked shape changes that correlated temporally with the start of enhanced prostacyclin production. Cytoschalasin B and D, vinblastine, and colchicine inhibited prostacyclin production, indicating involvement of the cytoskeleton in the cellular response to endotoxin and phorbol diester. The increase in prostacyclin production was prevented by trifluoperazine, an inhibitor of the Ca²⁺-calmodulin system, which is known to be involved in cytoskeletal function. Generation of prostacyclin was inhibited by cycloheximide and actinomycin D, indicating dependence on protein and ribonucleic acid synthesis. It is postulated that exposure to endotoxin or phorbol diester leads, via a series of reactions that involve RNA and protein synthesis and require intact cytoskeletal function, to the generation of toxic active intermediate(s) that stimulate the enzymes necessary for prostacyclin production.

Prostacyclin (PGI₂) is one of the most important biosynthetic products of endothelial cells. It is a powerful inhibitor of platelet aggregation and may also inhibit adhesion, thus influencing platelet participation in thrombosis and hemostasis. Prostacyclin synthesis is a readily quantifiable index of endothelial cell perturbation and thus serves as a marker for the identification of injurious stimuli. In the present study, the influence of two agents on prostacyclin synthesis by cultured bovine aortic endothelial cells was examined—bacterial endotoxin, which has been reported to induce endothelial cell damage both in vivo and in vitro, and 12-0-tetradecanoyl-phorbol-13-acetate, which induces prostacyclin synthesis in dog kidney cells and is widely known as a tumor promoter. Both of these agents had no immediate effect on prostacyclin production, but produced a powerful stimulatory effect after a lag period of several hours. Prostacyclin production was accompanied by marked morphological changes, leading finally to detachment from the culture dish and release of lactate dehydrogenase (LDH). The effects of agents that inhibit the contractile apparatus of the cells and of agents that inhibit protein or RNA synthesis on the stimulation of prostacyclin synthesis were also examined and are reported here.

MATERIALS AND METHODS

Cell Culture

Bovine aortic endothelial cells were isolated from calf aortas (a gift of the Great American Veal Co, Newark, NJ) as described by Schwartz and were grown in Dulbecco's modified Eagle's medium (DME) with penicillin-streptomycin (50 U/mL, 50 μg/mL, respectively) and 10% fetal calf serum (lot 100382, Hyclone, Sterile Systems, Logan, Utah). The cultures were maintained at 37°C in an atmosphere of 5% CO₂ in air. The cells were separated for subculture with 0.25% trypsin-0.05% EDTA (GIBCO, Rockville Centre, NY). Cells (passages 3 through 22) from different aortas were grown to confluence in 35-mm dishes (Corning, Fisher, Springfield, NJ), and at confluence, there were 1.2 to 1.5 x 10⁶ cells per cm². The cells were characterized by contact inhibition of growth and cobblestone appearance in monolayer; the presence of von Willebrand factor antigen using indirect immunofluorescence, and angiotensin-converting enzyme activity. Viability during the experiments was checked by trypan blue exclusion and was > 70%. For the experiments, medium was aspirated from the dishes and replaced with 1 mL of medium containing 10% fetal calf serum (lot 100382), endotoxin, or phorbol diester plus agents studied for inhibitory activity and incubated at 37°C. A single lot of serum was used for all experiments, as the quantitative response was variable with different lots. All supernatant samples were taken at specified time points and stored at -20°C.

The concentration of 6-keto-PGF₁α was determined by radioimmunoassay with the use of rabbit antiserum to 6-keto-PGF₁α, kindly provided by Dr J. Bryan Smith (Temple University, Philadelphia). The level of sensitivity was 4 pg/mL. LDH release was measured using a spectrophotometric assay purchased from Sigma (St Louis). Maximum release was determined by freezing and thawing cells three times. Spontaneous release was determined by incubation in DME without serum. Results were expressed as percent specific LDH release ([LDH test - LDH spontaneous]/LDH freeze-thaw x 100%) as described for ⁵¹Cr release. For those experiments, cells were kept in 35-mm dishes in DME at 37°C and 5% CO₂ for 12 hours in the absence of serum so that serum LDH would not interfere with measurement of cellular LDH. 6-Keto-PGF₁α [5,8,9,11,12,14,15-H(N)] was obtained from New

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Address reprint requests to Dr Peter P. Nawroth, Department of Medicine, Columbia University College of Physicians and Surgeons, 620 W 168 St, New York, NY 10032.

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cytochalasin B, cytochalasin D, vinblastine, colchicine, actinomycin D, and endotoxin (lipopolysaccharide prepared using phenolic extraction procedure from Escherichia coli (serotype 026:B6) lot 72F-4019) were obtained from Sigma. 12-0-Tetradecanoyl-phorbol-13-acetate (phorbol diester), the 12,13-diester of phorbol, was obtained from Consoliated Midland Corporation (Brewster, NY), and trifluoperazine was donated by Smith, Kline and French (Philadelphia). Acetylsalicylic acid was obtained from Aldrich Chemicals, and indomethacin, cycloheximide, actinomycin D, and endotoxin [lipopolysaccharide prepared using phenolic extraction procedure from Escherichia coli (serotype 026:B6) lot 72F-4019] were obtained from Sigma. 12-0-Tetradecanoyl-phorbol-13-acetate (phorbol diester), the 12,13-diester of phorbol, was obtained from Consolidated Midland Corporation (Brewster, NY), and trifluoperazine was donated by Smith, Kline and French (Philadelphia). Acetylsalicylic acid was obtained from Aldrich Chemicals, New York, NY. Ionophore A23187 was purchased from Boehringer Mannheim (West Germany).

RESULTS

Bovine aortic endothelial cells in culture generate only small amounts of 6-keto-PGF$_{10}$ unless perturbed. In the presence of culture medium containing 10% fetal bovine serum, the 6-keto-PGF$_{10}$ concentration of the culture medium increased from about 2 to 8 ng after 24 hours, and remained at this level for a further 24 hours (Fig 1). Endotoxin produced a dose-dependent increase in 6-keto-PGF$_{10}$ production that became evident only after six hours. Phorbol diester-induced production of 6-keto-PGF$_{10}$ was also dependent on both the dose and incubation time (Fig 2). As with endotoxin, most of the 6-keto-PGF$_{10}$ was produced after six hours of incubation. As already reported by other investigators for endotoxin, we also observed detachment of cultured bovine aortic endothelial cells from the culture dish with both agents tested. In the presence of 10 ng phorbol diester, 10% of all cells were detached at 12 hours, 20% at 24 hours, and 35% at 48 hours. In the presence of 100 ng, 15% were detached at 12 hours, 30% at 24 hours, and 50% at 48 hours. The experiments in Figs 1 and 2 were done on the same day, using batches of cells derived from the same aorta, in the same passage, and the same day after achieving confluence. The figures show representative experiments. The actual concentrations of 6-keto-PGF$_{10}$ observed in different experiments performed with different batches of cells varied considerably, although the pattern of change was reproducible. Cells derived from different aortas appeared to behave differently on a quantitative basis, and in general, cells in later passages appeared to be less sensitive to the perturbing effect of endotoxin and phorbol diester (data not shown). Variations in response with different batches of fetal calf serum were observed in initial studies, and thereafter, a single batch of serum was used throughout. To test whether the variability in response depended on various populations within a cell line, we tested a cloned line of bovine aortic endothelial cells, a generous gift of Dr G. Godman (Columbia University, New York). This cloned cell line also exhibited considerable differences in responsiveness to the perturbing agents in different experiments (data not shown). Morphological changes occurred in parallel with the release of 6-keto-PGF$_{10}$ (Fig 3). Endotoxin and phorbol diester-perturbed cells contracted and appeared thinner and more elongated with prominent processes. Finally, the cells rounded up and detached from the culture dish, as described by Harlan et al. In general, it appeared that more grossly altered cells, as judged by observation through the microscope, released more 6-keto-PGF$_{10}$ into the medium. The morphological changes prompted us to determine whether drugs affecting the contractile apparatus influenced 6-keto-PGF$_{10}$ production. Cytochalasin B and cytochalasin D, which act on actin, and vinblastine and colchicine, which act on microtubules, inhibited both phorbol

![Figure 1](https://www.bloodjournal.org)  
**Fig 1.** Concentration of 6-keto-PGF$_{10}$ in the culture medium of bovine aortic endothelial cells incubated with or without phorbol diester. The concentration of phorbol diester is shown in ng/mL. Cell viability was ≥ 80%, based on trypan blue exclusion. Values shown are the geometric mean ± 1 SE of duplicate samples from one representative experiment. The experiment was repeated four times with similar results.

![Figure 2](https://www.bloodjournal.org)  
**Fig 2.** Concentration of 6-keto-PGF$_{10}$ in the culture medium of bovine aortic endothelial cells incubated with or without phorbol diester. The concentration of phorbol diester is shown in ng/mL. Cell viability was ≥ 80%, based on trypan blue exclusion. Values shown are the geometric mean ± 1 SE from one representative experiment. The experiment was repeated four times with similar results.
diester and endotoxin-induced 6-keto-PGF$_{1a}$ production (Table 1). To exclude the possibility that the inhibitory effect was due to failure to release 6-keto-PGF$_{1a}$ from the cells rather than to inhibition of synthesis, all samples were obtained after cells had been frozen and thawed three times. In contrast, calcium ionophore-induced production of 6-keto-PGF$_{1a}$ was not affected by these drugs. Trifluoperazine, an inhibitor of calmodulin$^{213}$ and phospholipase A$_2$, blocked both endotoxin and phorbol diester-induced 6-keto-PGF$_{1a}$ production by the cultured cells in a dose-dependent manner (Fig 4).

The long time period required to generate 6-keto-PGF$_{1a}$ suggested that the stimulation of 6-keto-PGF$_{1a}$ production might depend on protein synthesis. Support for this suggestion was obtained by finding that cycloheximide completely inhibited the stimulation of 6-keto-PGF$_{1a}$ production (Table 2). Actinomycin D was also inhibitory, indicating that RNA synthesis was required as well. Neither cycloheximide nor actinomycin D inhibited stimulation of prostacyclin synthesis by the calcium ionophore A23187 (data not shown). Cell viability during the lag phase was necessary for stimulation of prostacyclin synthesis, as very high doses of endotoxin (1 mg), which led to detachment and cell death within two hours, induced 6-keto-PGF$_{1a}$ release only to the degree observed when endothelial cells were frozen and thawed (data not shown).

Inhibition of prostacyclin production by cycloheximide and actinomycin D could be due to inhibition of synthesis of an enzyme required in prostacyclin synthe-
sis, either directly or indirectly, or it could result from decreased production of a toxic metabolite when protein and RNA synthesis are inhibited. The latter possibility was tested by examining the effects of cycloheximide and actinomycin D on LDH release during stimulation of cells with endotoxin. Figure 5 shows that there is dose-dependent inhibition by actinomycin D and cycloheximide of endotoxin-induced release of LDH. The increase in LDH release at the high doses of actinomycin D and cycloheximide may be due to enhancement of the cytotoxic effect of endotoxin by the toxicity of the inhibitors. Similar results were obtained with phorbol diester (data not shown).

6-Keto-PGF₁α production by perturbed cells was blocked by the cyclooxygenase inhibitors, indomethacin and aspirin (Table 3).

**DISCUSSION**

Most substances reported to stimulate prostacyclin production by endothelial cells, such as arachidonic acid, ionophore A23187, thrombin, trypsin, and bradykinin, do so immediately. In contrast, in the present studies, increased prostacyclin production by cultured bovine aortic endothelial cells was evident only after a lag of about six hours, implying that endotoxin and phorbol ester cause the cells to generate a substance(s) that then stimulates arachidonic acid metabolism. That this hypothetical substance is toxic to the cells is indicated by LDH release from endotoxin-treated cells, as shown in Fig 5. Toxicity and stimulation of prostacyclin production are related, as inhibition of protein and ribonucleic acid synthesis (Table 2) also decreased LDH release from endotoxin-treated cells (Fig 5). Toxicity to the point of cell death, however, was not associated with enhanced prostacyclin synthesis. These findings are consistent with the decrease of detachment observed by Harlan and colleagues when endothelial cells were exposed to endotoxin at 4 °C at

![Graph A](image)

*Fig 4. Inhibition of 6-keto-PGF₁α production by (A) endotoxin or (B) phorbol diester-treated bovine aortic endothelial cells by trifluoperazine. (A) Cells were exposed to 1 μg endotoxin or (B) 400 ng phorbol diester for 12 hours in the presence of trifluoperazine. The concentration of trifluoperazine is shown on the abscissa. Curves shown are the best fit line determined by least squares analysis. Each point is the mean of duplicates from one representative experiment, and the experiment was repeated three times.*

![Graph B](image)

**Table 2. Influence of Cycloheximide and Actinomycin D on 6-Keto-PGF₁α Production by Phorbol Diester and Endotoxin-Perturbed Bovine Aortic Endothelial Cells**

<table>
<thead>
<tr>
<th>Agent (ng/mL)</th>
<th>6-Keto-PGF₁α (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phorbol diester, 400 ng/mL, + actinomycin D (125 nmol/L)</td>
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</tr>
<tr>
<td>Phorbol diester, 400 ng/mL, + cycloheximide (170 nmol/L)</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Endotoxin, 500 ng/mL, + actinomycin D (125 nmol/L)</td>
<td>318</td>
</tr>
<tr>
<td>Endotoxin, 500 ng/mL, + cycloheximide (170 nmol/L)</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

Confluent cells were exposed to phorbol diester or endotoxin over a period of 12 hours in the presence of serum containing medium with or without cycloheximide or actinomycin D. Each value is the mean from one representative experiment. The experiment was repeated twice.

![Graph C](image)

*Fig 5. Influence of the dose of actinomycin D and cycloheximide on the release of LDH by endotoxin-treated bovine aortic endothelial cells. Cells were exposed to 1 μg/mL endotoxin for 12 hours in DME. Values shown are the geometric means of duplicates from two experiments. (A) Actinomycin D in the presence of endotoxin; (B) actinomycin D alone; (C) cycloheximide in the presence of endotoxin; (X) cycloheximide alone.*
37 °C. In different species, Harlan et al failed to find detachment or prostacyclin synthesis in response to endotoxin. In particular, they did not observe an increase of prostacyclin production by cultured human umbilical vein endothelial cells grown in newborn calf serum in the presence of endotoxin. A possible explanation for the lack of effect of endotoxin under these conditions may be the alteration in fatty acid content of human umbilical vein endothelial cells grown in 20% fetal calf serum compared with cells grown in 20% human serum, as reported recently by Lagard and colleagues. The differences in response of bovine and human umbilical vein endothelial cells to endotoxin might also be due to differences in binding of endotoxin to the different cells or to different responses of the cells to the lipid A moiety of bound endotoxin.

More than a decade ago, endotoxin was reported to induce endothelial cell injury as manifested by morphological changes and desquamation. Marked changes in cell shape were observed in our perturbed cells (Fig 3), finally leading to detachment from the culture dish. These changes coincided with the increase in 6-keto-PGF₁α synthesis, suggesting that the cytoskeletal changes might be involved in the synthetic response to phorbol diester and endotoxin. Vinblastine and colchicine caused significant inhibition of endotoxin and phorbol diester effects on prostacyclin synthesis (Table 1), raising the possibility that microtubule rearrangement occurs during the shape change in perturbed endothelial cells and is involved in the increased prostacyclin synthesis. The possibility that actin polymerization is also involved in the response to endotoxin and phorbol diester is raised by the inhibitory effect of cytochalasins B and D (Table 1). Those data, however, do not prove a direct link between changes in the cytoskeletal arrangement and the generation of prostacyclin. Other effects of cytochalasins B and D, vinblastine, and colchicine, in addition to the interaction with the contractile apparatus, might be involved.

Inhibition of prostacyclin generation by trifluoperazine (Fig 4) suggests that function of the calcium-calmodulin complex is required for this response. Trifluoperazine, an inhibitor of Ca⁺⁺—calmodulin, has been shown to inhibit various effects of phorbol diester in different cell types, indicating that these effects may be mediated via Ca⁺⁺—calmodulin. Calmodulin-binding proteins in the cytoskeleton and actomyosin complex of platelets have been described, and trifluoperazine has been reported to interfere with the contractile apparatus in various cells. However, trifluoperazine has also been reported to inhibit phospholipase A₂, and thus, it could inhibit prostacyclin synthesis by preventing phospholipase A₂-induced release of arachidonic acid from cellular phospholipids, indicating that effects other than the interaction with the Ca⁺⁺—calmodulin system might be involved.

It is interesting that Ca ionophore-stimulated 6-keto-PGF₁α production, which occurs without a lag phase, is not inhibited by the drugs that interact with the cytoskeleton (Table 1), suggesting that endotoxin and phorbol diester act before the Ca ionophore-stimulated pathway of prostacyclin production. However, the inhibitory effects of aspirin and indomethacin on endotoxin and phorbol diester-stimulated 6-keto-PGF₁α production (Table 3) indicate that the stimulatory effects are dependent on cyclooxygenase.

Thus, these studies indicate that cultured bovine aortic endothelial cells must be viable and have intact biosynthetic processes, normal cytoskeletal function, and a normal Ca⁺⁺—calmodulin system in order to respond to phorbol diester and endotoxin with increased generation of prostacyclin. The increased generation of prostacyclin is associated with cell injury, as reflected by LDH release and detachment, but it is noteworthy that LDH release is suppressed by inhibition of protein and ribonucleic acid synthesis, indicating active production of a toxic intermediate(s) in the response to phorbol diester and endotoxin. Thus, these studies suggest that release of prostacyclin is a general response of endothelial cells to injurious agents.

ACKNOWLEDGMENT

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REFERENCES


Table 3. Influence of Indomethacin and Aspirin on 6-Keto-PGF₁α Production by Phorbol Diester and Endotoxin-Perturbed Bovine Aortic Endothelial Cells

<table>
<thead>
<tr>
<th>Agent</th>
<th>6-Keto-PGF₁α</th>
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<tr>
<td>Phorbol diester, 50 ng/mL, + indomethacin (10 μmol/L)</td>
<td>310</td>
</tr>
<tr>
<td>Phorbol diester, 100 ng/mL, + indomethacin (10 μmol/L)</td>
<td>329</td>
</tr>
<tr>
<td>Endotoxin, 50 ng/mL, + indomethacin (10 μmol/L)</td>
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</tr>
<tr>
<td>Endotoxin, 1 μg/mL, + aspirin (10 μmol/L)</td>
<td>166</td>
</tr>
<tr>
<td>+ indomethacin (10 μmol/L)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Endothelial cells were stimulated with endotoxin or phorbol diester over a period of 12 hours in serum containing medium with or without 20 μmol/L indomethacin or 10 μmol/L aspirin. Values shown are the means from one representative experiment. The experiment was repeated five times.
18. Gregory SH, Zimmerman DH, Kern M: The lipid A moiety of lipopolysaccharide is specifically bound to B cell subpopulations of responder and nonresponder animals. J Immunol 125:102, 1980
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