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Effects of Sizofiran on Endotoxin-Enhanced Cold Ischemia-Reperfusion Injury of the Rat Liver

M. KUKAN, Z. SZATMÁRY, M. LUTTEROVÁ, D. KUBA, K. VAJDOVÁ, J. HORECKÝ

Laboratory of Perfused Organs, SCOT, Institute of Preventive and Clinical Medicine, Bratislava, Slovak Republic

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Summary

Kupffer cells (KC), resident macrophages of the liver, have been strongly implicated in lipopolysaccharide (LPS)-induced liver graft injury. However, our recent study showed that sizofiran (schizophyllan glucan) (SPG), which activates KC, did not influence cold ischemia-reperfusion liver injury of LPS-exposed rats. Here we investigated some mechanisms by which SPG does not aggravate LPS-enhanced cold ischemia-reperfusion rat liver injury. Control and SPG-treated rats were exposed to LPS for 2 h prior to hepatectomy. The livers were cold-preserved in University of Wisconsin solution followed by reperfusion with Krebs-Henseleit buffer. We found that SPG dramatically inhibited LPS-induced increases of tumor necrosis factor-α (TNF-α) in the plasma and bile *in vivo*. Moreover, LPS-induced TNF-α release into the washout solution after cold ischemia was also abrogated by SPG pretreatment. However, SPG increased TNF-α release into the perfusate after reperfusion. On the other hand, SPG completely abolished expression of c-myc protooncogene, which is known to sensitize cells to TNF-α cytotoxicity. In conclusion, inhibition of both TNF-α release after LPS challenge and c-myc expression may explain why activation of KC with SPG does not aggravate endotoxin-enhanced cold ischemia-reperfusion liver injury.

Key words

Liver • Cold ischemia-reperfusion • Kupffer cells • Sizofiran (Schizophyllan glucan) • Tumor necrosis factor-α • c-myc protooncogene

Introduction

Kupffer cells (KC), resident macrophages of the liver, are activated after the exposure of rats to *Escherichia coli* lipopolysaccharide (LPS) (Sarphie *et al.* 1996) as well as upon reperfusion of cold-stored rat livers (Lemasters and Thurman 1997). Activated KC release vasoactive agents and highly active mediators (Decker 1990) and have thus been strongly implicated in LPS-induced graft injury. We recently provided direct evidence that LPS exposure of donor rats prior to liver harvest impaired hepatic graft function in a blood-free reperfusion model (Vajdová *et al.* 2000a). In addition, we showed that inactivation of KC or co-administration of LPS with drugs downregulating KC function (e.g. pentoxifylline or heparin) markedly protected the liver. Other studies showed that KC activation with schizophyllan glucan (SPG) increased tissue injury

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

following cold ischemia-reperfusion of the liver in endotoxin-free rats (Shibuya *et al.* 1997a,b). These findings suggested that 1) KC-dependent mechanism(s) were involved in both LPS-enhanced cold ischemia-reperfusion liver damage as well as in cold ischemia reperfusion liver injury, and that 2) an increase of KC activity by immunomodulators should potentiate cold ischemia-reperfusion liver injury in LPS-exposed liver donors.

However, a recent study had shown that the immunomodulator SPG, which activates rat hepatic macrophages (Noguchi *et al.* 1989), did not influence significantly LPS-enhanced cold ischemia-reperfusion rat liver damage (Kukan *et al*., unpublished results). Indeed, bile production (0.90±0.05 vs. 0.88±0.06 μl/min/g liver (mean of 6 livers ± S.E.M.)), an index of global hepatic function, and lactate dehydrogenase release (17.05±2.04 vs. 11.78±2.1 units/l/g liver), as a marker of hepato-cellular membrane integrity, were not significantly different between the LPS-enhanced cold ischemia-reperfusion and the SPG + LPS-enhanced cold ischemia-reperfusion groups of livers. Therefore, the aim of this study was to investigate some mechanisms of SPG in a model of LPS-enhanced cold ischemia-reperfusion liver injury in the rat.

Methods

Animals

Male Wistar rats, weighing 260-390 g, obtained from Velaz (Prague, Czech Republic) were used. The animals were housed in an air-conditioned room at 22 °C and fed standard rat chow and water *ad libitum* up to the time of the experiments. The study was approved by the local animal welfare committee.

Liver harvest and liver perfusion

Pentobarbital sodium (50 mg/kg) was given i.p. two hours after LPS treatment to induce anesthesia before surgery and the liver was prepared as described (Kukan 1999). Briefly, the bile duct was cannulated with polyethylene PE 50 tubing and 250 IU of heparin was injected intravenously. The portal vein was then cannulated with PE 240 tubing and the hepatic artery was ligated. The liver was flushed out *in situ* through the portal vein with 30 ml of cold (4 °C) University of Wisconsin solution (DuPont Pharmaceuticals, The Netherlands) at constant pressure of 12 cm H_2O. The liver was then excised and transferred to a polyethylene bag containing 40 ml of University of Wisconsin solution and stored at 1 °C for 18 h. It should be noted that 18-h cold ischemic model was chosen since our previous studies showed that liver function and liver injury are comparable to the control livers perfused immediately after hepatectomy (Kukan *et al.* 1997, Vajdová *et al.* 2000b). After cold ischemic storage, the liver was washed with 40 ml of warm (37 °C) Ringer-lactate solution (the washout solution was left for cytokine assay). The liver was then immediately perfused through the portal vein in a recirculating perfusion system (Kukan *et al.* 1997, Kukan 1999). As a perfusion medium, Krebs-Henseleit bicarbonate buffer (200 ml, pH=7.4), containing glucose (10 mM) and saturated with 95 % oxygen and 5 % carbon dioxide (inflow pO_2=400-500 mm Hg; assessed by 248 pH/Blood Gas Analyzer (Ciba Corning Diagnostics Limited, UK) was used. The liver was reperfused for 75 min. At the end of the reperfusion, liver samples were clamped using Wollenberger clamps precooled with liquid nitrogen and subsequently stored at –70 °C. Frozen tissue samples were homogenized by grinding to a powder in a mortar with pestle under liquid nitrogen for RNA isolation (see below).

Experimental design

To assess the effects of SPG on LPS-induced TNF-α (proinflammatory cytokine) and interleukine-10 (IL-10) (antiinflammatory cytokine) levels 2 h after LPS exposure, three groups of livers were studied: LPS (pretreated with saline 2 ml/kg i.v., 24 h later LPS 4 mg/kg i.v.) (n=5); SPG (pretreated with SPG 10 mg/kg i.v., 24 h later LPS 4 mg/kg i.v.) (n=5); and control (untreated rats) (n=4). Two hours after LPS treatment, rats were anesthetized by pentobarbital. The abdomen was opened and bile duct was cannulated. Bile samples were collected for a period of 10 min. Blood samples were taken from the vena cava and cytokines were determined in the plasma and bile samples as given below.

To determine the effects of SPG on LPS-induced TNF-α and IL-10 levels after 18-h cold ischemia (CI) and after reperfusion, the following groups of livers were investigated: CI (hepatectomy, cold storage for 18 h in UW solution followed by perfusion) (n=6); LPS + CI (4 mg/kg of LPS i.v., 2 h later hepatectomy, cold storage in UW solution for 18 h followed by perfusion) (n=6); and SPG + LPS + CI (SPG 10 mg/kg i.v., 24 h prior to 4 mg/kg of LPS i.v., 2 h later hepatectomy, cold storage in UW solution for 18 h followed by perfusion) (n=6);

LPS was dissolved in sterile saline and administered i.v. in doses of 2 ml/kg.

To assess the effects of SPG on long-term liver function after LPS challenge, two additional groups of livers were investigated in a third set of experiments: LPS (pretreated with saline 2 ml/kg i.v., 24 h later LPS 4 mg/kg i.v.) (n=5); and SPG (pretreated with SPG 10 mg/kg i.v., 24 h later LPS 4 mg/kg i.v.) (n=5). Twenty four hours after LPS administration, bile production, as a global sign of liver function, was measured during a 15-min period. Besides cytokine measurements, plasma was analyzed for aspartate aminotransferase activity, as a marker of liver injury.

Assays

TNF-α in the plasma, bile, the washout solution and in the reperfusion medium were measured by ELISA. Monoclonal hamster anti-murine TNF-α or purified anti-mouse/rat TNF-α monoclonal antibody was used as capture antibody, while biotinylated anti-mouse/rat TNF-α was used as detection antibody. Recombinant TNF-α was used as a standard. Disposable 96 well ELISA plates, easy wash™, (Corning Costar, New York, NY) were used in the procedure and ELISA tests were performed according to the manufacturer's instructions. The detection limit was 5 pg/ml.

IL-10 was determined using an ELISA set (catalogue No. 2611KI, Pharmingen, San Diego, CA) according to the manufacturer's instructions. The detection limit was 5 pg/mL.

RNA isolation was carried out according to the modified method of Chomczynski and Sacchi (1987). After the last precipitation, the obtained RNA was washed with a cold 75 % ethanol, dried in a vacuum and dissolved in DEPC-treated water. The amount of RNA was quantified in the Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, UK). The quality of the isolated RNA was checked by visualization of ethidium bromide stained 1 % agarose gels run in Tris-borate EDTA buffer, pH 7.8. Non-degraded RNA was selected for further processing.

Expressions of the cytokines and those of c-fos, c-jun, and c-myc mRNAs were performed by reverse-transcriptase polymerase chain reactions as described in details by Lutterová *et al*. (2000) using 1.3 μg of total cellular RNA.

Chemicals

Escherichia coli LPS, serotype 0128:B12 and sodium taurocholate were obtained from Sigma Chemical Co. (St. Louis, MO). Heparin was purchased from Léčiva (Prague, Czech Republic). Sizofiran (schizophyllan glucan) (SPG) was kindly provided by Kaken Pharmaceutical Co. Ltd., (Tokyo, Japan). Monoclonal hamster anti-murine TNF-α and biotinylated anti-mouse/rat TNF-α were purchased from Genzyme (Cambridge, UK). Purified anti-mouse/rat TNF-α monoclonal antibody and recombinant TNF-α were from Pharmingen (San Diego, CA). Horseradish peroxidase - streptavidin conjugate was purchased from GibcoBRL (Rockville, MD). All other chemicals and substances were of the highest commercially available purity.

Treatment of data and statistical analysis

Values are given as mean ± S.E.M. The data were analyzed by ANOVA followed by the Bonferroni correction test for multiple comparisons. A difference was considered significant when the $P<0.05$.

Results

Effects of SPG on LPS-induced TNF-α and IL-10 levels in rat plasma and bile in vivo

Plasma and biliary levels of TNF-α and IL-10 are shown in Figures 1A and 1B, respectively. As seen, plasma levels of TNF-α were dramatically reduced by KC activation with SPG (Fig. 1A). Similarly, biliary TNF-α decreased in SPG-treated animals. Interestingly, Figure 1AB also shows that changes in TNF-α levels mirrored changes of IL-10 concentrations.

Effects of SPG on TNF-α and IL-10 release by livers after cold ischemia in LPS-exposed rats

As seen in Figure 2, LPS-induced TNF-α release, while SPG blunted the LPS effect. There was no change in IL-10 levels, indicating that SPG minimized the ratio of TNF-α to IL-10 during cold ischemia.

Effects of SPG on TNF-α and IL-10 release by livers and on expression of mRNAs for cytokines and for c-fos, c-jun, and c-myc after cold ischemia-reperfusion in LPS-exposed rats

Figure 3A shows that LPS increases concentrations of both cytokines in the reperfusion medium after cold ischemia-reperfusion and that activation of KC with SPG potentiates the LPS effect. In accordance with these results, expression of mRNAs for cytokines was magnified mainly in the SPG + LPS + CI

group of livers (Fig. 3B). Expression of mRNAs for immediate early genes, c-fos, c-jun and c-myc was intensified in the LPS + CI group in comparison with the CI group. In the SPG + LPS + CI group, expression of mRNAs for both c-fos and c-jun was comparable to that of the CI group, while the expression of mRNA for c-myc was completely inhibited by SPG (Fig. 3B).

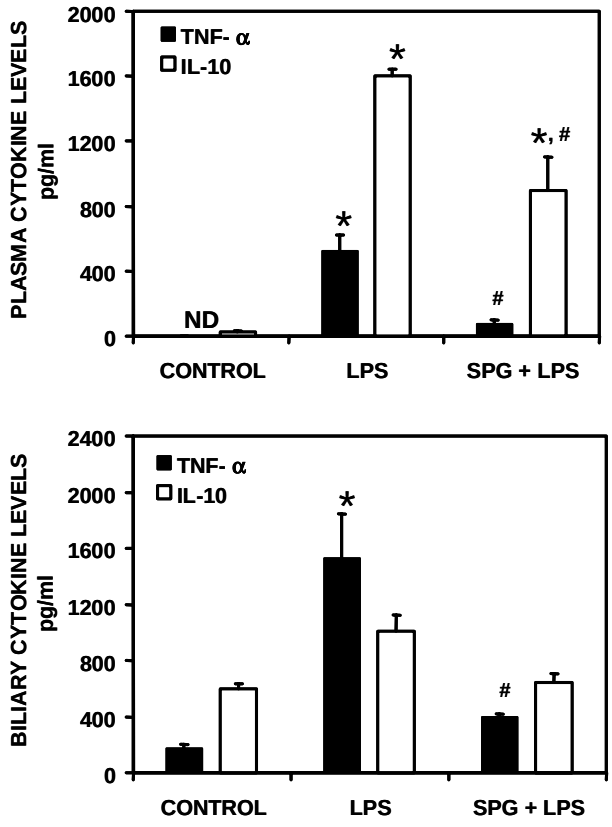

Fig. 1. TNF-α and IL-10 plasma (upper panel) and biliary (lower panel) concentrations in vivo 2 h after LPS challenge (just prior to liver harvesting). Data represent mean ± S.E.M. of 4-5 experiments. Significantly different: TNF-α: *(LPS or SPG + LPS vs. control), #(LPS + SPG vs. LPS). IL-10: *(LPS or SPG + LPS vs. control), #(LPS + SPG vs. LPS).

Effects of SPG on rat liver function in vivo 24 h after LPS exposure

To address the question whether SPG aggravates LPS-induced liver injury after long-term exposure, two groups of rats were investigated 24 h after LPS administration. As seen in Table 1, neither TNF-α nor IL-10 were detected after long-term LPS exposure. Aspartate aminotransferase, an index of hepatic injury *in vivo*, did not differ in the two groups studied. Neither was a difference found in bile production, suggesting that activation of KC does not aggravate liver injury after long-term LPS exposure.

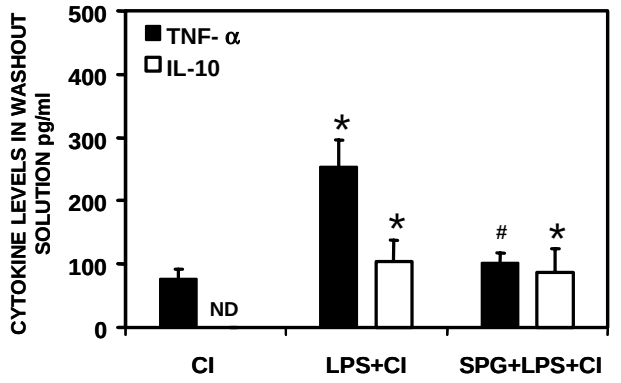

Fig. 2. TNF-α and IL-10 release into the washout solution after 18 h of cold ischemia. Livers were washed with 40 ml of Ringer lactate solution and were obtained from either untreated rats (CI) or pretreated rats with 4 mg/kg of LPS 2 h before hepatectomy. SPG was administered 24 h before LPS challenge. Data represent mean ± S.E.M. of 6 experiments. Significantly different: TNF-α: *(CI vs. LPS + CI or SPG + LPS +CI), #(SPG + LPS + CI vs. LPS + CI). IL-10: *(CI vs. LPS + CI or vs. SPG + LPS + CI).

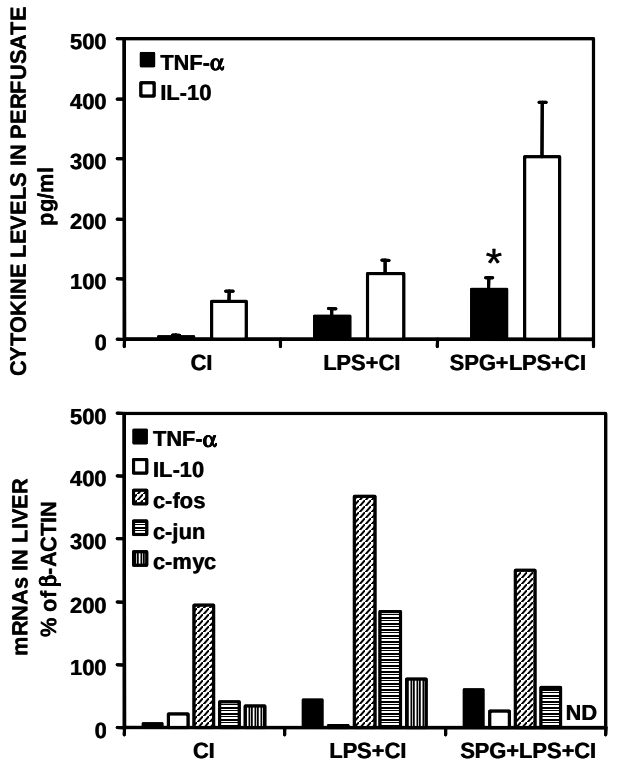

Fig. 3. TNF-α and IL-10 release into the perfusate (upper panel) and tissue expression of mRNAs for TNF-α, IL-10, c-fos, c-jun and c-myc (lower panel) in rat livers after cold ischemia-reperfusion. Livers were obtained from either untreated rats (CI) or rats pretreated with 4 mg/kg of LPS 2 h before hepatectomy and perfused after 18-h cold preservation in University of Wisconsin solution as described in Materials and Methods. SPG was administered 24 h before LPS challenge. Data represent mean ± S.E.M. of 6 experiments. Significantly different: TNF-α: *(SPG + LPS + CI vs. CI). IL-10: No significant differences were found between the groups studied.

Table 1. Effects of SPG on rat liver function in vivo 24 h after LPS exposure.

	TNF-α	IL-10	AST (units/l)	Bile production (μl/min/rat)
Control + LPS	N.D.	N.D.	25±3.9	16.1±0.79
SPG + LPS	N.D.	N.D.	26±2.5	16.4±0.89

Rats were pretreated with saline or SPG 24 h before LPS administration. Twenty-four hours after LPS administration, blood was withdrawn and analyzed for cytokines and aspartate aminotransferase (AST) levels. Bile production was measured over a 15-min collection period. Data represent mean ± S.E.M. of 5 experiments. N.D. not detected.

Discussion

The present results showed that SPG may exert its effects by decreasing TNF-α levels in plasma and bile following LPS challenge *in vivo* (Fig. 1) and by blunting TNF-α release during cold ischemia *ex vivo* (Fig. 2). In addition, the increased production of TNF-α in SPG-treated animals after reperfusion *ex vivo* (Fig. 3A) was compensated by abolishing c-myc protooncogene expression (Fig. 3B).

Since several of the pathophysiologic effects associated with LPS are mediated through TNF-α (Hewett *et al*. 1993, Jackson *et al*. 2000, Barton *et al*. 2001), here we tested the hypothesis that SPG decreased plasma TNF-α levels prior to cold ischemic storage of rat livers. The presented results are consistent with the findings of Vereschagin *et al.* (1998), Williams *et al.* (1999) and Bowers *et al.* (1989). In the former study, it was shown that KC activation with another soluble glucan, carboxymethyl-β-1,3-glucan, increased LPS clearance *in vivo* and reduced liver injury. Moreover, the immunomodulator was reported to decrease the LPS-induced TNF-α production in isolated macrophages *in vitro* (Vereschagin *et al.* 1998). In the latter studies, Bowers *et al.* (1989) and Williams *et al.* (1999) demonstrated that soluble glucan improved survival of animals in a model of polymicrobial sepsis. Importantly, one of the mechanisms by which glucan afforded protection to mice involved inhibition of mRNA synthesis for TNF-α in the liver after sepsis (Williams *et al.* 1999).

Our result showing that LPS increased TNF-α biliary levels suggests that LPS increased TNF-α expression in cells lining the biliary tree. This is consistent with the findings of other investigators. Indeed, Chen *et al.* (1997) demonstrated that LPS was capable of stimulating mRNA expression for TNF-α in isolated intrahepatic biliary epithelial cells and Jackson *et al.* (2000) showed that LPS increased TNF-α biliary levels 1.5 h after expossure of rats to LPS. A marked decrease in biliary TNF-α levels by SPG treatment suggests that SPG may protect the intestines from TNF-α-mediated toxicity. Two independent observations support this hypothesis. First, TNF-α infusion into the duodenal lumen caused intestinal damage similar to that elicited by intravenous LPS. Second, survival of rats treated with LPS was significantly increased after infusion of a TNF-α antibody into the duodenum (Jackson *et al.* 2000).

The liver was harvested 2 h after exposure of rats to LPS and reperfused following 18-h cold ischemia. This approach for testing LPS-enhanced cold ischemia-reperfusion injury of the liver seems to be appropriate as previous studies have shown that rat liver function is minimally altered 2 h after i.v. administration of 4 mg/kg of LPS (Moulin *et al*. 1996, Vajdová *et al.* 2000a) and after 18-h cold ischemic storage in University of Wisconsin solution (Kukan *et al.* 1997, Vajdová *et al.* 2000b). SPG was chosen to activate KC since it is a powerful inducer of macrophages in both rats and humans. Indeed, Noguchi *et al.* (1989) reported that rat hepatic macrophages were substantially activated 24 h after a 10 mg/kg SPG intravenous dose. Likewise, Chen *et al.* (1990) showed that SPG significantly increased the number of peritoneal macrophages by 24 h when given intramuscularly to humans.

Proteolysis occurs during cold ischemia and it may lead to TNF-α shedding from its transmembrane form (Mullberg *et al.* 2000). To study the effects of SPG on LPS-induced TNF-α shedding, we determined the cytokine level in the washout solution at the end of cold ischemia. Blunting TNF-α release with SPG suggests that KC may downregulate LPS-induced TNF-α proteolysis during cold ischemic storage of the rat liver.

The expression of immediate early genes has been hypothesized to play a key role in mediating cellular responses following injury to the liver (LaBrecque 1994). To see whether expression of immediate early genes was influenced by LPS in a blood-free perfusion model, we measured their tissue mRNAs. Exposure of rats to LPS prior to liver procurement potentiated expression of immediate early genes after cold ischemia-reperfusion, while SPG abolished LPS-induced effect. This result

suggests that KC may decrease expression of immediate early genes in a blood-free rat liver reperfusion model.

It is generally accepted that c-myc sensitizes cells to TNF-α cytotoxicity (Klefstrom *et al.* 1994). Blunting c-myc expression after cold ischemia-reperfusion also appears to be one of the mechamisms by which SPG may cause liver cells resistant to TNF-α cytotoxicity. This agrees well with our recent findings showing that high TNF-α release by the liver in the absence of c-myc expression is not able to induce liver damage in a blood-free perfusion model (Lutterová *et al.* 2000).

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Dr. Marián Kukan, Laboratory of Perfused Organs, SCOT, Institute of Preventive and Clinical Medicine, Limbová 14, 83301 Bratislava, Slovak Republic, Fax: +421-2-59369-195, e-mail: kukan@upkm.sk