Propylene-Glycol Aggravates LPS-Induced Sepsis through Production of TNF-α and IL-6

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ABSTRACT

Background: Propylene glycol (1,2-propanediol, PG) is a commonly used solvent for oral, intravenous, as well as topical pharmaceutical preparations. While PG is generally considered to be safe, it has been known that large intravenous doses given over a short period of time can be toxic. **Objective:** To evaluate the effect of PG in sepsis induced by the bacterial endotoxin lipopolysaccharide (LPS). **Methods:** Balb/c mice were treated with LPS (1 mg/kg b.w., i.p.) with or without PG (5 g/kg b.w. i.v.). The survival rate and the production of inflammatory cytokines were measured. In RAW264.7 mouse macrophages encoding NF-κB-luc reporter gene, the nuclear transcription factor kappa-B (NF-κB) activation was measured. **Results:** We found that intravenous PG increased the mortality rate in sepsis induced by the bacterial endotoxin lipopolysaccharide (LPS) in mice. In accordance with that, PG enhanced LPS-induced production of inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) *in vivo*. PG also increased the LPS-induced macrophage activation in vitro as detected by measuring NF-κB activation. **Conclusion:** Our results indicate that drugs containing high doses of PG can pose a risk when administered to patients suffering from or prone to Gram negative bacterial infection.

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INTRODUCTION

Propylene glycol (PG) is a colorless, odorless, slightly sweet, viscous liquid with a molecular weight of 76.1 Daltons. PG is a relatively efficient solvent of hydrophobic molecules, while it readily mixes with water, therefore, it can be used as a vehicle for administering poorly water soluble compounds. PG itself is practically non-toxic, however, ingestion of sizable amount (over 100 ml) may cause some gastrointestinal upset and temporary central nervous system depression. The LD50 of PG is very high in animals: i.e. rat (*oral*) = 20 g/kg, rat (*i.v.*) = 68 g/kg, dog (*oral*) = 22 g/kg. Furthermore, its primary metabolite, pyruvate is an endogenous molecule. PG was initially thought to be innocuous and is approved by the United States Food and Drug Administration for use as a solvent in food, drugs, and cosmetic products (1).

PG is an excellent vehicle for several *i.v.* drugs, including lorazepam, diazepam, etomidate, phenytoin, nitroglycerin, hydralazine, esmolol, phenobarbital, trimethoprim-sulfamethoxazole, and chlordiazepoxide, just to mention of few of them (2). Despite its outstanding reputation of safety, PG toxicity has been reported, mostly due to inappropriate dosage for exceptionally sensitive individuals. Among the noted adverse effects of PG, hyperosmolality, hemolysis, seizure, and in the worst cases, coma were recorded. In addition, PG toxicity can either mimic sepsis or systemic inflammation together with lactic acidosis, hypotension, and multisystem organ dysfunction (3). Patients are suspected of having propylene glycol toxicity if they have been exposed to high doses of lorazepam or other medications containing PG and show sepsis-like symptoms with negative infectious particles. PG is metabolized in the liver to lactate, acetate and pyruvate by a mechanism not entirely clarified, while the surplus is secreted unchanged in the urine. Upon exposure to excessive doses, proximal tubular secretion decreases and renal clearance slows down. Thus, risk factors for toxicity include a high infusion rate, large cumulative dose, concomitant use of other drugs containing PG, as well as pre-existing hepatic and renal insufficiency (4).

The bacterial lipopolysaccharide (LPS) found in the outer membrane of various Gram-negative bacteria is a typical example of endotoxin. The endotoxins are released upon bacterial cell lysis and elicit a strong response activating various components of the immune cells (5). LPS is thought to be responsible for initiating host responses leading to septic shock. Circulating endotoxin appears to be present in most patients who meet classical clinical criteria for sepsis (6). LPS administration in animals activates a signaling cascade analogous to sepsis. However, identification of mediators in animals, the blockade of which has been protective, has not translated into clinical efficacy in septic humans (7).

The origin of the paper was an observation rather than a theoretical consideration. Briefly, during our experiments we observed that high amounts of propylene glycol used as a solvent of experimental drug candidates interfered with the outcome of experimental sepsis in mice. In this paper our aim is to evaluate the interaction of PG and LPS in both *in vivo* and *in vitro* experiments.

MATERIALS AND METHODS

Reagents. PG, sodium lactate, sodium pyruvate, and LPS (*E. coli* 055:B5) were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in phosphate-buffered

saline (PBS). Recombinant human TNF-α, used as a positive control in our bioassays, was cloned and purified from E. coli in our laboratory (8), then it was aliquoted in 5×10^5 U/ml concentrations and stored at -85°C. One mg of human TNF-α protein is equivalent to 1×10^7 biological unit (U). One mg of mouse TNF-α protein is equivalent to 5×10^7 biological unit (U). 1 unit is defined as the amount of TNF-α required to mediate half-maximal cytotoxicity in WEHI 164 cells in the presence of actinomycin D. Recombinant human IL-6 was produced by a melanoma cell line in our laboratory. One mg of mouse IL-6 is equivalent with 10^8 biological unit (U). One unit of IL-6 activity is defined as the activity inducing half-maximal proliferation of *B9* cells.

Cell lines. NF-κB-luc/RAW264.7 stable cell line was obtained by transfecting RAW264.7 macrophages with pNF-κB-luc/Neo plasmids coding for an NF-κB reporter construct as described previously (9,10). NF-κB-luc/RAW264.7 cells were cultured in MIX MEM culture medium (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12) completed with 10% (v/v) FCS at 37°C in a humidified 5% CO_2-containing air environment. WEHI 164 mouse fibrosarcoma cell line, extremely sensitive to the cytotoxic effect of TNF-α, was cultured in MIX MEM culture medium. Murine IL-6-dependent hybridoma B9 cell line was cultured in RPMI 1640 medium with 5% FCS in the presence of IL-6 (100 pg/ml) and 50 μM mercaptoethanol (11).

Luciferase Assay. NF-κB-luc/RAW264.7 cells were seeded in 96 well plates (3 x 10^4 cells/well in MIX MEM, 10% FCS). Cells were treated with LPS (100 ng/ml) together with various concentrations of PG in 100 μl of the above medium per well. After 6 h incubation with LPS and/or PG, the medium was removed, and the cells were washed and lysed for 10 min at room temperature in Reporter Lysis Buffer (20 μl/well; Promega Bio-Science Hungary, Budapest, Budapest). We used 6 h incubation period, when, according to our preliminary experiments, the maximal luciferase expression was found (9). Then substrate was added (20 μl/well; Promega), and luciferase activity was measured in a Luminoskan Ascent (Thermo Electron Corporation, Waltham, MA) scanning luminometer. Other cells received only LPS (100 ng/ml) treatment or various concentrations of PG and were processed as mentioned above. Cell viability was routinely checked using Trypan blue exclusion test during the assays to make sure that assays were always carried out on viable cells.

Survival Studies. Two different mouse strains were used for toxicological experiments: the CD1 strain, widely used in toxicology experiments, and the inbred *Balb/c* strain, supposed to have an inherently lower variability. Female and male mice weighing, in average, 32 g, were maintained in a conventional animal facility under controlled environment (25 ± 2°C) and were provided standard animal food pellet and water *ad libitum*. All animal experimental protocols were performed in accordance with institutional and national animal welfare guidelines and approved by our institutional review committee, then the responsible governmental agency. The animals were housed individually in plastic cages. To estimate the LD50 values of LPS we used the conventional "up and down" procedure (12). Mice were treated with a LD50 dose of LPS *E. coli* 055:B5, 3 mg/kg b.w. *i.p.* for CD1 mice and 1 mg/kg b.w. *i.p* for *Balb/c* mice. Mice were also treated with either 2 g/kg b.w. *i.v.* PG or 5 g/kg b.w. *i.v.* PG, diluted at 1/1 with PBS. Survival diagram was recorded and the data were evaluated with the log rank test and Fisher's Exact test.

TNF-α and IL-6 Production in LPS-Treated Mice. To determine the kinetics of the TNF-α and IL-6 production, *Balb/c* mice were treated with LPS (1 mg/kg b.w., *i.p.*) and the serum was collected in 1, 2, 3 and 4 hrs after administration.

To assess the effect of PG on LPS-induced sepsis, m*ice* were separated into two groups*:* a) first group was treated with LPS alone (1 mg/kg b.w., *i.p.*) b) second group was treated with LPS (1 mg/kg b.w., *i.p.*) and PG (5 g/kg b.w., *i.v.*). Four mice were in each group. Mice were anesthetized with diethyl ether and blood samples were collected via cardiac puncture in 1.5 ml micro centrifuge tube. Blood samples were collected after 1 h of the treatment for TNF-α measurement and after 2 h of the treatment for IL-6 measurement. After the coagulation, the blood sera were separated by centrifugation and stored at 4°C for IL-6 and TNF-α detection.

TNF-α Cytotoxicity Assay. WEHI 164 mouse fibrosarcoma cells, extremely sensitive to TNF-α cytotoxicity, were used to measure the TNF-α bioactivity in the media from the RAW264.7 macrophages. WEHI cells were grown in 96-well plates at a density of 3×10^4 cells/well. After 24 h of incubation, serially diluted mouse sera were added to the cells in the presence of 1 μg/ml Actinomycin D. After 16-24 h of incubation the viability of cells was determined using the XTT colorimetric assay. TNF-α levels were estimated by evaluation of the sera dilution resulting in 50% reduction of cell survival and comparison with a concentration–cell survival curve for standard TNF-α.

B9 Hybridoma Proliferation Assay for IL-6. IL-6 levels in the samples from the sera of LPS-treated mice were determined by the IL-6-dependent leukemia B9 cell line. B9 cells were cultured in RPMI 1640 medium with 5% FCS in the presence of IL-6. For testing the IL-6 concentration, B9 cells were washed twice with IL-6-free medium and put into 96 well plates at a density of 3×10^4 cells/well. Different dilutions of IL-6 containing sera were added, and then cells were cultured for 72 h at 37°C. The proliferation of B9 cells after the incubation period was measured with the XTT colorimetric assay. The biological effect of IL-6 was estimated by evaluation of the sera dilution resulting in 50% increase in cell proliferation and comparison with a concentration-cell survival curve for standard TNF-α. The proliferation curve showing the half effective dose of IL-6 standard was used to determine the biological efficacy.

Determination of the Cytokine Profile. *Balb/c* mice (6-8 weeks old) were injected with graded concentrations of LPS *i.p.*, and 1 h later they received *i.v.* injections of PG diluted with PBS to at least 1:1. Six hours after the LPS injection the mice were anesthetized with diethyl ether, serum samples were collected and peritoneal cells were isolated according to the method described by Ray and Dittel (13). Peritoneal cells containing different immune cell populations (macrophages, T cells and B cells) were seeded at a density *of* 1 million cells*/ml in* 96-well plates and incubated at 37°C with 5% CO2 in RPMI medium supplemented with 10% FCS. After 24 h of incubation, the supernatants were collected. The amount of IL-6, IL-4, IL-10, IFN-γ and IL-17A was assessed by the flowcytometry-based cytokine bead array (CBA; BD Biosciences).

RESULTS

Mortality of Mice after LPS Injection. In the representative experiment performed on male and female CD1 mice, LPS caused the death of 11 animals out of 15 (Figure 1A). By the end of a one-week observation period all the surviving animals had completely recovered. The PG treatment accelerated the death of the LPS treated animals. By the third day, no one was living in this group. The difference between the survival times, as well as the ratio of surviving animals was statistically significant. The experiment was

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repeated with similar results in CD1, as well as in *Balb/c* mice (Figure 1B). PG treatment alone did not cause death.
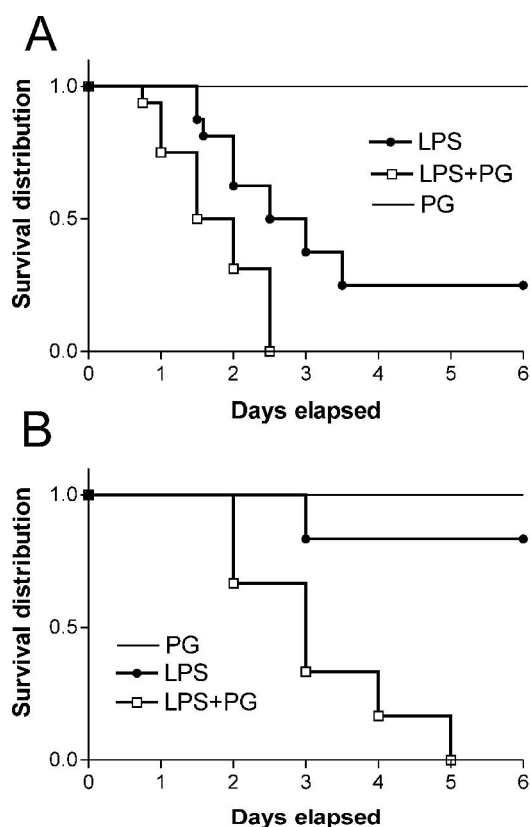
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B

Figure 1. The effect of PG on the LPS-induced lethality. A. Male and female CD1 mice were injected i.p. with 3 mg/kg b.w. LPS. One hour later, a group of mice received an additional intravenous injection of 2 g/kg b.w. PG diluted with PBS at 1/1 ratio, and then the mice were monitored. The proportion of mice surviving after treatment with LPS (n=15, thick line, black circle) LPS + PG (n=16, thick line, open square) or PG (n=6, thin line) was plotted. B. Male and female Balb/c mice were injected i.p. with 1 mg/kg b.w. LPS and/or 5 g/kg b.w. PG diluted with PBS at 1/1 ratio, and then they were monitored. The proportion of mice surviving after treatment with LPS (n=6, thick line, black circle) LPS + PG (n=6, thick line, open square) or PG (n=6, thin line) was plotted. In both cases, the survival analysis was performed with log rank test. The tests showed significant difference between the survival curves of mice treated with LPS versus LPS+PG. p<0.05. The ratio of the surviving and nonsurviving mice was also statistically different between the two groups (Fisher's Exact test, p<0.05). The experiment was repeated in both strains with similar results.

Measurement of NF-κB Activation in RAW264.7 Macrophages. LPS increased the activated NF-κB-dependent luciferase expression in pNF-κB-luc/RAW264.7 cells in a dose dependent manner up to 100 ng/ml LPS concentration (data not shown). Over the concentration of 100 ng/ml the additional LPS did not evoke further luciferase expression. However, PG could dose-dependently increase the maximum NF-κB activation. PG alone had minimal and not significant effect on NF-κB activation (Figure 2A).

PG administered to animals is oxidized by alcohol dehydrogenase to lactaldehyde, then to lactate by aldehyde dehydrogenase. Lactate is further metabolized to pyruvate, carbon dioxide, and water (14). The PG metabolites have minimal effect on LPS-induced NF-κB dependent luciferase activity (Figure 2B and -C). Both lactate and pyruvate treatments at 5 mg/ml concentrations led to small, but significant increase. However at the highest used concentration i.e., 10 mg/ml, we measured lower luciferase activity.
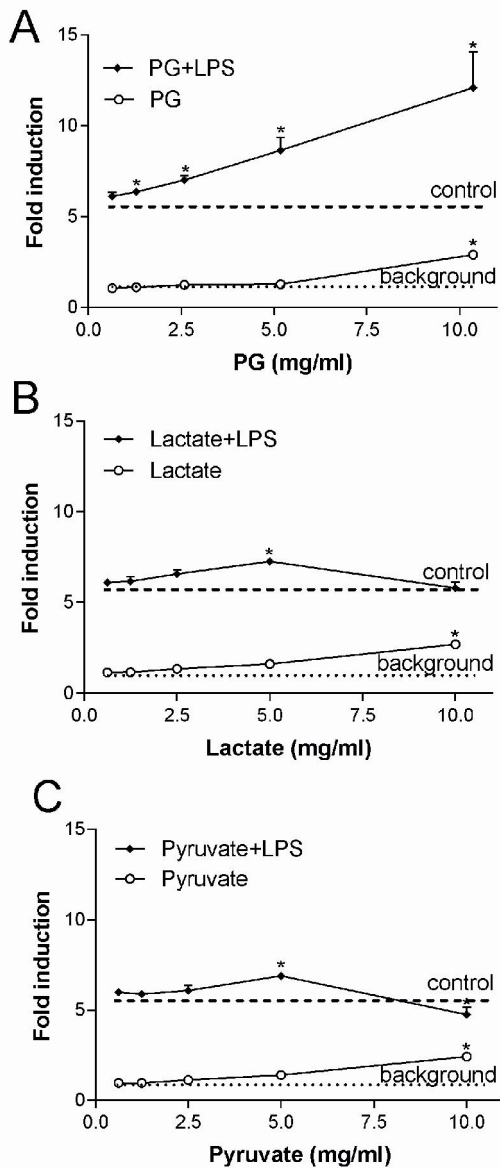
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Figure 2. NF-κB-dependent luciferase expression in macrophages. A. PG dose-dependly increased the 100 ng/ml LPS evoked luciferase expression after 6 h of treatment. Cells were treated with various concentrations of PG and 100 ng/ml LPS (black rhombus), with 100 ng/ml LPS (dashed line), with PG (open circle) or with vehicle (dotted line). PG alone has minimal and not significant effect on NF-κB activation compared with vehicle control. B. Lactate showed minimal effect on the LPS-induced NF-κB-dependent luciferase expression in NF-κB-luc/RAW264.7 cells after 6 h of treatment (black rhombus). 100 ng/ml LPS was used as control (dashed line). C. Pyruvat had a minimal effect on LPS- induced NF-κB activity after 6 h of treatment (black rhombus). 100 ng/ml LPS was used as control (dashed line). In all cases, data are mean±S.D. Statistical significance was determined by paired *t-test*. Asterisks (*) show significant changes (p<0.05).

Determination of TNF-α and IL-6 Levels in Serum. In mice treated with 1 mg/g body weight LPS, TNF-α production peaked 1h after *i.p.* LPS infusion, dropped significantly by 2 h, and was practically undetectable at the 3 and 4 h time points (data not shown). After 1 h of administration of LPS, serum TNF-α level were significantly higher in mice co-treated with PG (Figure 3A). Serum levels of IL-6, another pro-inflammatory cytokine, were also measured. IL-6 production had a 1-2 hours delay compared to TNF-α (data not shown). Similar result was presented by Fejer and coworkers (15). Serum IL-6 levels were significantly higher in animals treated with LPS and PG than in those treated with LPS alone (Figure 3B).
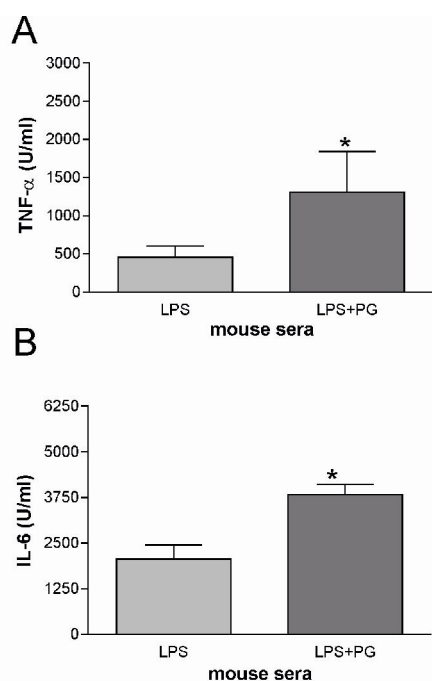
Figure 3. The effect of PG on the LPS-induced cytokine production in vivo. A. Mice were treated with LPS (1 mg/kg b.w., i.p.) alone or together with PG (5g/kg b.w., i.v.) for 1 h. The TNF-α content in serum was determined using WEHI 164 indicator cells. In both cases, data are mean ± S.D. (*p<0.05). B. Mice were treated with LPS (1 mg/kg b.w., i.p.) alone or together with PG (5 g/kg b.w., i.v.) for 2 h. The IL-6 content of the serum was determined with the B9 hybridoma proliferation assay (Figure 5B). In both cases, data are mean±S.D. (*p<0.05).

Cytokine Profile of the Treated Animals. We compared the cytokine production of BALB/c mice treated with graded amounts of LPS (0, 0.5, 1 and 5 mg/kg b.w.) and PG (0, 1, 2 and 3 g/kg b.w.). The cytokine profile was determined both in mice sera and the supernatants of peritoneal cells derived from the treated animals. Only IL-6, and not IL-2, IL-4, IL-10 and IL-17a, was consistently measurable in samples. The IL-6 secretion of isolated and overnight cultured peritoneal cells correlated with blood cytokine level of the tested animals. In this experiment, we also detected an aggravating effect of PG treatment on the LPS- induced IL-6 release (Figures 4A and 4B). Two sera and four supernatants of peritoneal cells out of 12 LPS-treated mice showed a slight but significant increase in IFN-γ content.
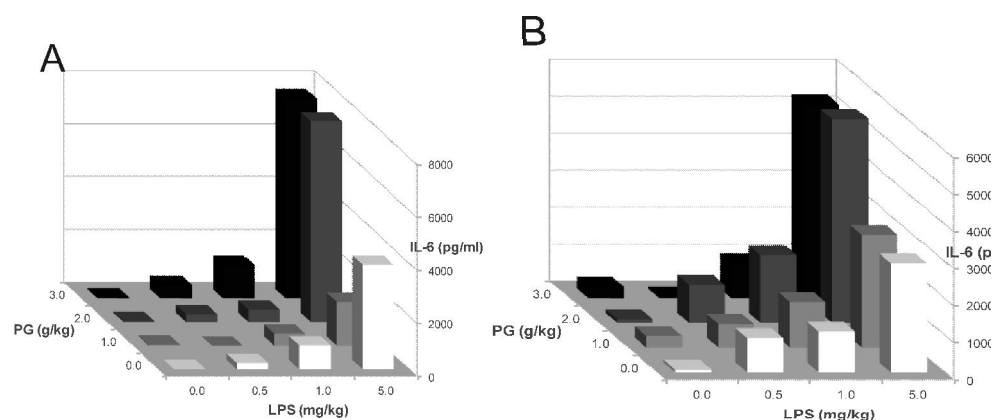

Figure 4. IL-6 secretion in mice treated with different concentration of PG and LPS. **A.** Three-dimensional bar graph represents the level of IL-6 production measured in mouse sera after 6 h of LPS administration. **B.** IL-6 production of peritoneal cells derived from treated mice. Each column represents the IL-6 secretion of one individual mouse. In both cases, PG dose-dependly increased the LPS-evoked IL-6 production.

DISCUSSION

In this paper we used *in vitro* and *in vivo* models and demonstrated that PG aggravates the effects of LPS inducing more excessive macrophage activation, more severe toxicity, earlier deaths, and a lower survival rate. Interestingly, the sole previous report on the connection between PG and macrophage activation has shown how PG binds a macrophage activating bacterial product, staphylococcal lipoteichoic acid, thus preventing TLR-2 dependent macrophage activation (16).

PG has been considered a safe and useful drug vehicle (17), However, PG toxicity can mimic either sepsis or systemic inflammatory response syndrome (SIRS). The SIRS with proven infection is referred to as sepsis (18). At this point it is difficult to distinguish PG toxicity from sepsis and SIRS. SIRS and sepsis are still an unmet medical need and one of the major causes of in-hospital morbidity and mortality (19). If PG-related hypotension is incorrectly diagnosed, the patient can rapidly progress toward acidosis, and eventually, multisystem organ dysfunction may occur.

The mechanism of high dose PG toxicity is still entirely unknown. When PG is administered *in vivo*, 55-75% is metabolized to pyruvate, acetate, and lactate (14,20,21), while 25-45% remains unchanged and appears in the urine. A possible point of convergence between sepsis and PG might be lactic acidosis. Gore *et al.* have demonstrated that the development of lactic acidosis during sepsis resulted from the markedly increased rate of pyruvate production (22). Both pyruvate and lactate are long known end products of PG (23). It was found that sodium lactate and LPS synergically increase NF-κB activation in U937 histiocytes (tissue macrophages) (24). Acid metabolites can shift extracellular pH and aggravate sepsis by release of other tissue damaging cytokines that may damage the kidney function and eventually result in multisystem organ dysfunction. It has been revealed that repeated exposure to PG causes cell injury in a primary culture of human proximal tubule cells (25) and may explain how prolonged use of PG can cause renal insufficiency *in vivo*. We found that PG increases the LPS-induced macrophage activation. This process can also contribute to the renal failure, because it has been shown that macrophage activation increasing the level of the circulating extracellular histon concentration leads to renal dysfunction (26). Out of the 128 patients who received continuous-infusion of lorazepam containing PG as a solvent, eight were diagnosed with increased concentrations of serum creatinine, a biomarker of worsening renal function (27). The fact that oral LD50 is lower than the i.v. LD50 for propylene glycol (rat (*oral*) = 20 g/kg, rat (*i.v.*) = 68 g/kg) may point to its effect on the gut mucosa. A mucosal damage and the following bacterial translocation might be a severe condition by itself, and it may also increase the severity of sepsis induced by external LPS (28).

LPS is one of the prominent endotoxin byproducts from gram-negative bacteria, which can induce sepsis, *in vivo*, and is also used in our models to induce systemic life-threatening SIRS. Still it was surprising how well the levels of circulating inflammatory cytokines such as TNF, IL-6, IL-1 correlated with the probability of sepsis and subsequent early death (29). We also found that PG increases the LPS-induced NF-κB activation, the signaling event leading to inflammatory cytokine production. Some of the above mentioned "inflammatory soup" components release NF-κB from the cytosol. The active NF-κB translocates to the nucleus and binds to promoter regions of genes coding for inflammatory mediators that include iNOS, COX-2, TNF-α, IL-1β, IL-6 and

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IL-8 (30). In addition to acute phase inflammatory response genes, NF-κB up-regulates various genes involved in immune response and in apoptosis (30).

Nevertheless, patients suffering from Gram negative bacteria-induced sepsis can be exposed to an unnecessary risk of toxicity or a potentially life-threatening condition if the drug is dissolved in PG.

ACKNOWLEDGEMENTS

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