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Tolerance of Mice to Lipopolysaccharide is Correlated with Inhibition of Caspase-3-mediated Apoptosis in Mouse Liver Cells

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Abstract Bacterial endotoxin lipopolysaccharide (LPS) often results in multiple organ failure. However, pre-exposure of mice to a sublethal dose of LPS renders the animal tolerant to a lethal dose of LPS. This study was designed to determine whether pre-exposure of a small dose of LPS was able to suppress apoptosis in mice when challenged with LPS in combination with *D*-galactosamine, and to investigate the expression changes of the apoptosis-associated molecules. The results showed that a characteristic apoptotic DNA fragmentation existed in mouse livers of the LPS-naive group, but not in control groups; and the mice of the LPS-naive group were all dead after 2 d. However, in the LPS-tolerance groups, both the lethal rate and apoptotic DNA fragmentation were suppressed after the mice were challenged with LPS/*D*-galactosamine, and the protection against the lethality and apoptotic reaction could be maintained for up to 7 d. In this period, significantly lower levels of caspase-3 and its mRNA appeared in LPS-tolerant groups compared to those of the LPS-naive group ($P<0.05$), and the caspase-3 activities gradually recovered as the observation was prolonged. Our findings suggest that LPS tolerance could suppress apoptosis in mouse liver cells, and the expression and activity of caspase-3 could be down-regulated.

Key words lipopolysaccharide; endotoxin; tolerance; apoptosis; caspase-3

Bacterial endotoxin lipopolysaccharide (LPS), a complex glycolipid, is composed of a hydrophilic polysaccharide moiety and a hydrophobic domain known as lipid A. LPS is a major component of the outer membrane of Gram-negative bacteria and one of the most potent microbial initiators of inflammation [1–3]. It has been shown that LPS activates monocytes and macrophages to produce proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), and interleukin (IL)-1, IL-6, IL-8, and IL-12. Macrophages also secrete, in response to LPS, a wide variety of other biological response mediators including platelet-activating factor, prostaglandins, enzymes, and free radicals, such as nitric oxide. Production of these inflammatory cytokines and mediators by monocytes/macrophages contributes to the efficient control of growth and dissemination of invading pathogens. However, excessive and uncontrolled production of these inflammatory cytokines and mediators may lead to serious systemic complications including microcirculatory dysfunction, liver and kidney damage [4,5], and septic shock with a high mortality [6]. When macrophages are treated with a sublethal dose of LPS, the cells become refractory to subsequent exposure to a high dose of LPS, which is known as endotoxin tolerance [7,8].

Apoptosis is an ATP-dependent cell death, morphologically characterized by chromatin condensation, nuclear fragmentation, cell shrinkage, and blebbing of the plasma membrane, and biochemically characterized by the activation of caspases, highly specific proteases that cleave a wide array of intracellular substrates. Activation of upstream caspases initiates a proteolytic cascade leading to DNA fragmentation and the cleavage of key regulatory proteins resulting in cell death. As an inflammatory factor [9,10], LPS plays an important role

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in pathogenesis through inducing apoptosis in several cell types [11,12].

We found in previous research that a characteristic apoptotic DNA fragmentation in the liver of affected mice was intimately correlated to lethality in mice that were challenged with LPS (0.05 mg) in combination with *D*-galactosamine (*D*-GalN) [13]. The exact molecular mechanisms attributed to the LPS-induced apoptosis, as well as LPS-induced tolerance, remain unknown.

For this reason, we intended to address, in this study, whether the tolerance of mice to LPS was correlated with the inhibition of caspase-3, which is believed to be involved in the induction of lethality and formation of tolerant status.

Materials and Methods

Reagents and mice

LPS was prepared from *Salmonella abortus equi* as previously described [14]. *D*-GalN hydrochloride was purchased from Sigma (St. Louis, USA). Six- to eight-week-old Kunming mice (18–20 g) of both sexes were obtained from the Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China) and used in this experiment. Mice were housed in stainless steel wire cages with free access to food and water. After a one-week equilibration period, the animals were randomly divided into different experimental groups. All experimental procedures were carried out in compliance with the regulations of the Shanghai Municipal Scientific and Technical Committee.

LPS-induced tolerant mouse model

The experimental mice were divided into nine groups (10 mice per group, except in the protection experiment). Mice in group 1–4 were injected with normal saline, 0.1 μg LPS, 2 μg LPS, or 20 mg *D*-GalN, respectively, as control groups. Those in the naive group (group 5) were challenged with LPS in combination with *D*-GalN directly. In LPS tolerance groups (group 6–9), mice were pretreated with a small dose of LPS (0.1 μg), and various periods later (3 h, 1 d, 2 d, and 7 d), they were challenged with LPS in combination with *D*-GalN. Six to seven hours after the last injection, the animal organs of interest were collected under ether anesthesia.

All reagents used in these experiments, including *D*-GalN and LPS, were dissolved in pyrogen-free normal saline. A standard volume of 200 μl was used throughout the study. Injections were usually intravenous in the lateral tail vein of the mice.

Protection of LPS tolerance

To detect the protection of LPS tolerance, mice were challenged with the reagent and grouped as above. The animals were continuously observed for 7 d, and the survival numbers of mice in different groups were counted. Thirty mice per group were used in this experiment.

Extraction of RNA-free DNA and electrophoresis

Mouse liver was homogenized in lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM EDTA, 0.5% sodium dodecyl sulfate) with 0.1 mg/ml of proteinase K. DNA was phenol/chloroform extracted and dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0) containing RNase (0.1 mg/L) at 37 ºC for 1 h. Subsequently, the DNA was extracted by phenol/chloroform and electrophoresed on 1% agarose in TAE buffer. The gels were photographed under UV light using the FR-980A electrophoresis image analysis system (FURI Science & Technology, Shanghai, China).

Caspase-3 activity

Caspase-3 activity was determined by a colorimetric activity assay kit (Chemicon, Temecula, USA). Briefly, after the animals' death at the indicated time points, murine livers were immediately dissected and frozen in liquid nitrogen. Frozen tissues were pulverized and lysed with lysis buffer provided by Chemicon. Caspase-3 activity was detected at 405 nm in a microtiter plate reader. In some assays, recombinant caspase-3 enzyme (Chemicon) was used as a positive control. Buffer alone and cell lysate alone were used as negative controls.

Extraction of total RNA and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from liver using RNArose reagent (Watson Biotech, Shanghai, China) following the manufacturer's instructions and treated with RNase-free DNase (TaKaRa, Dalian, China). For RT reaction, the avian myeloblastosis virus (TaKaRa) was applied. PCR amplification was carried out with *Taq* DNA polymerase (Shenenergy Biotech, Shanghai, China) for 30 cycles at 94 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 1 min in a PTC-100 PCR machine (MJ Research, Waltham, USA). The primers used for RT-PCR were: β-actin, 5′-TGGAAGCCTGTGGCATCCA-3′ and 5′-TAACAGTCCGCCTAGAAGCA-3′; and caspase-3, 5′-TGAAGGCAAGGT-

GCTAAA-3′and 5′-CTGGCTCAAACCACATTCTC-3′. All primers were obtained from Bioasia Biotech (Shanghai, China). Data were quantitated by scanning with an FR-980A electrophoresis image analysis system (FURI Science & Technology).

Statistical analysis

Results were obtained from at least three independent experiments and presented as mean±SD. Differences were established by Student's *t*-test and $P<0.05$ was considered significant.

Results

Protection of LPS tolerance

All mice in the control groups survived. From **Fig. 1**, we can see that the survival rate clearly increased after LPS-tolerance. The mice in the naive group were all dead 2 d after the LPS/*D*-GalN challenge. All mice in the LPS-tolerance 3 h and 1 d groups survived during the period of observation. In the LPS-tolerance 2 d group, the survival rate was 100% after 2 d, but gradually decreased to 90% after 7 d. In the LPS-tolerance 7 d group, mice died within 1 d, and a survival rate of 70% was observed after 7 d. The differences were significant ($P<0.05$).

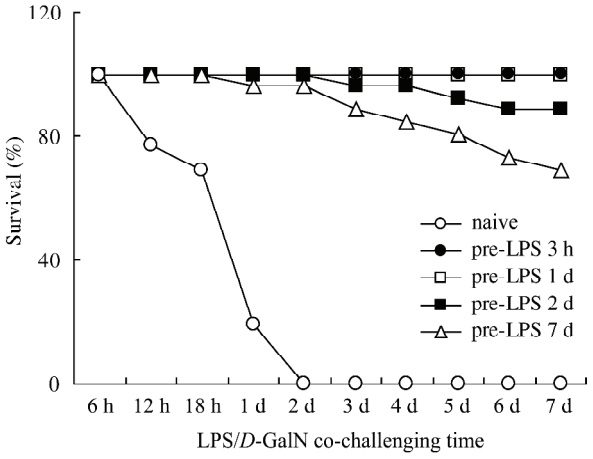

Fig. 1 Survival rates of lipopolysaccharide/*D*-galactosamine (LPS/*D*-GalN) challenged mice in LPS-tolerant and naive groups

The experimental mice were divided into nine groups (30 mice per group) as described in "Materials and Methods". The experimental mice were maintained and continuously observed for 7 d. The survival numbers of the mice in different groups were counted.

Suppression of apoptotic reaction in LPS-induced tolerant mice

DNA fragmentation was detectable in the mice of the naive group (**Fig. 2**, lane 5), whereas in LPS-induced tolerant groups, a clear suppression of DNA fragmentation (**Fig. 2**, lane 6–9) was visualized. This suppression of apoptotic reaction in terms of DNA fragmentation was capable of being sustained for up to 7 d. The integrity of the DNA was maintained in the control groups (**Fig. 2**, lane 1–4).

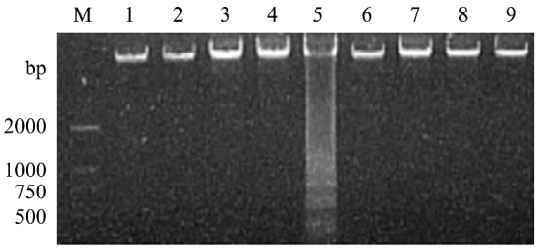

Fig. 2 Detection of DNA fragmentation in naive and tolerant mice after lipopolysaccharide/*D*-galactosamine (LPS/*D*-GalN) treatment

M, DL 2000 DNA marker; 1, DNA from normal saline control mouse; 2, DNA from a single injection of LPS (0.1 μg) only; 3, DNA from a single injection of LPS (2 μg) only; 4, DNA from a single injection of *D*-GalN (20 mg) only; 5, DNA from LPS (2 μg)/*D*-GalN (20 mg) co-challenged mouse; 6–9, DNA from LPS pre-treated mice (3 h, 1 d, 2 d and 7 d, respectively). DNA fragmentation was observed only in naive mice (lane 5).

Alterations of caspase-3 expression in LPS-induced tolerant mice

Caspase-3 mRNA measured by RT-PCR showed a higher transcription level in the naive group [**Fig. 3(A)**, lane 5] than those in tolerant [**Fig. 3(A)**, lane 6–9] and control groups [**Fig. 3(A)**, lane 1–4]. Densitometry of caspase-3 [**Fig. 3(B)**] showed a down-regulation then a gradual recovery in the LPS-induced tolerant mice. These decreases in tolerant mice, compared with naive mice, were statistically significant ($P<0.05$).

The expression of caspase-3 protein in LPS-induced tolerance was at its lowest activity at 3 h, then a gradual increase was found at 1 d, 2 d, and 7 d. In contrast, caspase-3 had higher activity in the naive group than in the LPS-induced tolerant and control groups ($P<0.05$) [**Fig. 3(C)**].

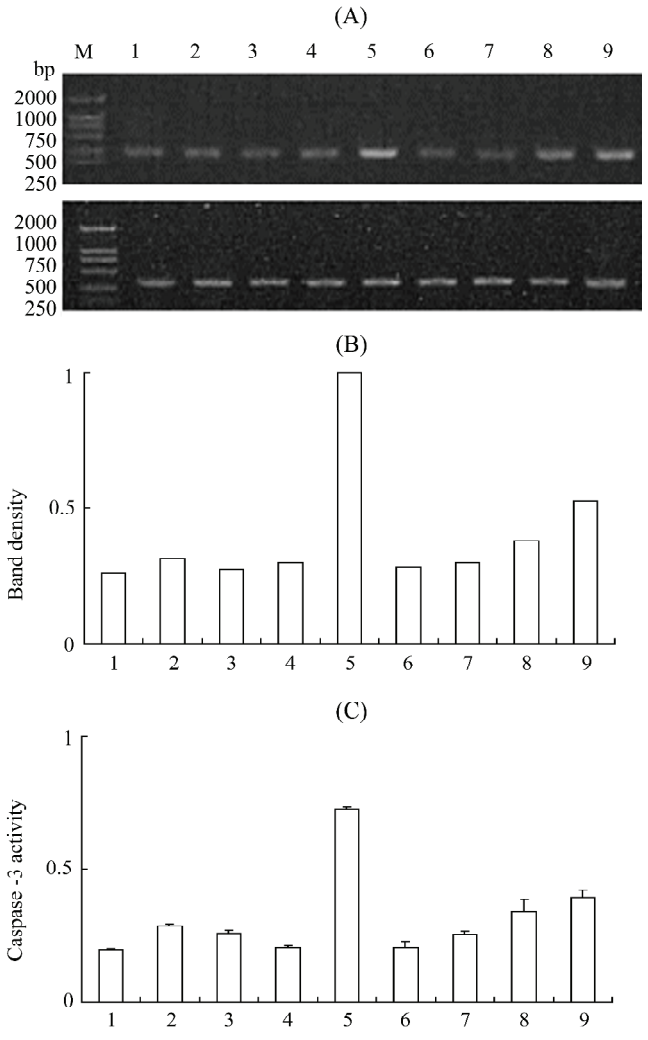

Fig. 3 Alterations of caspase-3 activity in mice after lipopolysaccharide/*D*-galactosamine (LPS/*D*-GalN) treatment

(A) Reverse transcription-polymerase cahin reaction detection of caspase-3 mRNA. Total RNA was extracted from experimental mouse liver and electrophoresed through 1% agarose gel. β-actin served as a sample-loading control. (B) Absorbance measurement of the bands on gel in (A). Differences were statistically significant between naive and LPS-induced tolerant mice ($P<0.05$). (C) Caspase-3 expression analysis. Caspase-3 activity in naive mice was higher than those in LPS-induced tolerant mice, and the differences were statistically significant between naive and LPS-induced tolerant mice ($P<0.05$). M, DL 2000DNA marker; 1, sample from normal saline control mouse; 2, RNA from a single injection of LPS (0.1 μg) only; 3, RNA from a single injection of LPS (2 μg) only; 4, RNA from a single injection of *D*-GalN (20 mg) only; 5, RNA from LPS (2 μg)/*D*-GalN (20 mg) co-challenged mouse; 6–9, RNAs from LPS pre-treated mice (3 h, 1 d, 2 d, and 7 d, respectively).

Discussion

LPS is one of the strongest stimulators of macrophages and leads to the secretion of nitrogen intermediates, prostaglandins and cytokines. The secretion of TNF-α, IL-1, IL-6, and IL-12 results in rapid induction and amplification of the host response to infection [15–18]. *In vitro*, LPS directly promotes apoptosis in macrophages, hepatocytes, ventricular myocytes and endothelial cells. *In vivo*, LPS has shown a pro-apoptotic effect on lymphocytes in Peyer's patches and thymocytes. In addition to direct apoptotic effects, LPS can also stimulate the recruitment of leukocytes and production of pro-inflammatory cytokines. Alikhani *et al*. [19] showed that LPS significantly increased caspase-8 and caspase-3 activities *in vivo*, which were dependent on TNF receptor signaling. But until recently [13,20], the relationship between LPS tolerance and apoptotic reaction in liver cells was not reported. In this study, we found that DNA fragmentation in mouse liver was more obvious in naive mice than in LPS-induced tolerant mice. More importantly, the suppression of apoptosis against later challenge of LPS could last up to 7 d, much longer than the previously reported 1 d [13,20]. In this period, the expression of an important executioner caspase, caspase-3, was also down-regulated in the LPS-induced tolerant group and the survival rate in LPS tolerant groups clearly increased. The mechanism might be as follows. (1) The expression of TNF receptor was altered after LPS tolerance. Previous research [20] also confirmed that the induced tolerance status was mediated not only by LPS but also by TNF, and the signaling of induction of tolerance and promotion of apoptosis was through TNF receptor 1. (2) LPS tolerance might stimulate activities of LPS receptor-Toll like receptor (TLR) 4 and its downstream molecular alteration. Any of these changes can lead to the alteration of the expression of caspase-3. In future research, we will use antagon of different molecules or gene knockout mice to ascertain the mechanism of suppressing apoptosis after LPS tolerance.

D-GalN impairs the biosynthesis of RNA, glycoproteins and glycogen, resulting in damage to hepatocytes. The biopharmacological effects of *D*-GalN used in this study are confined to hepatocytes, due to the lower level of uridine triphosphate in liver. Several studies [21] have demonstrated that mice treated with *D*-GalN were dramatically sensitized to LPS, allowing more than a 2500-fold reduction in the lethal dose of LPS. Single dose treatment with *D*-GalN showed no influence in caspase-3 mRNA transcription and protein levels, implying that LPS is the main factor in modulating caspase-3.

In conclusion, we found that pre-exposure of mice to small doses of LPS can suppress the apoptotic response for up to 7 d, much longer than the period of 1 d previously

reported [13,20]. The low mouse fatality rate was coincidental. Our findings suggest LPS tolerance could suppress apoptosis in mouse liver cells, and the activity and expression of caspase-3 are both down-regulated in this period.

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