Thromboxane A₂ from Kupffer cells contributes to the hyperresponsiveness of hepatic portal circulation to endothelin-1 in endotoxemic rats

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isolated liver perfusion; furegrelate; SQ-29548

ONE OF THE PATHOLOGICAL CHANGES in the early stage of endotoxemia is hepatic portal circulatory dysfunction, which leads to a reduction in sinusoidal perfusion and may ultimately result in hepatic failure (18). Growing evidence has shown that endothelin-1 (ET-1), a potent vasoconstrictive mediator, plays an important role under these conditions (17, 21, 29). Exogenous ET-1 causes reductions in sinusoid diameter and sinusoidal flow as well as increases in total portal resistance in the normal rat liver (3). In LPS-induced endotoxemia, ET-1 has been reported to be elevated in the portal and the systemic circulations (20). Studies from our laboratory and others have also demonstrated an upregulation of mRNA levels of ET-1 in the rat liver tissue in endotoxia (17, 25). In addition, the study by Pannen et al. (22) showed that the contractile response of the portal circulation to ET-1 was significantly potentiated by LPS-induced acute endotoxemia in the isolated perfused liver model, suggesting that enhanced ET-1 response, which occurs at sinusoidal and presinusoidal levels, may also contribute to endotoxin-induced hepatic microcirculatory failure. Indeed, our recent studies (4) have provided evidence showing that this portal hyperresponsiveness to ET-1 was responsible for severely reduced sinusoidal blood flow as well as impaired oxygen delivery in LPS-pretreated liver in rats. However, the underlying mechanism of this hyperresponsiveness is not fully understood. In the present study, we hypothesized that ET-1 triggers release of other vasoconstrictive mediators such as thromboxane A₂ (TXA₂) in the endotoxemic liver, which mediate the hyperresponsiveness of the portal circulation to endothelins.

TXA₂ has been shown to be a potent vasoconstrictor in the portal circulation (8, 27). Various studies have reported the increased release of TXA₂ in the liver (14, 16, 19) and other tissues (7) in endotoxemia. Inhibition of TXA₂ synthase and TXA₂ receptors provided a protective effect on the LPS-primed liver, suggesting an important role of TXA₂ in the pathogenesis of liver injuries during endotoxemia (13, 14). Furthermore, previous studies have shown that Kupffer cells are the major sources of TXA₂ production in diseased livers (30), and cultured Kupffer cells isolated from the rat liver were able to release TXA₂ in response to LPS administration (5, 15, 28).

Therefore, the goals of the present study were 1) to investigate whether TXA₂ plays any role in mediating the hyperresponsiveness of the portal circulation to ET-1 in endotoxemia and 2) to determine whether Kupffer cells are the major source of TXA₂ production in the endotoxemic liver in response to ET-1.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 200–250 g were housed in a temperature-controlled animal facility with alternating 12:12-h light-dark cycles and were fed standard lab chow ad libitum with free access to water. All procedures were performed in accordance with the National Institutional Animal Care and Use Committee of the University of North Carolina at Charlotte.

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**Experimental groups.** *Escherichia coli* LPS (1 mg/kg body wt; Sigma, St. Louis, MO) was injected intraperitoneally in vivo 6 h before experiments. In control groups, the same amounts of saline were injected. Saline- and LPS-treated animals were then assigned to 1) nontreatment groups: control (n = 9) and LPS (n = 10); 2) furegrelate-pretreatment groups: saline + Fure (n = 5) and LPS + Fure (n = 9); 3) SQ-29548-pretreatment groups: saline + SQ-29548 (n = 6) and LPS + SQ-29548 (n = 5); and 4) GdCl3-pretreatment groups: saline + GdCl3 (n = 5) and LPS + GdCl3 (n = 5).

**Isolated liver perfusion.** Six hours after pretreatment with saline or LPS, rats were subjected to isolated liver perfusion. The liver was exposed through a side transverse incision, and the portal vein was isolated. After the portal vein was cannulated with a PE-240 catheter, the liver was perfused with Krebs-Henseleit bicarbonate buffer (in mM: 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 0.1 EDTA, and 2.5 CaCl2, pH 7.4, 37°C, saturated with 95% O2 and 5% CO2) for 10 min to wash out the blood. In furegrelate-pretreatment groups, the specific TXA2 synthase inhibitor furegrelate [5-(3-pyridinyl-methyl) benzofuran-carboxylic acid, 1 mg/100 g body wt; Cayman Chemical, Ann Arbor, MI] was injected intraperitoneally 14 h before the experiment, and a boosting dose (5 μM) was added in the perfusion buffer during perfusion to inhibit TXA2 production. In SQ-29548-pretreatment groups, SQ-29548 (5-hepentic acid, 7-[3-[2-[(phenylamino) carbonyl]hydroxymethyl]-7-oxabicyclo; Cayman Chemical) was added into the perfusion buffer (1 μM) 10 min before ET-1 infusion to block the TXA2 receptor activity. In GdCl3-pretreatment groups, 7.5 mg/kg body wt GdCl3 in 0.5 ml saline was injected through the penile vein 24 h before the perfusion to inhibit the function of Kupffer cells. The isolated liver perfusion was performed by using a constant flow rate (100 ml/min), as described previously (6) with minor modification. Briefly, warmed perfusate was pumped from a reservoir into an overflow chamber and was oxygenated via a silicone tubing oxygenator (95% O2-5% CO2). The temperature of perfusate was maintained at 36–37°C by warming the reservoir in a water bath. A pressure transducer was placed in line immediately before the portal inlet cannula to monitor portal pressure.

The liver was perfused for 10 min in a nonrecirculating fashion to wash out the blood and stabilize the pressure. Then the perfusion was repeated with Krebs-Henseleit buffer containing 0.5 mM EGTA at a flow rate of 18 ml/min for 6 min. Subsequently, the liver was digested with GBSS containing 0.025% type IV collagenase (Sigma) and 20 μg/ml DNase I (Sigma) at a flow rate of 18 ml/min for 6 min. In the digestion procedure, we omitted pronase because pronase has been reported to destroy the LPS receptor CD14 on Kupffer cells during cell isolations (12). After digestion and removal of the capsule, the liver was excised and cut into small pieces in the GBSS-containing collagenase. Cells were suspended in GBSS and were centrifuged at 50 g at 4°C for 3 min to remove the hepatocyte fraction, followed by centrifugation at 700 g for 7 min to separate the nonparenchymal cell fraction. Kupffer cells were separated from the nonparenchymal cell fraction via centrifugal elutriation using a JB6 rotor (Beckman Instruments, Fullerton, CA). Kupffer cells were eluted at a constant rotor speed of 2,500 rpm and flow rate of 45 ml/min. Kupffer cell viability as assessed by trypan blue exclusion was >95%.

Cells were plated in 24-well plastic culture dishes (Corning, Corning, NY) at a concentration of 1 × 106 cells/well and were cultured in 500 μl RPMI 1640 medium (Sigma) supplemented with 25 mM HEPES, 20% fetal bovine serum and antibiotics (0.05% gentamycin sulfate). Nonadherent cells were removed by changing culture medium 2 h after plating. The purity of Kupffer cells is >95% of adherent cells, as indicated by a latex bead phagocytosis test. Cells were cultured at 37°C in 5% CO2 for 18 h. Then culture medium was replaced with fresh medium containing either PBS or LPS (final concentration, 50 ng/ml). After 6 h of incubation, cell medium was collected and cells were carefully washed with PBS three times, then 500 μl PBS containing either vehicle (PBS) or ET-1 at a final concentration of 10 nM was added to each well. After 10 min of incubation at 37°C, the medium was sampled for TXB2 assay. In this experiment, cells were divided into four groups (n = 4 for each group). In the LPS/ET-1 group, the cells were treated with PBS for 6 h, followed by vehicle incubation for 10 min. In the LPS−/ET− group, the cells were treated with PBS for 6 h, followed by ET-1 incubation for 10 min. In the LPS+/ET− group, the cells were treated with LPS for 6 h, followed by vehicle incubation for 10 min. Finally, in the LPS+/ET+ group, the cells were treated with LPS for 6 h, followed by ET-1 incubation for 10 min.

**Enzyme immunoassay for TXB2.** Enzyme immunoassay kits (Assay Designs, Ann Arbor, MI) were used to determine the concentration of TXB2 (a stable metabolite of TXA2 as an indicator of TXA2 release) in the perfusate. The samples were diluted 1:2 with Krebs buffer so that the highest concentration of TXB2 in the perfusate fell in the linear range of the standard curve. The level of TXB2 was expressed as picograms per milliliter.

**Statistical analysis.** Statistical significance in each group was tested by using one-way ANOVA, with individual means detected by Student-Newman-Keuls test. When criteria for parametric testing were violated, the appropriate nonparametric test (Mann-Whitney U-test) was used. The portal pressures within each group and among the groups were analyzed by using two-way ANOVA. A P value <0.05 was considered significant. All results are presented as means ± SE.

**RESULTS**

**Portal hemodynamic change in response to ET-1 after 6 h of LPS pretreatment.** After 6 h of LPS, administration of ET-1 for 10 min induced a peak increase in portal pressure in both LPS-pretreated and saline-pretreated groups during the isolated liver perfusion (Fig. 1). However, the response to ET-1 was much more pronounced in livers from animals pretreated with LPS compared with livers from saline-pretreated animals (9.1 ± 0.3 mmHg for LPS vs. 7.6 ± 0.4 mmHg for control at 10 min; P < 0.05).

**TXA2 released into the perfusate in response to ET-1 following LPS pretreatment.** TXA2 release was estimated by measuring the levels of TXB2 in the perfusate. Before ET-1 infusion, TXB2 levels in the perfusate of the LPS group were slightly higher than in the control group, but the difference did not reach statistical significance (P > 0.05; Fig. 2). After 10 min of ET-1 infusion, a dramatic increase of perfusate TXB2 (>8-fold) was observed in the LPS group compared with the initial level at 0 min (868.5 ± 73.3 vs. 144.9 ± 41.1 pg/ml at 0 and 10 min, respectively; P < 0.001; Fig. 2), whereas no change in TXB2 in the control group was observed. 

**Effect of furegrelate on the responsiveness of the LPS-primed liver to ET-1.** Furegrelate, a specific inhibitor for TXA2 synthase, was applied to block the production of TXA2 in response to ET-1. Pretreatment of furegrelate significantly
attenuated the ET-1-induced increase in portal pressure in the LPS group (absolute increase at 10 min: 4.9 ± 0.4 mmHg for LPS alone vs. 3.6 ± 0.5 mmHg for LPS + Furegrelate; P < 0.05). The portal pressure response with furegrelate showed no significant difference from that of the normal control group (saline only). Clearly, the TXA2 inhibitor completely prevented the LPS-induced portal pressure hyperresponsiveness to ET-1 (Fig. 3A). In contrast, the portal pressure in the saline + Fureg group was not significantly different from that of the saline-only group, indicating that furegrelate did not affect the portal resistance in the normal control group in response to ET-1.

At the end of the 10-min infusion of ET-1, the markedly increased perfusate TXB2 in LPS-primed livers was completely abolished by the pretreatment of furegrelate (886.6 ± 73.4 pg/ml for LPS alone vs. 110.8 ± 0.8 pg/ml LPS + Furegrelate; P < 0.05). The perfusate TXB2 levels after ET-1 in LPS livers treated with furegrelate were not significantly different from those of the saline + Fureg group or the levels before ET-1 in either group (Fig. 3B). The perfusate TXB2 levels of the control group (saline only) didn’t show any significant change after the administration of furegrelate.

Effects of SQ-29548 on the responsiveness of the LPS-primed liver to ET-1. To confirm the specific action of TXA2 in mediating the enhanced portal response by ET-1 in LPS primed livers, SQ-29548, a selective TXA2 receptor antagonist, was employed. The baseline portal pressure of both saline- and LPS-pretreated livers was not significantly altered during the 10-min preperfusion with SQ-29548 (data not shown). After 10 min of ET-1 infusion, the increase in portal pressure did not change significantly in control compared with saline + SQ-29548. However, the enhanced increase of portal pressure in response to ET-1 in LPS-primed livers was completely abolished by the administration of SQ-29548 (absolute increase at 10 min: 4.9 ± 0.4 mmHg for LPS vs. 2.6 ± 0.6 mmHg for LPS + SQ-29548; P < 0.05; Fig. 4A). Surprisingly,
the measurement of perfusate TXB2 showed that SQ-29548 significantly inhibited the ET-1-induced TXB2 increase in LPS-primed livers (at 10 min: 886.6 ± 73.4 pg/ml for LPS alone vs. 135.2 ± 45.2 pg/ml for LPS + GdCl3; \( P < 0.05 \); Fig. 5B).

**LDH releases.** In response to ET-1, LDH levels in the perfusate were significantly elevated in LPS-primed livers compared with control livers (10 min: 192.0 ± 86.8 vs. 32.8 ± 4.0 U/l; \( P < 0.05 \)); the administration of furegrelate completely prevented the ET-1-induced elevation in LDH levels in LPS-primed livers. Inhibition of ET-1-induced LDH increase was also observed with SQ-29548 and GdCl3, respectively (Fig. 6).

**Release of TXA2 from isolated Kupffer cells in response to LPS and ET-1.** To verify whether LPS-primed Kupffer cells release TXA2 in response to ET-1, we performed an in vitro study using isolated Kupffer cells. When Kupffer cells were pretreated with PBS for 6 h, no significant increase of TXA2 release was observed in response to 10 min of ET-1 treatment compared with the baseline level. However, for the Kupffer cells primed with LPS, incubation with ET-1 for 10 min induced a significantly greater production of TXA2, as indi-
cated by higher TXB₂ levels in the media compared with that from Kupffer cells pretreated without LPS (LPS−/ET− and LPS−/ET+ groups) or with LPS but without ET-1 (LPS+/ET− group) (Fig. 7).

**DISCUSSION**

Hepatic microcirculatory dysfunction is a severe and early complication in endotoxemia (18). It has been suggested that ET-1 plays a significant role in mediating hepatic microcirculatory dysfunction in endotoxemia (17, 21, 29). The study by Pannen et al. (22) showed that portal venous contractile response to ET-1 was significantly enhanced in the rat liver following the pretreatment with endotoxin. The sinusoids constricted with a greater magnitude in response to ET-1, which contributed to a dramatic decrease in the sinusoidal flow in LPS-primed animals compared with control animals. Our recent studies have also demonstrated that the enhanced response of the portal vascular system to ET-1 results in functional impairment in oxygen delivery, which causes focal areas of hypoxia in the liver, leading to local mismatches between the oxygen delivery and metabolic demands (4). Although the deleterious effect induced by the enhanced response to ET-1 on the hepatic microcirculation during endotoxemia has been recognized, the mechanism underlining LPS-induced hyperresponsiveness to ET-1 is still not clear. In the present study, we hypothesized that ET-1 triggers releases of other vasoconstrictive mediators such as TXA₂ in the endotoxemic liver, which mediate the hyperresponsiveness of the portal circulation to endothelins.

To explore the mechanism of the hyperresponsiveness of the portal circulation to ET-1 in acute endotoxemia, we employed the isolated liver perfusion system. After 6 h of pretreatment with LPS, the liver showed a slightly higher baseline portal pressure and significantly enhanced portal pressure in response to the exogenous ET-1 infusion compared with the control liver. The results confirmed Pannen et al.’s observation (22). This enhanced portal pressure increase in response to ET-1 in the LPS-pretreated liver was accompanied by more severe liver injury, as indicated by higher levels of LDH in the perfusate at the end of 10 min of ET-1 infusion, suggesting the detrimental effect of this potentiated portal response to ET-1 on hepatocellular function.

Several possible mechanisms may be responsible for the hyperresponsiveness of the portal circulation to ET-1 as a result of LPS priming. For example, releases of other vasoconstrictors such as TXA₂ in response to ET-1 could explain the enhanced portal pressor response seen in the LPS liver. In the present study, we sought to determine the possible role of TXA₂ in this LPS-induced hyperresponsiveness. A number of studies (9–11) have suggested the important role of TXA₂ in the portal system. Our recent study (30) has also shown that hepatic release of TXA₂ contributes to the early development of portal hypertension in the bile duct-ligated (BDL) liver (30). Interestingly, in the present study the levels of perfusate TXB₂, a stable metabolite of TXA₂, were found dramatically increased after 10 min of ET-1 infusion in LPS-pretreated livers but not in control livers, indicating that the hyperresponse to ET-1 was associated with the TXA₂ release into the portal...
circulation. These observations suggested that TXA2 might play a role in the hyperresponsiveness of the portal circulation to ET-1.

TXA2, derived from arachidonic acid, is synthesized by a specific enzyme: TXA2 synthase. To determine the role of TXA2 in the hyperresponsiveness of portal circulation to ET-1 during endotoxemia, we used furegrelate, a potent selective TXA2 synthase inhibitor, to block the production of TXA2 in the liver. Our recent data showed that furegrelate effectively suppressed the TXA2-mediated increase in portal pressure in BDL-induced early portal hypertension (30). In the present study, administration of furegrelate significantly attenuated the portal pressure elevation in response to 10 min of ET-1 infusion to a level similar to control, i.e., furegrelate eliminated the potentiated portal pressure response to ET-1 in the LPS-pretreated livers. The attenuated portal pressure response was accompanied by a substantial decrease of TXB2 release. LDH release measured at the end of 10 min of ET-1 infusion was also significantly decreased in the furegrelate-pretreated liver. These observations indicated that increased release of TXA2 into the portal system may account for the enhanced ET-1-induced portal pressure increase as well as liver injury in LPS-primed livers.

TXA2 and other vasoactive prostaglandins share a common biosynthesis pathway. It is possible that the inhibition of TXA2 synthesis with a TXA2 synthase inhibitor may affect the production of other vasoactive prostaglandins, thus confounding the results of our furegrelate experiment. To confirm the role of TXA2, we specifically blocked TXA2 receptors by using the TXA2 receptor antagonist SQ-29548. As a result, the inhibition of the TXA2 receptor eliminated the hyperresponsiveness of portal pressure to ET-1 in the LPS-pretreated liver. Similarly, the diminished portal pressure response was accompanied by amelioration of hepatic injury, as indicated by reduced perfusate levels of LDH. Clearly, the data demonstrate that TXA2 released from the liver in response to ET-1 plays a role in mediating the hyperresponsiveness of portal pressure during acute endotoxemia.

It is, however, noteworthy that SQ-29548 not only suppressed the enhanced portal response to ET-1 in endotoxemic rat livers, it also significantly reduced perfusate TXB2 levels (Fig. 4B). SQ-29548 has been shown to block TXA2 receptor activation by competing the receptor binding with TXA2 (1). This was an unexpected finding. We initially suspected that the compound SQ-29548 in the perfusate samples might cross-react with the antibodies of the enzyme immunoassay kits, thus interfering with the measurement of TXB2. We performed additional experiments using SQ-29548 only in the samples and found no cross-relativities of the enzyme immunoassay kits with the compound. Therefore, we currently do not have an explanation of why SQ-29548 inhibited TXB2 levels in the perfusate other than to speculate that TXA2 might provide positive feedback to regulate its production. Clearly, whether our speculation is true or not requires further investigation.

The role of TXA2 in the potentiated portal system’s response to ET-1 during acute endotoxemia raises the question of the source of the increased release of TXA2. A number of studies have demonstrated that activated Kupffer cells were able to produce TXA2 in response to LPS stimulation in vitro (5, 15, 28). We recently have shown (30) that an increased amount of TXA2 is likely released from Kupffer cells in the early BDL-induced portal hypertension model. With Kupffer cell inhibitor GdCl3 in the present study, we were able to completely eliminate the increased production of TXA2 in the portal circulation, indicating that Kupffer cells are the major source of the TXA2 release. Indeed, our in vitro study provided direct evidence showing that Kupffer cells pretreated with LPS release a significantly elevated amount of TXA2 in response to ET-1 stimulation. Furthermore, inhibition of TXA2 production from Kupffer cells also abolished the enhanced portal response to ET-1 and decreased hepatocellular injury, as indicated by reduced perfusate levels of LDH, suggesting a pathogenic role of Kupffer cells in the ET-1-induced microcirculatory dysfunction during endotoxemia.

The precise mechanism of the significantly increased production of TXA2 by Kupffer cells during 10 min of ET-1 infusion is not clear. Although several studies have shown that LPS pretreatment itself may induce TXA2 synthesis (10, 14), and our data with Western blot analysis also revealed upregulations of PLA2 and COX-2 expression after 6 h of LPS (data not shown), the measurement of TXB2 levels in perfusate before ET-1 administration, which indicates the baseline release of TXA2 induced by LPS, was not significantly higher than in the control group, suggesting that at the early stage of LPS-induced endotoxemia, LPS may only “prime” the TXA2 synthesis pathway. The question is how the LPS-primed liver responds to ET-1 and rapidly increases the production of TXA2 within minutes. Apparently, activation of the key enzymes (such as PLA2, COX, or TXA2 synthase) by ET-1 likely precedes the rapid increased release of TXA2. However, the precise mechanism of ET-1-induced activation of the TXA2 synthesis pathway remains to be elucidated.

Nevertheless, our results demonstrate that release of TXA2 during acute endotoxemia likely plays an important role in the hyperresponsiveness of the portal circulation to ET-1. However, other mechanisms most likely also contribute to the LPS-induced hyperresponsiveness to ET-1. Recent work from our lab (2) has shown that although ETB receptors that can mediate dilation through activation of endothelial nitric oxide synthase (eNOS) in endothelial cells are upregulated, their coupling to eNOS activation is impaired. Moreover, inhibition of eNOS sensitizes the liver microcirculation to ET-1 and impairs oxygen delivery in a manner similar to that seen with LPS treatment (23). These results indicate that suppression of eNOS activation also contributes to the hypersensitivity to endothelin following LPS. Further studies will be needed to elucidate the interaction between increased thromboxane production and decreased nitric oxide release in the hypersensitivity to ET-1.

In summary, the present study demonstrates that the hyperresponse of portal pressure to ET-1 in acute endotoxemia is at least partly mediated by TXA2, which suggests an important role of thromboxane in the pathogenesis of portal circulatory dysfunction during acute endotoxemia. In addition, Kupffer cells are likely the major source of thromboxane production in LPS-induced endotoxemia.

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