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Interleukin (IL)-8 and Growth Related Oncogene-α in Severe Endotoxemia and the Effects of a Tumor Necrosis Factor-α/IL-1β Inhibitor on These Chemokines

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FR167653 inhibits the production of tumor necrosis factor (TNF)-α and interleukin (IL)-1β, powerful inducers of CXC chemokines IL-8 and growth related oncogene (GRO)-α. The production of IL-8 and GRO-α was investigated and the effects of FR167653 were examined in a rabbit model of endotoxin shock. Male New Zealand rabbits were given endotoxin at a dose sufficient to induce DIC. Three groups of rabbits received FR167653 at different doses. TNF-α, IL-1β, IL-8, and GRO-α levels were measured, several pathologic features were evaluated, and the results were compared with those obtained in control rabbits, which received only endotoxin. Endotoxin increased serum levels of IL-8 and GRO-α, which were associated with hypotension, renal dysfunction, and mortality, peaking at 4 h. FR167653 improved mortality, an event that was associated with decreased levels of not only TNF-α and IL-1β but also IL-8 and GRO-α. TNF-α peaked at 2 h, at a time point before IL-8 and GRO-α reached their peak, and the TNF-α level was tightly correlated with that of IL-8 and GRO-α. Altogether, these data suggest the possible involvement of IL-8 and GRO-α in endotoxin shock, and FR167653 may foster a beneficial outcome in part by modulating the chemokines level by inhibiting TNF-α and IL-1β.

Key Words: endotoxin shock; interleukin-8; growth related oncogene-α; tumor necrosis factor-α; interleukin-1β; FR167653.

INTRODUCTION

Sepsis or septic shock is the systemic response to infection, causing hypotension, coagulopathy, and multiple organ failure that are fatal to the host. Endotoxin or lipopolysaccharide (LPS), a component of Gram-negative bacteria, plays a central role in septic shock by triggering the overzealous production of inflammatory mediators that include cytokines [1]. Among cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-1β are regarded as functioning as proximal inflammatory cytokines [2]. TNF-α and IL-1β have been detected in patients with sepsis and in animal models of endotoxin shock [3, 4]. TNF-α or IL-1β blockade with antibodies or receptor antagonist improved the outcome of endotoxin shock in experimental animals [5–9]. Accumulating evidence indicates that TNF-α and IL-1β set a cascade of inflammatory response to motion, leading to the generation of a wide variety of mediators that orchestrate the pathological response during endotoxin shock [1, 2]. Thus, therapeutic intervention directing TNF-α and IL-1β appears to be a promising strategy for the treatment of endotoxin shock. FR167653, a low-molecular-weight compound that inhibits the production of TNF-α and IL-1β *in vitro* [10], is shown to ameliorate endotoxin shock in experimental animals [10, 11].

Chemokines are chemotactic cytokines that constitute a large supergene family of 8- to 10-kDa basic heparin-binding proteins. CXC chemokines are a subfamily of chemokines that preferentially attract and activate neutrophils [12]. IL-8/CXCL8 and growth related oncogene-α (GRO-α/CXCL1) belong to the CXC chemokine subfamily and have been detected in human endotoxemia [13, 14]. Because of their biological properties, these chemokines are considered to be involved in the pathogenesis of endotoxin shock [15]. Since IL-8 and GRO-α can be induced by LPS directly and indirectly via the production of TNF-α and IL-1β [16–20], it is tempting to speculate that FR167653 may modulate the production of these chemokines, resulting in a therapeutic effect in endotoxin shock. However, the production of IL-8/GRO-α and its relationship with TNF-α/IL-1β during endotoxin shock remains unclear. To address this, a lethal dose of LPS was intravenously infused in

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rabbits, and the rabbits were treated with various concentrations of a TNF-α and IL-1β inhibitor, FR167653. In the present study, we show the possible involvement of IL-8 and GRO-α in an animal model of endotoxin shock. FR167653 may exert a beneficial effect in part by modulating the chemokines level by inhibiting TNF-α and IL-1β.

MATERIALS AND METHODS

Experimental Protocol

Male New Zealand white rabbits (weight 2.8–3.5 kg) were used. Rabbits were anesthetized by an intramuscular injection of 30 mg/kg ketamine hydrochloride and 0.002 mg/kg xylazine hydrochloride followed by ketamine hydrochloride injection during experiments to maintain anesthesia. Endotoxin shock was induced by iv infusion of 100 μg/kg/h LPS for 6 h in saline (10 ml/h) through the marginal ear vein. The rabbits were simultaneously infused with FR167653 (kindly provided by Fujisawa Pharmaceutical Co., Osaka, Japan) in saline at 0 (LPS group, 11 rabbits), 0.25 (9 rabbits), 0.5 (9 rabbits), and 1 (8 rabbits) mg/kg/h for 6 h through the other marginal ear vein. As controls, rabbits were not infused with LPS but with saline (five rabbits) or FR167653 at 1 mg/kg/h (two rabbits) for 6 h. Before LPS challenge and at 2, 4, and 6 h after the challenge, mean arterial pressure (MAP) was recorded and artery blood was obtained. Rabbits were monitored for 24 h, after which rabbits were killed by an overdose of Nembutal (60 mg/kg iv, Abbott Laboratories, Abbott Park, IL). Kidney and lung were removed for histological analysis.

Leukocyte Count

The number of peripheral leukocytes was counted with a Counter STKS automatic analyzer (Coulter Corp., Hialeah, FL).

Mean Arterial Pressure

MAP value was calculated as follows: MAP (mm Hg) = [2 $\times$ diastolic arterial pressure (mm Hg) + systolic arterial pressure (mm Hg)]/3. The value at time 0 was not used in the study because it was strongly influenced by the anesthesia needed for femoral artery and ear vein cannulation.

Renal Function

Serum creatinine levels were measured using a direct colorimetric method [21]. Data were expressed as the percentage increase compared to the baseline value.

Measurements of Cytokines and Chemokines

TNF-α activity was quantified by L929 cell (ECACC, Salisbury, UK) cytotoxic assay as described previously [22]. Known concentrations of recombinant human TNF-α (Genzyme, Cambridge, MA) were used as a standard. IL-1β and IL-8 were measured by specific sandwich ELISA, as described elsewhere [16]. Measurements of GRO-α were carried out by time-resolved fluoroimmunoassay as described [19]. The detection limit for TNF-α, IL-1β, IL-8, and GRO-α was 10, 10, 30, and 30 pg/mL, respectively. The antibodies employed in the assay did not cross-react with other available cytokines/chemokines. To normalize the data, the baseline value of each rabbit was subtracted from each value.

NOx Analysis

Serum levels of the nitric oxide (NO) metabolites, NO_2^- and NO_3^- (NOx), were determined by nitric oxide colorimetric assay kit (Roche, Mannheim, Germany). Data were expressed as a percentage compared to the baseline value.

Histological Examination

Kidney and lung were fixed in 10% formalin and embedded in paraffin, and the sections were stained with Masson's trichrome and examined by a pathologist unaware of the experimental design.

Statistical Analysis

ANOVA followed by Dunnett's test or Kruskal–Wallis followed by the Mann–Whitney U test was applied for group comparison. To compare survival data, the Mann–Whitney U test was used. The Pearson correlation coefficients were calculated to analyze the association between different parameters. The linear by linear association χ^2 test was applied to evaluate the differences in the mortality rate between groups.

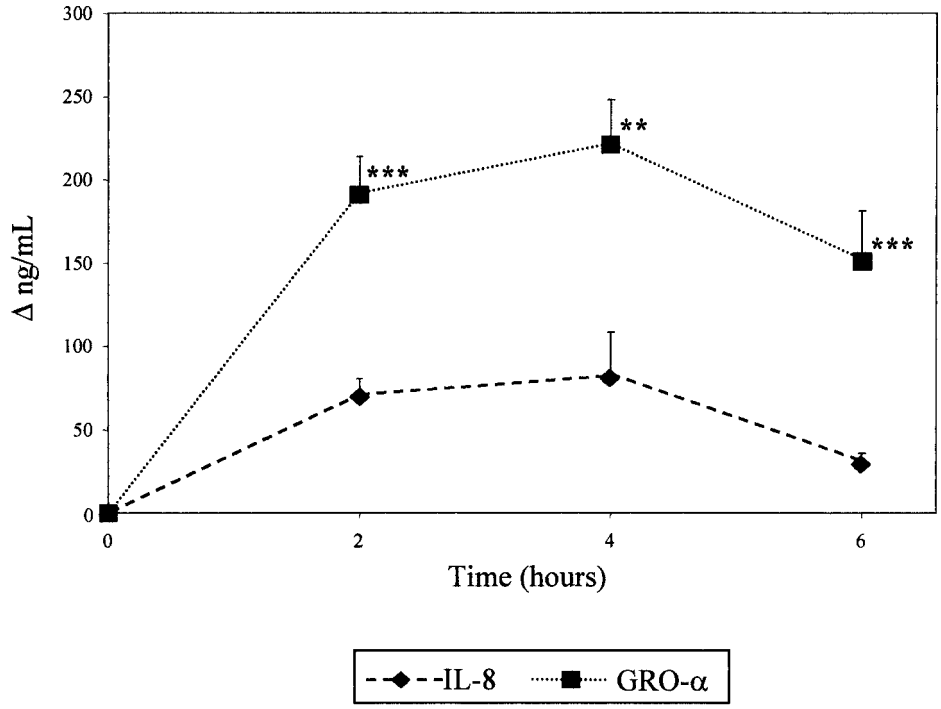

FIG. 1. Serum IL-8 and GRO-α increment (Δ ng/mL) at 2, 4, and 6 h in the LPS group. Results are mean $\pm$ standard error of the mean (SEM); $^{**}P < 0.01$, $^{***}P < 0.001$, with respect to the IL-8 levels. Statistical analysis: The Mann–Whitney *U* test was used for comparison between IL-8 and GRO-α levels.

RESULTS

Control Experiments

Rabbits receiving saline or 1 mg/kg/h FR167653 without LPS challenge did not show alterations in any of the analyzed parameters (data not shown) and all survived for 24 h.

Production of IL-8 and GRO-α and Effects of FR167653 on the Levels

Infusion of LPS resulted in an increase in the serum level of IL-8 and GRO-α (Fig. 1). Production of IL-8 and GRO-α peaked at 4 h [IL-8, 83.0 $\pm$ 25.3 ng/mL; GRO-α, 221.7 $\pm$ 26.3 ng/mL, mean $\pm$ standard error of the mean (SEM)], followed by a decrease at 6 h after LPS infusion. Levels of GRO-α were significantly higher than those of IL-8 ($P < 0.001$ at 2 and 6 h, $P < 0.01$ at 4 h; Fig. 1). Thus, IL-8 and GRO-α were induced during endotoxin shock.

Correlation between IL-8/GRO-α and Parameters of Endotoxin Shock

To understand the involvement of IL-8 and GRO-α in this model of endotoxin shock, all data from rabbits challenged with LPS were pooled and analyzed ($n = 37$). Levels of IL-8 and GRO-α were inversely correlated with MAP at 4 h [IL-8-MAP, $r = -0.650$, $P < 0.01$ (Fig. 2A); GRO-α-MAP, $r = -0.615$, $P < 0.01$ (Fig. 2B)], suggesting that elevated IL-8 and GRO-α were associated with hypotension. There was a negative correlation between creatinine and MAP ($r = -0.442$, $P < 0.01$; Fig. 2C), suggesting that renal dysfunction was due to the hypotension. IL-8 and GRO-α levels were positively correlated with creatinine levels [IL-8-creatinine at 4 h, $r = 0.581$, $P < 0.01$ (Fig. 2D); GRO-α-creatinine at 4 h, $r = 0.475$, $P < 0.01$ (Fig. 2E)]. Thus, elevated IL-8 and GRO-α appear to be related to renal dysfunction through hypotension.

At 24 h after LPS infusion, 29 rabbits were alive and 8 rabbits were dead. When data were analyzed comparing survivors and nonsurvivors, there was a trend toward an increase in the level of IL-8 in nonsurvivors compared to

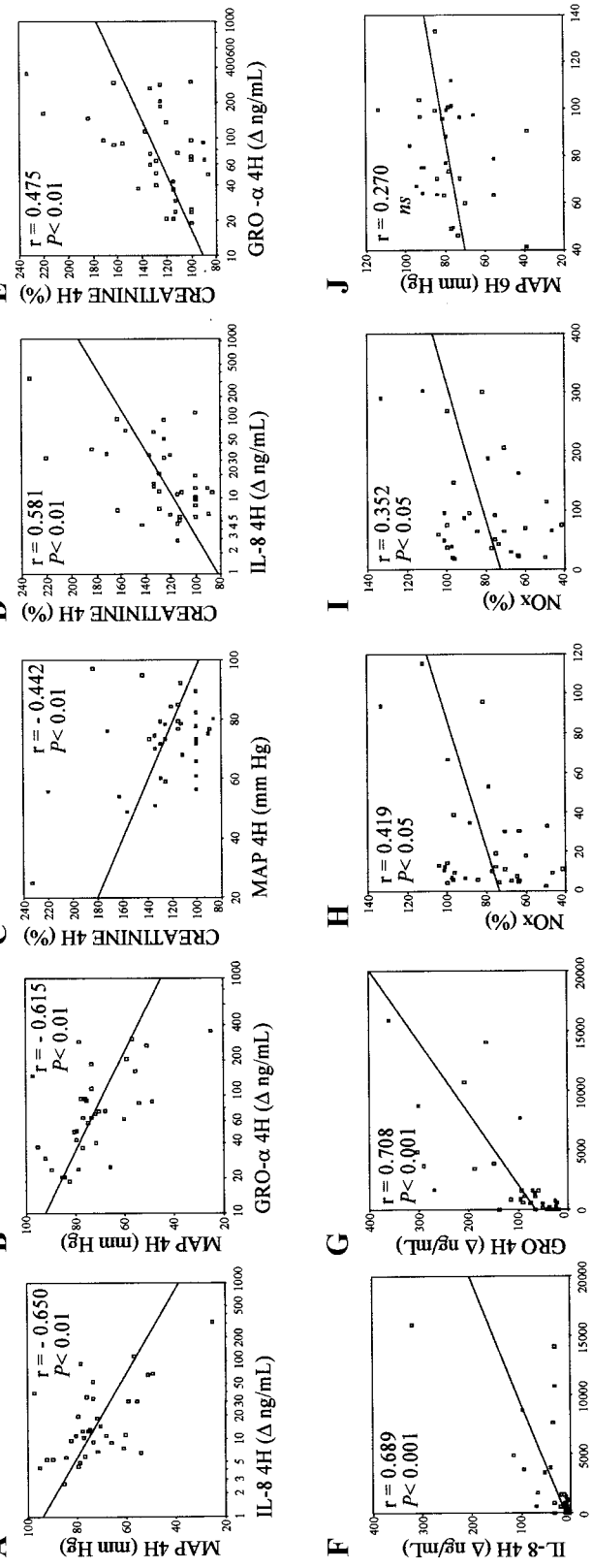

FIG. 2. Correlation between variables, pooling all LPS-challenged rabbits ($n = 37$). The Pearson correlation coefficient and the corresponding P value are indicated in each panel. Statistical analysis: The Pearson correlation coefficient was calculated to analyze the association between the variables.

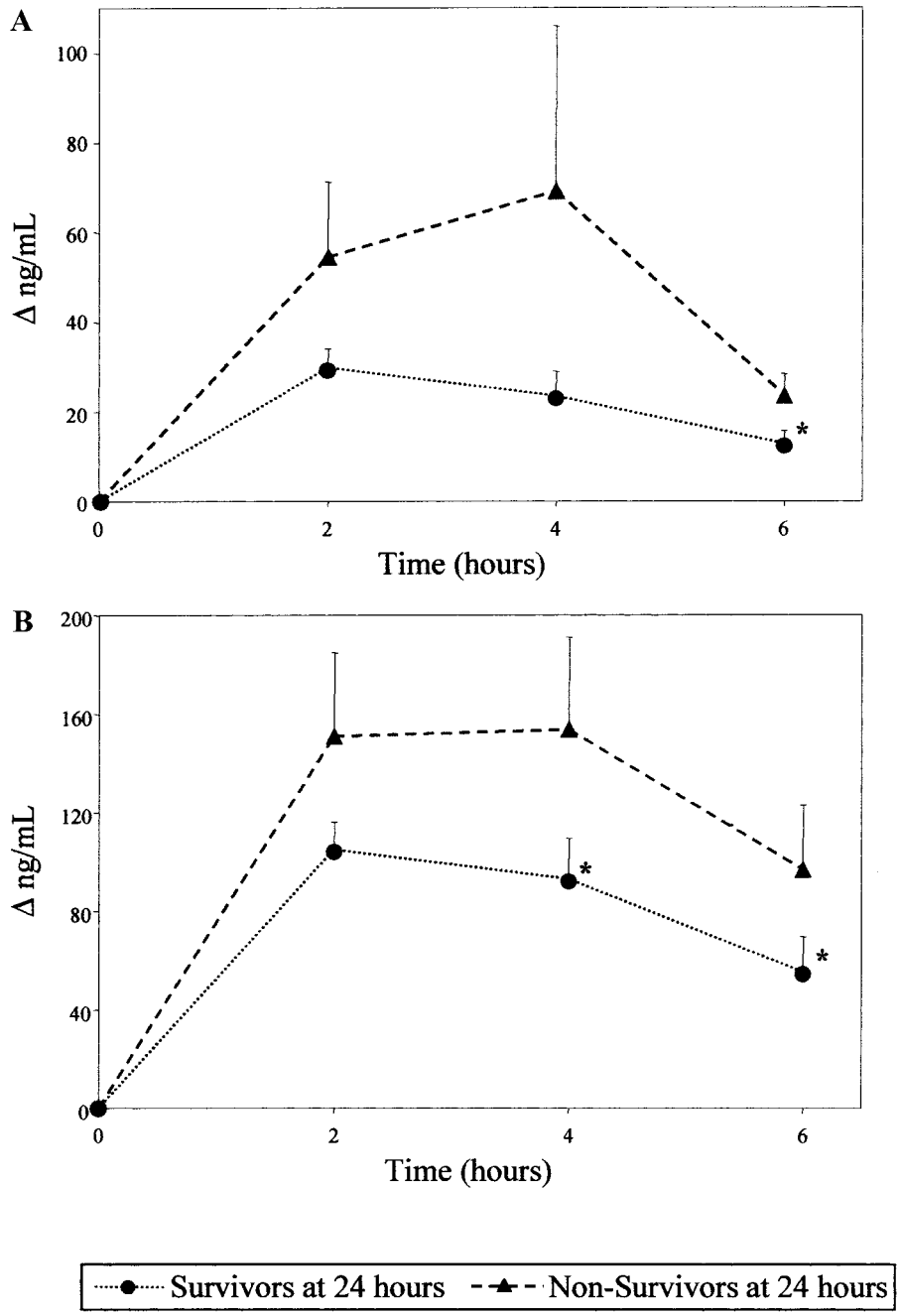

FIG. 3. IL-8 (A) and GRO-α (B) increment (Δ ng/mL) in survivor and nonsurvivor rabbits. Results are mean $\pm$ standard error of the mean (SEM); *$P <$ 0.05 with respect to the nonsurvivors group. Statistical analysis: The Mann–Whitney *U* test was used for comparison between survivors and nonsurvivors.

survivors. The difference was statistically significant at 6 h after LPS infusion ($P < 0.05$; Fig. 3A). Likewise, the GRO-α level was significantly higher in nonsurvivors at 4 and 6 h than in survivors ($P < 0.05$; Fig. 3B). These data suggest that IL-8 and GRO-α may exert a deleterious role in endotoxin shock.

Modulation of IL-8 and GRO-α Level by FR167653 Treatment

Previous studies demonstrated that FR167653 treatment improved mortality during endotoxin shock [11]. In the present study, 36% rabbits (4 of 11 rabbits) were dead at

24 h after LPS infusion. The mortality rate was dose-dependently reduced by FR167653 treatment (linear by linear association χ^2 test: $P = 0.035$; Fig. 4A). All rabbits treated with 1 mg/kg/h FR167653 were alive (Fig. 4A). Consistent with a previous report [11], decreased MAP and increased creatinine levels were significantly improved by FR167653 treatment [MAP, $P < 0.05$ at 4 h with FR167653 at high dose (Fig. 4B); creatinine, $P < 0.01$ at 4 h and $P < 0.05$ at 6 h with FR167653 at medium and high doses (Fig. 4C)]. In this situation, chemokine levels were examined and the data were compared to the LPS group. As shown in Fig. 5, FR167653 treatment dose-dependently inhibited the production of IL-8 and GRO-α during endotoxin shock. Levels of IL-8 and GRO-α were inhibited by 1 mg/kg/h FR167653 by 91 and 88%, respectively, at the peak (4 h after LPS infusion). Thus, production of IL-8 and GRO-α was significantly inhibited by FR167653.

Relationship between TNF-α/IL-1β and IL-8/GRO-α

Since TNF-α and IL-1β are potent inducers of IL-8 and GRO-α and FR167653 is a dual inhibitor of TNF-α and IL-1β, it is speculated that FR167653 inhibited the production of IL-8 and GRO-α through the inhibition of TNF-α and IL-1β. To confirm this speculation, levels of TNF-α and IL-1β were examined in all rabbits receiving LPS infusion ($n = 37$). As shown in Fig. 6, the TNF-α level increased rapidly, peaked at 2 h (LPS group, 6871.5 $\pm$ 1506.8, mean $\pm$ SEM), and returned to basal level by 4 h. Consistent with the previous results [10, 11], the TNF-α level at 2 h was completely inhibited by the treatment with 1 mg/kg/h FR167653 ($P < 0.001$; Fig. 6). IL-1β was detected in 2 of 11 rabbits after LPS infusion and peaked at 4 h after LPS infusion (20 and 350 pg/mL). No IL-1β was detected after FR167653 treatment.

There was a positive correlation between IL-8 and TNF-α ($r = 0.689$, $P < 0.001$; Fig. 2F) and GRO-α and TNF-α ($r = 0.708$, $P < 0.001$; Fig. 2G). Although it was not possible to analyze the correlation between IL-1β and IL-8/GRO-α because IL-1β was detected in very few rabbits after LPS infusion, the rabbit with a high IL-1β level after LPS infusion exhibited a much higher level of IL-8 and GRO-α than the other rabbits (IL-8, 319.4 ng/mL; GRO-α, 359.6 ng/mL at 4 h). Thus, there appears to be a link between proinflammatory cytokines TNF-α and IL-1β and CXC chemokines IL-8 and GRO-α, and TNF-α and IL-1β may contribute to the production of these chemokines.

CXC Chemokines and NOx Levels

There was a positive correlation between IL-8 and GRO-α at 4 h and serum NOx levels at 6 h [IL-8-NOx, $r = 0.419$, $P < 0.05$ (Fig. 2H); GRO-α-NOx, $r = 0.352$, $P < 0.05$ (Fig. 2I)]. No inverse correlation was observed between NOx and MAP at 6 h ($r = 0.270$; Fig. 2J). Interestingly, survivor rabbits showed higher levels of NOx at 6 h compared to nonsurvivors at marginal statistical significance [83.9 $\pm$ 3.8% vs 67.2 $\pm$ 9.4% (mean $\pm$ SEM), $P = 0.069$]. These data suggest that NO may play a beneficial role in endotoxin shock.

Peripheral Leukocyte Counts

The leukocyte counts were decreased after LPS infusion ranging from 25 to 40% compared to baseline. There were no significant differences in the number of leukocytes after treatment with FR167653 (not shown).

Histological Examination

After LPS infusion, fibrin deposition was present in glomeruli and tubules of the kidney. Alveolar septa of the lung showed a mild diffuse widening. These histological changes were not altered remarkably after FR167653 treatment (not shown).

DISCUSSION

CXC chemokines IL-8 and GRO-α have been described as potent contributors to recruitment and activation of neutrophils in a variety of LPS-induced inflammation models [16–20]. Their role in LPS-induced sepsis is not totally elucidated. We have studied IL-8 and GRO-α involvement in a model of LPS-induced severe sepsis in rabbits and their TNFα/IL-1β dependence by using increasing doses of FR167653, an inhibitor of TNF-α/IL-1β production. IL-8 and GRO-α experienced a notable increase in serum after the LPS infusion, especially at 4 h, shortly after TNF-α activity had reached its peak. FR167653 treatment efficiently decreased the serum levels of CXC chemokines IL-8 and GRO-α in a dose-dependent manner, as had happened with TNF-α. As levels of TNF-α, IL-8, and GRO-α correlated highly during the experiment, it is suggested that the up-regulation of both chemokines in severe endotoxemia is driven by TNF-α, as is the case in other LPS-based *in vivo* models [18, 20, 23]. The remarkable IL-8 and GRO-α

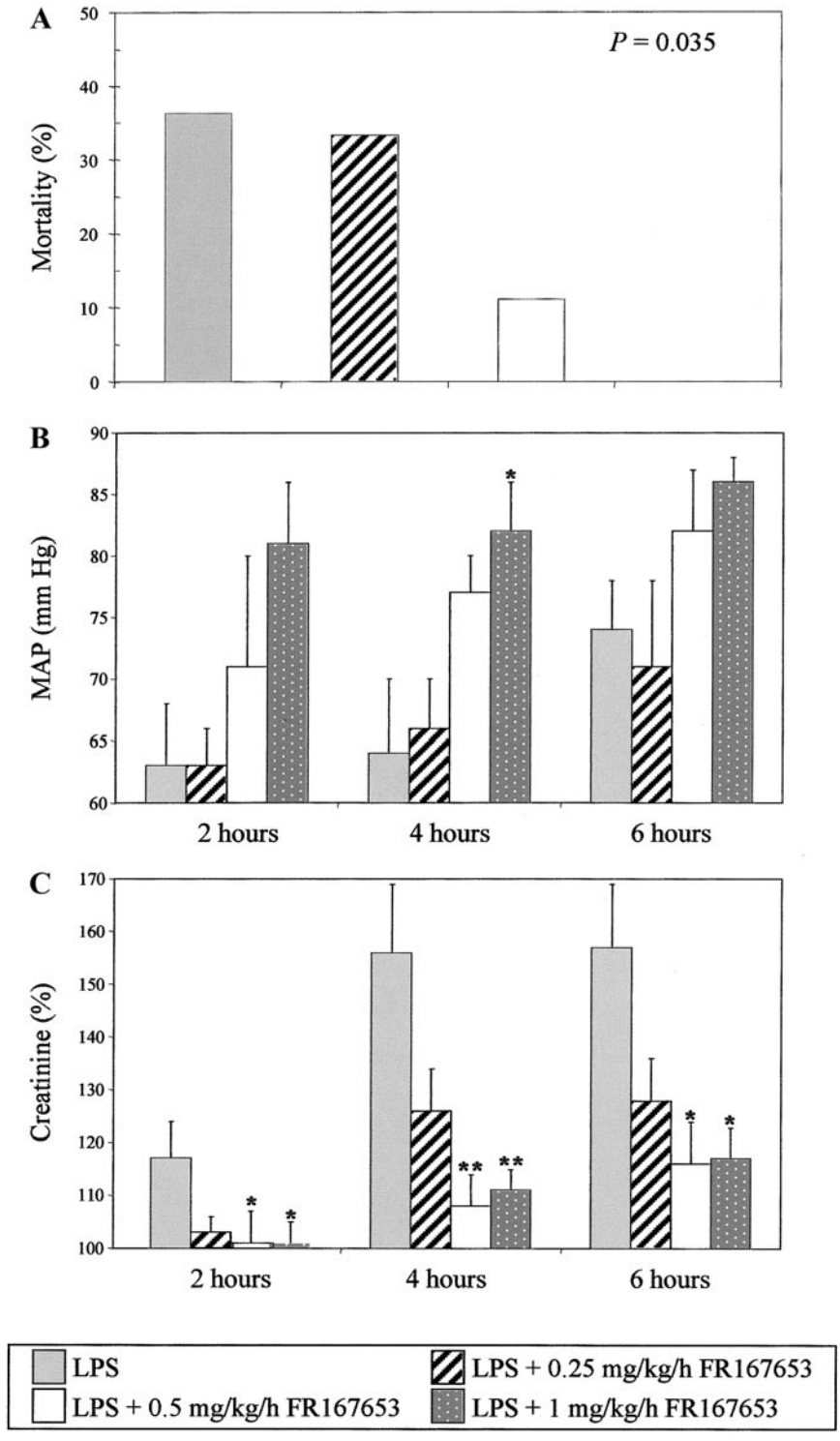

FIG. 4. Mortality rate at 24 h in the LPS and the FR167653 groups (A, expressed as the percentage of nonsurvivors with respect to the number of animals in each group); MAP (mm Hg, B), and creatinine (percentage with respect to basal values, C) at 2, 4, and 6 h in the LPS and the FR167653 groups. Results are mean ± SEM; $*P < 0.05$, $**P < 0.01$, with respect to the LPS group. Statistical analysis: Linear by linear χ^2 test for analysis of mortality; ANOVA followed by Dunnett's test for comparison between the LPS and the FR167653 groups.

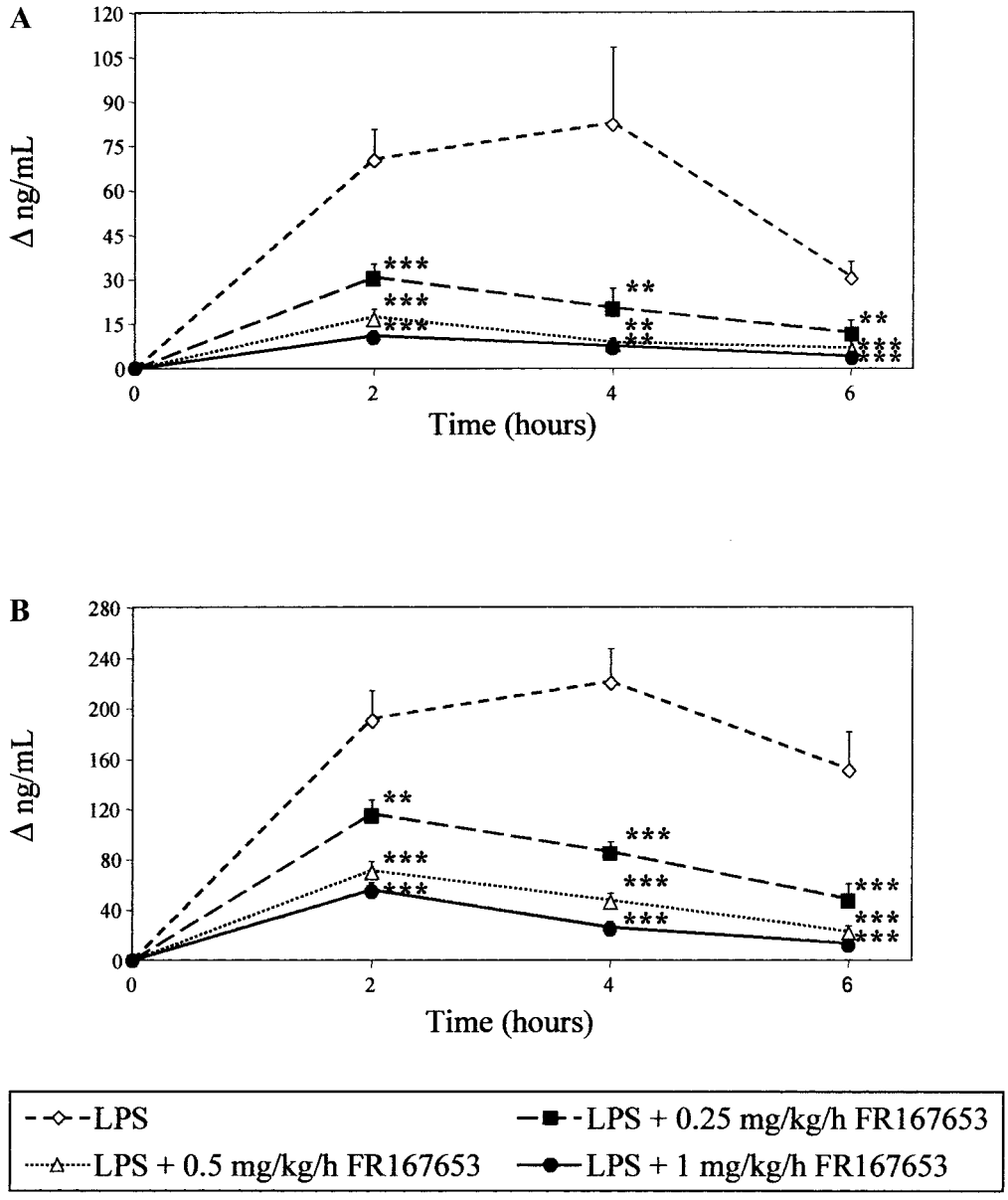

FIG. 5. IL-8 (A) and GRO-α (B) increment (Δ ng/mL) at 2, 4, and 6 h in the sera of the LPS and the FR167653 groups. Results are mean ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$, with respect to the LPS group. Statistical analysis: ANOVA followed by Dunnett's test was used for comparison between the LPS and the FR167653 groups.

increase observed in the rabbit showing significant IL-1β levels would suggest that this cytokine could enhance the effect of TNF-α on CXC chemokines. The very high levels of IL-8 and GRO-α in nonsurvivors together with their important correlation with MAP and renal dysfunction suggest that these two CXC chemokines could be involved in the development of severe sepsis and septic shock. Although it is known that CXC chemokines play key roles in the recruitment and activation of neutrophils [24, 25], many other chemokine functions must be elucidated [26]. Our data suggest that attenuation of IL-8 and GRO-α seems to improve the outcome of sepsis by preventing hypotension and the subsequent organ failure rather than by reducing neutrophil recruitment and activation: first, leukopenia, in agreement with previous endotoxemia studies using FR167653 or other TNF-α inhibitors [10, 27, 28], could not

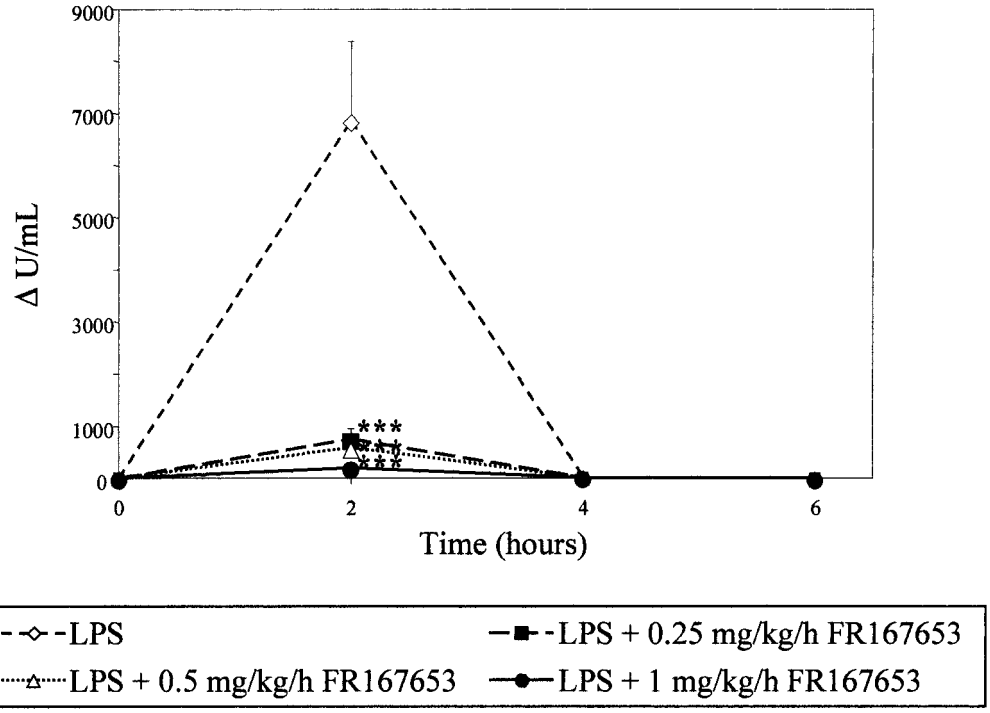

FIG. 6. TNF-α activity increment (Δ U/mL) at 2, 4, and 6 h in the sera of the LPS and the FR167653 groups. Results are mean $\pm$ SEM; $^{***}P < 0.001$ with respect to the LPS group. Statistical analysis: The Kruskal–Wallis test followed by the Mann–Whitney *U* test was used for comparison between the LPS and the FR167653 groups.

be reversed despite preventing IL-8 and GRO-α production; second, no noticeable differences in the histological appearance of the kidney and lung between LPS group and FR167653 treated rabbits could be seen. In this context, our results are coherent with those of a recent work by Matsukawa *et al.* in a sepsis model in which the therapeutic control of the CXC chemokines macrophage inflammatory protein-2 and KC levels improved the renal function without a noticeable modification in the renal neutrophil influx [29]. Altogether, these experiments support the notion that renal injury in sepsis is likely to be caused by renal hypoperfusion rather than inflammation [30]. Although evidence exists as to the beneficial effect of inhibiting IL-8 activity on hemodynamics [31, 32], further work is needed to elucidate how IL-8 and GRO-α would be involved in the development of hypotension. Nevertheless, it can be said that our data do not support the idea that their involvement is related to an increase in the NO levels, not only because a negative correlation between NOx and MAP could not be demonstrated but also because nonsurvivors showed lower NOx levels than survivors, rather supporting the idea that lowering the NO production in septic shock could be harmful [33].

We conclude that IL-8 and GRO-α contribute to hypotension, leading to renal hypoperfusion and subsequent renal failure in this model of endotoxin shock. The production of IL-8 and GRO-α appears to depend on TNF-α and IL-1β. FR167653 may contribute to a beneficial outcome in part by modulating the chemokines level by inhibiting TNF-α and IL-1β.

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