Refractory Period Phenomenon in the Induction of Tissue Factor Expression on Endothelial Cells

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Tissue factor (TF), a membrane-bound glycoprotein, initiates the extrinsic pathway of blood coagulation by serving as the receptor and essential cofactor for factor VII and VIIa. Once formed, the factor VIIa-TF complex activates factor IX and X, leading ultimately to thrombin generation and fibrin accumulation. TF is constitutively expressed at different cellular sites that are not in contact with blood. By contrast, TF is not usually expressed by cells within the vasculature. However, both monocytes and endothelial cells (ECs), two cell types that are in direct contact with the blood, may be stimulated to express TF by various inflammatory agents. On the basis of these observations, it has been proposed that constitutively expressed TF, whose contact with blood is limited to events following trauma and tissue destruction, is involved in hemostasis; by contrast, TF induced on monocytes and ECs may be critical in the thrombogenic states associated with different pathologies. TF activity can be induced in ECs by immune complexes, tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), throbmin, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). The regulatory mechanisms controlling the induction of TF expression in ECs treated with LPS, TNF-α, and PMA operate at both transcriptional and posttranscriptional levels.

One characteristic feature of TF activity on monocytes and ECs is its transient induction, a peak occurring at 4 to 8 hours, followed by a return to basal levels after 24 hours. Interestingly, it has been reported that human umbilical vein ECs (HUVECs) that had been pretreated for 24 hours with IL-1β became refractory to the reinduction of TF by a second IL-1β stimulation. Such refractory behavior may have important pathophysiologic implications, because it may serve to limit the extent of endothelium-related intravascular coagulation in the presence of an inflammatory agent. However, the mechanisms underlying this insensitivity to further stimulation are poorly understood. To shed new light on the mechanisms underlying the role of TF in thrombogenesis, we evaluated the effects of inflammatory mediators known to induce TF activity in ECs, such as TNF-α, LPS, or PMA; in addition, such mediators present a refractory period phenomenon with respect to the reinduction of TF activity. To identify common pathways in TF induction, we studied the reinduction of TF activity by one inflammatory agent on cells pretreated by an alternative agent. Finally, we examined the mechanisms involved in the IL-1β–induced refractory period.

MATERIAL AND METHODS

Reagents. Fibronectin and collagenase type I were from Sigma (La Verpillière, France). Human platelet-free plasma and serum were purchased from the Centre National de Transfusion Sanguine (Les Ulis, France). Cell culture media were obtained from Gibco (Cergy-Pontoise, France), LPS from Escherichia coli serotype 0128:B12, and rabbit brain thromboplastin from Sigma. Goat antiserum against human TF was generously provided by Dr J.H. Morrissey (Oklahoma Medical Research Foundation, Oklahoma City, OK). Limulus assay was from Kabivitrum (Stockholm, Sweden). Recombinant human TNF-α (specific activity, 2 × 10⁷ U/mg) was supplied by Genzyme (Boston, MA) and recombinant human IL-1β (specific activity, 2.2 × 10⁶ U/mg) by the Glaxo Institute for Molecular Biology (Geneva, Switzerland). All radiolabeled compounds and Hybond N nylon membranes were from Amersham (Les Ulis, France). 6-keto-prostaglandin F₁α (6-keto PGF₁α) was from Sigma and 6-keto-PGF₁α antiserum from Biosys (Compiegne, France).

was performed. Release of PGI₂ into the medium was quantitated as well as the time required to reach a fixed optical density. Milliunits sequentially frozen and thawed twice in 10 mmol/L Tris-HCl, 1 mmol/L EDTA pH 7.4 were tested. They were then rinsed twice with calcium-free PBS and antibiotics. Cells were recovered by centrifugation and resuspended in culture media supplemented with 20% HS and antibiotics; such cells were plated in 24-cm² tissue culture flasks precoated with purified human fibronectin (1 to 5 µg/cm²). When cultured monolayers were confluent, cells from different cords (three to seven) were trypsinized and pooled. For experiments, second passage cells (HUVECs-P2) were subcultured into fibronectin-coated 96-well plates for the study of TF activity or into 75-cm² tissue culture flasks for RNA extractions. The cells were characterized by their cobblestone appearance in monolayers and by positive indirect immunofluorescence for detection of von Willebrand factor (vWF). All tested agents were added onto confluent cell monolayers in serum-free medium or in culture medium supplemented with serum. Cell viability was assessed by visual inspection and confirmed in some experiments by vital staining of the cells and cell counting.

**TF assay.** Procoagulant activity (PCA) was measured in a single-stage clotting assay performed by robot (Biomek; Beckman, Gagny, France). Briefly, HUVEC-P2 were grown to confluency in 96-well microplates and subsequently incubated with the agents to be tested. They were then rinsed twice with calcium-free PBS and sequentially frozen and thawed twice in 10 mmol/L Tris-HCl, 1 mmol/L EDTA pH 7.4 (80 µL/well) to lyse the cells. To each well, 80 µL of normal human citrated platelet-free plasma and 80 µL of 30 mmol/L CaCl₂ were added. Clot formation in the well was monitored by determination of the optical density at 540 nm at intervals of 35 seconds. The clotting time was determined for each well as the time required to reach a fixed optical density. Milliunits (mU) of TF were arbitrarily defined by conversion from standard curves (log-log plot) developed with a preparation of rabbit brain thromboplastin. PCA was totally TF-dependent as assessed by use of factor VII-deficient plasma instead of normal plasma or by preincubation of cell lysates with anti-TF antibodies.

**Prostacyclin (PGI₂) assay.** Samples of culture media were collected after cell incubation and stored at −20°C until assay of PGI₂ was performed. Release of PGI₂ into the medium was quantitated by radioimmunoassay (RIA) of its stable breakdown product, 6-keto PGF₁α. Briefly, samples were diluted in RIA buffer (0.1 mol/L phosphate buffer, 0.1% gelatin, pH 7.4) and incubated with 15 pg of [H]-labeled 6-keto PGF₁α (157 Ci/mmol) and 6-keto PGF₁α antiserum. After 30 minutes at room temperature followed by an overnight incubation at 4°C, bound and free antigen were separated by absorption of the free antigen to activated charcoal (1% charcoal, 0.1% dextran in RIA buffer) for 10 minutes at 4°C. The absorbed antigen was then separated by centrifugation at 400g for 15 minutes at 4°C. The radioactivity of the supernatants was measured in a β counter after addition of scintillation fluid. The amount of 6-keto PGF₁α was calculated from a standard curve analyzed in parallel.

**Dissociation of IL-1β from its receptor.** Receptor-bound IL-1β was removed by brief treatment (1 minute) of HUVEC monolayers with an acidic buffer (0.15 mol/L NaCl, 50 mmol/L glycine, pH 3.2).

**Estimation of protein synthesis.** Protein synthesis in HUVECs was quantified by determination of the incorporation of 35S-methionine (>1,000 Ci/mmol) into trichloroacetic (TCA)-precipitable radioactivity. Briefly, cells grown to confluency in 96-well plates were incubated with 5 µCi/well of 35S-methionine in normal medium containing 5% fetal calf serum (FCS), in the presence or absence of the agents to be studied. After 4 hours, the cells were washed three times and proteins precipitated by addition of 15% ice-cold TCA to each well. Wells were then washed three times and allowed to dry completely. All TCA-precipitated material was solubilized in 1 mol/L NaOH, transferred to vials containing scintillation fluid, and radioactivity counted in a β counter.

**TF messenger RNA (mRNA) analysis.** Total RNA was prepared from 6 to 12 x 10⁶ cells using the guanidine thiocyanate/cesium chloride method, and then electrophoresed and transferred onto membranes as previously described. Complementary DNA for human TF was isolated from a human placental cDNA library in λgt11 screened with an oligonucleotide probe. A total of 84 positive clones was obtained. A 830-bp EcoRI fragment (up to the first EcoRI site of the TF cDNA)²² was subcloned into pGEM1 and used as a probe. This TF-containing plasmid was radiolabeled to a specific activity in excess of 10⁶ dpm/µg using dCTP 5'-32P (3,000 Ci/mmol) by the random priming method (Multiprime DNA labeling system; Amersham). Hybridization was performed for 3 hours at 65°C with 8 ng/mL of probe in the rapid hybridization buffer (Amersham). The membranes were sequentially washed in 2X standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) (twice for 10 minutes), 1X SSC, 0.1% SDS (once for 15 minutes), 0.7X SSC, 0.1% SDS (once for 15 minutes), and finally in 0.5X SSC, 0.1% SDS (once for 15 minutes). Finally, the membranes were exposed (4 to 16 hours) for autoradiography using intensifying screens.

**RESULTS**

**Induction of TF activity by inflammatory agents.** Unstimulated HUVECs displayed very low levels of PCA in our standard clotting assay (<2 mU/4x10⁶ cells). Stimulation of the cells with IL-1β transiently increased PCA. Such procoagulant activity was essentially due to TF induction because this activity was not detectable when factor VII-deficient plasma was used in the PCA assay. Moreover, treatment of HUVEC-P2 lysates with antihuman TF antibodies completely prevented the IL-1β–induced PCA and also blocked the low basal PCA expressed in unstimulated cells (results not shown).

Kinetic analysis showed that TF activity attained a peak between 4 to 8 hours and then declined to near basal levels after 24 hours (Fig 1A). These results are in agreement with the observations of Bevilacqua et al. Short exposure (from 2 to 30 minutes) of HUVECs to 5 U/mL of IL-1β, followed by washing and incubation of the cells in IL-1β–free medium, resulted in a significant increase in TF activity by 4 hours (Fig 1B). These findings indicate that, after a short incubation period, IL-1β is able to transduce a cascade of intracellular events leading ultimately to increased TF activity. IL-1β–induced TF activity was dose-dependent, stimulation was observed with as little as 0.05 U/mL and was maximal at ~5 U/mL (Fig 1C). In addition to the effect of IL-1β, we examined the effects of other inflammatory mediators on TF activity. Our results are summarized in Table 1. We observed that, under our assay conditions, TNF-α, LPS, and PMA induced TF-related PCA. These data agree with earlier observations (see for TNF-α, Bevilacqua et al. and Nawroth and Stern, for LPS, Colucci et al, and for PMA, Lyberg et al.¹)
Table 1. Effect of Inflammatory Agents on the TF Activity of HUVECs

<table>
<thead>
<tr>
<th>Agents</th>
<th>Fold Stimulation</th>
<th>Concentration*</th>
<th>Time (h)$^t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>5–30</td>
<td>5 U/mL</td>
<td>4–8</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5–30</td>
<td>10 ng/mL</td>
<td>4–6</td>
</tr>
<tr>
<td>LPS</td>
<td>5–30</td>
<td>1 μg/mL</td>
<td>4–6</td>
</tr>
<tr>
<td>PMA</td>
<td>20–50</td>
<td>10 nmol/L</td>
<td>4–8</td>
</tr>
</tbody>
</table>

*To determine the maximal effective concentration of different inflammatory agents on TF activity induction, confluent HUVEC monolayers were incubated for 4 hours in medium containing 20% HS alone or supplemented with IL-1β, TNF-α, LPS, or PMA at various concentrations.

$^t$The time at which the effect is maximal is determined by incubating HUVECs with the indicated agent at its maximal effective concentration for various periods up to 72 hours.

All these experiments were performed in the presence of 20% HS. However, comparable results were obtained in the presence of 5% FCS or with serum-free medium. In addition, none of the measured TF activities were due to endotoxin contamination of the incubation media because less than 12 pg/mL of LPS could be detected in incubation samples by the Limulus lysate assay.

Effect of inflammatory agents on TF mRNA content. Unstimulated cells contained undetectable levels of TF mRNA. Exposure of cells for different times to either IL-1β or TNF-α or LPS or PMA resulted in the induction of a major 2.2-kb TF mRNA transcript. Induction of another minor TF transcript of 3.1 kb was occasionally detected (Fig 2), in agreement with previous observations. The kinetics of induction were similar for IL-1β and LPS with a peak at 1 hour, followed by a progressive decline in TF mRNA levels. Incubation of cells with TNF-α and PMA gave maximal TF mRNA induction at 2 and 4 hours, respectively. Thus, the transient increase in TF steady-state mRNA levels was reflected some 3 to 5 hours later in a transient increase in TF activity, observed after incubation of HUVECs with inflammatory mediators. Treatment of cells with cycloheximide alone (10 μg/mL) did not increase TF mRNA level. Cycloheximide, in combination with LPS, enhanced TF mRNA levels with respect to LPS alone (data not shown). This may be due to stabilization of TF mRNA. Observations consistent with this mechanism have been reported.

Effect of repeated stimulation on TF expression: refractory state phenomenon. The ability of activated ECs to respond...
to a second stimulation by an inflammatory agent was then evaluated. Addition of fresh IL-1β for 4 hours to cells that had been pretreated with IL-1β for 48 hours yielded a significantly reduced restimulation, suggesting that ECs had become "refractory" to the stimulatory signal (Fig 3A). This phenomenon has also been observed by Bevilacqua et al.16

To determine the concentration of the primary dose necessary to render cells hyporesponsive, cells were incubated for 48 hours with different concentrations of IL-1β (from 0.02 U/mL to 5 U/mL), washed, and then restimulated with a fixed dose of IL-1β (1 U/mL) (Fig 3B). The induction of a refractory state was concentration-dependent, with clear-cut hyporesponsiveness at doses of IL-1β ≥ 1 U/mL during the first 48 hours of incubation. Therefore, a primary dose from 1 to 5 U/mL of IL-1β was used in all subsequent experiments.

The kinetics of recovery from the hyporesponsive state after removal of the primary IL-1β dose were also determined. After an initial exposure of 48 hours to IL-1β, cells were incubated for varying lengths of time with medium alone and then rechallenged with IL-1β for 4 hours. Under these conditions, cells maintained in medium alone for 24 and 48 hours were still not able to fully respond to a second IL-1β stimulation (49% and 64% of a primary 4-hour IL-1β stimulation, respectively; results not shown).

The kinetics of the development of TF hyporesponsiveness were subsequently examined. To observe a refractory state on IL-1β restimulation at 24 hours, a primary incubation with IL-1β for at least 7 hours, followed by incubation in IL-1β-free medium, was required. Maximal hyporesponsiveness was observed after an initial treatment of 24 hours with IL-1β. Comparable hyporesponsiveness was observed after 24 or 48 hours of initial treatment with IL-1β (data not shown).

We have examined whether other inflammatory agents presented a similar refractory phenomenon with respect to TF expression. Continuous incubation of HUVECs with LPS for 48 hours led to a state of hyporesponsiveness when the cells were rechallenged (4 hours) with fresh LPS (30% of the primary stimulation). The same phenomenon was observed with TNF-α (39% of the primary stimulation). Among the inflammatory agents tested, PMA induced the strongest refractory state that we have observed (only 7% of a primary stimulation; Table 2).

The refractory state at the level of TF mRNA was then studied. For all the inflammatory agents tested, decreased TF mRNA steady-state levels were detected in restimulated cells when HUVECs pretreated with a specific factor were compared with sham pretreated cells (Fig 4). Thus, the observed refractory state, previously observed at the level of TF activity, was accounted for by decreased induction of TF mRNA.

Effect of sequential stimulations with two different inflammatory agents on TF expression. The preceding experiments showed that cells pretreated with an inflammatory agent "A" became refractory to the same agent with respect to reinduction of TF activity. We also examined whether this effect could be observed in response to stimulation with an agent "B" on cells pretreated with agent "A." Table 2

Fig 2. Effect of inflammatory agents on TF mRNA content in HUVECs. Confluent HUVEC monolayers were incubated in medium containing 20% HS alone (control cells, C) or supplemented either with IL-1β (A, 5 U/mL), TNF-α (B, 10 ng/mL), LPS (C, 1 μg/mL), or PMA (D, 5 x 10^-9 mol/L) for the indicated times. Monolayers were then washed and total RNA extracted from the cells. Equal amounts of RNA (10 μg/track) were analyzed by Northern blot and the filter hybridized to a 32P-labeled TF cDNA probe.
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Fresh IL-1s (1 U/mL). TF activity was measured in cell lysates at the end of the second incubation. The concentration of IL-1β in our experiments was 2.6 to 13 pmol/L (1 to 5 U/mL), whereas the dissociation constant of IL-1β on HUVECs is 81 pmol/L (N. Smithers, personal communication, October 1990). Thus, it is unlikely that the observed refractory state was due to a saturation of all IL-1β receptor sites. This suggestion was confirmed by treating HUVECs with an acidic buffer to remove receptor-bound IL-1β before a second stimulation. Under these conditions, HUVECs were unable to synthesize TF on restimulation with IL-1β (Fig 6).

To assess the possible involvement of a negative feedback regulation of IL-1β receptor in the induction of hyporesponsiveness, we characterised the expression and the binding properties of IL-1β receptors in untreated HUVEC cultures and in cultures treated for 24 hours with 1 U/mL of IL-1β. Unstimulated and stimulated cells showed no differences in IL-1β receptor mRNA levels (R. Lenstra, personal communication, July 1990). Moreover, a preliminary experiment involving Scatchard analysis showed that a comparable number of IL-1β receptors with a similar affinity was present at the surface of both untreated and treated cells, thereby ruling out modulation of the receptor as a significant mechanism in the induction of the refractory state by IL-1β (N. Smithers, personal communication, October 1990).

Alternatively, IL-1β–induced hyporesponsiveness could be due to a nonfunctional IL-1β receptor, which would be expressed at the same level and with the same affinity as the functional one. If this was the case, we would then expect that other biologic functions mediated by the binding of IL-1β to its receptor would be affected. IL-1β induces PGI₂.

Table 2. Hyporesponsiveness of HUVECs After Restimulation With Inflammatory Agents

<table>
<thead>
<tr>
<th>Pretreatment (48 h)</th>
<th>LPS</th>
<th>TNF-α</th>
<th>IL-1β</th>
<th>PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>LPS</td>
<td>30 ± 17*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TNF-α</td>
<td>114 ± 16*</td>
<td>39 ± 16*</td>
<td>63 ± 13†</td>
<td>141 ± 40†</td>
</tr>
<tr>
<td>IL-1β</td>
<td>80 ± 10</td>
<td>66 ± 3*</td>
<td>14 ± 8*</td>
<td>132 ± 75</td>
</tr>
<tr>
<td>PMA</td>
<td>55 ± 17†</td>
<td>99 ± 14</td>
<td>56 ± 15†</td>
<td>7 ± 10*</td>
</tr>
</tbody>
</table>

HUVECs were pretreated for 48 hours with an inflammatory agent, washed twice, and then restimulated for 4 hours with the same or a different agent. Doses used during pretreatment and restimulation periods were identical (LPS 0.5 µg/mL; TNF-α 10 ng/mL; IL-1β 1 U/mL; PMA 5 × 10⁻⁸ mol/L). Results are expressed as a percentage of a primary stimulation. Values represent mean ± SD of four to seven independent experiments. Comparison between TF induction by an inflammatory agent in mediator-pretreated cells and in sham-pretreated cells was performed with Student’s t-test.

Abbreviation: ND, not determined.

*Significantly different P ≤ .001.
†Significantly different P ≤ .01.
Fig 4. Effect of repeated stimulation with an inflammatory agent on TF mRNA content. Confluent HUVEC monolayers were incubated in medium containing 20% HS alone or supplemented with IL-1β (A, 5 U/mL), LPS (B, 1 μg/mL), or PMA (C, 5 × 10⁻⁵ mol/L). After 20 to 24 hours, cells were washed and reincubated for 2 to 3 hours as indicated, in medium containing the same agent at the same dose used during the first incubation. Total RNA was extracted and analyzed by Northern blot using 10 μg/track of RNA. The filter was hybridized to a 32P-labeled TF cDNA probe.

production by ECs after a few hours of exposure. It has been hypothesized that such production results from the de novo synthesis of enzymes involved in the pathway for PGI₂ synthesis. To evaluate PGI₂ production by HUVECs, we measured 6-keto PGF₁α in culture media after a primary and a secondary stimulation by IL-1β. Figure 7 shows that the content of PGI₂ in culture supernatants after IL-1β treatment for 4 hours was increased about fivefold. Cells cultured sequentially with IL-1β for 48 hours and then in the absence of IL-1β for 4 hours produced more PGI₂ than unstimulated cells, indicating that the effect of a primary IL-1β stimulation persisted for at least 52 hours without requiring the continuous presence of this cytokine. HUVECs pretreated for 48 hours with IL-1β produced more PGI₂ than untreated cells after 4 hours of restimulation with IL-1β, thus showing the absence of cell hyporesponsiveness to restimulation. In addition, this latter observation indicates that IL-1β receptors are still functional under these culture conditions as repeated IL-1β stimulation is able to further trigger PGI₂ synthesis.

To assess whether protein synthesis is required for induction of the refractory state during initial stimulation, cells were incubated for the first 24 hours with IL-1β in the presence of 0.5 μg/mL cycloheximide, and then thoroughly washed before a second stimulation with the same dose of IL-1β (Table 3). Inhibition of protein synthesis during the first 24 hours was approximately 50%, as measured by 35S-methionine incorporation. HUVECs treated for 24 hours with IL-1β plus cycloheximide were still refractory to the IL-1β-induced restimulation of TF activity. This finding was not accounted for by a general toxic effect on biologic functions of ECs nor by inhibition of IL-1β receptor synthesis, as untreated HUVECs and HUVECs pretreated for 24 hours with cycloheximide alone synthesized comparable levels of protein after 4 hours of incubation (see radiolabeling results and data on expression of TF activity after IL-1β stimulation).

These results suggested that de novo protein synthesis is not required during the initial incubation for a refractory period to be observed in conjunction with reinduction of TF.

**DISCUSSION**

The inflammatory agents IL-1β, TNF-α, LPS, and PMA induced a transient increase in TF activity in HUVECs, which attained a peak at 4 to 8 hours. Such activity was preceded by a transient increase in the steady-state levels of TF mRNA, with a maximum at 1 to 2 hours after stimulation for IL-1β, TNF-α, and LPS, and at 4 hours for PMA. The time-courses of the induction of TF activity by these agents are in agreement with previous reports. However, some discrepancies exist concerning TF mRNA induction. Thus, Scarpati and Sadler observed a maximal response after 3 hours with both TNF-α and PMA on TF.
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mRNA levels in HUVECs. Moreover, they found that PMA was only effective at micromolar doses, contrasting with the effect of PMA on our cells at nanomolar doses. In the same cellular model, Crossman et al. found a time-course of induction of TF mRNA by LPS and PMA that was almost identical, with a peak occurring during the first 2 hours after stimulation. In addition, they found that cycloheximide alone was able to induce an increase in TF mRNA levels after 1 hour, thereby contrasting with its lack of effect in our experiments and those of Scarpati and Sadler.

The differences between our data and those reported previously may be explained, at least in part, by differences in EC culture conditions or in the number of passages that the cells have undergone. Scarpati and Sadler, as well as Crossman et al., used medium supplemented with EC growth factor (EGCF) and heparin. This aspect may constitute an important difference because it has been reported that growth of HUVECs in the presence of ECGF and heparin decreases the inducibility of TF activity on these cells. The relatively moderate effects measured by Scarpati and Sadler with PMA and TNF-α on TF mRNA levels (eightfold and threefold of increase, respectively), as well as the relative insensitivity of their cells to PMA (only effective at micromolar ranges), could be due to extensive passaging of the cells (cells were used between passages 15 and 20). Indeed, we have also observed that at these passages cells were far less responsive with respect to TF induction (data not shown).

We have shown that the transient TF induction seen after an initial exposure of the cells to either IL-1β, TNF-α, LPS, or PMA is followed by partial (for TNF-α and LPS) or total (for IL-1β and PMA) hyporesponsiveness for TF reinduction by a second homologous stimulation.

We have also examined the question as to whether pretreatment of the cells with an agent “A” (IL-1β, TNF-α, or LPS) leads to a refractory state with respect to the reinduction of the expression of TF by a second agent “B” (heterologous stimulation). In this latter case, partial but significant hyporesponsiveness has been observed after IL-1β stimulation on TNF-α-pretreated cells, and conversely after TNF-α stimulation of IL-1β-pretreated cells.

The experiments that we performed on PMA-pretreated cells are of interest with respect to the mechanism of action of IL-1β, TNF-α, or LPS. It is well known from studies of different biological responses that phorbol ester treatment of the cells is followed by a refractory period during which a second treatment has little, if any, effect. This loss of responsiveness is accounted for by downregulation of protein kinase C (PKC), the major cellular receptor for phorbol esters. The fact that TF induction by IL-1β and LPS was significantly decreased on PMA-pretreated cells with respect to untreated cells suggests a potential involvement of PKC in the IL-1β and LPS pathways. By contrast, TNF-α was fully active on cells that had become completely unresponsive to PMA, suggesting that this cytokine acts through a PKC-independent mechanism. Similar conclusions can be drawn regarding the secondary messenger pathways for IL-1β and TNF-α from studies of Pober et al. on the induction of endothelial leukocyte adhesion molecule-1 (ELAM-1) in HUVECs.

Because the observed hyporesponsiveness is essentially a phenomenon of self-hyporesponsiveness (cells pretreated with agent “A” are refractory with respect to TF induction by the same agent “A,” but rarely and only to a limited extent by a second agent “B”), we have focused our attention on the self-hyporesponsiveness induced by IL-1β. This phenomenon is time-dependent (7 hours of incubation with IL-1β is necessary to detect a refractory state to a second IL-1β stimulation at 24 hours) and dose-dependent.
were confirmed by a preliminary Scatchard analysis that induction of intracellular inhibitor(s) seems rather unlikely in view of the very low amounts that are synthesized. Finally, HUVECs that were refractory with respect to IL-1\(\beta\)-induced TF expression continued to express functional receptors, because they produced enhanced PGI\(_2\) levels on restimulation. A downmodulation of IL-1\(\beta\) receptor expression seems rather unlikely, because TF mRNA levels were downmodulated whereas IL-1R mRNA levels remained constant during hyporesponsiveness (R. Lenstra, personal communication, July 1990). These data were confirmed by a preliminary Scatchard analysis that indicated that IL-1\(\beta\)-treated HUVECs presented a similar number of receptors and a comparable binding affinity (N. Smithers, personal communication, October 1990). Induction of intracellular inhibitor(s) seems rather unlikely because preincubation of HUVECs with IL-1\(\beta\) and cycloheximide did not prevent the refractory phenomenon. We have no clues concerning the possible occurrence of differential TF mRNA stability, or of an alteration in the secondary messenger pathway for IL-1\(\beta\).

The refractory period phenomenon is not restricted to the expression of TF on HUVECs. Other surface and secreted molecules, which are characteristic phenotypic markers of ECs, also present a refractory phenomenon with respect to their induction. The regulation of the expression of the cell adhesion molecule ELAM-1 by inflammatory agents is comparable with our observations on TF induction. ELAM-1 is transiently expressed (presenting a peak at 4 to 6 hours) at the cell surface of IL-1\(\beta\)-, TNF-\(\alpha\)-, or FMA-stimulated HUVECs. As in the case of TF, cells pretreated for 24 hours with one inflammatory agent were hyporesponsive to the same agent with respect to ELAM-1 reinduction. When two different agents were used, cross-inhibitions were observed with some combinations but not with others, as in the case of TF. Comparable patterns of cross-suppression are observed for TF and ELAM-1. These findings suggest that the refractory period involved in ELAM-1 and in TF expression may be comparable. Histamine establishes a desensitized state to further stimulation of tissue-type plasminogen activator release or to further stimulation of PGI\(_2\) production by ECs. The refractory period phenomenon observed for the modulation of various functions by inflammatory agents is not restricted to ECs. For example, it has recently been reported that LPS induces a hyporesponsive state with respect to its own effect on TNF-\(\alpha\) production by a macrophage-like cell line. This observation can be related to the phenomenon of endotoxin tolerance whereby animals treated with low doses of endotoxin become resistant to lethal doses. This observation is at the basis of therapeutic strategies to prevent the occurrence of septic shocks, and illustrates the general interest of understanding the regulatory mechanisms responsible for the onset of the refractory period phenomenon in response to inflammatory agents. In the case of TF, a clear understanding of the refractory period phenomenon might lead to new ways of limiting extensive thrombogenesis.

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Refractory period phenomenon in the induction of tissue factor expression on endothelial cells

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