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Influence of Heat Shock Protein 70 and Metallothionein Induction by Zinc-bis-(DL-Hydrogenaspartate) on the Release of Inflammatory Mediators in a Porcine Model of Recurrent Endotoxemia

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ABSTRACT. The manipulation of stress gene expression by heavy metals provides protection against the lethal effects of endotoxemia in murine models of septic shock. Recent *in vitro* studies with alveolar macrophages or monocytes show that induction of the stress response in these cells is followed by a decreased liberation of major cytokines [tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1)] after endotoxin challenge. These findings suggest that the increased resistance to endotoxin *in vivo* after stress protein induction could be explained by an altered pattern of inflammatory mediator release. Therefore, we measured the time course of thromboxane-B2 (TxB2), 6-keto-PGF1α, platelet activating factor (PAF), TNFα, interleukin-1β (IL-1β), and interleukin-6 (IL-6) formation with and without induction of the stress response in an established porcine model of recurrent endotoxemia (Klosterhalfen *et al.*, *Biochem Pharmacol* **43:** 2103–2109, 1992). Induction of the stress response was done by a pretreatment with Zn^{2+} (25 mg/kg zinc-bis-(DL-hydrogenasparate = 5 mg/kg Zn^{2+}).

Pretreatment with Zn^{2+} prior to lipopolysaccharide (LPS) infusion induced an increased heat shock protein 70 and metallothionein expression in the lungs, liver, and kidneys and increased plasma levels of TNFα, IL-1β, IL-6, and TxB2 as opposed to untreated controls. After LPS infusion, however, pretreated animals showed significantly decreased peak plasma levels of all mediators as opposed to the untreated group. The time course of mediator release was identical with the decreasing and increasing three peak profiles described previously. Hemodynamic data presented significantly decreased peak pulmonary artery pressures and significantly altered hypodynamic/hyperdynamic cardiac output levels in the pretreated group.

In conclusion, the data show that the induction of stress proteins by Zn^{2+} could be a practicable strategy to prevent sepsis. BIOCHEM PHARMACOL 52;8:1201–1210, 1996.

KEY WORDS. zinc; heat shock protein 70; metallothionein; lipopolysaccharide; cytokines; sepsis prevention

In spite of the enormous progress in modern intensive care medicine, septic shock is still a feared complication after major surgery and in multitraumatized patients, with mortality rates remaining high. In recent decades, numerous animal models of human septic shock were designed to study the underlying pathophysiological aspects of the disease. Research was focused on hemodynamic alterations due to septic shocklike states [1–4] and on particular biochemical alterations. The underlying pathobiochemical alterations constitute a dramatic increase in eicosanoid production [5] and PAF# acether [6–10], a release of cytokines (in particular, IL-1, IL-6, and TNFα) [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, and fibrinolysis [17], and complement activation [18].

What has become obvious in the years since the introduction of new immunotherapies, including the use of IL-1

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Abbreviations: LPS, lipopolysaccharide; TxA2/B2, thromboxane A2/B2; PGI2, prostacyclin; TNFα, tumor necrosis factor-α; PAF, platelet activating factor; IL-1β, interleukin-1β; IL-6, interleukin-6; PAP, pulmonary artery pressure; CO, cardiac output; HSP70, heat shock protein 70; MT, metallothionein.

receptor antagonist, mono- and polyclonal antibodies to TNF, TNF receptors linked to IgG, and mono- and polyclonal antibodies to LPS [19–26], is that the sepsis biochemical cascade is much more complex than initially envisaged. Single antibody therapies targeting unique pathways, therefore, may easily fail. Furthermore, the cytokines and other mediators released in sepsis are rapidly deployed and hit their cellular targets quickly. Thus, effective immunotherapies will have to be given early or administered prophylactically to patients identified to be at high risk of developing septic shock.

A new aspect in prevention of septic shock is the stress gene manipulation to protect animals against the lethal effects of LPS. At the level of a single cell, nature has evolved a system that allows the cell and virtually all organ systems, from bacteria to mammals, to tolerate stresses that might otherwise be lethal. This defense mechanism is commonly referred to as the stress response and can be initiated by a wide variety of different agents including ischemia, several types of metabolic stress, and hyperthermia [27–30]. The general theme of the stress response in all organisms is the rapid and almost exclusive synthesis of a small number of intracellular proteins, the so-called stress proteins, including HSPs and MTs.

Recent studies have shown that induction of HSP70 by sodium arsenite and heat [31, 32] reduced the mortality rate and organ damage in a sepsis-induced lung injury rat model. Ryan *et al.* [33] reported that acute heat stress can protect rats against the lethal effects of LPS. Singh *et al.* [34] investigated the effect of zinc on immune functions, host resistance against infection, and tumor challenge in Swiss albino mice. In addition, this group reported an increased resistance against endotoxin after zinc acetate application. Recent *in vitro* studies have demonstrated a close relationship between cytokine production and stress protein expression [35, 36] on the one hand and cytokine liberation, LPS, and zinc [37, 38] on the other.

Until now, the influence of stress protein induction on cytokine release and the endotoxin-dependent profile of mediator liberation after stress protein expression *in vivo* have yet to be elucidated. In addition, hemodynamic data to our knowledge have not been reported before in the literature.

Accordingly, we have modified an established porcine model of human septic shock [39] to investigate the effect of pretreatment with Zn^{2+} on the induction of HSP70 and MT and on the plasma levels of the arachidonic acid products TxB2, 6-keto-PGF1α, and PAF and the cytokines TNFα, IL-1β, and IL-6. The modification of hemodynamics and liberation of the aforementioned mediators after LPS infusion were also examined.

MATERIALS AND METHODS

Animal Model

Minimal disease domestic pigs (28–32 kg) were kept in quarantine for at least 1 week and treated with an antibiotic (100 mg doxycyclin/day; Vibravenös®, Pfizer, Germany) to prevent infections of the respiratory tract. Experimentation was performed in a sterile surgical room at 22°C. All manipulations and surgical procedures were done under sterile conditions. Animals were divided into five groups: (I) saline pretreatment only for 24 hr (n = 3), (II) Zn^{2+} pretreatment only for 24 hr (n = 3), (III) saline pretreatment and saline treatment (n = 5), (IV) saline pretreatment and LPS treatment (n = 5), and (V) Zn^{2+} pretreatment and LPS treatment (n = 5). Pretreatment with Zn^{2+} was done 24 hr before the first LPS infusion with 25 mg/kg zinc-bis-(DL-hydrogenasparate) ≈5 mg/kg Zn^{2+} (Unizink®, Köhler Pharma, Germany), and LPS treatment was performed by intravenous injection of the *Escherichia coli* serotype W0111:B4 (0.5 μg/kg over 30 min) in three 5-hr intervals.

Zn^{2+} infusion and saline solution, respectively, were administered in all groups after intramuscular premedication of 3 mg/kg azaperon (Stresnil®). Blood samples in groups I and II (saline or Zn^{2+} pretreatment only) were taken 12 hr and 24 hr after Zn^{2+} administration, respectively. After 24 hr, groups I and II (saline or Zn^{2+} pretreatment only) were killed by KCl i.v. to determine the induction of HSP70 and MT by saline or Zn^{2+}, respectively.

After pretreatment with Zn^{2+} or saline solution and an overnight fast, the pigs in groups III–V were premedicated with 3 mg/kg azaperon intramuscularly (Stresnil®, Janssen, Germany), followed by induction with 5 mg/kg etomidate (Hypnodil®, Janssen, Germany) 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®, Janssen, Germany; 0.5 mg/kg/hr). To maintain an arterial P_{CO_2} of 35–40 mmHg, the pigs were ventilated with room air with a volume-cycled respirator (Dräger AV1) at a tidal volume of 10–15 mL/kg and a respiratory rate of 15–20 min^{-1}.

Maintenance fluid was administered with constant infusion of 3–6 mL/kg/hr of Ringer's solution. Arterial and venous catheters were inserted for blood sampling, for infusion of all drugs and endotoxin, systemic arterial pressure (SAP), central venous pressure (CVP), and heart rate (HR). PAP, pulmonary arterial wedge pressure (PCWP), and CO were measured with a 5F pediatric Swan-Ganz catheter advanced through the right jugular vein into an interlobular pulmonary artery. CO was measured in triplicate by a thermodilution method by using a cardiac output computer and 5-mL injections of 0.9% ice-cold saline. Strain gauges were zeroed to the level of the midaxilla. Body temperature was recorded from the thermister in the pulmonary artery.

After surgical preparation, the animals were allowed to recover for 60 min to stabilize. Domestic pigs are hemodynamically stable for several days on the anesthesia and fluid regimen used in this animal model. Baseline values were recorded immediately before i.v. infusion of endotoxin. Before and after the start of the infusion, blood samples for TxB_2, 6-keto-$PGF_{1\alpha}$, PAF, TNF_{α}, IL1β, and IL-6 were

taken at 0, 15, 30, 45, 60, 120, 180, 300, 420, 600, 720, 780, 900, and 1080 min. The overall duration of the LPS experiments was 18 hr. At the end, the animals were killed by i.v. KCl application. The organs were immediately removed and processed for further investigation as described below.

Preparation of Endotoxin

Half a milligram of the *E. coli* endotoxin W0111:B4 (Sigma Chemicals, Germany) was diluted in 500 mL 0.9% saline. The endotoxin dose administered was 0.5 μg/kg (=1.5 mL) over 30 min in three 5-hr intervals.

Antibodies

Antibodies included monoclonal mouse anti-MT E9, M639, 1:200 (Dako, Hamburg, Germany); polyclonal rabbit anti-HSP 70 A500, 1:200 (Dako); and monoclonal anti-HSP70/HSC70 SPA-820, 1:200 (Biomol, Hamburg, FRG). Anti-HSP70 reacts strongly with the two major HSP70s (HSP72 and HSP73). Anti-MT is directed against the products of two separate groups of genes in the human genome, MT-1 and MT-2. The products are a group of molecular weight proteins (~6 kD) containing a single chain of 61 amino acids, which is folded doubly within two domains, A and B.

Light Microscopy and Immunohistochemistry

Tissue samples were fixed in 10% formalin, embedded in paraffin, and sections were stained with hematoxylin and eosin (H&E) and periodic-acid Schiff (PAS) plus diastase and Alcian blue for mucin. Immunohistochemistry was performed on the paraffin-embedded material by using the avidin-biotin complex method, with diaminobenzidine as chromogen. The same staining methodology was used in all test and control animals, and each strain was performed twice on separate days.

Protein Detection

HSP70 and MT were detected by Western immunoblotting. Organs were removed and immediately frozen in liquid nitrogen. Tissues were subsequently thawed, homogenized in cold phosphate buffered saline, and centrifuged at 10,000*g* for 15 min. The supernatants were collected and protein concentration measured with a colorimetric reaction with bicinchoninic acid protein assay reagent (Pierce Chemical, Rockford, IL, USA). The samples were then suspended in sodium dodecyl sulfate–glycerol sample buffer. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, with 50 μg total protein loaded per lane. After gel electrophoresis, the proteins were transferred to nitrocellulose membrane and labeled with the primary antibodies HSP70 and MT at 1:200 dilution. After secondary labeling with goat, antimouse, or antirabbit IgG, respectively, conjugated with alkaline phosphatase at 1:2000 dilution, the protein was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium.

Preparation of Blood Samples

Blood samples were taken with polypropylene syringes (Monovetten®, Saarstedt-Nümbrecht, Germany) containing EDTA (2 mg/mL blood) and acetylsalicylic acid (Aspisol®, Bayer, Leverkusen, Germany; 0.5 mg/mL blood) dissolved in 0.6 mL of 0.9% saline. The samples were carefully tilted twice and immediately centrifuged at 2500*g* for 10 min at 4°C. The platelet-poor plasma (PPP) was divided into 1.0-mL portions in polypropylene reaction tubes (Brand, Wertheim, Germany; model 780.500) and stored at −70°C.

Radioimmunoassay

Plasma concentrations of thromboxane B2 (stable metabolite of thromboxane A2), 6-keto-PGF1α (stable metabolite of PGI2), TNFα, IL-1β, and PAF were measured by radioimmunoassay. The radioimmunoassay was performed according to the supplier's instructions (Amersham, Braunschweig, Germany). TxB2, 6-keto-PGF1α, and PAF were extracted from plasma and separated from other lipids prior to the assay, whereas the TNFα assay and IL-1β assay were directly performed on plasma (recovery rates: TxB_2, 92.3 ± 2.5%; 6-keto-$PGF_{1\alpha}$, 94.4 ± 2.2%; IL-1β, 89.2 ± 4.1%; TNFα, 88.3 ± 3.9%; PAF, 90.2 ± 3.6%).

IL-6 Assay

The murine hybridoma cell line B9 was grown in RPMI 1640 or DMEM, supplemented with 2 mM glutamine, 60 μM 2-mercaptoethanol, penicillin (100 units/mL), streptomycin (100 μg/mL), and 10% fetal calf serum (Gibco, Eggenstein, Germany). The assay for IL-6 with the murine hybridoma cell line B9 was performed essentially as described by Ribeiro *et al.* [35]. In a total volume of 200 μL, 5000 cells per well (flat-bottom microtiter plates, 96 wells) were incubated at 37°C for 72 hr in the presence of the PPP to be tested. During the last 4 hr, the cells were labeled with

TABLE 1. Influence of Zn^{2+} on mediator release *in vivo*

	GI (12 hr)	GI (24 hr)	GII (12 hr)	GII (24 hr)
TNFα	20 ± 12	22 ± 10	56 ± 22*	42 ± 14*
IL-1β	23 ± 12	33 ± 11	51 ± 19*	65 ± 21*
IL-6	21 ± 11	0 ± 0	33 ± 18	153 ± 57*
TXB2	115 ± 45	98 ± 39	355 ± 82*	468 ± 154*
6-k-PGF1α	67 ± 36	100 ± 47	82 ± 40	121 ± 42
PAF	21 ± 12	29 ± 21	33 ± 15	38 ± 26

Changes in mean plasma levels of TNFα (fmol/mL), IL-1β (pg/mL), IL-6 (units/mL), TxB2 (pg/mL), 6-keto-PGF1α (pg/mL), and PAF (pg/mL) after saline or Zn^{2+} pretreatment for 12 or 24 hr, respectively; group I (GI) = saline pretreatment only; group II (GII) = Zn^{2+} pretreatment only; *$P < 0.05$ group I vs. group II.

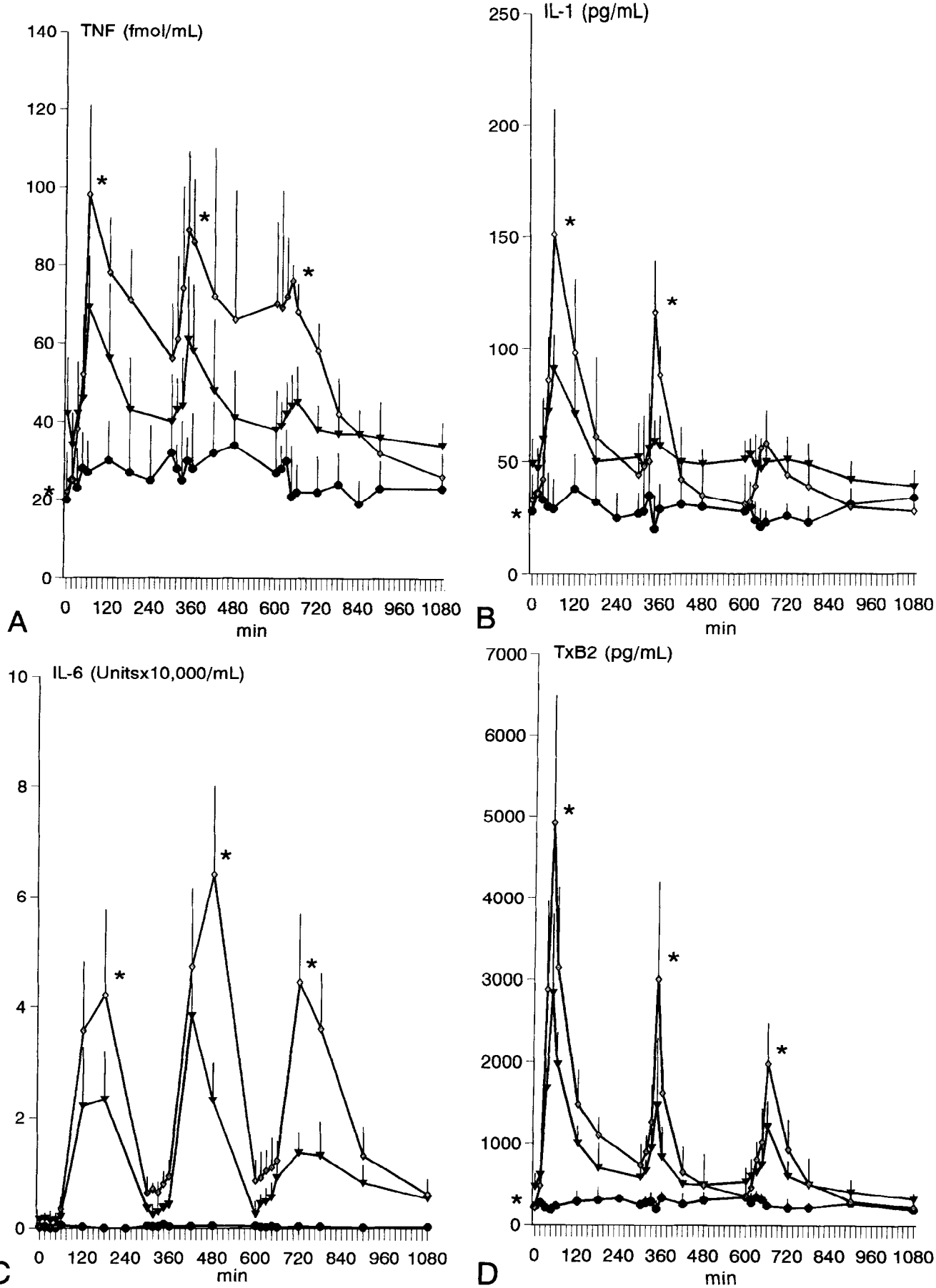

FIG. 1. Changes in mean plasma levels of TNFα (A), IL-1β (B), IL-6 (C), TxB2 (D), 6-keto-PGF1α (E), and PAF (F). *$P < 0.05$ group IV (saline pretreatment, LPS treatment) vs. group V (Zn^{2+} pretreatment, LPS treatment); rhombus, group IV: triangles, group V; circles, group III (saline pretreatment, saline treatment).

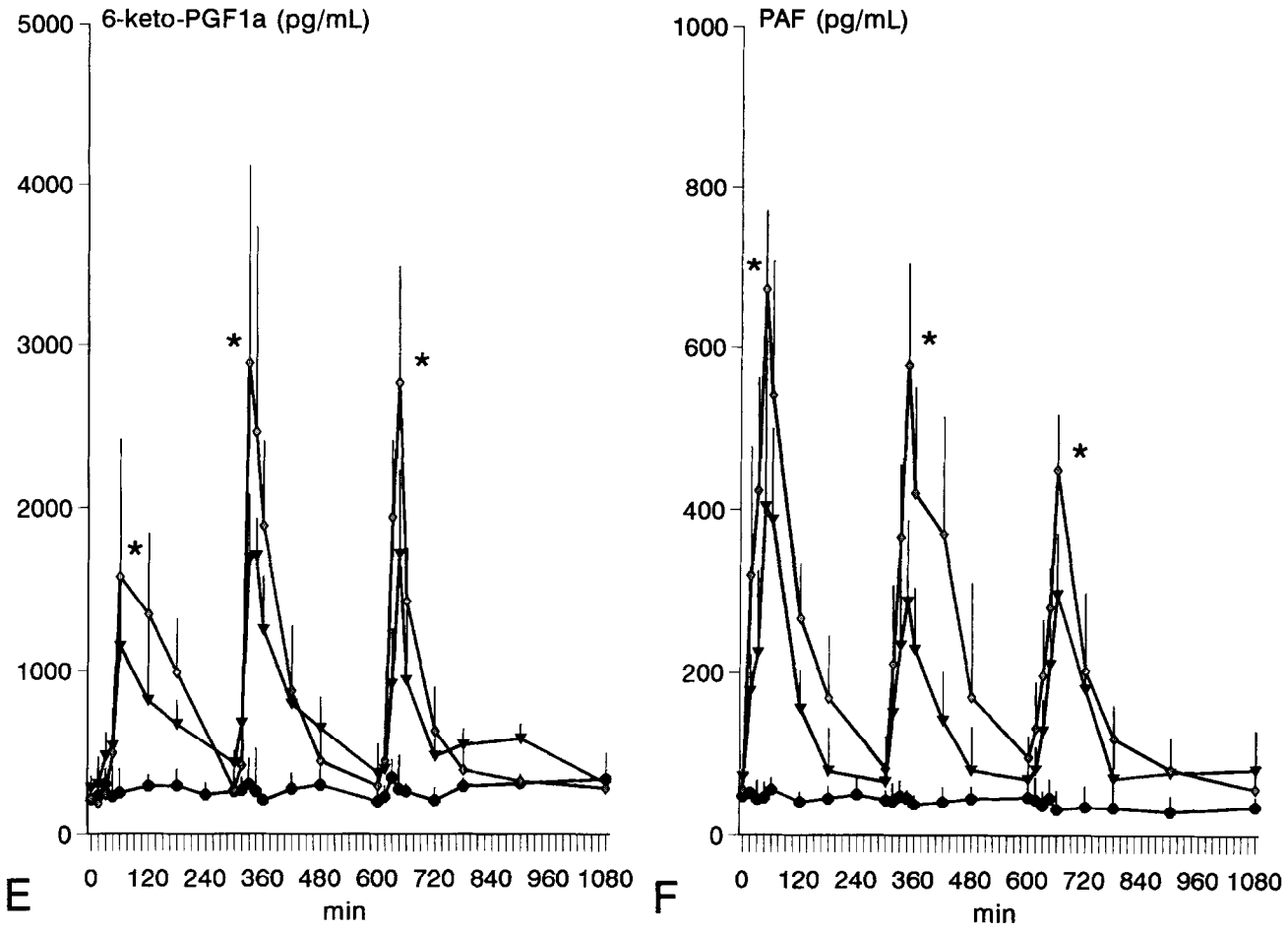

FIG. 1. Continued.

0.5 μCi of [^{3}H]thymidine, and the radioactivity incorporated into the nuclei was counted. One unit/mL is defined as the amount of IL-6 that causes a 50% decrease in maximal [^{3}H]thymidine incorporation. RhIL-6 (prepared from recombinant *E. coli*; specific activity of approximately 3.8×10^8 units/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 results and hemodynamic data were analyzed for statistical significance by using analysis of variance for repeated measures, followed by paired *t*-tests when significant differences ($P < .05$) were indicated. All data are expressed as mean ±SD values.

RESULTS

Mediator Levels after Zn^{2+} Pretreatment (Table 1)

Pretreatment with Zn^{2+} significantly increased plasma levels of TNFα, IL-1β, and TxB2 after 12 hr and plasma levels of TNFα, IL-1β, IL-6, and TxB2 after 24 hr. 6-Keto-PGF1α and PAF showed only insignificantly increased plasma levels after Zn^{2+} pretreatment.

Mediator Profiles after LPS Treatment (Fig. 1A–F)

In group IV (saline pretreatment, LPS treatment) the first mediators detected after the first LPS infusion in the plasma were TxB2, TNFα, IL-1, PAF, and 6-keto-$PGF_{1\alpha}$. An increase in the plasma levels of these mediators was measured 15–30 min after the start of the LPS infusion. TxB2 and PAF plasma concentrations significantly peaked after 45 min and TNFα, IL-1β, and 6-keto-PGF1α plasma levels after 69 min. IL-6 plasma levels increased after 120 min and showed maximal values after 180 min.

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. TxB2, PAF, TNFα, IL-1β, and 6-keto-PGF1α now significantly peaked after 30–60 min. IL-6 maximally peaked after 120 min. Thus, the liberation of these inflammatory mediators was accelerated after the second and third LPS administrations.

The mediators TxB2, PAF, IL-1β, and TNFα showed a decreasing three-peak profile after subsequent LPS administration. In contrast, in the case of 6-keto-PGF1α and IL-6,

an increase in peak size could be detected after repeated LPS injections.

Biochemical parameters of control group III (saline pretreatment, saline treatment) showed no significant alterations. Thus, the common time course of inflammatory mediator release in this study was identical to the data previously described [39].

In contrast with group IV (saline pretreatment, LPS treatment), group V (Zn^{2+} pretreatment, LPS treatment) animals showed significantly decreased plasma peak levels after Zn^{2+} pretreatment. The kinetics and profile of mediator liberation due to LPS administration was similar to that in group IV.

PAP and CO (Fig. 2A–B)

The changes in the hemodynamic parameters in group IV (saline pretreatment, LPS treatment) without Zn^{2+} pretreatment can be classified as a septic shocklike state characterized by features such as systemic hypotension, marked hypodynamic circulatory state, and low systemic vascular resistance. LPS applications at different times to this group caused a significant, sustained rise in systolic, mean, and diastolic PAPs. However, the peak in PAP after the second LPS injection was not as marked as that after the first one. CO indicated hypodynamic values in the first 10 hr of the experiments. From the 10th hour onward, CO increased steadily, thereby indicating a hyperdynamic circulatory state towards the end of the experiment.

In contrast with group IV, group V with Zn^{2+} pretreatment showed significantly decreased peaks in PAP and significantly increased CO levels after the first two LPS injections. After the third LPS administration, CO levels changed and showed slightly increased hyperdynamic values as compared with group III (saline pretreatment, saline treatment). Baseline values of PAP in this group showed significantly increased values compared with saline-pretreated group IV, which may be explained by the significantly increased plasma levels of TxB2 (Fig. 1D).

Hemodynamic parameters of group III (saline pretreatment, saline treatment) showed no significant alterations.

Induction of HSP70 and MT (Figs. 3, 4A–B)

Zn^{2+} pretreatment in group II (Zn^{2+} pretreatment only) induced increased tissue levels of HSP70 and MT as assessed in the lungs, liver, and kidneys by Western blotting (Fig. 3). Only small amounts of either stress protein could be detected by Western blotting in group I (saline pretreatment only). LPS treatment without Zn^{2+} pretreatment in group IV (saline pretreatment, LPS treatment) also increased HSP70 and MT expression. Group V (Zn^{2+} pretreatment, LPS treatment) showed decreased HSP70 and MT tissue levels as compared with group II (Zn^{2+} pretreatment only) but increased values as compared with group IV (saline pretreatment, LPS treatment).

Immunohistochemistry of group I (saline pretreatment only) showed only occasional positive cells without specific topographical distribution in the investigated organs (Fig. 4A). Group II (Zn^{2+} pretreatment only) animals, however, expressed HSP70 and MT intracytoplasmatically in the alveolar, vessel, and bronchic wall structures of the lungs and in the parenchymatous cell structures of the liver. In contrast with the diffuse expression pattern in the lungs and liver, HSP70 and MT showed a pronounced expression in the proximal renal tubules of the kidney (Fig. 4B).

DISCUSSION

The major findings of this study are that a single Zn^{2+} application increases plasma levels of different inflammatory mediators, in particular the proinflammatory cytokines TNFα, IL-1β, and IL-6, and induces an increased expression of both HSP70 and MT in the lungs, liver, and kidneys. As a result of the increased inflammatory mediator plasma levels and increased expression of HSP70 and MT after Zn^{2+} pretreatment, subsequent treatment with recurrent sublethal LPS infusions results in significantly decreased peak plasma levels of these mediators and an attenuated hemodynamic reaction.

The data presented in this study, which show significantly increased cytokine and arachidonic acid plasma baseline values after Zn^{2+} pretreatment compared with untreated sham animals, are in agreement with other *in vitro* and *in vivo* studies. Driessen *et al.* [38] described the induction of IFN-γ, IL-1β, IL-6, TNFα, and sIL-2R by Zn^{2+} ions added as $ZnSO_4$ in human peripheral blood mononuclear cells and separated monocytes in a concentration-dependent manner. Other *in vitro* studies, however, have shown that induction of HSP70 attenuates cytokine production in monocytes and alveolar macrophages [35, 36]. Exposure of mice to whole body hyperthermia increases IL-1 production within 24 hr [40]. These are important findings because pretreatment of rodents with low doses of LPS, rTNF, or rIL-1 can protect these animals against the lethality produced by either the subsequent administration of LPS or rTNF [41] or subsequent infection with gram-negative bacteria [42]. However, animals tolerant to LPS are completely unable to respond to LPS exposure with TNF release [43, 44]. This phenomenon is attributed to an impairment of macrophages in releasing TNF [45–47]. Furthermore, Waage [48] described TNF release recovering 7 days after LPS administration, which coincides with the disappearance of tolerance to LPS [49].

Induction of HSP70 and MT is another mechanism by which Zn^{2+} pretreatment may provide protection against the toxic effects of LPS. This presumes that elevated cellular levels of HSP70 and MT can directly provide protection against the cytotoxic effects of LPS and TNF [50, 51] or indirectly provide protection by inhibiting the induction of cytokine and arachidonic acid production in LPS-activated cells [52, 53]. If the development of cross resistance to LPS is at least partially dependent on elevated stress protein levels, the phenomenon of cross resistance to LPS may become detectable within hours and may last for days. HSP70 can be increased in numerous tissues within 2

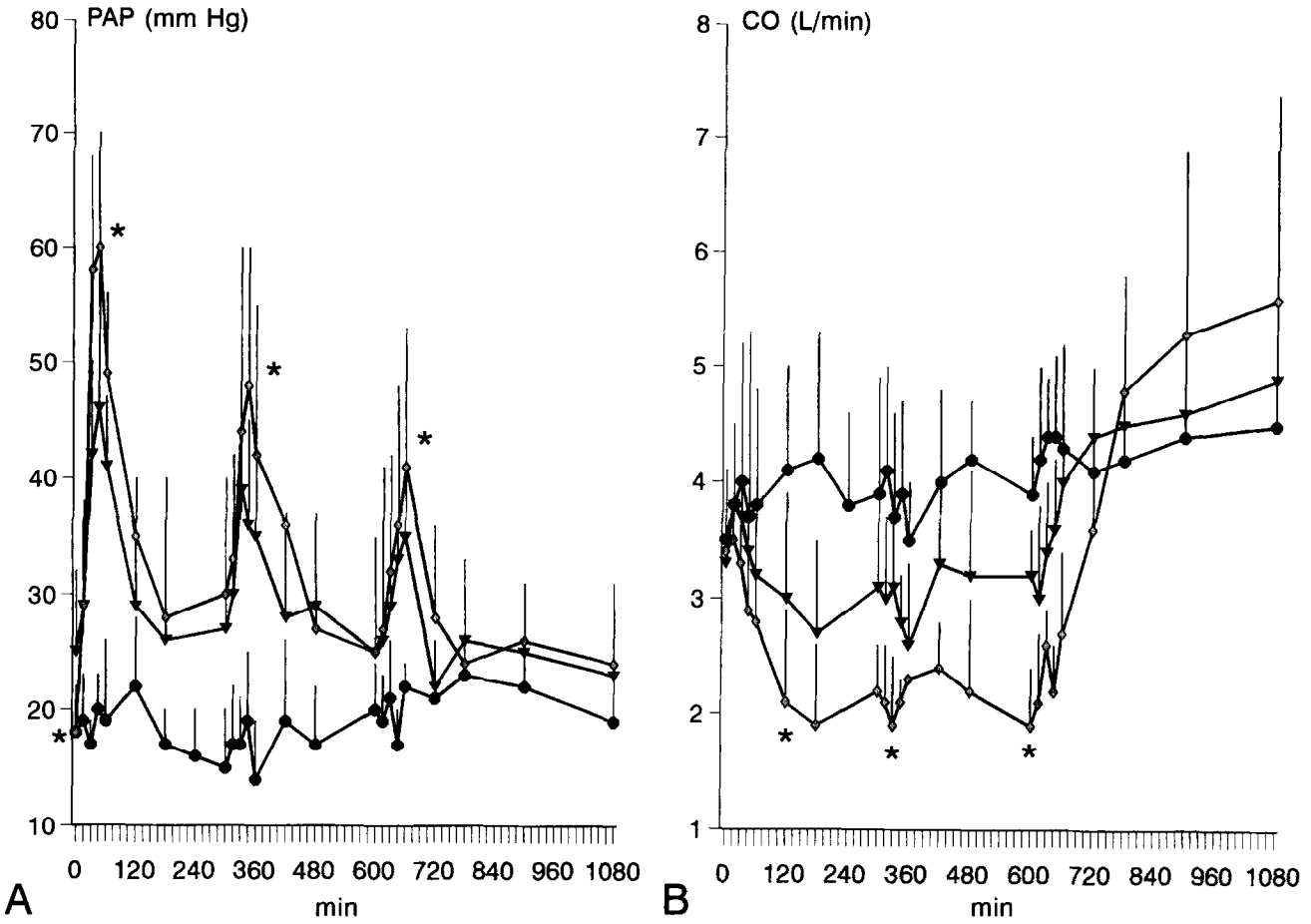

FIG. 2. Changes in PAP (A) and CO (B). *P < 0.05 group IV (saline pretreatment, LPS treatment (vs. group V (Zn^{2+} pretreatment, LPS treatment); rhombus, group IV; triangles, group V; circles, group III (saline pretreatment, saline treatment).

or 3 hr and can remain at elevated levels for 8–16 days [54]. This observation is an interesting parallel to the disappearance of LPS tolerance after 7 days [49].

As a consequence of decreased inflammatory mediator plasma levels after Zn^{2+} pretreatment and subsequent LPS treatment, hemodynamic alterations due to LPS administration are significantly diminished. For example, PAP shows a direct correlation to TxB2/6-keto-PGF1α plasma-level ratios [55]. This response seems to be LPS dose related because it was not seen with either continuous low-dose LPS infusions [56–60], which had no hemodynamic effects and only a mild effect on TxB2 plasma levels, or after bolus injections of supralethal LPS doses, which led to an irreversible increase in the PAP and TxB2, resulting in death. The data presented here demonstrate that Zn^{2+} pretreatment attenuates hemodynamic alterations at the same dose of LPS, which is a direct consequence of the altered immunology.

This investigation shows that, as Zn^{2+} pretreatment rendered pigs more resistant to the effects of LPS within 24 hr, it may therefore be useful in preventing sepsis in high-risk patients. This acquired resistance may have been due to direct and indirect effects of Zn^{2+}. Direct effects of Zn^{2+} result in (1) an increased expression of HSP70 and MT and

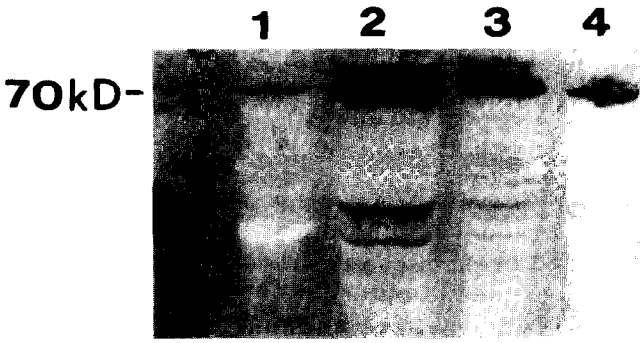

FIG. 3. Representative Western blot analysis of HSP70 from lung homogenates (two animals/lane); lane 1 = group I (saline pretreatment only): note the very weak band by 70 kD; lane 2 = group II (Zn^{2+} pretreatment only): note the intensely positive band by 70 kD, indicating an increased HSP70 expression in the lungs after Zn^{2+} treatment for 24 hr and the additional bands, which may represent HSP70 fragments; lane 3 = group V (Zn^{2+} pretreatment, LPS treatment): note the positive band that is not as clear as in lane 2 (group I, Zn^{2+} treatment only), indicating a decreased HSP70 expression in the lungs of animals with prior Zn^{2+} treatment after LPS treatment; lane 4 = group IV (saline pretreatment, LPS treatment): note again the positive band indicating LPS as an inducer of HSP70 in the lungs *in vivo*.

FIG. 4. Immunohistochemical specimen of the kidney of group I (A; saline pretreatment only) and group II (B; Zn^{2+} pretreatment only); after Zn^{2+} pretreatment, MT is expressed mainly in proximal renal tubules (B), whereas the untreated animals show no MT expression in renal tubules (A). G, glomerulus; arrow, renal tubules. Phase-contrast, 100×.

(2) a low liberation of cytokines and arachidonic acid products, with slightly increased baseline values of treated animals compared with untreated sham animals. Indirect effects are (1) a reduction of the responsiveness of cells, most likely mononuclear phagocytic cells, to activation by LPS with subsequently decreased cytokine liberation; (2) an enhanced resistance to the cytotoxic effects of LPS and its potentially toxic mediators, such as TNF and IL-1; and (3) a cross resistance or tolerance to LPS similar to prior application of heat stress, LPS, rTNF, or rIL-1. The overall effect is an attenuated course of septic shock, with significantly altered hemodynamical parameters of the treated animals.

However, further research is needed in the use of Zn^{2+} in septic shock and in the field of potential inducers of stress proteins. Our data show that Zn^{2+} is an inducer of both HSP70 and MT, whereas arsenite or heat have only been described as inducing HSP70 [31, 32]. It remains unclear whether the further induction of MT by Zn^{2+} reveals an additional effect in the prevention of septic shock. More investigation is necessary.

Chandra [61] reported that excessive intake of zinc impairs the immune response in healthy men. However, the deleterious effects were observed after ingestion of 150 mg of elemental zinc twice a day for 6 weeks, which is not comparable to a single bolus therapy done to prevent sepsis in high-risk patients, as in this experiment. Furthermore, preventive administration of Zn^{2+} should take into consideration that serum Zn^{2+} generally decreases after surgery, accidental trauma, or thermal injury, which are high-risk precursor diseases of sepsis [62, 63]. Another general problem is an impaired immune response due to Zn^{2+} deficiency, especially in elderly patients. Prasad *et al.* [64] reported that approximately 30% of elderly subjects had deficient Zn^{2+} levels in both granulocytes and lymphocytes. In this group, plasma copper was increased and IL-1 production significantly decreased. Zn^{2+} supplementation corrected Zn^{2+} deficiency, normalized plasma copper levels, and significantly increased IL-1 production. These data and those of Driessen *et al.* [37] clearly show that Zn^{2+} and other inducers share biologic activities with LPS itself, in particular the elevation of highly biologic active cytokines such as TNF, IL-1, and IL-6. Therefore, it seems unlikely that treating already septic patients with Zn^{2+} would be useful. However, the data of this study show that prophylactic administration of Zn^{2+} to prevent sepsis in patients identified to be at high risk could be a practicable therapeutic strategy.

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