Impact of endothelin-1 in endotoxin-induced pulmonary vascular reactions

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Impact of endothelin-1 in endotoxin-induced pulmonary vascular reactions

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Objectives: **Elevated endothelin-1 (ET-1) levels have been detected during sepsis. The aim of the study was to examine the role of thromboxane A_2 (TXA_2) and ET-1 in pulmonary vascular reactions after endotoxin (LPS) challenge.**

Design: **Prospective experimental study in rabbits.**

Setting: **Experimental laboratory in a university teaching hospital.**

Subjects: **Twenty-four adult rabbits of either sex.**

Interventions: **Experiments were performed on 30 isolated and ventilated rabbit lungs, which were perfused with a saline solution containing 10% autologous blood.**

Measurements and Main Results: **Pulmonary arterial pressure and lung weight gain were continuously registered. Perfusate samples were drawn intermittently to determine ET-1, TXA_2, and prostacyclin (PGI_2) concentrations. LPS isolated from *Escherichia coli* (0.5 mg/mL; n = 6) was added to the perfusate. A marked pulmonary arterial pressure increase followed by massive edema formation after 60 mins was observed after LPS injection. At the same time, elevated TXA_2 and PGI_2 levels in the perfusate were measured. ET-1 was detected 30 mins after LPS infusion (13.4 ± 2.6 fmol/L). Pretreatment with the ET_A receptor antagonist LU135252 (10^{-6} M; n = 6) almost completely suppressed the pressure reaction after endotoxin injection ($p < .01$ at 50 and 60 mins) and reduced edema formation ($p < .05$). The cyclooxygenase inhibitor diclofenac (10 μg/mL; n = 6) was as effective as LU135252 in preventing vascular reactions after LPS injection.**

Conclusions: **Pretreatment with the ET_A receptor antagonist LU135252 and the cyclooxygenase inhibitor diclofenac reduced pulmonary vascular reactions after LPS challenge. Based on the current data, we conclude that the pulmonary arterial pressure increase and edema formation after LPS injection are related to an ET-1- and TXA_2-dependent mechanism. (Crit Care Med 2000; 28: 2851–2857)**

Key Words: Edema; endothelin receptor antagonist; endotoxin; LU135252; thromboxane; sepsis; systemic inflammatory response syndrome; lung; pulmonary hypertension; LU135252; diclofenac

Septic shock and multiple organ failure continue to be major causes of death in critically ill patients (1). In cardiovascular reactions under shock conditions, depressed intestinal barrier function has been observed, caused by reduced perfusion of the gut (2, 3) followed by spreading of endotoxin and bacteria into the circulating blood, with translocation into other organs. Pulmonary complications, such as pneumonia, pulmonary hypertension, and the development of adult respiratory distress syndrome (4–6), are the main complications during shock conditions. This study focuses on whether endotoxin influences pulmonary vascular reactions in the isolated rabbit lung. To analyze potential mechanisms of action, involvement of the arachidonic acid metabolite thromboxane (TX) A_2 and endothelin (ET)-1 in pulmonary vascular reactions was investigated after endotoxin challenge. TXA_2 is known to be an important vasoconstrictor in the pulmonary circulation (7). ET-1, a vasoactive peptide produced by vascular endothelial cells (8), has been reported to mediate pulmonary vascular reactions during septic shock (9, 10).

The hypothesis of the study was that endotoxin (LPS) challenge leads to pulmonary hypertension via the release of ET-1. ET-1-induced release of arachidonic acid metabolites has been seen during inflammatory reactions after selective activation of granulocytes (11). Thus, the role of eicosanoids was studied by the use of the cyclooxygenase inhibitor diclofenac. ET_A receptor-related pulmonary vasoconstriction has been shown in previous experiments (11). Therefore, we examined the potential role of ET-1 in the endotoxin-induced pulmonary vascular reactions using the selective ET_A receptor antagonist LU135252 (12, 13).

MATERIALS AND METHODS

Lung Model. The techniques of preparing and perfusing isolated rabbit lungs have been previously described in detail (14, 15). Rabbits of either sex, weighing 2900 ± 185 g (mean ± SD), were anesthetized with pentobarbital sodium (60–80 mg/kg) and anticoagulated with heparin sodium (1000 IE/kg body weight). The isolated lungs, suspended from an electronic weight balance (Hottinger, Baldwin Meβtechnik Type U1, Darmstadt, Germany) in a temperature-controlled (37°C) and humidified chamber, were perfused with a saline solution enriched with 10% of autologous blood

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a at constant flow of 200 mL/min in a recirculating system (circulating volume, 200 mL). The perfusate was collected into a reservoir after lung passage and then reinfused. Ventilation was performed with 4% co_2 in air (frequency, 25/min; tidal volume, 30 mL; positive end-expiratory pressure, 0.5–1.0 cm H_2O). Pulmonary arterial pressure, airway pressure, and the weight of the isolated lung were recorded continuously by means of pressure and weight transducers. Because of the constant perfusion flow, alterations in perfusion pressure directly reflect alterations in pulmonary vascular resistance. Intermittently, samples of perfusate were taken for measurements of Po_2, Pco_2, oxygen saturation (ABL 330, Radiometer Copenhagen, Copenhagen, Denmark), and oncotic pressure (Onkometer BMT 921, Dr. Karl Thomae GmbH, Biberach, Germany). Initially, the lungs were perfused with a saline-bovine serum albumin solution, with low flow rates in the opened circulatory system. The perfusion fluid was then exchanged for fresh buffer via two separate perfusion circuits, 2 mins after the beginning of extracorporeal circulation and again after increasing the flow to 200 mL/min (30 mins). Within 10 mins, 20 mL of autologous blood was added to the perfusate. The addition of blood to the saline solution did not alter pulmonary arterial pressure or lung weight. The perfusion was able to maintain the integrity of the microcirculation for >5 hrs in our model. Homogeneous capillary organ perfusion and the absence of structural endothelial damage (e.g., vacuolization, mitochondrial disintegration, or hydropic swelling of endothelial cells) were verified by light and electron microscopy controls. No relevant alterations in vascular tone (<±2 mm Hg), permeability (weight increase, <1.5 g), or mediator release occurred during this observation period. Entry criteria for the present study consisted of a homogeneous appearance of the lungs, with no signs of hemostasis or edema formation (weight gain, 0 g/min), and no changes in vascular resistance (≤±1 mm Hg) during the 30-min equilibration period.

Experimental Protocol. A total of 24 lung preparations were randomly assigned to four groups containing six lungs each. Six lungs without intervention served as the sham group. After a 30-min equilibration period, endotoxin (100 mg; final concentration, 0.5 mg/mL) was injected into the pulmonary artery (control, n = 6). This dose was able to induce reproducible reactions in the pulmonary circulation, as assessed in pilot studies. In the other experimental groups, either LU135252 (10^{-6} M; n = 6) or diclofenac (10 μg/mL; n = 6) was added to the perfusate 10 mins before LPS application. Immediately before and at defined time points (10, 15, 30, and 60 mins) after endotoxin injection, samples were taken to determine TXA_2 and prostacyclin (PGI_2) concentrations.

Radioimmunoassay of TXB_2 and 6-Keto-Prostaglandin Factor$_{1\alpha}$. TXB_2 and 6-keto-prostaglandin (PG) factor $(F)_{1\alpha}$ were assayed serologically from 100 μL of recirculating Krebs-Henseleit-hydroxyethyl starch buffer solution as stable hydrolysis products of TXA_2 and prostacyclin by radioimmunoassay, according to a method described by Peskar et al (16). Radioactivity was quantified with a Philips PW 4700 liquid scintillation counter (Philips, Kassel, Germany). Results were obtained by standard constructed dose-response curves. The cross-reactivity of TXB_2-antiserum with prostaglandin D_2 was 2.7% and 0.05% with 6-keto $PGF_{1\alpha}$, PGE_2, PGE_1, $PGF_{1\alpha}$, 13,14-dihydro-15-keto PGE_2, and 13,14-dihydro-15-keto $PGE_{2\alpha}$, respectively. The cross-reaction of 6-keto-$PGF_{1\alpha}$ antiserum was 0.05% with TXB_2 and the aforementioned prostaglandins.

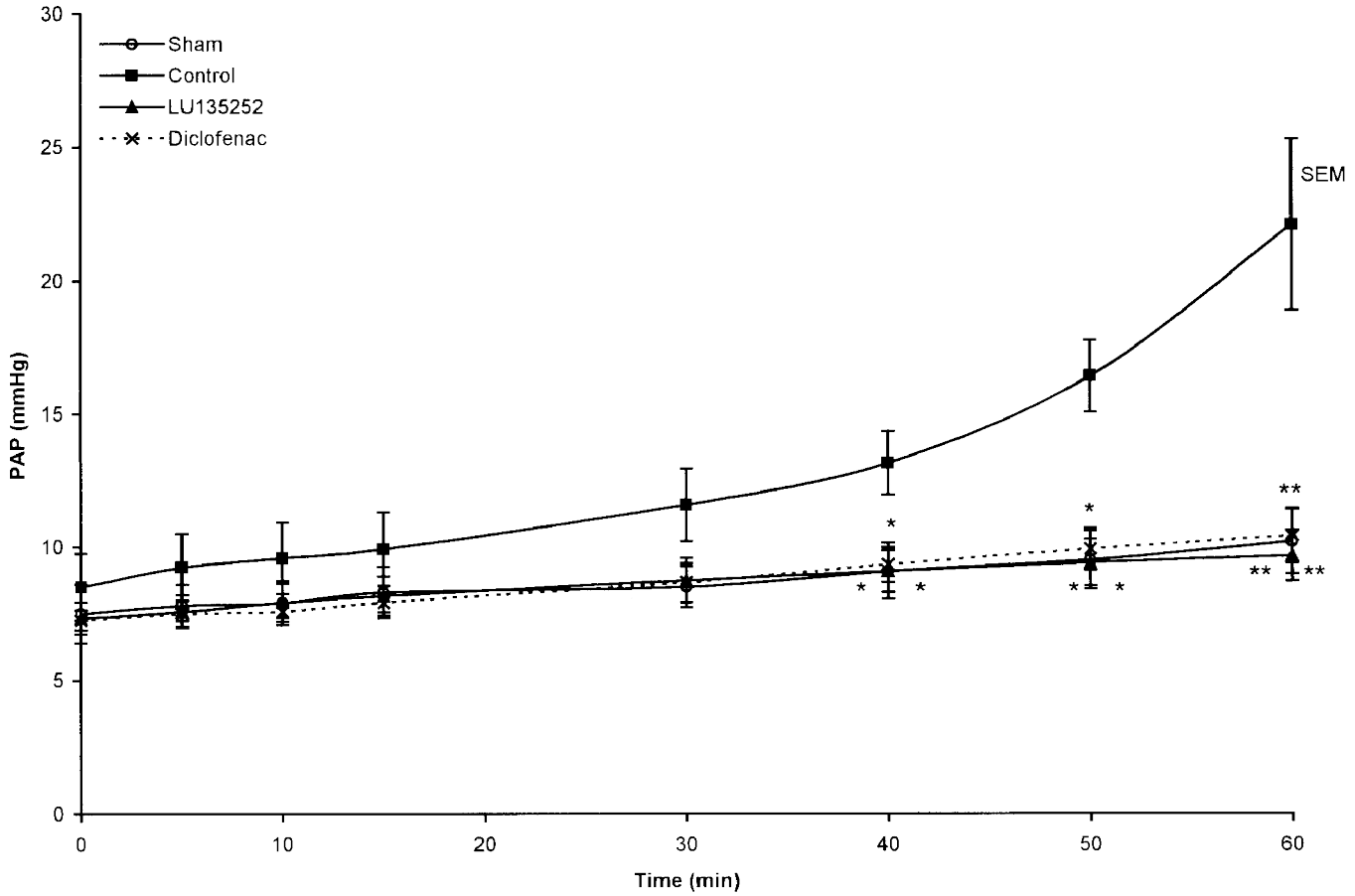

Figure 1. Changes in pulmonary arterial pressure (*PAP*) after endotoxin injection (0.5 mg/mL) in untreated controls (n = 6) and in groups pretreated with the ET_A receptor antagonist LU135252 (10^{-6} M; n = 6) or with the cyclooxygenase inhibitor diclofenac (10 μg/mL; n = 6). Sham operated lungs without any intervention are also presented (n = 6). The PAP increase during endotoxemia was significantly reduced by LU135252 and diclofenac. Data are presented as mean ± SEM. *$p < .05$; **$p < .01$ (analysis of variance).

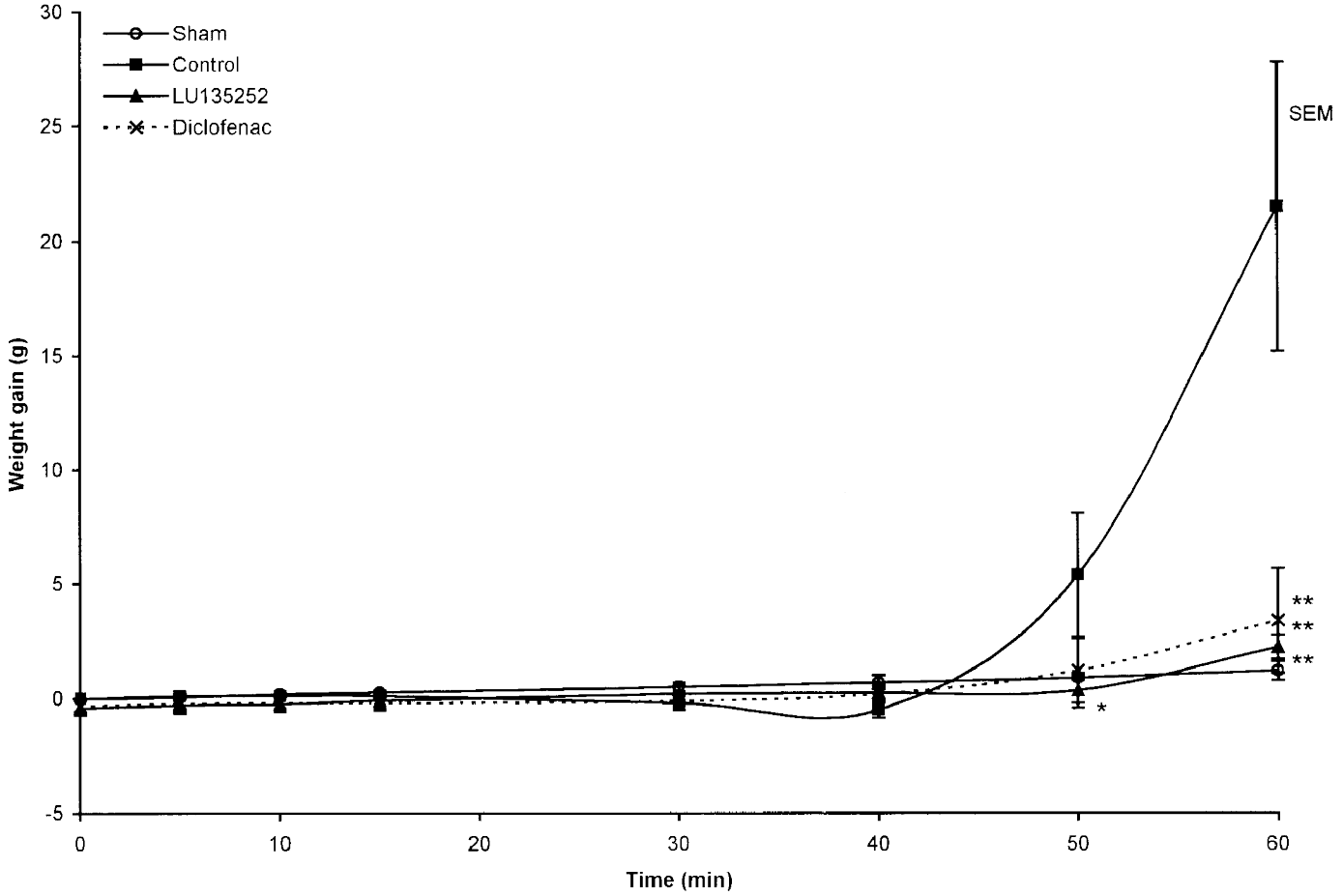

Figure 2. Lung weight gain reflecting edema formation after endotoxin injection (0.5 mg/mL) in controls (n = 6) and in experiments with the additional application of the ET_A receptor antagonist LU135252 (10^{-6} M; n = 6) and the cyclooxygenase inhibitor diclofenac (10 μg/mL; n = 6). Sham treated lungs without any intervention are also presented (n = 6). Significant suppression of edema formation was seen in the LU135252 group at 50 and 60 mins, whereas weight gain in the diclofenac group was significantly reduced only at 60 mins. Data are presented as mean ± SEM. *$p < .05$; **$p < .01$ (analysis of variance).

Enzyme-Linked Immunosorbent Assay of ET-1. ET-1 was assayed from 100 μL of perfusion fluid by enzyme immunoassay. The cross-reactivity of anti-ET-1 antibody was 100% with ET-1, >100% with ET-2, <.001% with ET-3, .07% with big ET-1, and <.0006% with Sarafotoxin 6b.

Materials. The saline solution contained 2.4 mmol/L calcium chloride dihydrate, 1.3 mmol/L calcium hydrogen phosphate, 4.3 mmol/L potassium chloride, 1.1 mmol/L potassium hydrogen phosphate, and 125 mmol/L sodium chloride, and 2.5 g/L glucose enriched with 1 mg/mL bovine serum albumin (Serva, Heidelberg, Germany). To adjust the pH to 7.4, $NaHco_3$ 8.4% was used. Twenty milliliters of autologous blood was acquired after ligation and cannulation of the vena cava inferior. Based on dose-response studies, diclofenac sodium (Voltaren, Ciba-Geigy, Wehr, Germany) was given in a concentration (10 μg/mL) shown to completely inhibit the generation of cyclooxygenase products. The cyclooxygenase pathway was already completely blocked when diclofenac was added to the perfusion fluid, 3 mins before direct stimulation of arachidonic acid metabolism by injection of arachidonic acid or calcium ionophore (unpublished data). The selective ET_A receptor antagonist LU135252 (Knoll AG, BASF Pharma, Ludwigshafen, Germany) is a substituted propionic acid derivative with ET_A receptor affinity in the low nmol/L range (12, 13). The dose of LU135252 (10^{-6} M) was based on dose-response studies (10^{-6}-10^{-9} M) that showed a complete inhibition of vascular reactions after injection of ET-1 in the concentration used in our model (unpublished data). Experiments with LU135252 and diclofenac in untreated lungs have been performed to exclude potential direct effects of these substances on pulmonary vascular tone, permeability, and mediator release (11). Furthermore, it has been demonstrated that LU135252 does not inhibit the cyclooxygenase pathway (11, 12). Additional experiments (n = 4) have been performed to exclude an effect of LU135252 on cyclooxygenase. Arachidonic acid (100 μM) was injected into the pulmonary circulation, followed by increased TXA_2 (342 ± 36 pg/mL) and PGI_2 (433 ± 52 pg/mL) levels 30 mins thereafter. This reaction was not significantly influenced by pretreatment with LU135252 (10^{-6} M; n = 4) 10 mins previously (TXA_2, 326 ± 46 pg/mL; PGI_2, 446 ± 32 pg/mL).

Endotoxin from *Escherichia coli* 0111 was donated by R. Urbaschek (Department of Immunology and Serology, Institute of Medical Microbiology, Faculty of Clinical Medicine Mannheim, University of Heidelberg). Rabbit anti-TXB_2 and rabbit anti-6-keto-$PGF_{1\alpha}$ were purchased from Paesel (Frankfurt, Germany); ^{3}H-labeled TXB_2 and ^{3}H-labeled-6-keto-$PGF_{1\alpha}$ were from New England Nuclear (Dreiech, Germany); and precipitating goat anti-rabbit antibodies were from Calbiochem-Behring (Frankfurt, Germany). An enzyme-immunoassay test kit from Amersham (Braunschweig, Germany) was used to measure ET-1 concentrations.

Statistical Analysis. Data are presented as mean ± SEM (SE). Differences between groups were tested by one-way analysis of variance followed by Scheffé's multiple range test (Statgraphics Plus for Windows; Manugistics, Rockville, MD). Statistical significance was considered as $p < .05$. Linear regression anal-

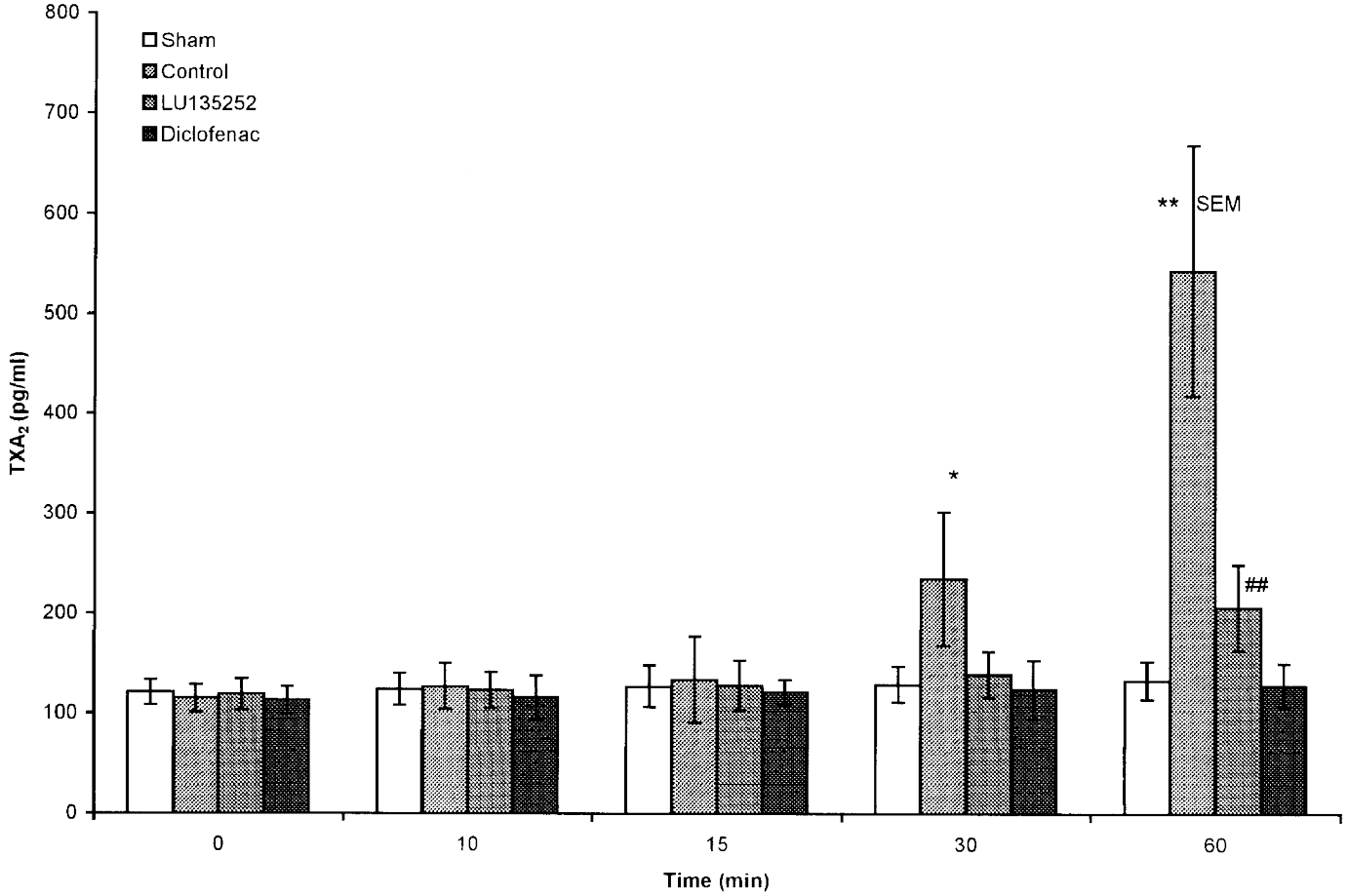

Figure 3. Thromboxane A_2 (TXA_2) concentrations in the perfusate in sham operated lungs (n = 6) and after endotoxin injection (0.5 mg/mL; control, n = 6). The generation of TXA_2 was significantly enhanced 60 mins after endotoxin application ($p < .01$ vs. time 0 and $p < .05$ vs. 30 mins). Pretreatment with the ET_A receptor antagonist LU135252 (10^{-6} M; n = 6) reduced the increase in TXA_2 concentrations ($p < .01$ at 60 mins vs. control). An increase in TXA_2 concentrations occurred in the LU135252 group, but statistical significance was not reached compared with baseline levels and TXA_2 concentrations at 60 mins ($p = .062$). Data are presented as mean ± SEM. *$p < .05$; **$p < .01$ vs. previous values in the control group; ##$p < .01$ between the control and the LU135252 groups (analysis of variance).

ysis was performed with Origin 5.0 software (Microcal, Northampton, NY).

This study was approved by the Animal Subject Protection Committee of the University of Giessen. The care and handling of animals conformed to the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society.

RESULTS

Pulmonary Vascular Reactions After Endotoxin Injection. In the sham group, pulmonary arterial pressure remained between 7.5 ± 1.1 mm Hg (baseline) and 10.2 ± 1.2 mm Hg (Fig. 1). In addition, lung weight was unaltered during the observation period (Δg = ± 1.2 g; Fig. 2). No increase in TXA_2 (121 ± 13 pg/mL at 0 mins; 133 ± 19 pg/mL at 60 mins) and PGI_2 (129 ± 16 pg/mL at 0 mins; 140 ± 23 pg/mL at 60 mins) levels was seen during the observation period (Figs. 3 and 4). The injection of endotoxin (0.5 mg/mL) resulted in an initial smooth increase in pulmonary arterial pressure within 30 mins, followed by an enhanced increase in pulmonary vascular pressure from 11.6 ± 1.4 mm Hg at 30 mins to 22.1 ± 3.2 mm Hg at 60 mins (Fig. 1). At the same time, massive edema formation was revealed by a lung weight increase of >20 g (Fig. 2). Initially, ET-1 was not detectable, but at 30 mins, very small amounts (13.4 ± 2.6 fmol/L) were noted. Furthermore, massive generation of TXA_2 (543 ± 125 pg/mL at 60 mins; $p < .01$ vs. 0 mins) (Fig. 3) and PGI_2 (623 ± 178 pg/mL at 60 mins; $p < .05$ vs. 0 mins) (Fig. 4) was measured at the end of the observation period. There was a direct correlation between pulmonary arterial pressure and TXA_2 ($r^2 = .988$) and PGI_2 ($r^2 = .982$) concentrations during the observation period (Fig. 5).

Effects of the ET_A Receptor Antagonist LU135252 on Pulmonary Arterial Pressure After Endotoxin Injection. Pretreatment with the ET_A receptor antagonist LU135252 (10^{-6} M) significantly reduced the pulmonary arterial pressure increase during endotoxemia. Significant differences were first observed after 40 mins (9.1 ± 0.8 mm Hg compared with 13.2 ± 1.2 mm Hg in the control group; $p < .05$), and the peak pressure reached only 9.7 ± 1.0 mm Hg at 60 mins (vs. 22.1 ± 3.2 mm Hg in the control group; $p < .01$) (Fig. 1). The generation of TXA_2 (206 ± 43 pg/mL; $p < .01$) (Fig. 3) and PGI_2 (275 ± 67 pg/mL; $p < .01$) (Fig. 4) was also reduced compared with the control group. Furthermore, edema formation was effectively suppressed (Fig. 2). A significant difference in the control group was reached from 30 mins ($p < .05$) to 60 mins (2.2 ± 0.5 g; $p < .01$).

Effect of the Cyclooxygenase Inhibitor Diclofenac. Similar to the ET_A receptor antagonist LU135252, the cyclooxygenase

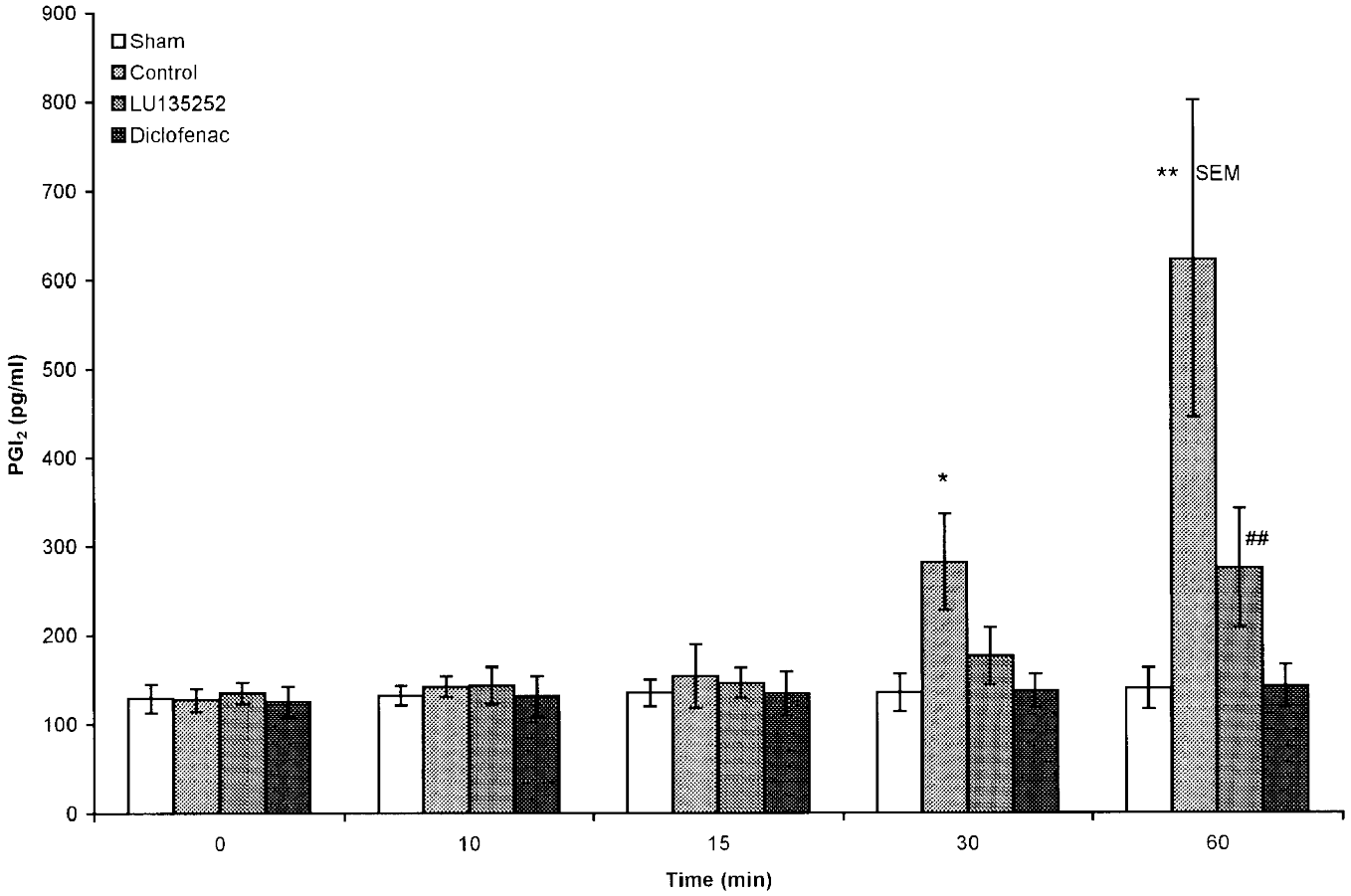

Figure 4. Prostacyclin (*PGI_2*) concentrations in the perfusate in sham treated lungs (n = 6) and after endotoxin injection (0.5 mg/mL; control, n = 6). The generation of PGI_2 was significantly enhanced 60 mins after endotoxin application ($p < .01$ vs. time 0 and $p < .05$ vs. 30 mins). Pretreatment with the ET_A receptor antagonist LU135252 (10^{-6} M; n = 6) reduced the increase in PGI_2 concentrations ($p < .01$ at 60 mins vs. control). An increase in PGI_2 concentrations occurred in the LU135252 group, but statistical significance was not reached compared with baseline levels and PGI_2 concentrations at 60 mins ($p = .057$). Data are presented as mean ± SEM. *$p < .05$; **$p < .01$ vs. previous values in the control group; ##$p < .01$ between the control and the LU135252 groups (analysis of variance).

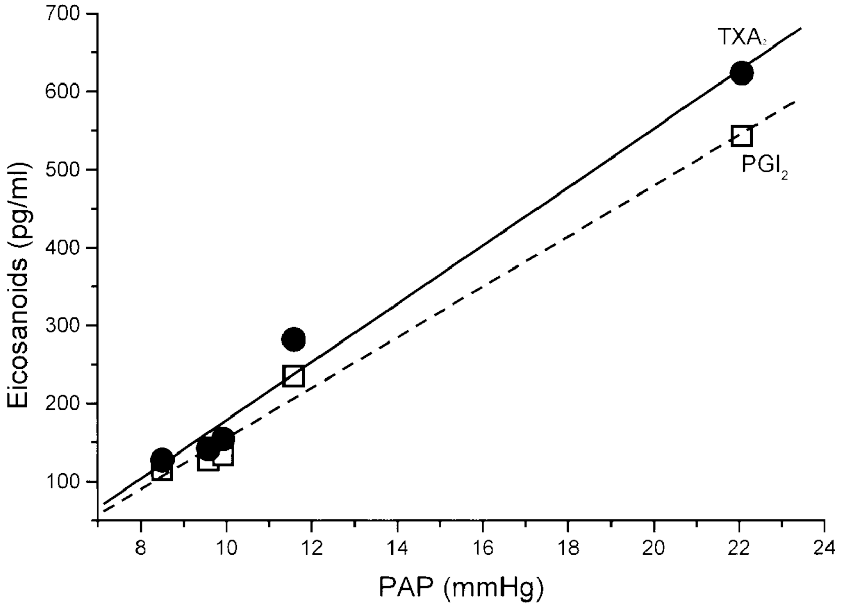

Figure 5. Correlation between pulmonary arterial pressure (*PAP*) and thromboxane A_2 (*TXA_2; circles*) and prostacyclin (*PGI_2; squares*) levels during the experimental period at 0, 10, 15, 30, and 60 mins. Data are presented as mean values and the linear correlation is given ($r^2 = .988$ between PAP and TXA_2 levels and $r^2 = .982$ between PAP and PGI_2 levels).

inhibitor diclofenac (10 μg/mL; n = 6) significantly reduced the LPS-induced increase in pulmonary perfusion pressure beginning at 40 mins (9.3 ± 0.7 vs. 13.2 ± 1.2 mm Hg in the control group; $p < .05$) (Fig. 1). At the end of the observation period, a >50% reduction in pulmonary arterial pressure (10.4 ± 1.0 mm Hg) was noted in this group compared with the control group (22.1 ± 3.2 mm Hg; $p < .01$). Edema formation was also reduced (3.4 ± 2.3 g; $p < .01$) (Fig. 2). Levels of TXA_2 (113 ± 14 pg/mL at 0 mins and 128 ± 22 pg/mL at 60 mins) and PGI_2 (127 ± 17 pg/mL at 0 mins and 142 ± 24 pg/mL at 60 mins) did not significantly differ from the beginning of the experiments until the end of the observation period (Figs. 3 and 4).

DISCUSSION

Severe injury predisposes the host to an increased susceptibility to infection,

Pulmonary vascular reactions during endotoxemia seem to be mediated via endothelin-1 and thromboxane A_2.

which often leads to adult respiratory distress syndrome and multiple organ failure (1). The hemodynamic changes in shock contribute to reduced intestinal barrier function, resulting in bacteremia and endotoxemia (17, 18), followed by the colonization of organs (19). The lung is often the primary target organ because of the large alveolar and capillary bed. Endotoxin is known to induce alterations in pulmonary vascular function, which may contribute to pulmonary damage followed by the development of adult respiratory distress syndrome. Therefore, we examined the effects of endotoxin on pulmonary vascular resistance and edema formation with respect to the potential involvement of ET-1 and TXA_2 as mediators of LPS-induced actions. Elevated circulating ET-1 levels in patients with severe sepsis have been found to be two- to sevenfold higher than those in healthy volunteers (20, 21). Serial measurements have indicated that ET-1 levels are elevated in the initial phase of sepsis (22). Thus, the potential involvement of ET-1 in the pressure reaction evoked by endotoxin was examined. ET-1 is well known to induce the elevation of pulmonary vascular resistance (23, 24) and edema formation (25, 26). The effects of ET-1 in the pulmonary circulation have been shown to be mainly mediated via ET_A receptors (11, 27, 28). In view of the therapeutic consequences, the selective ET_A receptor antagonist LU135252 (12, 13) was used to block putative ET-1 effects. Because the synthesis of TXA_2 has been reported to be induced by ET-1 (29), we examined TXA_2 effects using the cyclooxygenase inhibitor diclofenac.

The injection of endotoxin into the pulmonary artery induced an increase in pulmonary arterial pressure after 30 mins, which was followed by massive edema formation. The vascular reactions were paralleled by the detection of ET-1 in the perfusate and an increase in TXA_2 and PGI_2 concentrations. Pretreatment with the ET_A receptor antagonist LU135252 significantly reduced the pressure reaction and edema formation induced by endotoxin. Furthermore, the release of TXA_2 and PGI_2 into the perfusate was significantly reduced. The important role of ET-1 during LPS-induced pulmonary vascular reactions was evidenced by the detection of ET-1 after 30 mins. ET-1 was detected only in a very low concentration in the present setting. It is not surprising that detection of ET-1 was difficult, because it was released in small amounts as a paracrine substance diluted in 200 mL of perfusate. In addition, with its well-known clearing capacity, the pulmonary circulation is able to clear ~85% of ET-1 by first pass (30), especially in the presence of granulocytes, with their high potency to degrade ET-1 by proteases (31). The hypothesis of an ET-1-related mechanism of endotoxin action is supported by experimental results of elevated ET-1 levels in pulmonary lymph and lung tissue after endotoxin infusion (32, 33). These studies have been performed in intact animals, and so it could not be excluded in contrast to the present work that ET-1 has been produced in other tissues as the lung. A correlation between ET-1 levels and the increase in pulmonary arterial pressure during endotoxemia has also been reported (34). In contrast to this finding, pulmonary arterial rings showed a reduced sensibility to ET-1 after pretreatment with *Salmonella enteritidis* endotoxin (35), indicating that endotoxin may not only induce ET-1 release, but also influence ET receptor activity.

The relevance of elevated TXA_2 levels after endotoxin injection in the present study was evidenced by the significant reduction of pulmonary arterial pressure after pretreatment with the cyclooxygenase inhibitor diclofenac. The role of cyclooxygenase products as "second messengers" of the ET-1-induced actions has been previously reported by Del Basso and Argiolas (29). They inhibited the ET-1-induced vascular reactions by pretreatment with a cyclooxygenase inhibitor and a TX receptor antagonist. ET-1-induced release of TXA_2, and PGI_2 has also been postulated after lung embolism, which was investigated by our group (36). In contrast, other investigators have described a TXA_2-independent mechanism of ET-1 actions in the isolated lamb lung (37) using ET-1, a cyclooxygenase inhibitor, and a TX analog together. A marked increase in TXA_2 concentrations was observed in isolated rabbit lungs after ET-1 injection (24), indicating interaction between ET-1 and arachidonic acid metabolites. PGI_2 concentrations were increased in parallel to the TXA_2 levels. The release of the vasodilator PGI_2 may represent an internal feedback mechanism, which has been seen often in previous experiments (11, 38), but the effects of the vasoconstrictor TXA_2 were dominant on PGI_2 actions in the experiments (36). Based on the current results, the impact of PGI_2 release could not be determined. Detailed analyses with selective TX receptor antagonists will be performed in the future to investigate the function of PGI_2 in the regulation of vascular tone during inflammation.

From the current data, we conclude that pulmonary vascular reactions during endotoxemia seem to be mediated via ET-1 and TXA_2. Based on data from the literature and the present results, it can be postulated that LPS leads to the initial release of ET-1, which may induce the generation of cyclooxygenase products.

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