ET activates the phospholipase C signaling pathway. In both hepatocytes (14) and Kupffer cells (51), as well as in the constituent cells of the liver, spleen, pancreas, stomach, uterus, testis, and bone (47, 53), as well as in the constituent cells of the vascular system. The tissue distribution of ET is nearly identical to that of its receptor(s), indicating the potential importance of paracrine or autocrine signaling pathways (19). Studies from our laboratory have shown that in the intact perfused liver, ET elicits sustained vasoconstriction and an increased hepatic glucose output (15) and that hepatocytes (14), Kupffer cells (51), and liver endothelial cells (9) possess functional receptors for ET. In both hepatocytes (14) and Kupffer cells, ET activates the phospholipase C signaling pathway. Other workers have shown that in cultured Ito cells, ET-1 binds to ETα and ETβ receptors, causing contraction of cells attached to a collagen matrix (23). Additionally, the contraction of liver sinusoids in vivo after infusion of ET has been localized to the Ito cells lining the sinusoids (58).

Under normal physiological conditions the low basal levels of ET found in the circulation are likely not sufficient to activate cellular processes (36). However, in conditions such as renal failure, congestive heart failure, and endotoxic shock the level of circulating ET has been shown to increase significantly (35). During sepsis the liver is a key organ in the pathophysiological response to endotoxin [lipopolysaccharide (LPS)]; early liver dysfunction is indicated by abnormal release of hepatic enzymes into the circulation, and prolonged exposure to LPS leads to liver failure (21). The first report that plasma ET levels increase in response to LPS (52) has been confirmed in different models of endotoxin exposure; these reports (10, 30, 42) confirm also that the baseline plasma immunoreactive ET-1 (irET-1) level is quite low. In pigs, a 2-h infusion of LPS caused a significant elevation in irET-1 both in the hepatic portal vein and systemically; this elevation persisted for several hours after termination of the infusion (40). We have shown recently that a large increase in the hepatic expression of ET-1 occurs during periods of endotoxin exposure in the rat and that liver endothelial cells are the primary cell type involved in this increased expression (10). Endothelial cells in culture are generally accepted to increase ET production in direct response to LPS. For example, calf pulmonary artery endothelial cells increase ET-1 production in response to treatment with as little as 10 ng/ml LPS (42), and microvascular pulmonary endothelial cells also respond to treatment with LPS by increasing ET-1 mRNA levels within 1 h of exposure (17). The current study was designed to identify the mechanism by which LPS exposure caused increased hepatic ET synthesis.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (SASCO, Omaha, NE) weighing between 225 and 300 g were used as a source of primary cultured cells for these studies. The rats were fed a standard rat chow and water ad libitum and were handled in compliance with the Animal Welfare Act and according to the guidelines set forth by United States Department of Agriculture. All protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio, which is accredited by the American Association for the Accreditation of Laboratory Animal Care.

Reagents. Collagenase (type IV from Clostridium histolyticum), protease E (type XIV from Streptomyces griseus), and BSA (fraction V and essentially fatty acid free) were pur-
Isolation and culture of liver cells. Rat sinusoidal endothelial cells and Kupffer cells were isolated as described previously by Knook and Sleyster (26). Kupffer cells (2 × 10^6 cells/ml) were plated at 4 ml/60-mm dish or 2 ml/35-mm well, using RPMI 1640 containing 10% FCS and 5,000 U/ml penicillin and 5,000 µg/ml streptomycin, and placed in an incubator at 37°C in an atmosphere of 95% air-5% CO₂. The media were changed after 24 h in culture, and the cells were used after the third day in culture. After two rinses with serum-free RPMI containing 0.1% BSA, Kupffer cells were cultured in low-serum RPMI (containing only 2% FCS) during experiments.

Liver endothelial cells were suspended in RPMI 1640 media containing 0.01% heparin, 2 mM L-glutamine, and 5,000 U/ml penicillin and 5,000 µg/ml streptomycin (RPMI) supplemented with 20% iron-supplemented calf serum. Aliquots of the cell suspension (2 × 10^6 cells/ml) were plated at 4 ml/60-mm dish or 2 ml/35-mm well, on dishes coated previously with rat tail collagen (UBI, Lake Placid, NY), which were incubated at 37°C in an atmosphere of 95% air-5% CO₂. After up to 4 h had been allowed for attachment, the cells were placed in serum-free medium containing 0.1% BSA, and other additions were made as indicated in the various figure legends. Plating efficiency (75–80%) was determined by counting adherent cells, using a phase-contrast microscope after staining with 0.1% crystal violet. Functionality of liver endothelial cells was confirmed by their ability to internalize acetylated low-density lipoprotein, which was labeled with the fluorescent tag Dil. Within the rat liver, endothelial cells are known to be the predominant site of internalization of acetylated low-density lipoprotein (41).

Coculture of Kupffer cells and liver endothelial cells. Kupffer cells were plated on Transwell inserts contained within a six-well plate and maintained in standard culture as described for 3 days before coculture with liver endothelial cells. The liver endothelial cells were plated onto the surface of a standard six-well plate and allowed to attach for 2–4 h as described previously. One hour before coculture the Kupffer cells were placed in RPMI containing 2% fetal bovine serum with or without 50 ng/ml LPS. Immediately before coculture, liver endothelial cells were placed in RPMI medium containing 0.1% BSA. The 6-h coculture interval was initiated by transferring the inserts containing the Kupffer cells into the wells containing liver endothelial cells.

Northern analyses. Total RNA samples were prepared from cultured liver endothelial cells or Kupffer cells using the method of Chomczynski and Sacchi (5). Northern blot analyses were performed on total RNA samples using a cDNA either to rat preproendothelin-1, kindly provided by Dr. M. Yanagisawa (47), or to rat TGF-β1, a generous gift of Dr. Lynda Bonewald (7). RNA was separated on 1% formaldehyde/agarose gels, transferred to nylon membranes, and subsequently hybridized with cDNA, which was random-primer labeled using [α-32P]dCTP. Stringency washes were performed sequentially as follows: at room temperature for 20 min in 2× saline-sodium citrate (SSC) with 1% SDS, 30 min at 60°C, also in 2× SSC with 1% SDS, 30 min at 60°C in 1× SSC with 0.5% SDS, and 30 min at 50°C in 0.1× SSC with 0.05% SDS. Differences in the amounts of mRNA were quantitated using a Molecular Dynamics PhosphorImager, and variation in sample loading was adjusted relative to the level of sample hybridization to an 18S RNA probe.

RIA of ET-1. The level of irET-1 was measured in media obtained from cultured liver endothelial cells. After centrifugation to remove debris from the culture media, the media were extracted using a C₁₈ column to reduce the effects of proteins on the assay, using a modification of the technique described by Cernacek and Stewart (4). The RIA was adapted from a commercial protocol and was sensitive over the range of 1–128 pg irET-1/ml. The samples were incubated overnight in assay buffer [0.1 M sodium phosphate (pH 7.4), 0.05 M sodium chloride, 0.1% BSA, 0.01% sodium azide, and 0.1% Triton X-100] with an anti-ET-1 antibody. According to the manufacturer, the antibody used in the RIA has only minimal cross-reactivity with big endothelin (<15%) and ET-3 (<5%). A second overnight incubation was performed with 125I-ET-1. The immune complex was precipitated with goat anti-rabbit IgG and normal rabbit serum, and the pellet was collected by centrifugation and counted using a gamma-scintillation counter.

TGF-β1 assay. Kupffer cells were plated and treated with LPS for 6 h as described previously. Media were removed from the cells and stored at −70°C until use, at which time they were acid-activated by adjusting the pH to 2 using 1 N HCl, and then returned to pH 7.4 using 1 N NaOH. Samples were diluted 1:2, 1:4, and 1:8 and subjected to analysis using a commercially available TGF-β1 ELISA kit according to the protocol supplied by the manufacturer.

Statistics. Results are expressed as means ± SE. Data were analyzed for significance using ANOVA and Newman-Keuls tests with P < 0.05 considered significant.

RESULTS

Effect of LPS treatment on ET-1 secretion. Hepatic endothelial cells were established in culture as described and then incubated in RPMI plus 10% iron-supplemented calf serum containing LPS at concentrations ranging from 10 ng/ml to 1 mg/ml for 24 h. At the end of the incubation period the media were removed and the level of irET-1 was measured by RIA. There was no significant elevation in the amount of irET-1 released into the culture media at any LPS concentration tested (Fig. 1). The highest LPS concentration (1 mg/ml) resulted in decreased levels of irET-1 in the culture media (56% of the control value), which is consistent with a toxic effect of LPS at this concentration. This finding suggested that acute effects of LPS on ET-1 production by the liver endothelial cells occurred through an indirect mechanism.

LPS stimulation of TGF-β synthesis by Kupffer cells. Cultured Kupffer cells were stimulated with LPS, and total RNA was isolated to assess the change in TGF-β1 mRNA levels. Exposure to LPS for 6 h increased the level of TGF-β1 mRNA by 53% relative to control cells.
The culture media taken from Kupffer cells treated with LPS contained 2.78 ± 0.22 ng/ml TGF-β1, and the media samples from untreated Kupffer cells contained 1.78 ± 0.15 ng/ml TGF-β1 (Fig. 3). These data confirm the ability of short-term cultured Kupffer cells to increase expression and secretion of TGF-β1 in response to LPS treatment.

Stimulation of ET-1 secretion by Kupffer cell-conditioned media. Culture medium (RPMI medium with 10% fetal bovine serum) containing 50 ng/ml LPS or vehicle was incubated for 24 h with cultured Kupffer cells (conditioned medium) or in empty culture plates (control medium). These media then were added immediately to liver endothelial cells, which had been allowed to equilibrate for 2–3 h after plating. After the liver endothelial cells were incubated for an additional 24 h, the media were removed and the level of irET-1 was determined by RIA. Incubation of the liver endothelial cells with medium conditioned by LPS-treated Kupffer cells resulted in a 60% increase in the level of irET-1 relative to cells treated with control media without LPS. Neither control media containing LPS nor conditioned media without LPS resulted in a statistically significant increase in the amount of irET-1 detected. Therefore, the capacity of LPS to increase ET-1 synthesis and release from liver endothelial cells likely occurs via a paracrine signaling mechanism involving increased cytokine synthesis by the Kupffer cells.

Effect of various agonists on ET-1 secretion. Because Kupffer cells stimulated with LPS have been shown to increase production of different mediators (38, 45), liver endothelial cells were treated with various agonists that are associated with endotoxin challenge of the liver. After incubation for a 24-h period, the culture media were collected to determine the amount of irET-1 released by the endothelial cells. Table 1 shows that 100 pM TGF-β resulted in greater than a 2.5-fold increase in irET-1, whereas the other agonists tested, TNF-α, platelet-activating factor (PAF), and LPS, failed to cause a statistically significant increase in the irET-1 secreted relative to controls. This lack of significant increase in irET-1 was observed at concentrations of agonist spanning several orders of magnitude and several different incubation intervals (data not shown).

Table 1: Effect of various agonists on ET-1 secretion.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>irET-1 Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>&gt;2.5-fold</td>
</tr>
<tr>
<td>TNF-α</td>
<td>&lt;2.5-fold</td>
</tr>
<tr>
<td>PAF</td>
<td>&lt;2.5-fold</td>
</tr>
<tr>
<td>LPS</td>
<td>&lt;2.5-fold</td>
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</tbody>
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This lack of significant increase in irET-1 was observed at concentrations of agonist spanning several orders of magnitude and several different incubation intervals (data not shown).
and total RNA was collected at the time of addition as well as 4, 6, 8, 12, and 18 h later. The level of ET-1 mRNA was assessed by Northern analysis with a cDNA probe for ET-1. Elevation of ET-1 mRNA was first noted at 10 pM TGF-β, and the increase appeared to be maximal at 100 pM (Fig. 6, A and B). TGF-β stimulated the secretion of irET-1 from liver endothelial cells in a concentration-dependent manner, which was maximal at 100 pM, 6.4 ± 1.1 pg/10⁶ cells (Fig. 7). A marked increase in the secretion of ET-1 occurred after 24 h in response to as little as 25 pM TGF-β (3.2 pg/10⁶ cells compared with 2.4 pg/10⁶ cells in the control). The maximal level of irET-1 was that measured at 24 h of exposure. However, even after 12 h of treatment with TGF-β a detectable difference in the amount of irET-1 released into the culture medium was apparent (data not shown). This lag in irET-1 synthesis after stimulation has been observed in porcine aortic endothelial cells (29). Taken together, the present results clearly demonstrate the ability of TGF-β to stimulate the production of ET-1 in liver endothelial cells and implicate a paracrine signaling mechanism operating between hepatic sinusoidal cells. Involved in this intercellular signaling mechanism, LPS-treated Kupffer cells release the cytokine TGF-β, which stimulates ET-1 synthesis in the endothelial cell.

TGF-β-neutralizing antibody inhibits ET-1 production by liver endothelial cells cocultured with LPS-stimulated Kupffer cells. Liver endothelial cells cocultured with LPS-treated Kupffer cells contained approximately twofold more ET-1 mRNA than cells cocultured with untreated Kupffer cells (Fig. 8, A and B). When a panspecific neutralizing antibody to TGF-β was included in the medium the increase in ET-1 mRNA was diminished, indicating that a substantial portion of the LPS-mediated Kupffer cell stimulation of ET-1 synthesis by liver endothelial cells was a consequence of TGF-β generation by the Kupffer cells. The amount of neutralizing antibody was in sufficient excess to have completely blocked any effect of the TGF-β1 detected in the Kupffer cell medium (2.7 ng/ml),

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>irET-1, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>0</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>100 pM</td>
<td>6.4 ± 1.1*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>PAF</td>
<td>500 ng/ml</td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>3.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>100 pM</td>
<td>5.1 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means ± SE from at least 3 separate experiments. Liver endothelial cells were stimulated with stated doses of transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), or platelet-activating factor (PAF) for 24 h. Culture media were collected and assayed for immunoreactive endothelin-1 (irET-1). *P < 0.0005 vs. controls at each time point.

**Table 1. Stimulation of ET-1 secretion by TGF-β and other mediators**
and a 10-fold increase in the amount of antibody used resulted in no further diminution in the level of ET-1 mRNA (data not shown). The effect of coculture on the release of irET-1 was not measured because media collected after only 6 h in culture would have accumulated insufficient irET-1 for differences in synthesis and release to be detected. Additionally, we anticipated that binding of released ET to Kupffer cells during the coculture interval would further decrease measurable irET-1.

**DISCUSSION**

In pathophysiological episodes such as sepsis, ischemia-reperfusion injury, renal failure, and congestive heart failure the plasma levels of ET-1 are elevated, reflecting presumptive increases in ET-1 synthesis (2, 10, 18, 32). It is generally accepted that ET itself is not stored within the cell and that increased levels of peptide secretion usually are accompanied by elevated levels of ET mRNA. However, it has been suggested recently (46) that low LPS concentrations might promote ET-1 release by a posttranscriptional mechanism located upstream of big ET-1, without altering the level of ET message. Therefore, in the present study we have measured both the release of irET-1 and the levels of mRNA for preproendothelin-1.

Production of ET or TGF-β in response to LPS. Tissue-specific differences in mRNA for ET-1 in rat models of sepsis have been noted, with heart and lung (24) and liver (10) exhibiting an elevation of ET-1 mRNA, whereas kidney or skeletal muscle shows little change (24). Endothelial cells from nonhepatic tissues show increased ET-1 production in direct response to LPS (17, 42). However, treatment of cultured liver endothelial cells with LPS did not significantly increase either irET-1 detected in the culture media or cellular ET-1 mRNA (data not shown). These observations led to the hypothesis that LPS action must involve an
intermediate agonist possibly acting through another cell type in the liver. Kupffer cells, the resident macrophage of the liver, are colocalized with endothelial cells within the sinusoid and are the primary hepatic site of LPS sequestration (55). Cultured rat Kupffer cells metabolically process LPS (12), and the ability of the Kupffer cell to respond to LPS has been well documented (21). Moreover, in the intact LPS-treated rat, Kupffer cells are thought to modulate both the functional and ultrastructural properties of sinusoidal endothelial cells (48).

The production and significance of TGF-β during long-term hepatic changes (e.g., fibrosis and regeneration) have been extensively investigated. However, there is a surprising paucity of data regarding the acute hepatic production of TGF-β in response to LPS. Cultured rat Kupffer cells are known to produce increased amounts of TGF-β in response to LPS or to bacterial cell wall preparations (31); in these experiments 3.5 × 10⁶ mixed sinusoidal cells were plated and the Kupffer cell population was selected by their rapid adherence to the culture surface [≈1.2 × 10⁶ Kupffer cells (54)]. Exposure to 100 ng/ml LPS for 24 h yielded 2.5 ng active TGF-β in the medium. Unstimulated cells secreted only 0.18 ng TGF-β, so the increase in this period was 1.9 ng active TGF-β/10⁶ cells. Our experiments used 4 × 10⁶ Kupffer cells stimulated with 10 ng/ml LPS for a shorter (6 h) stimulation period, and we obtained an increase of 1 ng/ml TGF-β (Fig. 3); this represents an increase of 0.25 ng TGF-β/10⁶ cells. Taken together, these data suggest that within 6 h of LPS exposure Kupffer cells secrete sufficient TGF-β to cause increased expression of preproendothelin-1 mRNA in liver endothelial cells (1 ng/ml TGF-β = 40 pM; see Fig. 6B) and that continuing secretion of TGF-β readily yields enough mediator (2.5 ng/ml TGF-β = 100 pM) to cause the observed increase in ET-1 production by liver endothelial cells (see Fig. 7). These effects are directly confirmed by our coculture data (Fig. 8).

**Hepatic production of TGF-β: isoforms and activation state.** In cells from normal rat liver, Kupffer cells express more TGF-β₁ mRNA than any other cell type (1, 8). Although signals for TGF-β₂ and TGF-β₃ are much weaker than for TGF-β₁, Kupffer cells also express more mRNA for TGF-β₂ than any other cell type (1, 8) and express as much TGF-β₃ mRNA as Ito cells (1). In the present study TGF-β₁ levels were elevated in Kupffer cells when exposed to the same concentration of LPS used by us to achieve maximal increases in the synthesis of the lipid mediator PAF.
Further neutralization studies are necessary, or TGF-\(\beta\) in response to elevated levels of TGF-\(\beta\) that Kupffer cells additionally synthesize and release.

The neutralizing antibody used in (59) and the expression of the inducible form of nitric oxide synthase (39). The neutralizing antibody used in the present coculture experiments does not distinguish between the different isoforms of TGF-\(\beta\); it is possible that Kupffer cells additionally synthesize and release elevated levels of TGF-\(\beta2\) or TGF-\(\beta3\) in response to LPS. Further neutralization studies are necessary, using antibodies specific for other isoforms of TGF-\(\beta\) and for other cytokine(s) (e.g., interleukins 1 and 6) known to be released by the Kupffer cell during endotoxic episodes (45).

Unstimulated Ito cells secrete TGF-\(\beta1\) (33), so it is possible that these cells may also contribute to the level of ET synthesis and release by liver endothelial cells. Two factors influencing such effects remain to be clarified. First, it is not clear how much LPS passes the Kupffer cells and sinusoidal endothelial cells to reach the Ito cells. Second, to our knowledge there is no report that LPS increases TGF-\(\beta\) secretion by Ito cells.

TGF-\(\beta\) and TNF-\(\alpha\) as secretagogues for ET. TGF-\(\beta\) was first reported to increase both the expression of mRNA for preproendothelin1 and the secretion of ET from, porcine aortic endothelial cells (29). Since then, exogenously added TGF-\(\beta\) has been confirmed as a secretagogue for ET in a number of different cell types, most recently in astrocytes (56) and human decidualized endometrial cells (28). Antibodies or antisense oligodeoxynucleotides against TGF-\(\beta\) can ablate the effects of endogenous TGF-\(\beta\), suggesting that autocrine production of the cytokine can also modulate ET production (49). Pharmacological studies in the intact rat are consistent with the concept that TGF-\(\beta1\) stimulates the synthesis and release of ET (16). The secretagogue control mechanism has yet to be fully characterized; in the MDCK cell line treatment with TGF-\(\beta\) increases the half-life of preproendothelin mRNA, suggesting that this is one mechanism for increased ET secretion (22). It has been suggested also that TGF-\(\beta\) may directly stimulate expression of the gene for ET-1 (27).

TNF-\(\alpha\) activates ET-1 synthesis in human amnion cells (3) and in bovine endothelial cells of aortic (34) and cerebral (11) origin. The lack of a significant increase in ET-1 synthesis with TNF-\(\alpha\) treatment again distinguishes the liver endothelial cell from endothelial cells derived from other tissues.

TGF-\(\beta\) effects on ET binding. The binding of ET to its receptors is rapid and essentially irreversible, which means that substantial downregulation of ET receptors occurs when peptide levels are elevated; this downregulation can be autologous (13). Although endotoxemia elevates systemic ET levels (see above), treatment of rats with LPS enhances rather than decreases the contractile responses of the hepatic portal vein to ET (43). This suggests that in the intact animal LPS itself or an evolved cytokine might directly upregulate ET receptors. Treatment with TGF-\(\beta\) has been reported both to increase binding of ET-1 in cultured rabbit costal chondrocytes (25) and to decrease the maximal binding for ET-1 binding (in A617 cells, a vascular smooth muscle-derived cell line (6)). We have detected no substantial change in the binding of ET-1 to liver endothelial cells as a result of TGF-\(\beta\) treatment (9).

Intercellular interactions involving TGF-\(\beta\) and ET. These interactions have been investigated in cardiovascular models. The ability of platelets to stimulate the production of ET-1 by vascular endothelial cells is thought to be mediated predominantly by platelet release of TGF-\(\beta\) (37). Moreover, TGF-\(\beta\) causes cardiac...
hypertrophy in vivo but not directly in cultured cardiomyocytes; in coculture TGF-β stimulates nonmyocyte cardiomyocytes to secrete ET (20), which is directly hypertrophic. To our knowledge, there is only one other report investigating comparable hepatic interactions. Rieder et al. (44) showed that ET production from guinea pig sinusoidal endothelial cells is elevated both by TGF-β and by conditioned medium from LPS-stimulated Kupffer cells (maximal increase ~50% in both cases). We have extended these findings by measuring ET gene expression in addition to ET production, by obtaining a greater (2-fold) increase in ET production using coculture experiments, and by using antibodies to confirm that TGF-β is a major effector produced by Kupffer cells.

The present studies suggest a model of paracrine signaling to describe the mechanism for the elevation of hepatic ET-1 levels after endotoxin exposure. LPS released into the portal circulation challenges the liver where it is bound and cleared by Kupffer cells, resulting in the production of several inflammatory mediators, including the cytokine TGF-β. Mediators released by Kupffer cells bind to receptors on liver endothelial cells and instigate an increase in the expression and release of ET-1. ET-1 synthesized in this fashion may exhibit actions in various cell types of the liver, causing contraction of the hepatic sinusoids, increased glycogenolysis in hepatocytes, and further synthesis of inflammatory mediators.

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