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TIME COURSE OF VARIOUS INFLAMMATORY MEDIATORS DURING RECURRENT ENDOTOXEMIA

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Abstract—The time course of thromboxane B_2 (TxB_2), 6-keto-$PGF_{1\alpha}$ (stable metabolite of prostacyclin), tumor necrosis factor-α (TNF_α), platelet activating factor (PAF), and interleukin-6 (IL-6) formation after three lipopolysaccharide (LPS) infusions was studied in pigs over an 18-hr, period. The *Escherichia coli* endotoxin W0111:B4 was injected i.v. into 10 of the test group pigs at a dose of 0.5 μg/kg over 30 min at 0, 5 and 10 hr of the experiment. Three pigs injected with physiological saline served as controls. At defined time points before and after each LPS administration venous blood was withdrawn (0, 15, 30, 45, 60, 120, 180 min) and plasma levels of TxB_2, 6-keto-$PGF_{1\alpha}$, PAF, TNF_α and IL-6 were determined. Pulmonary artery pressure (PAP) and cardiac output (CO) were measured every 15 min. TxB_2 and PAF peaked significantly between 30 and 45 min, TNF_α and 6-keto-$PGF_{1\alpha}$ between 30 and 60 min, and IL-6 between 120 and 180 min after each LPS injection. The mediators PAF, TNF_α and TxB_2 showed a decreasing three-peak profile whereas 6-keto-$PGF_{1\alpha}$ exhibited an increasing one. IL-6 plasma concentrations increased after each LPS injection. The peak after the third LPS administration, however, was surprisingly low compared to the previous two. The first LPS infusion in our test group led to a significant, sustained rise in mean PAP. After recurrent LPS injections the peak in PAP was not as marked as after the first infusion, indicating the development of a tolerance towards LPS. Initially, CO showed hypodynamic values, whereas the end stage of the experiment was characterized by hyperdynamic CO levels. In conclusion, we believe this porcine model of septic shock to be one of the first large animal models to describe in detail the time-course of various important inflammatory mediators.

The clinical syndrome of septic shock is characterized by various hemodynamic and biochemical features. The hemodynamic pattern during the course of septic shock consists of a hyperdynamic phase with low peripheral resistance and an increased cardiac output [1]. With the loss of volume the circulatory response becomes similar to hypovolemic shock. Cardiac output remains depressed because of decreased blood volume, as well as decreased venous return. Thus, the clinical syndrome of septic shock is divided into two phases, an early hyperdynamic and a late hypodynamic phase [2–4]. The underlying pathobiochemical alterations comprise a dramatic increase in eicosanoid production [5] and platelet activating factor (PAF‡) acether [6–10]; a release of cytokines, in particular, of interleukin (IL)-1, IL-6 tumour necrosis factor-α (TNF_α [11–14]) the formation of oxygen-centered free radicals from granulocytes and macrophages [15]; a release of leucocyte elastase [16] accompanied by oxidative inactivation of serum protease inhibitors; an activation of the plasmatic coagulation cascade, as well as fibrinolysis [17]; and complement activation [18].

In the past decade, several different animal models have been used to study the pathophysiological mechanisms behind the development of septic shock [19]. The pathophysiological aspects leading to the clinical picture of septic shock, especially the mode of action and kinetics of various mediators as well as their interactions, are not well understood at present and need to be investigated. Although in the past "septic shock" was induced by bolus injections of large amounts of bacteria or endotoxin, which led to marked and irreversible hemodynamic alterations and death of the animals, experimental designs nowadays should imitate a human septic shock-like state [20], using small amounts of lipopolysaccharide (LPS). One way of imitating human septic shock is the repeated injection of sub-lethal LPS doses.

The objective of this study was to extend our previous experiments [21], in which plasma levels of prostacyclin (PGI_2), thromboxane A_2 (TxA_2) and IL-6 were determined after two LPS infusions and an experimental duration of 48 hr. In the present paper we describe a modified model with an experimental duration of 18 hr and three sub-lethal LPS infusions. The time intervals between taking the blood samples have been reduced, in order to detect even smaller differences in mediator formation after a single LPS administration. In addition, we extended the number of measured inflammatory

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‡ Abbreviations: LPS, lipopolysaccharide; TxA_2/B_2, thromboxane A_2/B_2; TNF_α, tumor necrosis factor-α; PAF, platelet activating factor; PGI_2, prostacyclin; IL, interleukin; PAP, pulmonary artery pressure.

mediators. Besides PGI_2 and IL-6, plasma levels of PAF and TNF_α were determined. The rationale for our experiments is based on the consideration that the kinetics of the above mentioned eicosanoids, TNF_α, PAF, and IL-6, have been found to play an important role in recurrent endotoxemia [21–30]. The time-dependent kinetics of PAF in response to repeated endotoxin administration have, not to our knowledge, been reported.

MATERIALS AND METHODS

Animal model. Domestic pigs* (28–32kg) were quarantined for at least 1 week and treated with an antibiotic (100 mg doxycyclin/day; Vibravenös®) to prevent infections of the respiratory tract. After an overnight fast, the pigs were premedicated i.m. with 3 mg/kg azaperon (Strensnil®), followed by induction using 5 mg/kg etomidate (Hypnodil®), and intratracheal intubation. Anesthesia was maintained with a mixture of etomidate (Hypnodil®; 0.06 mg/kg/hr), pancuronium (Pancuronium organon®; 0.4 mg/kg/hr) and piritramide (Dipidolor®); 0.5 mg/kg/hr). To maintain an arterial PCO_2 of 35–40 mm Hg the pigs were ventilated with room air using a volume-cycled respirator (Dräger AV1) at a tidal volume of 10–15 mL/kg and a respiratory rate of between 15 and 20 L/min.

Maintenance fluid was administered using constant infusion of 3–6 mL/kg of Ringer's solution. Arterial and venous catheters were inserted for blood sampling, for infusion of all drugs and endotoxin, and for continuous monitoring of hemodynamic data. Pulmonary pressure and cardiac output were measured using a 5F pediatric Swan-Ganz catheter, advanced via the right jugular vein into an interlobular pulmonary artery. Cardiac output was measured in triplicate by a thermodilution method using a cardiac output computer and 5-mL injections of 0.9% ice-cold saline.

After surgical preparation, the animals were allowed to recover for 60 min to stabilize the hemodynamic parameters. To exclude an overlapping effect between single LPS administrations we selected time intervals such that the hemodynamic response and the mediator plasma levels returned to near control levels between administrations. The test group (N = 10) was given three LPS infusions (0.5 μg/kg over 30 min; approximate LD_{50} 1.0 μg/kg over 30 min) at t = 0 hr, 5 hr, and 10 hr; the duration of the experiments was 18 hr. The control group (N = 3) received same amounts of physiological saline solution. Baseline values were recorded immediately before i.v. infusion of endotoxin. After the start of the infusion in the test and control groups, venous blood plasma samples for TxB_2 (stable metabolite of TxA_2), 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2), TNF_α, PAF and IL-6 were taken at defined time points (0, 15, 30, 45, 60, 120, 180, 300, 315, 330, 345, 360, 420, 480, 600, 615, 630, 645, 660, 720, 780, 900, 1080 min).

Preparation of endotoxin. Escherichia coli endotoxin W0111:B4 (0.5 mg; Difco Laboratories, Detroit, MI, U.S.A.) was diluted in 500 mL 0.9% saline. An endotoxin dose of 0.5 μg/kg was administered over 30 min to the test group animals.

Radioimmunoassay. Plasma concentrations of TxB_2, 6-keto-$PGF_{1\alpha}$, TNF_α, and PAF were measured by radioimmunoassay [31–34]. Blood samples were collected in pyrogen-free polypropylene tubes containing EDTA and acetysalicylacid. Specimens were vortexed, centrifuged at 2500 *g* for 10 min at 4° and the supernatant was used for analysis. Radioimmunoassay was then performed according to the supplier's instructions (Amersham, Braunschweig, F.R.G.). TxB_2, 6-keto-$PGF_{1\alpha}$ and PAF were extracted from plasma and separated from other lipids prior to assay, whereas the TNF_α assay was performed on plasma directly (recovery rates: TxB_2, 92.3% ± 2.5; 6-keto-$PGF_{1\alpha}$, 94.4% ± 2.2; PAF, 90.2% ± 3.6).

IL-6 assay. The murine hybridoma cell line B9 was grown in RPMI 1640 or Dulbecco's modified Eagle's medium, supplemented with 2 mM glutamine, 60 μM 2-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% fetal calf serum (Gibco, Eggenstein, F.R.G.). The assay for IL-6 using the murine hybridoma cell line B9 was performed essentially as described by Aarden *et al.* [35]. In a total volume of 200 μL, 5000 cells/well (flat-bottom microtiter plates, 96 wells) were incubated at 37° for 72 hr in the presence of the platelet-poor plasma to be tested. During the last 4 hr the cells were labelled with 0.5 μCi of [^{3}H]-thymidine and the radioactivity incorporated into the nuclei was counted. One unit per millilitre is defined as the amount of IL-6 that caused a 50% decrease in maximal [^{3}H]thymidine incorporation. RhIL-6 (prepared from recombinant *E. coli;* sp. act. of approximately 3.8×10^8 U/mg in the B9-cell proliferation test; kindly provided by Drs T. Kishimoto and T. Hirano, Osaka, Japan) was used as an internal standard.

Statistical analysis. All biochemical and hemodynamic results were analysed for statistical significance using analysis of variance for repeated measures, followed by paired *t*-tests when significant differences ($P < 0.05$) were indicated. Control and test group values, as well as baseline values in each group and the estimated values during the experiment, were compared. Significance was set at a 0.05 probability level. All data are expressed as mean values ± SEM.

RESULTS

In the 18-hr experiments, kinetic features of various determined inflammatory mediators were as follows: the mediators which could be detected after the first LPS infusion in the plasma were TxB_2 (Fig. 1A), TNF_α (Fig. 1B), PAF (Fig. 1C) and 6-keto-$PGF_{1\alpha}$ (Fig. 2A). An increase in the plasma levels of these mediators was measured 15–30 min after the start of the LPS infusion. Plasma levels of TxB_2 and PAF peaked significantly after 45 min, and of TNF_α and 6-keto-$PGF_{1\alpha}$ after 60 min. IL-6 (Fig. 2B) plasma levels increased after 120 min and showed maximal values after 180 min.

§ This animal study was performed in adherence to the NIH guidelines for the use of experimental animals.

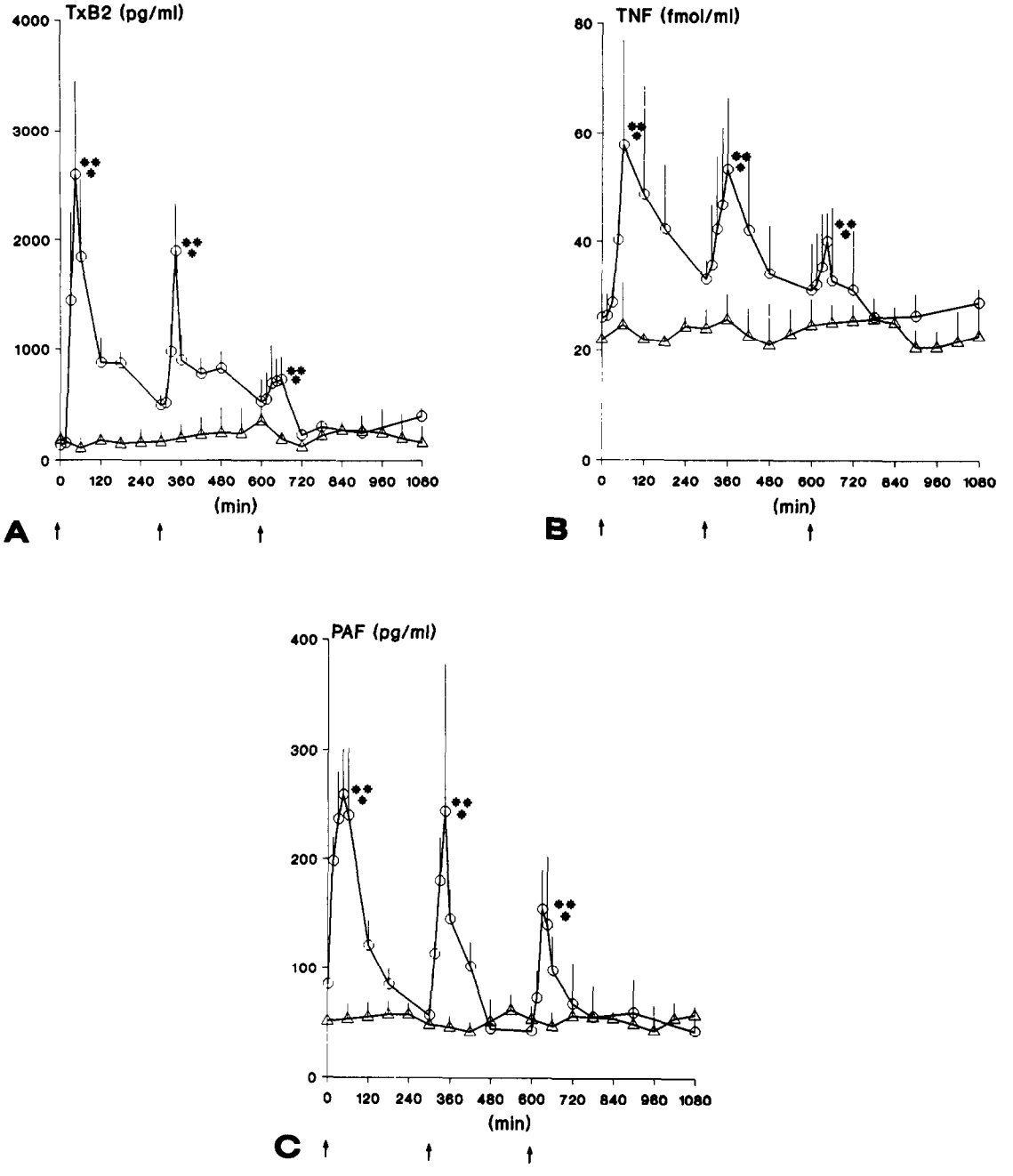

Fig. 1. Changes in mean plasma levels of TxB_2 (A), TNF_α (B), PAF (C). * $P < 0.05$ vs baseline value, ** $P < 0.05$ vs control group; arrowheads, time of LPS infusion; circles, test group; triangles, controls.

With repeated LPS injections plasma levels of all mediators with the exception of IL-6 increased within 15 min of the start of the infusion. TxB_2, PAF, TNF_α and 6-keto-$PGF_{1\alpha}$ now peaked significantly after 30–60 min. IL-6 peaked maximally after 120 min. Thus, the liberation of these inflammatory mediators was accelerated after the second and third LPS administrations.

The mediators TxB_2, PAF and TNF_α showed a decreasing three-peak profile after subsequent LPS administrations (Fig. 1A–C). In contrast, in the case of 6-keto-$PGF_{1\alpha}$ an increase in peak size could be detected after repeated LPS injections. IL-6 plasma levels increased after two subsequent LPS injections. The peak after the third LPS injection, however, was surprisingly modest in comparison to the first two (Fig. 2A and B). Biochemical parameters of the control group showed no significant alterations.

The changes in the hemodynamic parameters in the test group can be compared with a hyperdynamic septic shock in man, characterized by features such as systemic hypotension, hyperdynamic circulatory

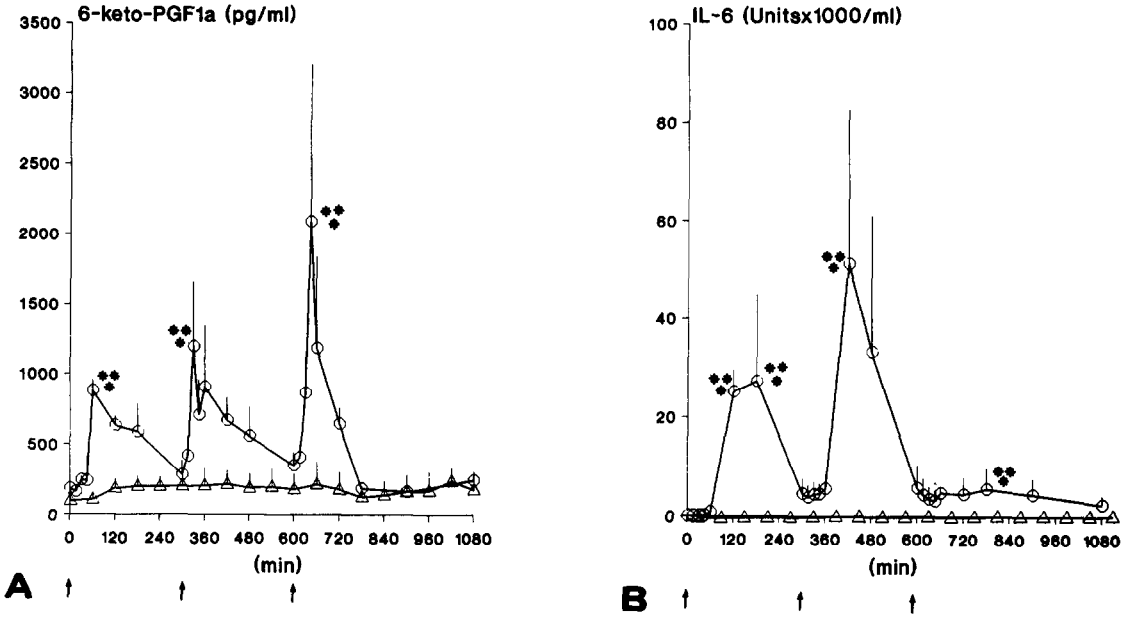

Fig. 2. Changes in mean plasma levels of 6-keto-$PGF_{1\alpha}$ (A) and IL-6 (B). * $P < 0.05$ vs baseline value, ** $P < 0.05$ vs control group; arrowheads, time of LPS infusion; circles, test group; triangles, controls.

state and low systemic vascular resistance. Endotoxin application at various times to our test group caused a significant, sustained rise in systolic, mean and diastolic pulmonary artery pressure (PAP). However, the peak in PAP after recurrent LPS administrations was not as marked as after the first one (Fig. 3A). Initially, cardiac output indicated hypodynamic values, whereas the end stages of the experiments were characterized by hyperdynamic cardiac output levels (Fig. 3B). Hemodynamic parameters of the control group showed no significant alterations.

DISCUSSION

Effects of recurrent endotoxemia have been reported for TxB_2, 6-keto-$PGF_{1\alpha}$, TNF_α and IL-6 [21–30], but not for PAF, which is known to show increased plasma levels after LPS bolus

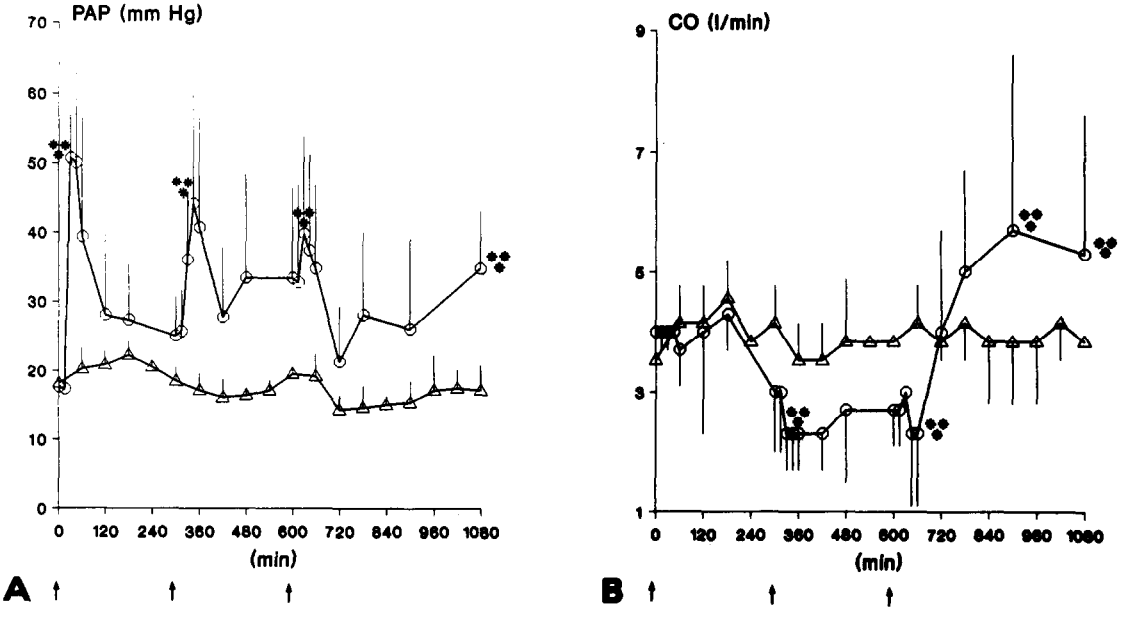

Fig. 3. Changes in PAP (A) and CO (B). * $P < 0.05$ vs baseline value, ** $P < 0.05$ vs control group; arrowheads, time of LPS infusion; circles, test group; triangles, controls.

administrations [6–10]. Most of these studies have been performed with animals (e.g. rats, mice, rabbits) under laboratory conditions. The study of shock-related phenomena with these experimental designs has often been criticized with regard to the atypically high LPS dosages tolerated by these species. Although, rats and mice respond qualitatively to LPS in a manner similar to that of sheep, pigs and primates, and also release identical lipid mediators and cytokines upon LPS injection, the choice of animal models for septic shock research should be made nowadays with regard to the clinical situation. The basis of such a model should be the use of animals with a similar pathophysiology to man, an experimental design identical to critical care unit conditions and the administration of LPS or bacteria in the same quantities as reported in septic patients [36–38]. Therefore, in this study and in a previous work [21] we simulated the situation of a septic patient in an intensive care unit by using pigs as an experimental animal model. In addition, the quantities of LPS used in the animal model approach those reported in septic patients.

Demling *et al.* [22–24] studied the effects of repeated LPS applications on systemic and pulmonary hemodynamic parameters and on TxB_2 and 6-keto-$PGF_{1\alpha}$ levels in plasma and lung lymph in the adult sheep. The main findings after the first two endotoxin administrations were a concomitant systemic hypodynamic state with an increased systemic vascular resistance index and an decrease in cardiac output. In parallel, a marked increase in both circulating and lung lymph TxB_2 and 6-keto-$PGF_{1\alpha}$ levels was detected after the first dose, and a smaller TxB_2 peak and essentially no significant release of 6-keto-$PGF_{1\alpha}$ after the subsequent eight LPS injections. During the whole experiment, the ratio of TxB_2 to 6-keto-$PGF_{1\alpha}$ remained increased. This finding is in contrast to our observation that TxB_2 responses decreased while those of 6-keto-$PGF_{1\alpha}$ increased.

TNF_α formation due to recurrent endotoxemia is characterized by decreasing plasma levels of this cytokine. Animals tolerant to LPS are completely unable to respond to LPS exposure with TNF_α release [11, 25]. This phenomenon is attributed to an impairment of macrophages in releasing TNF_α [28, 39, 40]. Furthermore, Waage [41] describes TNF_α release upon LPS administration recovering after 7 days which coincides with the disappearance of tolerance to LPS [29].

In our model the response to the first LPS injection can be subdivided into a rapid and marked liberation of TxA_2, PAF, TNF_α, and PGI_2 and a delayed and limited liberation of IL-6. The hemodynamic response is characterized by a markedly increased PAP and a hypodynamic circulatory state with the predominance of the vasoconstrictive mediator TxA_2.

Subsequent LPS infusions led to a more rapid liberation of all mediators with a predominance of PGI_2 and IL-6. Hemodynamic data for this stage show an increased PAP and a hyperdynamic circulatory state. The hemodynamic pattern after the second and third LPS applications in the end stage of the experiment cannot be explained by the action of the measured vasoactive mediators. Effects of trauma of surgery, anaesthesis and repeated bleeding, and the hemodynamic effects of other vasoactive mediators such as leukotrienes or bradykinin have to be considered, as well as injury to various organs during the course of recurrent LPS insults.

The marked elevation of PAP and the release of TxB_2, TNF_α and PAF after the first LPS infusion was reduced greatly after the second and third infusions. In 1988 Godsoe *et al.* [42] described the same phenomena for TxB_2 and the PAP. This response seemed to be dose related, since it was not seen with either continuous low-dose LPS infusions [43–47], which had no hemodynamic effects and only a mild effect on TxB_2 plasma levels, or after bolus injections of supra-lethal LPS doses, which led to an irreversible increase in the PAP and TxB_2 resulting in death. On the other hand, plasma levels of 6-keto-$PGF_{1\alpha}$ and IL-6 after the second (6-keto-$PGF_{1\alpha}$, IL-6) and third (6-keto-$PGF_{1\alpha}$) LPS injections increased. This difference in expression of the mediator responses after recurrent endotoxemia with a decrease in TNF_α, PAF and TxB_2 on the one hand and an increase in 6-keto-$PGF_{1\alpha}$ and IL-6 on the other hand allows a continuous evaluation of the pathophysiological role of the inflammatory mediators during endotoxemia.

PGI_2 and IL-6 are believed to exhibit beneficial effects and are of systemic importance during endotoxemia or sepsis [48, 49]. This hypothesis is confirmed by prostacyclin analogs, which prevented LPS/galactosamine-induced liver necrosis in mice [50] and increased the survival of LPS-treated rats [51]. Injected IL-6 induces the synthesis of acute phase proteins by the liver [52]. Many acute phase proteins are protease inhibitors [53] supposed to prevent tissue destruction by proteolytic enzymes such as leucocyte elastase [16], cathepsin G or collagenase. In addition, IL-6 has been demonstrated to induce a hyporesponsiveness to LPS in mice [54]. Furthermore, it inhibits the release of TNF_α in human U937 cells and monocytes *in vitro* and in mice *in vivo* [55]. In contrast, TxB_2, PAF and TNF_α are believed to be "aggressive" mediators of lethality in sepsis and septic shock. The efficacy of neutralizing antibodies against TNF_α [27], antagonists against PAF [6, 8, 10] and inhibitors of the cyclooxygenase pathway [56, 57] in the treatment of septic shock gives further support to this idea.

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