Endotoxin Tolerance Inhibits Lipopolysaccharide-Initiated Acute Pulmonary Inflammation and Lung Injury in Rats by the Mechanism of Nuclear Factor-κB

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Abstract

In this study, the effect of endotoxin tolerance on lipopolysaccharide (LPS)-initiated pulmonary inflammation, the local production of tumour necrosis factor-α (TNF-α) and the cytokine-induced neutrophil attractant (CINC), as well as the activation of nuclear factor-κB (NF-κB) and its subunit composition, were examined *in vivo*. Endotoxin tolerance was reproduced by four consecutive daily intraperitoneal injections of 0.6 mg/kg of *Escherichia coli* 055:B5 LPS. Compared with control rats, endotoxin-tolerant rats failed to increase the permeability of pulmonary microvascular or recruit neutrophil to lung tissue upon restimulation with 6 mg/kg of LPSs. Pretreatment with LPSs inhibited the protein level of TNF-α in bronchoalveolar lavage fluid (BALF) and mRNA expression of CINC in lung tissue in response to subsequent LPS stimulation. These changes were accompanied by the suppression of activation of NF-κB, including the low level of total amount of DNA-binding activity and high percentage of non-transactive p50 homodimers. These data demonstrate that endotoxin tolerance can alleviate the LPS-induced acute neutrophilic pulmonary inflammation in rats and can inhibit the proinflammatory cytokines in lung and suggest that endotoxin tolerance might result from the unresponsiveness of NF-κB and persistent high percentage of p50 homodimers. Therefore, the phenomenon of endotoxin tolerance might be used as a strategy for the prevention or treatment of LPS-associated acute respiratory distress syndrome in which excessive or dysregulated inflammation leads to acute lung injury.

Introduction

Endotoxins (lipopolysaccharides, LPSs) are major and integral components of the outer membrane of Gram-negative bacteria. Furthermore, they possess proinflammatory activities and play an important role in the pathogenesis and manifestation of Gram-negative bacteria infection [1, 2]. Severe endotoxaemia is an important risk factor for the development of acute respiratory distress syndrome (ARDS). In the recent decades, numerous treatments aimed at LPSs and LPS-stimulated cytokines, such as anti-LPS antibody, anti-CD14 antibody, recombinant bactericidal/permeability-increasing protein segment and anti-tumour necrosis factor-α (TNF-α) antibody, have been developed to prevent and treat severe endotoxaemia; however, none showed significant effect in clinical trials.

Endotoxin tolerance refers to a state in which endotoxin-triggered responses are at least partially abrogated by prior exposure to endotoxin [3]. The endotoxin-tolerant monocytes and macrophages are characterized by the inhibition of LPS-stimulated proinflammatory cytokine production, such as TNF-α [4–6]. As endotoxaemia is an important cause of ARDS and they have some pathophysiologic features in common, including neutrophil activation and a plethora of proinflammatory cytokines, we hypothesized that the phenomenon of endotoxin tolerance might be exploited as a preventive or therapeutic strategy for ARDS associated with Gram-negative sepsis. Although several studies *in vivo* showed that endotoxin-tolerant animals had a marked reduction in the systemic response to endotoxaemia, including mortality, weight loss, fever and serum cytokine level [4, 5, 7, 8], the pulmonary inflammation and local cytokine production under

endotoxin-tolerant status remain unclear. Moreover, the mechanism of endotoxin tolerance is not fully defined.

The ability of LPSs to stimulate cellular responses is initiated through Toll-like receptor 4 (TLR4), a signal-transducing receptor which is essential for the recognition of LPSs. Once TLR4 is activated, it recruits the adapter protein MyD88. Then, interleukin-1R (IL-1R)-associated kinase is phosphorylated and associated with tumour necrosis factor receptor (TNFR)-activated factor 6 (TRAF-6). Oligomerization of TRAF-6 activates a member of MAP3K family and IκB kinase sequentially, which ultimately leads to the induction of a wide variety of inflammatory and immune-response genes via nuclear translocation and activation of nuclear factor-κB (NF-κB) [9, 10]. NF-κB is a critical transcription factor for elevated expression of many cytokines that are involved in the pathogenesis of inflammatory disease [11, 12], and activation of NF-κB has been observed in ARDS [13]. It has been demonstrated that the nuclear translocation and activation of NF-κB is reduced in endotoxin-tolerant cells, which indicates the important role of NF-κB in endotoxin tolerance [14, 15]. The NF-κB/Rel family consists mainly of p65 and p50. Compared with p65, p50 lacked a transactivation domain. The p50 homodimers could block the transactivation function by occupying the relevant DNA motif [16].

In order to see whether endotoxin tolerance could alleviate the acute lung injury and cytokine production in endotoxaemia, we reproduced endotoxin-tolerant rat model and focused on the endotoxaemia-initiated lung inflammation as well as on the local production of TNF-α and expression of cytokine-induced neutrophil chemoattractant (CINC), which was a potent mediator of neutrophil infiltration. To elucidate the role of NF-κB in the effect of endotoxin tolerance on lung inflammation, we also studied the activation of NF-κB and its subunit composition in lung tissue.

Materials and methods

Animal model. Male Sprague–Dawley rats weighing between 170 and 200 g were used for all experiments. For the induction of endotoxin tolerance, rats were injected intraperitoneally with a dose of 0.6 mg of *Escherichia* LPSs (serotype 055:B5; Sigma, St. Louis, MO, USA) per kg body weight on each of four consecutive days. Normal control rats were injected intraperitoneally with the same volume of pyrogen-free 0.9% sodium chloride. On the fifth day, rats were injected intraperitoneally with 6 mg/kg of LPSs to induce endotoxaemia and lung inflammation. Blood, left bronchoalveolar lavage fluid (BALF) and right lung were collected before and 2, 6, 24, 48 and 72 h after the high-dose injection of LPSs (six rats for each time point), according to our published protocol [17]. The middle lobe of the right lung was sectioned and stained with haematoxylin and eosin (H&E) for pathological observation. The other lobes were quickly frozen in liquid nitrogen tank and stored at −80 °C.

Cytological examination of blood and BALF. Several drops of blood were obtained for leucocyte counts and differentials. BALF was centrifuged at 1000 r.p.m. (≈110×*g*) for 10 min after leucocyte counts. The supernatants were aliquoted and stored at −80 °C, whereas the pellets were resuspended in 0.9% sodium chloride to make cytocentrifuge slides on which differential cell counts were enumerated by counting 300 cells in cross section after Wright stain.

Quantitation of albumin in BALF. The supernatants of BALF were used for the determination of albumin in a sandwich enzyme-linked immunosorbent assay (ELISA). Goat anti-rat albumin antibody, rat serum albumin, biotinylated anti-rat albumin antibody, streptavidin–horse radish peroxidase and O-phenylenediamine (OPD) were a kind gift from Prof Ou Zhouluo (Cancer Hospital, Fudan University, Shanghai, China). The detection limit was 1 ng/ml.

TNF-α determination. BALF levels of TNF-α were measured with a commercially available ELISA kit (Jingmei Company, Shanghai, China) according to the manufacturer's guidelines. The detection limit was 62.5 pg/ml.

Quantitative analysis of CINC mRNAs. Total RNA was extracted from the frozen lung tissue by the guanidinium–thiocyanate–phenol–chloroform method [18], and relative quantification of CINC mRNAs was carried out by the reverse transcriptase-polymerase chain reaction (RT-PCR) method, in which the target mRNA was amplified by PCR after the reverse transcription of mRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Table 1 Leucocyte count and differentials of blood

	Normal control group		Tolerant group	
	0 h	24 h	0 h	24 h
WBC ($\times 10^9$/l)	6.1 ± 1.8	4.5 ± 1.4	5.1 ± 1.4	6.0 ± 1.5
PMN percentage	31.2 ± 19.3	63.5 ± 19.1*	27.4 ± 8.2	26.3 ± 4.6†
PMN ($\times 10^9$/l)	1.9 ± 1.4	3.0 ± 1.7	1.5 ± 0.8	1.6 ± 0.5

Values are presented as mean ± SD, $n = 6$. PMN, polymorphonuclear neutrophil; WBC, white blood cell.
*$P < 0.05$, when compared with the value before high-dose injection of lipopolysaccharides.
†$P < 0.01$, when compared with the same time point of normal control group.

mRNA was amplified as internal control. The primers for the gene analysed in this study were as follows: CINC (sense) 5′-AAC AGA GCA CCA TGG TCT-3′, (antisense) 5′-GAC GCC ATC GGT GCA ATC TA-3′; GAPDH (sense) 5′-CTC TAC CCA CGG CAA GTT CAA-3′, (antisense) 5′-GGG ATG ACC TTG CCC ACA GC-3′. The optimal cycle number for each gene was determined empirically and was defined as the number of cycles that results in detectable PCR-amplified products under non-saturating conditions. Each cycle consisted of 30 s at 95 °C, 1 min at a gene-specific annealing temperature and 30 s primer extension at 72 °C. The annealing temperatures and optimal cycle numbers (shown in parentheses) for each primer set were as follows: 60 °C for GAPDH (30 cycles) and 54 °C for CINC (37 cycles). PCR products were run on a 1.2% agarose gel and visualized with ethidium bromide under ultraviolet illumination. The intensity of bands was quantified by image analysis software (Pharmacia Biotech ImageMaster system, Piscataway, NJ, USA). The ratio of CINC mRNA to GAPDH mRNA was calculated for each sample.

Nuclear protein extraction. Nuclear protein extracts of lung tissue were prepared by the method of Deryckere and Gannon [19] with some modifications. Briefly, about 100 mg frozen lung tissues were homogenized in 2 ml of solution A (0.6% Nonidet P-40, 150 mmol/l NaCl, 10 mmol/l HEPES, pH 7.9, 1 mmol ethylenediaminetetraacetic acid (EDTA), 0.5 mmol phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotonin, 10 μg/ml soybean trypsin inhibitor and 1 μg/ml pepstatin). About 250 μl of the homogenate was transferred to a 1.5 ml tube and centrifuged for 1 min at 2000 r.p.m. ($\approx 450\times g$) at 4 °C. The supernatant was obtained, incubated on ice for 5 min and centrifuged for 10 min at 5000 r.p.m. ($\approx 2400\times g$) at 4 °C. Nuclear pellets were then resuspended in 75 μl of solution B (25% glycerol, 20 mmol/l HEPES, pH 7.9, 420 mmol/l NaCl, 1.2 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 0.5 mmol/l dithiothreitol, 0.5 mmol/l phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotonin, 10 μg/ml soybean trypsin inhibitor and 1 μg/ml pepstatin) and incubated on ice for 30 min prior to centrifugation for 1 min at 14,000 r.p.m. ($\approx 19{,}000\times g$) at 4 °C. The supernatants containing nuclear proteins were aliquoted and stored at −70 °C. Protein concentrations were determined by Bradford assay using bovine serum albumin (Sigma) as a reference standard.

Electrophoretic mobility shift assays. The NF-κB gel shift assay was performed using a kit obtained from Promega (Madison, WI, USA). Double-stranded NF-κB consensus oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was end labelled with [λ-^{32}P]ATP (5000 Ci/mmol at 10 mCi/ml) by T4 polynucleotide kinase, as recommended by the manufacturer.

Nuclear protein (10 μg) was mixed with a binding buffer (4% glycerol, 1 mmol/l $MgCl_2$, 0.5 mmol/l EDTA, 0.5 mmol/l dithiothreitol, 50 mmol/l NaCl, 10 mmol/l Tris–HCl, pH 7.5 and 0.05 μg/ml poly dIdC) and incubated for 10 min at room temperature, and then 50,000 cpm of labelled oligonucleotide was added to each sample for binding reaction at room temperature for 20 min. Reaction products were separated on a 6% non-denaturing polyacrylamide gel in ×0.25 Tris-borate-EDTA (TBE) buffer at 150 V, and gels were analysed by autoradiography. The ratio of densitometric unit of total amount of NF-κB of each sample to that of Hela cells was calculated to semiquantitively represent the DNA-binding activity of NF-κB. The ratio of the intensity of p50 homodimers to that of p65/p50 heterodimers in the same lane was calculated to represent the balance of p65/p50 heterodimers and p50 homodimers (Pharmacia Biotech ImageMaster system).

The protein extract of Hela cells (Promega) was used as negative control and positive control. Specific competition experiment using the same nuclear extracts incubated with a 50-fold excess of unlabelled NF-κB oligonucleotide and non-specific competition experiment using unlabelled AP-1 oligonucleotide were performed to test the specificity of the labelled NF-κB oligonucleotide and to confirm the identity of NF-κB.

Statistics. Statistics was performed by SPSS 10.0 software. All values were expressed as mean ± SD. The unpaired *t*-test and a one-way analysis of variance using the Dunnetts' analysis were adopted to calculate statistical differences between and within groups, respectively. Differences were considered significant at the level of $P < 0.05$.

Results

Endotoxin tolerance and its beneficial effect on endotoxaemia-initiated pulmonary inflammation

After the intraperitoneal injection of 6 mg/kg of LPSs, normal control rats showed less activity, food intake and faster respiratory rate. No abnormality was found in endotoxin-tolerant rats.

During the first 24 h after the high-dose injection of LPSs, we found a loss of up to 8.9 ± 3.1% of the basal body weight ($P = 0.044$) in normal control rats and the weight loss reached 11.5 ± 6.0% ($P = 0.019$) till 72 h. In contrast, only 1.0 ± 2.1% of weight variation in the first 24 h, followed by a steady increase, was seen in tolerant rats (Fig. 1) (data not shown).

In normal control rats, BALF concentration of albumin increased by 14.47 ± 5.30 mg/l ($P = 0.042$) at 2 h after injection of 6 mg/kg of LPSs, which was highest at 6 h after injection (24.15 ± 5.30 mg/l elevated, $P < 0.001$), reflecting the increased permeability of the alveolar capillary membrane. In tolerant rats, BALF concentration of albumin did not increase.

There was no statistically significant difference of the total white cell in blood and BALF before injection and

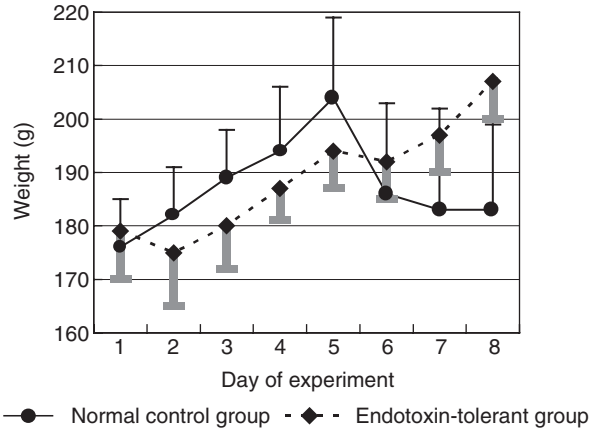

Figure 1 Weight loss induced by high intraperitoneal dose of lipopolysaccharides (LPSs) on the fifth day. Values of normal control group and endotoxin-tolerant group are presented as means + SD and means − SD, respectively. $n = 8$.

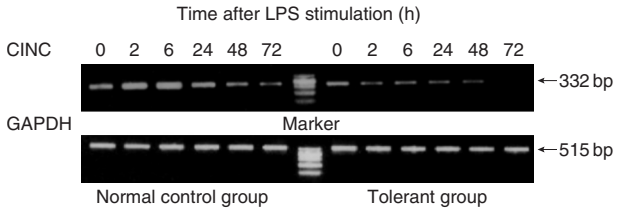

Figure 2 Effect of 6 mg/kg of lipopolysaccharides (LPSs) on the pulmonary expression of mRNA for cytokine-induced neutrophil attractant (CINC) in normal control and tolerant rats. Products of reverse transcriptase-polymerase chain reaction (RT-PCR) were separated on a 1.2% agarose gel with ethidium bromide staining. The middle bands are the electrophoretogram of 101, 200, 299, 398 and 520 bp DNA marker. Numbers on the left indicate the sizes of PCR products of CINC and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

24 h after injection in either normal control group or tolerant group (Table 1). In normal control group, the differentiation of white blood cell shifted from lymphocyte to polymorphonuclear neutrophil leucocyte (PMN), and PMN percentage in BALF also increased from 0.443 ± 0.345 to $8.000 \pm 2.896\%$ as its peak at 24 h after injection ($P < 0.001$), according with the pulmonary pathological study showing that PMN sequestered in lung tissue and exudates at 24 h after injection. In tolerant rats, there was no elevation in PMN percentage either in blood or in BALF, and the acute neutrophil-infiltrating pulmonary inflammation was not found (Table 2).

Endotoxin tolerance prevented pulmonary production of TNF-α and mRNA expression of CINC

There was a little TNF-α production in BALF and CINC mRNA expression in lung tissue before high-dose LPS challenge in both the groups. In normal control group, the level of TNF-α and CINC mRNA rapidly increased after challenge. TNF-α reached its peak at 6 h after injection, with the level achieved at 10.79 ± 3.53 μg/l ($P = 0.001$), while CINC mRNA showed a peak between 2 and 6 h, with the comparative value of about 1.6 ($P < 0.001$). In contrast, the TNF-α level in BALF and CINC mRNA expression of lung tissue in tolerant rats were not elevated (Fig. 2 and Table 3).

LPS-stimulated pulmonary NF-κB activation was elevated in normal control group

There were mainly two retardation bands in the gel shift assay. The upper bands indicate p65/p50 heterodimers and the lower p50 homodimers (Fig. 3). The intensity of bands of p65/p50 heterodimers, p50 homodimers and total amount of NF-κB were measured by computer-analysed densitometry. There was little DNA-binding activity of NF-κB before 6 mg/kg of LPS stimulation in normal control group, and p50 homodimers predominated (Fig. 3B, lane 2). After the stimulation, activation of NF-κB dramatically increased, reaching summit during 2–6 h, and the nuclear NF-κB protein consisted mainly of p65/p50 heterodimers (Fig. 3B, lane 3 through lane 7). The ratio of p50 homodimers to p65/p50 heterodimers decreased to nadir accordingly.

Table 2 Comparison of dynamical variation of polymorphonuclear neutrophil leucocyte (PMN) percentage and albumin level in bronchoalveolar lavage fluid (BALF) between endotoxin-tolerant rats and normal control rats

	Percentage of PMNs in BALF (%)		Albumin concentration in BALF (mg/l)	
	Normal control group	Tolerant group	Normal control group	Tolerant group
0 h	0.443 ± 0.345	0.665 ± 0.422	21.09 ± 3.23	18.30 ± 7.95
2 h	3.556 ± 1.693*	0.500 ± 0.351§	35.56 ± 6.47*	10.27 ± 3.55¶
6 h	4.222 ± 2.063†	0.388 ± 0.253§	45.24 ± 16.66‡	12.59 ± 10.26
24 h	8.000 ± 2.896‡	0.610 ± 0.330§	27.15 ± 10.67	9.23 ± 3.19§
48 h	4.168 ± 1.697†	0.498 ± 0.459§	18.14 ± 4.36	5.44 ± 4.89†§
72 h	2.167 ± 0.809	0.667 ± 0.59§	15.08 ± 6.56	7.69 ± 3.76*,**

Values are presented as mean ± SD, $n = 6$.
*$P < 0.05$, †$P < 0.01$ and ‡$P < 0.001$, when compared with the value before high-dose injection of lipopolysaccharides.
§$P < 0.01$; ¶$P < 0.001$ and **$P < 0.05$, when compared with the same time point of normal control group.

Table 3 Kinetics of tumour necrosis factor-α (TNF-α) in bronchoalveolar lavage fluid (BALF) and cytokine-induced neutrophil attractant (CINC) mRNA in lung tissue

	TNF-α level in BALF (μg/l)		Expression of CINC mRNA in lung tissue	
	Normal control group	Tolerant group	Normal control group	Tolerant group
0 h	4.12 ± 1.14	5.32 ± 2.26	0.388 ± 0.257	0.589 ± 0.181
2 h	8.68 ± 3.78*	4.78 ± 1.42	1.610 ± 0.113*	0.468 ± 0.145§
6 h	10.79 ± 3.53†	5.04 ± 2.10‡	1.672 ± 0.211*	0.623 ± 0.337‡
24 h	6.75 ± 1.85	6.14 ± 1.06	1.349 ± 0.266*	0.545 ± 0.114§
48 h	5.87 ± 2.36	5.96 ± 1.89	0.569 ± 0.378	0.360 ± 0.043
72 h	5.47 ± 1.31	4.22 ± 1.60	0.322 ± 0.102	0.309 ± 0.063

Values are presented as mean ± SD, $n = 6$.
*$P < 0.05$ and †$P < 0.01$, when compared with the value before high-dose injection of lipopolysaccharides.
‡$P < 0.05$ and §$P < 0.001$, when compared with the same time point of normal control group.

Pulmonary activation of NF-κB was inhibited in endotoxin-tolerant group

Figure 3C shows the gel shift assay of NF-κB in tolerant group. The activation of NF-κB before injection of 6 mg/kg of LPSs in tolerant rats was the same as that in normal control rats, although the ratio which represented the percentage of p50 homodimers was lower than that in normal control rats ($P < 0.05$). However, neither an increase of the total amount of NF-κB activation nor a decrease of the ratio of p50 homodimers to p65/p50 heterodimers was observed after high dose of LPS stimulation. The difference between the two groups was significant statistically (Table 4).

Discussion

In this experiment, we have reproduced a rat model of endotoxin tolerance by four consecutive daily intraperitoneal injections of 0.6 mg/kg of LPSs. Its presence has been demonstrated in three ways compared with normal control rats. First, the changes of behaviour such as less activity and food intake did not appear in endotoxin-tolerant group after high dose of LPS stimulation. Second, the weight increase did not obviously be impeded in the tolerant group. Third, the shift from lymphocyte to PMN in blood was not found.

Next, we have reconstructed a picture of the pulmonary inflammation in endotoxin-tolerant rats. The reconstruction, which incorporates and confirms the observation of previous investigators, includes several new observations that provide a more complete understanding of the benefit of endotoxin tolerance on LPS-initiated pulmonary inflammation. As observed in our experiment, the pulmonary inflammation in endotoxin-tolerant group induced by 6 mg/kg of LPS was far less severe than that in normal control group. Here, we evaluated pulmonary inflammation from three aspects. The first was the albumin concentration of BALF, which reflected the permeability of microvascular. LPS could damage pulmonary vascular endothelial cells directly and indirectly, which could result in altered pulmonary vascular permeability, pulmonary oedema and increased albumin concentration in alveolar cavity [20, 21]. The results reported here show

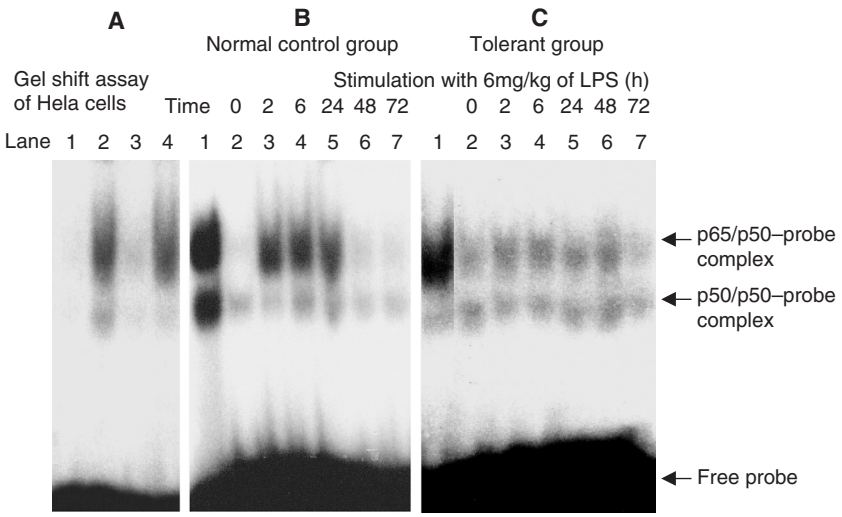

Figure 3 Gel shift assay examining the effect of endotoxin tolerance on lipopolysaccharide (LPS)-mediated activation of nuclear factor-κB (NF-κB). (A) Lane 1, the negative control; lane 2, the positive control; lane 3, specific competition experiment; lane 4, non-specific competition experiment. (B) Time course of NF-κB activation after injection of 6 mg/kg of LPSs in normal control rats. (C) Time course of NF-κB activation after injection of 6 mg/kg of LPSs in tolerant rats. The left lane shows the positive control from Hela cells. The right six lanes show the activity of NF-κB at different time point (data summarized in Table 4).

Table 4 Effect of endotoxin tolerance on lipopolysaccharide (LPS)-stimulated pulmonary activation of nuclear factor-κB (NF-κB)

	Activation of NF-κB in lung tissue		Ratio of p50/50 to p65/p50	
	Normal control group	Tolerant group	Normal control group	Tolerant group
0 h	0.697 ± 0.169	0.657 ± 0.106	1.500 ± 0.341	0.684 ± 0.092†
2 h	1.112 ± 0.251	0.511 ± 0.260†	0.401 ± 0.077§	0.672 ± 0.098‡
6 h	1.213 ± 0.272*	0.426 ± 0.274‡	0.454 ± 0.086§	0.750 ± 0.092†
24 h	0.812 ± 0.286	0.467 ± 0.311	0.648 ± 0.128§	0.673 ± 0.184
48 h	0.730 ± 0.188	0.375 ± 0.278	0.774 ± 0.055§	0.780 ± 0.096
72 h	0.757 ± 0.256	0.359 ± 0.132†	0.820 ± 0.024§	0.898 ± 0.105

Values are presented as mean ± SD, $n = 6$.
*$P < 0.05$ and §$P < 0.001$, when compared with the value before high-dose injection of LPSs.
†$P < 0.05$ and ‡$P < 0.01$, when compared with the same time point of normal control group.

that the immediate elevation of pulmonary vascular permeability induced by 6 mg/kg of LPS stimulation is inhibited in endotoxin-tolerant rat.

The second was the pulmonary sequestration of PMNs. Stimulated by LPSs, PMNs would quickly penetrate the pulmonary vessel wall into the tissue, and activated PMNs may contribute substantially to local inflammatory reactions [22]. In normal control rats, the percentage of PMNs in BALF was significantly increased, and PMNs infiltration in lung was found by histopathological examination. Whereas in endotoxin-tolerant rats, the acute neutrophilic pulmonary inflammation was not found.

The final was the blockade of production of TNF-α and the mRNA expression of CINC in lung in tolerant rats. In accordance to the well-demonstrated role of TNF-α as a mediator of LPS toxicity [23, 24] and CINC as a mediator of PMNs local sequestration [25–27], associated with the sequential peak of PMNs percentage in BALF and cytokine expression in lung tissue observed in normal control rats, these results suggest that the non-responsiveness of TNF-α and CINC may be one of the causes of diminished neutrophilic pulmonary inflammation in endotoxin-tolerant rats.

Thus, we could come to the conclusion that the elevated cytokine production and acute lung injury can be inhibited in endotoxin-tolerant rats. The cytokine incapability in lung suggested that endotoxin tolerance might be beneficial in the prevention of ARDS arising from not only sepsis but also other clinical settings, such as trauma.

Previous studies that assessed the mechanisms of endotoxin tolerance have demonstrated that tolerant macrophages exhibit great alterations in several LPS-signal molecules, including NF-κB [14, 15, 28–30]. Our present findings in endotoxin-tolerant rats show that the nuclear translocation and DNA-binding activity of NF-κB are not elevated after high dose of LPS stimulation, which are consistent with the observation of previous studies.

When stimulated with 6 mg/kg of LPS, the DNA-binding activity of p65/p50 heterodimers do not vanish and shift greatly to p50 homodimers, as observed in the *in vitro* study of Ziegler-Heitbrock *et al.* [31–33]. Instead, we observed that the ratio of p50 homodimers to p65/p50 heterodimers remained at a constant level as before the stimulation. But in normal control rats, in which acute pulmonary inflammation developed after the stimulation, the ratio sharply decreased and p65/p50 heterodimers were predominant. Therefore, the percentage of p50 homodimers in tolerant rats is higher than that in normal control rats. Higher percentage of p50 homodimers may neutralize the transactivity of p65/p50 heterodimers in tolerant rats, and the activity of NF-κB is counteracted. As a result, the production of proinflammatory cytokine is inhibited and pulmonary inflammation is alleviated.

We observed that there is a constitutive small amount of nuclear translocation of NF-κB, consisting almost completely of p50 homodimers, in the rats without any stimulation of LPSs. Interestingly, after four consecutive injections of 0.6 mg/kg of LPS, there is also a small amount of nuclear translocation of NF-κB, with the ratio decreased from 1.500 ± 0.341 to 0.684 ± 0.092; however, it was not accompanied with significant elevated level of TNF-α in BALF, mRNA expression of CINC in lung, pulmonary PMNs recruitment and inflammation.

Taken together, these observations suggest that the transactivity of NF-κB is associated with not only the total amount of nuclear NF-κB, but also the percentage of the p50 homodimers. Furthermore, the ratio of p50 homodimers to p65/p50 heterodimers may be useful to evaluate the activation of nuclear NF-κB.

In summary, our results indicate that endotoxin tolerance can alleviate the acute neutrophilic pulmonary inflammation and impair the production of proinflammatory cytokines such as TNF-α and CINC induced by endotoxaemia. These findings in rats imply that endotoxin tolerance might be used as a strategy for the prevention or treatment of LPS-associated ARDS. The silent reaction to high dose of LPS may be mediated by unresponsiveness of NF-κB and the increased percentage of p50 homodimers in NF-κB nuclear protein.

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References

1 Morrison DC, Ryan JL. Bacterial endotoxins and host immune responses. Adv Immunol 1979;28:293–450.
2 Morrison DC, Ryan JL. Endotoxins and disease mechanisms. Annu Rev Med 1987;38:417–32.
3 West MA, Heagy W. Endotoxin tolerance: A review. Crit Care Med 2002;30:S64–73.
4 Sanchez-Cantu L, Rode HN, Christou NV. Endotoxin tolerance is associated with reduced secretion of tumor necrosis factor. Arch Surg 1989;124:1432–5.
5 Flohe S, Dominguez Fernandez E, Ackermann M, Hirsch T, Borgermann J, Schade FU. Endotoxin tolerance in rats: expression of TNF-α, IL-6, IL-10, VCAM-1 and HSP 70 in lung and liver during endotoxin shock. Cytokine 1999;11:796–804.
6 Liu H, Sidiropoulos P, Song G *et al.* TNF-α gene expression in macrophage: regulation by NF-κB is independent of c-Jun or C/EBP β. J Immunol 2000;164:4277–85.
7 Greisman SE, Hornick RB. Mechanism of endotoxin tolerance with special reference to man. J Infect Dis 1973;128:265–76.
8 Golub ES, Weigle WO. Studies on the induction of immunologic unresponsiveness: I. Effects of endotoxin and phytohemagglutinin. J Immunol 1967;98:1241–7.
9 Mackay I, Rosen FS. Innate immunity. N Engl J Med 2000; 343:338–43.
10 Medzhitov R, Kopp EB. The Toll-receptor family and control of innate immunity. Curr Opin Immunol 1999;11:11–8.
11 Christman JW, Lancaster LH, Blackwell TS. Nuclear factor κ B: a pivotal role in the systemic inflammatory response syndrome and new target for therapy. Intensive Care Med 1998;24:1131–8.
12 Blackwell TS, Christman JW. The role of nuclear factor-κB in cytokine gene regulation. Am J Respir Cell Mol Biol 1997;17:3–9.
13 Kelley J. Cytokines of the lung. Am Rev Respir Dis 1990;141:765–88.
14 Wahlstrom K, Bellingham J, Rodriguez JL, West MA. Inhibitory κBα control of nuclear factor-κB is dysregulated in endotoxin tolerant macrophages. Shock 1999;11:242–7.
15 Medvedev AE, Kopydlowski KM, Vogel SN. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: Dysregulation of cytokine, chemokine, and Toll-like receptor 2 and 4 gene expression. J Immunol 2000;164: 5564–74.
16 Bohuslav J, Kravchenko VV, Parry GC *et al.* Regulation of an essential innate immune response by the p50 subunit of NF-κB. J Clin Invest 1998;102:1645–52.
17 Qu J, He L, Rong Z *et al.* Alteration of surfactant proteins A and D in bronchoalveolar lavage fluid of *Pneumocystis carinii* pneumonia. Chin Med J (Engl) 2001;114:1143–6.
18 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162:156–9.
19 Deryckere F, Gannon F. A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. Biotechniques 1994;16:405.
20 Haimovitz-Friedman A, Cordon-Cardo C, Bayoumy S *et al.* Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. J Exp Med 1997;186:1831–41.
21 Harlan JM, Harker LA, Reidy MA, Gajdusek CM, Schwartz SM, Striker GE. Lipopolysaccharide-mediated bovine endothelial cell injury in vitro. Lab Invest 1983;48:269–74.
22 Hangen DH, Segall GM, Harney EW, Stevens JH, McDougall IR, Raffin TA. Kinetics of leukocyte sequestration in the lungs of acutely septic primates: a study using ^{111}In-labeled autologous leukocytes. J Surg Res 1990;48:196–203.
23 Zhong WW, Burke PA, Hand AT, Walsh MJ, Hughes LA, Forse RA. Regulation of cytokine mRNA expression in lipopolysaccharide-stimulated human macrophages. Arch Surg 1993;128:158–63.
24 Ghofrani HA, Rosseau S, Walmrath D *et al.* Compartmentalized lung cytokine release in response to intravascular and alveolar endotoxin challenge. Am J Physiol 1996;270:L62–8.
25 Suzuki H, Suematsu M, Miura S *et al.* Rat CINC/gro: a novel mediator for locomotive and secretagogue activation of neutrophils in vivo. J Leukoc Biol 1994;55:652–7.
26 Ulich TR, Howard SC, Remick DG *et al.* Intratracheal administration of endotoxin and cytokines. VI. Antiserum to CINC inhibits acute inflammation. Am J Physiol 1995;268:L245–50.
27 Shanley TP, Schmal H, Warner RL, Schmid E, Friedl HP, Ward PA. Requirement for C-X-C chemokines (macrophage inflammatory protein-2 and cytokine-induced neutrophil chemoattractant) in IgG immune complex-induced lung injury. J Immunol 1997;158: 3439–48.
28 Blackwell TS, Blackwell TR, Christman JW. Induction of endotoxin tolerance depletes nuclear factor-κB and suppresses its activation in rat alveolar macrophages. J Leukoc Biol 1997;62:885–91.
29 Senftleben U, Karin M. The IKK/NF-κB pathway. Crit Care Med 2002;30 (Suppl. 1):S18–26.
30 Takasuka N, Matsuura K, Yamamoto S, Akagawa KS. Suppression of TNF-αmRNA expression in LPS-primed macrophages occurs at the level of nuclear factor-κB activation, but not at the level of protein kinase C or CD14 expression. J Immunol 1995;154:4803–12.
31 Ziegler-Heitbrock HW, Wedel A, Schraut W *et al.* Tolerance to lipopolysaccharide involves mobilization of nuclear factor κB with predominance of p50 homodimers. J Biol Chem 1994;269:17001–4.
32 Ziegler-Heitbrock HW, Petersmann I, Frankenberger M. P50 (NF-κB1) is upregulated in LPS tolerant P388D1 murine macrophages. Immunobiology 1997;198:73–80.
33 Wedel A, Frankenberger M, Sulski G *et al.* Role of p52 (NF-κB2) in LPS tolerant in a human B cell line. Biol Chem 1999;380:1193–9.