Thrombin, TNF-α, and LPS exert overlapping but nonidentical effects on gene expression in endothelial cells and vascular smooth muscle cells

Sheng-Qian Wu and William C. Aird
Division of Molecular and Vascular Medicine, Center for Vascular Biology Research, Beth Israel Deaconess Medical Center, Boston, Massachusetts

Submitted 27 September 2004; accepted in final form 6 April 2005

Wu, Sheng-Qian, and William C. Aird. Thrombin, TNF-α, and LPS exert overlapping but nonidentical effects on gene expression in endothelial cells and vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 289: H873–H885, 2005. First published April 15, 2005; doi:10.1152/ajpheart.00993.2004.—Thrombin, TNF-α, and LPS have each been implicated in endothelial cell and vascular smooth muscle cell (VSMC) activation. We wanted to test the hypothesis that these three agonists display mediator and/or cell type-specific properties. The addition of thrombin to human pulmonary artery endothelial cells resulted in upregulation of PDGF-A, tissue factor (TF), ICAM-1, and urokinase-type plasminogen activator (u-PA), whereas TNF-α and LPS failed to induce PDGF-A. These effects were mimicked by protease-activated receptor-1 activation. In VSMC, thrombin induced expression of TF and PDGF-A but failed to consistently induce ICAM-1 or u-PA expression. In contrast, TNF-α and LPS increased expression of all four genes in this cell type. Inhibitor studies in endothelial cells demonstrated a critical role for PKC in mediating thrombin, TNF-α, and LPS induction of ICAM-1, TF, and u-PA and for p38 MAPK in mediating thrombin, TNF-α, and LPS induction of TF. Taken together, these results suggest that inflammatory mediators engage distinct signaling pathways and expression profiles in endothelial cells and VSMC. The data support the notion that endothelial cell activation is not an all-or-nothing phenomenon but rather is dependent on the nature of the extracellular mediator.

MATERIALS AND METHODS

Materials. PD-98059, SB-203580, H-7, H-89, genistein, TMB-8, and LY-294002 were obtained from Calbiochem (La Jolla, CA). Thrombin, TNF-α, LPS, staurosporine, rottlerin, U-0126, and GF-109203X were obtained from Sigma (St. Louis, MO). TFFLR-amide was from Peptides International (Louisville, KY).

Cell culture. Human pulmonary artery endothelial cells (HPAECl (Clonetics, San Diego, CA) were grown in endothelial basic medium-2 (EBM-2; Clonetics) supplemented with endothelial cell growth medium-2 (EGM-2) MV SingleQuots containing hydrocortisone, FGF-2, VEGF, ascorbic acid, EGF, gentamicin sulfate, and 5% FBS. Human coronary artery smooth muscle cells were cultured in smooth muscle basic medium (SmBM, Clonetics) supplemented with insulin, FGF-2, EGF, gentamicin sulfate, and 5% FBS. Cells were grown to confluence, serum starved overnight in starvation medium (EBM-2 for endothelial cells and SmBM for VSMC, both containing 0.5% FBS but no other supplement), and incubated in the absence or presence of thrombin, TNF-α, or LPS at the concentrations and for the times indicated. In inhibitor studies, serum-starved cells were preincubated for 10 min (in μM) with PD-98059, 10 U-0126, 10 SB-203580, 15 LY-294002, 1 staurosporine, 2 GF-109203X, 10 rottlerin, 50–300 genistein, 100 TMB-8, 20 H-7, and 20 H-89 or 1.5–5 U/ml hirudin and then treated with thrombin (1 U/ml), TNF-α (20 ng/ml), or LPS (50 ng/ml) for the times indicated (inhibitors are

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

THE ENDOTHELIUM is a metabolically active organ that is involved in a wide array of homeostatic processes, including the control of cellular trafficking, the maintenance of blood fluidity, the regulation of vasomotor tone, and the balance of pro- and anti-inflammatory mechanisms. In the healthy state, endothelial cells are heterogeneous in structure and function. In disease states, the phenotype of the endothelium may change in ways that differ from one vascular bed to another (2).

Endothelial cell heterogeneity in health and disease is mediated largely by the extracellular milieu. Indeed, the endothelium functions like a biosensor, sensing changes within the microenvironment and responding in ways that are usually beneficial but sometimes harmful to the host.

In this study we asked whether thrombin, TNF-α, and LPS result in identical or similar changes in gene expression profiles in endothelial cells. Thrombin binds to protease-activated receptors (PAR) expressed on the surface of endothelial cells (45); TNF-α binds to two receptors, namely, TNF-α receptor (TNFR)-1 and TNFR-2 (50), and LPS binds to the Toll-like receptor-4 (38). All three receptor classes are coupled to similar downstream signaling pathways, including PKC, phosphatidylinositol 3-kinase (PI3K), Akt, and NF-κB. Moreover, each receptor has been implicated in endothelial cell activation (3, 19).

However, endothelial cell activation is not an all-or-nothing phenomenon. Indeed, there is increasing evidence that while inflammatory mediators may trigger common downstream events, each mediator engages in a unique dialogue with endothelial cells. In this study we asked whether thrombin, TNF-α, and LPS result in identical or similar changes in gene expression profiles in endothelial cells. Rather than choosing DNA microarray experiments as a global readout of mediator signaling, we decided to focus on a panel of five target genes: early growth response (Egr)-1, tissue factor (TF), PDGF-A, ICAM-1, and urokinase-type plasminogen activator (u-PA).

In VSMC, we were able to systematically study both the kinetics of activation and the signaling pathways involved. Finally, we extended the studies to ask whether these mediators signaled differently in vascular smooth muscle cells (VSMC) compared with endothelial cells. Our results show that for a given cell type, there are mediator-specific differences in downstream signaling and gene expression, whereas for a given mediator there are cell type-specific differences in responses.
Table 1. Summary of inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>Concentration, µM</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-98059</td>
<td>MEK1/2</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>U-0126</td>
<td>MEK1/2</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>SB-203580</td>
<td>p38 MAPK</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>LY-294002</td>
<td>PI3K</td>
<td>15</td>
<td>65</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Protein kinase (PKC, PKA, PKG)</td>
<td>1</td>
<td>59</td>
</tr>
<tr>
<td>GF-109203X</td>
<td>Classic and novel PKC isoforms</td>
<td>2</td>
<td>62</td>
</tr>
<tr>
<td>Rottlerin</td>
<td>PKC-8</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>Genistein</td>
<td>Protein tyrosine kinase</td>
<td>50–300</td>
<td>6</td>
</tr>
<tr>
<td>TMB-8</td>
<td>Intracellular Ca(^{2+}) release</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>H-7</td>
<td>Serine/threonine kinase (PKA, PKC, PKG)</td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td>H-89</td>
<td>PKA</td>
<td>20</td>
<td>19</td>
</tr>
</tbody>
</table>

See text for abbreviation definitions.

**Probes for RNase protection assays.** The following fragments were subcloned into the pPCR-Script Amp SK(+) cloning vector by using PCR-Script AMP Cloning Kit (Stratagene, La Jolla, CA) (in bp): 440 TF, 376 PDGF-A, 334 Egr-1, 283 ICAM-1, and 210 u-PA human RT-PCR fragments. The cDNA inserts were verified by automated sequencing. The plasmids containing the respective cDNA fragments were amplified and purified using Qiagen HiSpeed Plasmid Midi Kit (Valencia, CA). The plasmids were linearized and used as in vitro transcription templates. The cDNA plasmid template for β-actin was purchased from Ambion (Austin, TX).

**RNA isolation and RNase protection assays.** Endothelial cells were harvested for RNA by adding RNA-STAT reagent (TEL-TEST, Friendwood, TX) directly into the wells. Total RNA was extracted according to the manufacturer’s instructions. α-[\(^{32}\)P]UTP riboprobes

Fig. 1. Agonist-mediated induction of tissue factor (TF), PDGF-A, ICAM-1, and urokinase-type plasminogen activator (u-PA) in human pulmonary artery endothelial cells (HPAEC). HPAEC were grown to confluence, serum starved in 0.5% serum overnight, and treated in the absence or presence of thrombin, TNF-α, or LPS at the concentrations and for the times indicated. Total RNA was extracted, and mRNA levels for TF, PDGF-A, ICAM-1, and u-PA were assayed by RNase protection. A: time course with thrombin (IIa, 1.0 U/ml), TNF-α (20 ng/ml), or LPS (50 ng/ml). B: dose response with thrombin, TNF-α, or LPS. C and D: mean fold induction (±SD) relative to control (untreated) based on densitometry of RNase protection assays from three independent experiments: thrombin (in U/ml), 0.005 (+); 0.025 (+++); 0.1 (+++); 0.5 (+++++); and 2.5 (+++++); and TNF-α and LPS (in ng/ml), 0.1 (+); 0.5 (++); 2.5 (+++); 10 (+++++); and 50 (+++++). Concentrations used for time course: 1.0 U/ml thrombin, 20 ng/ml TNF-α, and 50 ng/ml LPS. Dose response was carried out using a 4-h time point.
Fig. 1. (Continued)
were synthesized from the cDNA templates, by using either T3 or T7 RNA polymerase (Ambion), and subsequently gel purified. RNase protection assays were carried out using the RPA III kit (Ambion). Briefly, the riboprobes were mixed with 5 μg total cellular RNA in a volume of 10 μl and hybridized at 42°C overnight in hybridization buffer. The unhybridized RNAs were digested at 37°C for 30 min with RNase A-RNase T1 mix. The protected fragments were precipitated by adding the RNase Inactivation-Precipitation III solution and separated on a 4% acrylamide-8 M urea gel. The gels were dried and autoradiographed. The intensity of the bands was analyzed by National Institutes of Health Image software. Statistical analyses were carried out using the Student’s t-test.

RESULTS

Comparison of the effect of thrombin, TNF-α, and LPS on gene expression in HPAEC. To determine the effect of different inflammatory mediators on target gene expression in endothelial cells, early passage HPAEC were serum starved in 0.5% FBS overnight and incubated in the absence or presence of thrombin, TNF-α, or LPS at varying concentrations and for different times. As shown in Fig. 1, the addition of thrombin resulted in a 31.7-fold induction of TF mRNA, with maximal levels occurring at a concentration of 0.5 U/ml at 2 h; a 2.6-fold induction of PDGF-A mRNA, with maximal levels occurring at a concentration of 0.1 U/ml at 4 h; a 3.5-fold induction of ICAM-1 mRNA, with maximal levels occurring at a concentration of 0.5 U/ml at 4 h; and a 3.3-fold induction of u-PA, with maximal levels occurring at a concentration of 0.5 U/ml at 4 h. TNF-α resulted in a 23.8-fold induction of TF mRNA, with maximal levels occurring at a concentration of 10 ng/ml at 2 h; no change in PDGF-A mRNA; a 4.4-fold induction of ICAM-1 mRNA, with maximal levels occurring at a concentration of 2.5 ng/ml at 2 h; and a 2.6-fold induction of u-PA, with maximal levels occurring at a concentration of 10 ng/ml at 1–2 h. LPS resulted in an induction of TF, ICAM-1, and u-PA but not PDGF-A. The effect of LPS was much lower compared with thrombin or TNF-α.

The PAR-1-specific agonist TFLLR-amide (30 μM) resulted in a similar pattern of gene induction compared with thrombin (Fig. 2). Hirudin inhibited the effect of thrombin but not TFLLR-amide. These data suggest that thrombin exerts its effect through PAR-1.

We have previously reported that thrombin induces Egr-1 expression in human umbilical vein endothelial cells (HUVEC) and HPAEC (65). In this study, we wanted to extend these observations by studying the relative effect of thrombin, TNF-α, and LPS on Egr-1 expression in HPAEC. Like thrombin (65), TNF-α and LPS induced Egr-1 mRNA expression with peak levels occurring at 1 h (Fig. 3). Maximum induction was observed with 0.5 U/ml thrombin (4.0-fold), 10 ng/ml TNF-α (6.3-fold), and 50 ng/ml LPS (4.3-fold) (data not shown).

Analysis of signaling pathways involved in mediating the effect of thrombin, TNF-α, and LPS on gene expression in HPAEC. To determine the signaling pathways involved in transducing the above responses, HPAEC were pretreated in the presence or absence of chemical inhibitors of signaling, including PD-98509 [inhibitor of mitogen-activated protein kinase 1/2 (MEK1/2)], SB-203580 (inhibitor of p38 MAPK), LY-294002 (inhibitor of PI3K), staurosporine (inhibitor of PKC and other kinases), rottlerin (inhibitor of PKC-δ), H-7 (inhibitor of PKA, PKC, and PKG), H-89 (inhibitor of PKA), genestein (inhibitor of PTK), and TMB-8 (inhibitor of intracellular Ca²⁺ release) (Table 1).

As shown in Fig. 4, thrombin-mediated induction of TF was inhibited by SB-203580, staurosporine, rottlerin, H-7, H-89, genestein, and TMB-8. Thrombin stimulation of PDGF-A was inhibited by PD-98509, staurosporine, rottlerin, H-7, H-89, genestein, and TMB-8. The effect of thrombin on ICAM-1 and u-PA was inhibited by staurosporine, rottlerin, H-7, H-89, genestein, and TMB-8. The effect of thrombin on ICAM-1 and u-PA was inhibited by staurosporine, rottlerin, H-7, H-89, genestein, and TMB-8. The effect of thrombin on ICAM-1 and u-PA was inhibited by staurosporine, H-7, and genestein. LPS effect on TF was inhibited by SB-203580, staurosporine, H-7, genestein, and TMB-8 and was accentuated by LY-294002 and H-89. LPS-mediated induction of ICAM-1 and u-PA was inhibited by staurosporine, H-7, genestein and TMB-8.

To confirm the effect of MAPK in mediating agonist signaling, cells were pretreated with another MEK1/2 inhibitor, U-0126 (10 μM). In these experiments, thrombin resulted in only minimal induction of PDGF-A. These data point to significant experiment-to-experiment variability in the PDGF-A response and preclude meaningful conclusions about the role for MEK1/2 in this pathway (Fig. 5). U-0126 failed to inhibit thrombin-, TNF-α-, or LPS-mediated induction of TF, ICAM-1, or u-PA. Because staurosporine blocks a wide spectrum of kinases, we tested the effect of another PKC inhibitor, GF-109203X (2 μM). GF-109203X inhibited thrombin and TNF-α-mediated induction of TF as well as thrombin-mediated induction of ICAM-1 and u-PA (Fig. 5).

To deplete endothelial cells of classic and novel PKC isoforms, HPAEC were incubated with PMA for 24 h. Long-term
PMA treatment resulted in increased basal levels of PDGF-A, attenuation of the thrombin effect on TF, ICAM-1, and u-PA, and superinduction of TNF-α- and LPS-mediated increases in TF expression (Fig. 6).

Thrombin-, TNF-α-, and LPS-mediated induction of Egr-1 was completely blocked by pretreatment of HPAEC with PD-98509, staurosporine, H-7, or genistein and partially inhibited by H-89 (Fig. 7). TMB-8 pretreatment resulted in superinduction of Egr-1 expression.

Comparison of the effect of agonists on HPAEC and VSMC. Endothelial cells and VSMC share certain features in common. They represent critical cellular components of the vessel wall. They communicate with one another through paracrine signaling networks. Many of the same genes are expressed in both cell types. However, there are also important differences between these two vascular cell types. To determine the effect of different inflammatory mediators on target gene expression in VSMC, early passage human coronary artery VSMC were serum starved in 0.5% FBS overnight and incubated in the absence or presence of thrombin, TNF-α, or LPS at varying concentrations and for different times. Compared with endothelial cells, VSMC demonstrated higher basal levels of TF (Fig. 8). The addition of thrombin resulted in a small but significant (1.5-fold) induction of TF mRNA, with maximal levels occurring at a concentration of 0.5 U/ml at 2 h; a 1.8-fold induction of PDGF-A mRNA, with maximal levels occurring at a concentration of 0.5 U/ml; and no or little change in ICAM-1 and u-PA mRNA expression (Fig. 8). TNF-α resulted in a 1.5-fold induction of TF mRNA, with maximal levels occurring at a concentration of 2.5–10 ng/ml at 1–2 h; a 1.4-fold induction of PDGF-A mRNA; a 2.5-fold induction of ICAM-1 mRNA, with maximal levels occurring at a concentration of <100 ng/ml at 1–2 h; and a 2-fold induction of u-PA, with maximal levels occurring at a concentration of <100 ng/ml at 1–2 h.

In the time-course study shown in Fig. 8, thrombin resulted in a slight upregulation of ICAM-1 at 4 and 8 h (Fig. 8A). In contrast, thrombin had no such effect in the dose-response
study at 4 h (Fig. 8B). Quantitation of three independent time-course experiments revealed a high degree of variation in ICAM-1 mRNA levels (Fig. 8C). Thus, based on the available data, it is difficult to conclude whether or not thrombin induces ICAM-1 expression in VSMC.

In inhibitor studies, TNF-α and LPS-mediated induction of ICAM-1 was attenuated by staurosporine and rottlerin (Fig. 9). Because thrombin, TNF-α, and LPS had minimal effects on TF and PDGF expression in VSMC, preincubation with chemical inhibitors did not result in significant changes (Fig. 9).

The major findings were as follows: 1) basal expression of TF was high in VSMC; 2) each of the three mediators had less effect on TF in VSMC compared with endothelial cells; 3) PDGF was induced (albeit at low levels) in response to all three mediators (in contrast to endothelial cells, in which only thrombin had an effect; and 4) in contrast to endothelial cells, thrombin had minimal or no effect on ICAM-1 or u-PA in VSMC.

DISCUSSION

Pober and Gimbrone (53) were the first to demonstrate that a well-defined stimulus (lectin phytohemagglutinin) could induce the expression of an endothelial cell marker (Ia-like antigen). Through a series of elegant biochemical, molecular, and cellular studies, Gimbrone and colleagues (12, 15, 51) identified the first inducible endothelial cell-specific leukocyte adhesion molecule (ELAM-1; later designated E-selectin). Gimbrone’s group (10, 11, 13, 14) went on to show that numerous inflammatory mediators, including endotoxin, TNF-α, and IL-1, induced the expression of new antigens (so-called “activation antigens”) on the surface of HUVEC and was correlated with the expression of proadhesive, antigen-presenting, and procoagulant activities. Similar findings were reported by other laboratories (30, 57).

Pober and Cotran (52) proposed that “activation” reflects the capacity of endothelial cells to perform new functions without
any evidence of cell injury or cell division. In 1986, Cotran et al. (20) described activation of the endothelium in vivo. In the latter study, a murine monoclonal antibody, which had been shown to bind an antigen in IL-1-stimulated HUVEC (51) (later identified as ELAM-1 or E-selectin), was found to bind to the microvascular endothelium of human skin in delayed hypersensitivity reactions but not to normal skin (20).

Although endothelial cell activation has at times been described as a binary response (on or off; quiescent or activated), there is now ample evidence to the contrary. First, the phenotypic spectrum of an endothelial cell follows a continuum. As evidenced in this study, the addition of increasing concentrations of thrombin, TNF-α, or LPS resulted in a graded, though nonlinear, increase in expression of downstream target genes. Second, what constitutes activation for one cell type at a particular moment in time may not meet the definition of activation for another site or at another moment in time (5).

Third, as initially pointed out by Pober et al. (54), not all inflammatory mediators or endothelial cell activators are created equal. More recently, several groups including our own (45, 63, 67) have employed DNA microarrays to delineate the effect of one or another agonist on endothelial cell gene expression. In this study, we demonstrate that under identical culture conditions, TNF-α, thrombin, and LPS exert overlapping, yet distinct effects on downstream signaling and gene expression. Finally, at the risk of stating the obvious, the terms “activation” and “activity” are not synonymous. Normal endothelium is by its very nature highly active, constantly sensing and responding to alterations in the local extracellular environment, as might occur in the setting of transient bacteremia, minor trauma, and other common daily stresses.

Whereas agonist-specific differences in cell signaling argue against a simple on-off mechanism of endothelial cell activation, the overlap in expression patterns points to the importance
of cross talk between disparate receptor systems and/or the pleiotropic functions of downstream signaling intermediates. Indeed, existing data suggest that the many endothelial agonists are transduced by the same transcription factors. For example, IL-1 (36), IL-18 (47), TNF-α (63), LPS (38), hypothermia (56), high glucose (37), relaxin (26), oxidized HDL (41), fibroennin (35), C-reactive protein (46), thrombin (43), VEGF (1), and granulocyte-macrophage colony-stimulating factor (27) have each been shown to activate NF-κB; IL-6 (48), IL-10 (16), platelet-activating factor (25), leptin (60), oxidized phospholipids (66), VEGF (9), and FGF-2 (24) are coupled to STAT-3; and hypoxia (39), LPS, high glucose (32), insulin (32), oxidized phospholipids (34), VEGF (40), and hepatocyte growth factor (22) have all been reported to induce Egr-1 in endothelial cells. The above studies were carried out using varying types of endothelial cells and assay systems. This limitation notwithstanding, the results suggest that many extracellular mediators ultimately converge at the level of cell signaling, helping to explain their tendency to activate similar, though nonidentical, gene programs.

Endothelial cells and VSMC, which lie in close approximation to one another in the blood vessel wall, are both susceptible to activation and dysfunction. Whereas endothelial cells and VSMC share many cell surface receptors, signaling pathways, expression profiles, and activation phenotypes, each cell uniquely couples input with output. For example, we (42) have previously shown that thrombin induces VCAM-1 in endothelial cells, but not in VSMC, despite the presence of functional thrombin receptors on both cell types and despite the ability of other mediators such as IL-4 to induce VCAM-1 expression in VSMC. Others have employed DNA microarrays to demonstrate the differences in TNF-α/IL-1-responsive gene expression in human coronary artery endothelial cells and VSMC (8). In this study, we have shown significant differences in both basal activity and agonist response between endothelial cells and VSMC. For example, constitutive levels of TF were higher in VSMC compared with endothelial cells. These results are consistent with previous studies (23, 28) that compared TF protein in VSMC and endothelial cells. Others (17) have shown that TF is expressed at basal levels even after 48 h or serum deprivation and that expression is further increased with serum. TF is not normally expressed in VSMC in vivo but may become upregulated in response to arterial injury or atherosclerosis (59, 62). In the current study, VSMC were serum starved overnight with 0.5% FBS before treatment with an agonist. It is possible that the cells were not fully quiescent under these conditions and that a synthetic phenotype contributed to the high basal levels of TF expression.

Most notable among the differences in agonist response was the observation that TNF-α and LPS induce PDGF-B in VSMC but not in endothelial cells, whereas thrombin induces ICAM-1 and u-PA primarily in endothelial cells. These data confirm the
notion that endothelial cells and VSMC, at least under in vitro conditions, differ in how they transduce activation signals.

One limitation of the current study is the use of chemical inhibitors to block signaling pathways in endothelial cells. As a general rule, these inhibitors have nonspecific effects. To minimize this problem, we employed two inhibitors of MEK1/2 (PD-98059, U-0126), two inhibitors of PKC (staurosporine and GF-109203X), and different concentrations of genistein. Moreover, we carried out long-term PMA treatment to deplete endothelial cells of classic and novel PKC isoforms. However, further studies employing dominant negative or small interfering RNA approaches will be required to prove a role for these pathways in mediating signal transduction.

Our results suggest that for a single vascular cell type, different activation agonists are coupled to target genes through distinct intracellular pathways (Fig. 10). For example, in endothelial cells, an inhibitor of PKA attenuated thrombin-mediated activation of TF, PDGF-A, ICAM-1, and u-PA, yet it had no effect on TNF-α- and LPS-mediated gene induction. On
Fig. 8. Agonist-mediated induction of TF, PDGF-A, ICAM-1, and u-PA in VSMC. Human coronary artery smooth muscle cells were grown to confluence and serum starved in 0.5% serum overnight. Thrombin, TNF-α, or LPS was added at the time and concentrations indicated. Total RNA was extracted, and mRNA levels for TF, PDGF-A, ICAM-1, and u-PA were assayed by RNase protection. A: time course of thrombin (1.0 U/ml), TNF-α (20 ng/ml), or LPS (50 ng/ml). B: dose response of HPAEC to thrombin, TNF-α, or LPS. Cells were incubated with thrombin, TNF-α, or LPS at the concentrations indicated for 4 h. C: mean fold induction (±SD) of ICAM-1 relative to control (untreated) based on densitometry of RNase protection assays from three independent experiments. *P < 0.05 compared with untreated control.
the other hand, inhibition of Ca\(^{2+}\) release blocked the effects of thrombin and LPS, but not TNF-\(\alpha\), on target gene expression. As an example of target gene-specific signaling, thrombin has been shown, both here and in previous studies (42, 55), to regulate two highly related adhesion molecule genes VCAM-1 and ICAM-1 through distinct mechanisms. Thrombin-mediated activation of VCAM-1 is dependent on new protein synthesis, is sensitive to the redox potential of the endothelial cell, and involves PI3K-PKC-\(\gamma\)-p65 NF-\(\kappa\)B, PI3K-PKC-\(\gamma\)-GATA-2-dependent pathways. In contrast, thrombin stimulation of ICAM-1 does not require new protein synthesis, is insensitive to reactive oxygen species, and is mediated by a PKC-\(\delta\)-p65 NF-\(\kappa\)B-dependent pathway. These data suggest that by targeting one or another signal intermediate, one may selectively modulate the activation phenotype.

Severe sepsis is often associated with endotoxemia and leads to activation of coagulation and inflammatory pathways (4). As a result, the endothelium is exposed to myriad activators, including thrombin, TNF-\(\alpha\), and LPS. An important goal in sepsis therapy is to design drugs that will dampen the host response. The failure of single modality agents to improve survival in this syndrome may reflect the remarkable pleiotropic and redundant nature of cell activation. Adding to the complexity is the notion that each agonist is coupled to cell type-specific responses via distinct signaling pathways. The future success of therapies aimed at attenuating endothelial cell activation and dysfunction in syndromes such as severe sepsis will be contingent on a better understanding of the spatial and temporal dynamics of these pathways.

**GRANTS**

This work was supported by National Heart, Lung, and Blood Institute Grant HL-36028.

---

**Fig. 9.** Signaling pathways involved in mediating induction of TF, PDGF-A, ICAM-1, and u-PA in VSMC. VSMC were serum starved overnight and pretreated with or without inhibitors for 10 min before cells were incubated with thrombin (1.0 U/ml), TNF-\(\alpha\) (20 ng/ml), or LPS (50 ng/ml) for 4 h. Total RNA was harvested, and TF, PDGF-A, ICAM-1, and u-PA mRNA levels were examined using RNase protection. Inhibitors used consisted of (in \(\mu\)M) 20 PD-98059, 10 SB-203580, 15 LY-294002, 1 staurosporine, or 10 rottlerin.

**Fig. 10.** Schematic of signaling pathways. Positive and negative pathways are indicated next to the signal (thrombin, TNF-\(\alpha\), or LPS). There is no information from these studies as to which signaling pathways lie proximal or distal for any given agonist-target gene axis. As discussed in RESULTS, the role of MEK1/2 in mediating thrombin stimulation of PDGF-A is not conclusive.
REFERENCES


