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EFFECTS OF LIPOPOLYSACCHARIDES ON TLR4 EXPRESSION IN INS-1 RAT INSULINOMA CELLS

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Abstract

The aim of the study was to obtain insight into the mechanism of sepsis-induced hyperglycemia, to explore the expression of toll-like receptor 4 (TLR4) on INS-1 cells, the effects of lipopolysaccharide (LPS) on TLR4 expression and cell viability. The expression of TLR4 on INS-1 was detected by both RT-PCR and Western blot assays. After being intervened by LPS of various concentrations (0.01, 0.1, 1, 5, 10mg/L) for a certain time, the effects of LPS on TLR4 expression and cell viability were detected by quantitative real-time reverse-transcriptase polymerase chain reaction, western blotting and CCK-8 assay. Then INS-1 cells were stimulated by LPS (0.1, 1mg/L) together with anti-TLR4 antibody, cell viability and TLR4 expression were detected again. TLR4 expressed in INS-1 cell line. Its expression was up-regulated by the stimulation of LPS higher than 0.1mg/L for 12h (P<0.05). However, there was a little down regulation of TLR4 between the LPS treated groups and controls with further LPS treatment for 24 and 48h (P>0.05). In certain concentrations(0.1～10mg/L), viability of INS-1 cells was inhibited by LPS in a dose dependent manner (P<0.05). These effects could be blocked by anti-TLR4 antibody partially. These results suggest that LPS may act directly on the pancreatic β cells via TLR4 on the β-cell membrane. LPS increased TLR4 expression in the early short period of time and caused injury to INS-1 cells after a certain time. It could be one of the mechanisms that hyperglycemia occurs in the early stage of sepsis.

Key words: Toll like receptor 4, lipopolysaccharide, INS-1 cell line, cell viability.

Article information's

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Abbreviations: **TLR4:** toll-like receptor 4; **LPS**: lipopolysaccharides; **FCS**: Fetal calf serum; **CCK-8**: Cell Counting Kit-8; **TNF**: tumor necrosis factor; **IRF 3**: interferon regulatory factor 3.

INTRODUCTION

Hyperglycemia is common in critical illness such as serious infection, sepsis and septic shock and it is associated with poor outcome. The secretion of endogenous catecholamine is increased so that patients may develop insulin resistant and result in hyperglycemia, even in the absence of a diagnosis of diabetes. This condition is termed stress hyperglycemia and is often seen during such situations as mentioned above. The incidence was about 50% in the patients with moderate to severe infection who had no diabetes before. Insulin secretion normally decreases at a rate of approximately 0.7% per year with aging; this decrease in beta-cell function is accelerated about two-fold in people with impaired glucose tolerance-first phase to a greater extent than second phase (20). Hyperglycemia results from beta-cell dysfunction in critically ill patients (16),

but many questions remain about the cause of stress hyperglycemia. Lipopolysaccharides (LPS) are found in the outer membrane of Gram-negative bacteria, act as endotoxins. Toll-like receptor 4 (TLR4) expressed in many cells functions as the transmembrane component of the LPS receptor complex.

In the present study, we investigated whether TLR4 expressed on pancreatic β cells and the direct effects of LPS on its expression and on cell viability. We aimed to reveal the endocrinological basis of infection induced stress hyperglycemia.

MATERIALS AND METHODS

Reagents

RPMI1640, Fetal calf serum (FCS) and 2-mercaptoethanol were purchased from Gibco Company (Gibco, USA). Cell lysate was purchased from Cell Signaling Technology (USA). Trizol reagent was obtained from Invitrogen (San Diego, CA). Rabbit anti-rat TLR4 primary antibody and HRP-conjugated goat anti-rabbit secondary antibody were purchased from Abcam (UK). Anti-GAPDH antibody was obtained from Novus Biologicals. LPS, Cell Counting Kit-8 (CCK-8), SDS-PAGE gel preparation kit and DAB Horseradish Peroxidase Color Development Kit were purchased from Beyotime (Biotechnology, China).

Cell culture

Pancreatic β-cell line rat insulinoma cells (INS-1) were kindly provided by professor Xiao-Ying Li of Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The cells were cultured on 25cm dishes and propagated in RPMI medium 1640 supplemented with 2 mM L-glutamine, 10% heat-inactivated FCS, 1 mM Na-pyruvate, 10mM Hepes, 50μM 2-mercaptoethanol, and 100 U/mL penicillin, 100μg/mL streptomycin in a humidified 5% CO_2/95% O_2 atmosphere at 37 °C. The culture medium was removed and replaced with fresh media every other day. At 70-80% confluence, cells were treated with the indicated concentrations of LPS for 6, 12, 24, or 48h respectively with only a minimum (0.5%) of FCS added.

The INS-1 cells were grouped as follows: (1) basal: normal medium group; (2) medium with different LPS concentrations.

Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-PCR) analysis

INS-1 cells in growth medium were equally seeded into 6-well clusters at a density of 5×10^5 /well. The growth medium was replaced with RPMI1640 containing 0.1% serum when cells had grown to approximately 90% confluence for 24 h. The subconfluent cells were then exposed to fresh medium containing LPS at various concentrations for a certain time. Total RNA was isolated with Trizol reagent according to the manufacturer's instructions and then subjected to cleanup using RNase-Free DNase Set and the RNeasy Mini kit (Qiagen). Quantitative real-time RT-PCR was performed. The primers were designed by Primer Premier5.0 and Primer Design software and synthesized by Sangong Company (Shanghai, China). Real-time fluorescence detection was carried out using an ABI PRISM 7900 Sequence Detector (Applied Biosystems). PCR primers for TLR4 were 5'–TTCCTCTCCTGCGTGAGAC-3' and 5'-TTCATAGGGTTCAGGGACAG-3'. PCR primers for GAPDH were 5'-TACCAACTGGGACGACAT-3' and 5'-AGAAGGAAGGCTGGAAAA-3'. PCR amplification was performed for 32 cycles at 94℃ for 1min, 67℃ for 1min and 72℃ for 2min. Amplification products were electrophoresed on 1.2% (WV) agarose gels containing 0.5μg/ml ethidium bromide and the sizes of the products were determined by comparison to 100 bp DNA ladder marker. The intensity of each band amplified by real-time PCR was analyzed using Bio-Rad Quantity One software and normalized to GAPDH mRNA in corresponding samples.

Western blotting

INS-1 cells in six-well plates were washed twice with ice-cold PBS and placed immediately in lysis buffer. Lysates were gently mixed for 10 min at 4°C, and then centrifuged at 14,000 x *g* for 15 min at 4°C. The protein concentration of the extracts was determined according to the method of bicinchoninic acid, using BSA as the standard. Samples were separated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences, UK). After blocking nonspecific binding sites with 1%bovine serum albumin in TBS for 1 h, the specimens were incubated overnight at 4°C with the rabbit anti-rat TLR4 primary antibody (1:1000), followed by incubation with the HRP-conjugated goat anti-rabbit antibody (1:5000) for 1 h at room temperature. As an internal control, GAPDH expression was detected using GAPDH antibody. Visualization was detected with chemiluminescence reagent, using the ECL Western blotting analysis system. Protein bands were analyzed by densitometric analyses and normalized against the intensity of GAPDH.

CCK-8 Assay for cellular activity

Cell viability was determined using the CCK-8 assay, which is based on the conversion of water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] to a water- soluble formazan dye upon reduction in the presence of an electron carrier by dehydrogenases (9). Briefly, 100μl cell suspension (containing 2×10^4 cells) was plated in each well of a 96-well plate. After overnight culture, to allow reattachment, the cells were incubated in the absence or presence of various concentrations of LPS for a certain time. At the end of each experiment, the cell proliferation reagent CCK-8 was added to each well, and the cells were incubated at 37 °C for 1.5 h. A_{450nm} was measured using a kinetic plate reader. Cell viability was expressed as the OD value.

Statistical analysis

Each experiment was repeated at least three times with triplicate values within each group. Data are presented as the mean ± standard deviation (SD). Analyses were performed using SPSS 11.5 software. The significance of the results was analyzed using Student's two tailed **t**-test. **P** values less than 0.05 were considered statistically significant.

RESULTS

TLR4 expression on INS-1 cells

The mRNA and protein levels of TLR4 were measured in the extracts from the INS-1 cells. They were detected by both RT-PCR and Western blot analysis in the rat insulinoma cell line INS-1, GAPDH was used as control. Bp sizes are indicated. Results are from a representative experiment. Both RT-PCR and Western blot analysis showed that there was expression of TLR4 mRNA and TLR4 protein on INS-1 cells (Fig 1A, 5).

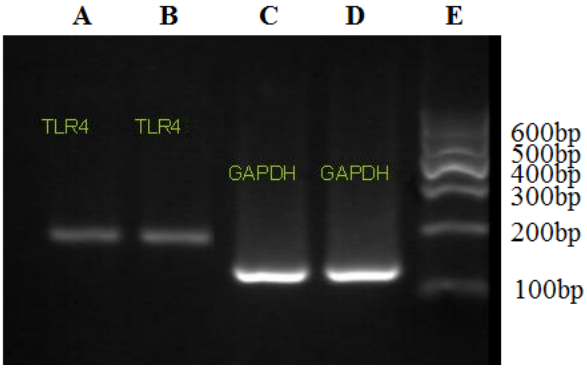

Figure 1. RT-PCR analysis of TLR4 mRNA. GAPDH was used as control. Lane A and B: TLR4; Lane C and D: GAPDH; Lane E: marker.

Effects of LPS on INS-1 cells viability

First, we examined the effects of LPS on INS-1 cells growth. To evaluate these effects on INS-1 cells viability, CCK-8 assay was used. Together, these data demonstrated that LPS over a certain concentration caused injury to INS-1 cells after a certain time.

The effect of LPS concentration on INS-1 cells viability

As shown in Fig 2, the OD values decreased after INS-1 cells were incubated with LPS at the concentration of 0.01, 0.1, 1, 5, 10mg/L for 24h, but the OD values decreased significantly when the cells were stimulated by LPS at the concentration higher than 0.1 mg/L compared with normal culture (**P*<0.05). These stimulatory effects were observed obviously above 1mg/L LPS concentration (***P*<0.01). The viability of INS-1 cells was inhibited by LPS in a dose dependent manner.

The effect of time course on INS-1 cells viability

As shown in Fig 3, the INS-1 cells were exposed to LPS at the concentration of 1mg/L for 6, 12, 24, 48h. No statistically significant decrease in OD value was observed in INS-1 cells stimulated by 1mg/L LPS for 6 or 12h compared with that in basal control groups. Exposure of the cells for 24 or 48h induced a significant decrease in OD value compared with normal culture (**P*<0.05).

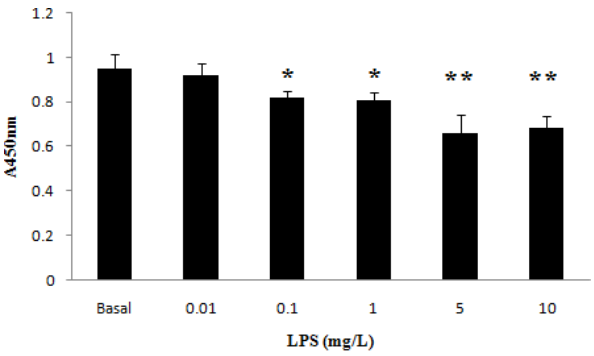

Figure 2. Cell viability of INS-1 exposed to different concentrations of LPS for 24h（n=4）Versus control: * *P*<0.05; ***P*<0.01

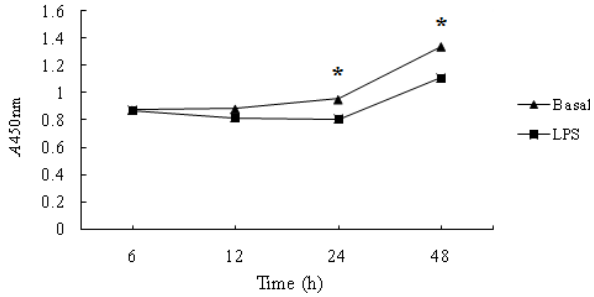

Figure 3. Cell viability of INS-1 exposed to 1mg/L LPS for different time（n=4, $\overline{X}$ ±s） Versus control: **P*<0.05

Effects of LPS on TLR4 expression in INS-1 cells

Effects of LPS on TLR4 mRNA levels

To investigate the effects of LPS on TLR4 expression in pancreatic βcells, LPS at the concentration of 0.1mg/L was used for 6h. RT-PCR analysis of TLR4 mRNA levels showed that the expression of TLR4 mRNA on INS-1 cells was markedly increased with the stimulation of LPS (Fig. 4A). To demonstrate the effects of LPS concentrations on INS-1 cells, the mRNA levels of TLR4 were measured in the extracts from the three groups at different concentrations of LPS. Quantification of RT-PCR products was normalized to internal GAPDH band intensity using Bio-Rad Quantity One software. Significant difference in the mRNA expression of TLR4 was observed in INS-1 cells. The mean expression of TLR4 mRNA was higher in 0.1mg/L and 1mg/L LPS treated groups, being 2- or 3-fold higher than in control groups, the difference was statistically significant (**P*<0.05) (Fig. 4B). By quantitative RT-PCR analysis, it showed that the mRNA expression of TLR4 was up-regulated with the stimulation of 0.1mg/L LPS for 12h and

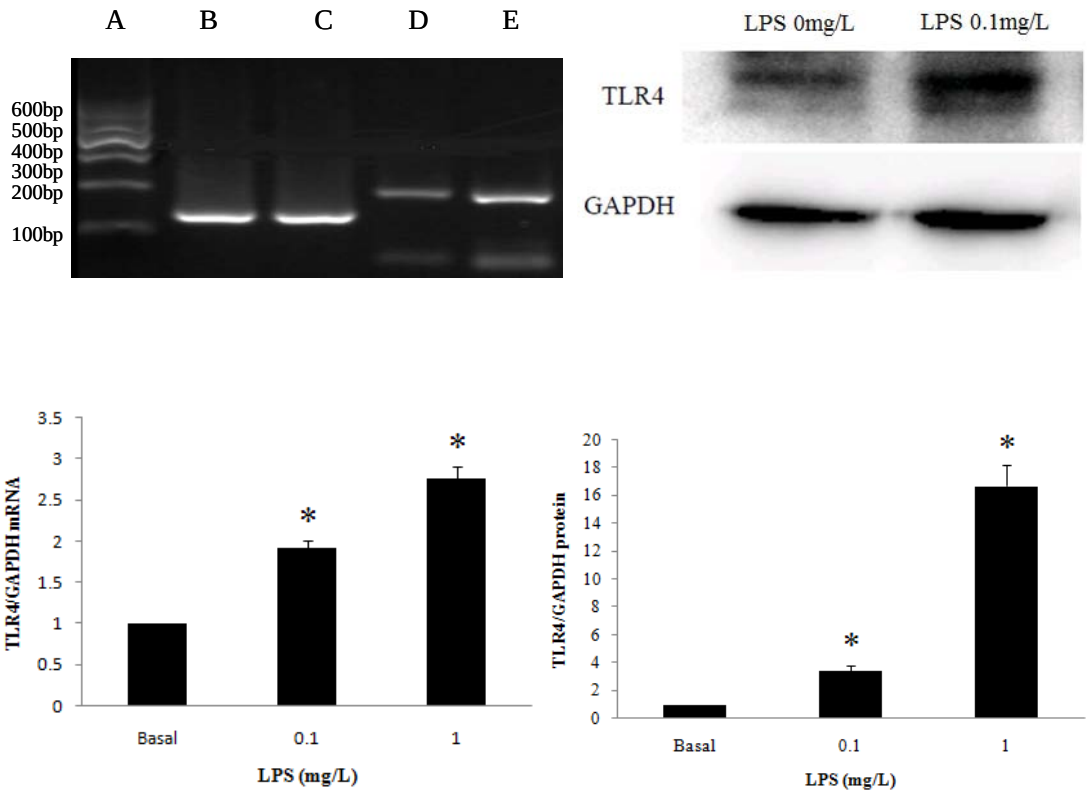

Figure 4. TLR4 mRNA and protein expression and effects of LPS concentrations on INS-1 cells (quantitative real-time PCR and western blotting analysis). GAPDH was used as control. The results were corrected for GAPDH expression within each sample. Versus control: **P*<0.05.
Lane A: marker; Lane B: GAPDH Control, LPS 0; Lane C: GAPDH LPS 0.1mg/L; Lane D: TLR4 Control, LPS 0; Lane E: TLR4 LPS 0.1mg/L.

the difference was statistically significant. Whereas there was a little down regulation of TLR4 between the LPS treated groups and controls with further LPS treatment for 24 and 48h, no statistical significance was observed (Fig. 4C).

Effects of LPS on TLR4 protein levels

To analyze TLR4 expression at the protein level, we performed Western blot studies on INS-1 protein extracts. It showed that the expression of TLR4 protein levels was increased with the stimulation of LPS (Fig. 5A). Quantification of TLR4 protein levels was normalized to GAPDH control using Bio-Rad Quantity One software. To determine the effects of LPS on TLR4 expression, INS-1 cells were exposed to LPS at the indicated concentrations for 12h. The mean expression of TLR4 protein levels was slightly higher in 0.1mg/L LPS treated groups, being almost 3-fold to that of control groups, and was significantly higher in 1mg/L LPS treated groups, being more than 16-fold higher than in control groups, these stimulatory effects were LPS concentration dependent, the difference reached statistical significance (Fig. 5B).

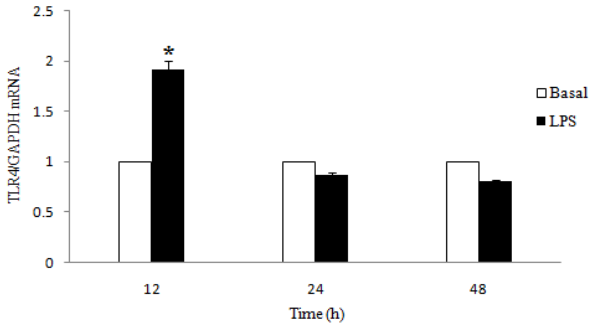

Figure 5. Effects of time course on mRNA expression for TLR4 with the stimulation of 0.1mg/L LPS. Versus control: * *P*<0.05

Effect of anti-TLR4 antibody on LPS stimulated INS-1 cells

We next studied whether anti-TLR4 antibody modulated constitutive or inhibited TLR4 expression in INS-1 cells stimulated by LPS.

After INS-1 cells were pretreated with 1mg/L anti-TLR4 antibody for 1h, cells from each group were then stimulated by LPS at the concentration of 1mg/L with or without anti-TLR4 antibody for 24h. By CCK-8 assay we observed that after INS-1 cells were pretreated

with 1mg/L anti-TLR4 antibody for 1h, the cells viability was not significantly decreased in the incubation with LPS (**P*<0.05). Furthermore, anti-TLR4 antibody does not modify the normalized expression of TLR4 under basal, non-stimulated conditions in INS-1 cells (Fig 6).

To observe the effects of anti-TLR4 antibody on LPS induced TLR4 expression, INS-1 cells were exposed to 0.1mg/L LPS with/without anti-TLR4 antibody for 12h. By real-time PCR analysis, it showed that the mRNA expression of TLR4 was upregulated with the stimulation of LPS. But anti-TLR4 antibody could significantly decreased LPS induced TLR4 mRNA expression in INS-1 cells (**P*<0.05) (Fig 7).

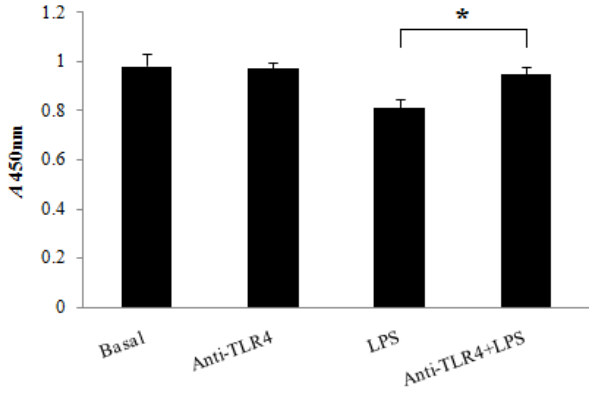

Figure 6. Cell viability of INS-1 exposed to LPS 1mg/L with/without anti-TLR4 antibody for 24h（n=4） * *P*<0.05

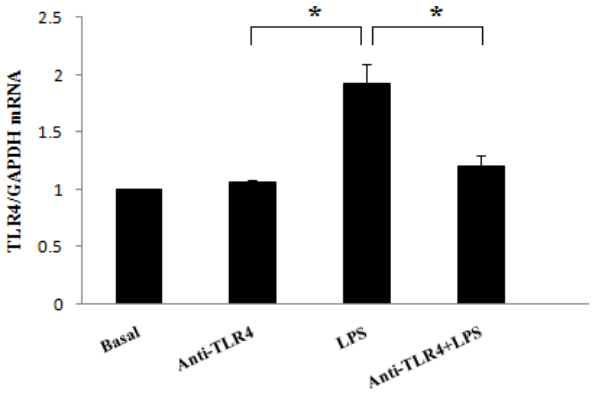

Figure 7. Quantification of TLR4 mRNA expression in INS-1 cells exposed to 0.1mg/L LPS with/without anti-TLR4 antibody for 12h * *P*<0.05

DISCUSSION

Severe sepsis is the cause of 9% to 22% intensive care unit admissions and is associated with a mortality rate up to 50% (12). Bacterial antigens trigger the initial cytokine response to infection, which is necessary for the clearance of invading pathogens, but overwhelming activation of immune cells, with excessive production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. LPS (endotoxin) is the constituent of outer leaflets of Gram negative bacteria, such as *E. coli* or *S typhi* (23). It is a potent activator of the immune system. The presence of large amounts of LPS leads to excessive release of these mediators, resulting in septic shock.

Stress hyperglycemia is frequent in critically ill patients. Impaired glucose homeostasis has been well documented in patients with sepsis. Hyperglycemia is the most common blood glucose abnormality seen early in the course of bacterial sepsis. Islet β cells injury is an important reason for hyperglycemia after infection, but the exact mechanism is not clear. The causes of stress hyperglycemia after infection include the presence of excessive counterregulatory hormones (glucagon, growth hormone, catecholamine, and glucocorticoid, either endogenous or exogenous), high circulating or tissue levels of cytokine (in particular TNF-α and IL-1). This metabolic milieu results in failure of insulin to suppress hepatic gluconeogenesis. However, there have not been relevant reports whether LPS induced pancreatic β cells dysfunction, increased oxidative stress via the direct effect on TLR4 expression in islet cells.

Toll is a type I transmembrane protein in Drosophila and plays important roles in the host defense. Toll-like receptors (TLRs) are expressed primarily in immune cells but also in solid organs including brain, heart, lung, liver, and kidney (2,3,5,13,22). TLRs recognize structurally conserved microbial products and mediate the initiation of inflammatory and immune defense responses (21). To date, 11 members of the TLR family have been identified in humans, TLR4 being the first to be described (1,4,14). TLR4 has been identified in human β cells and β cell lines such as HP62 while the expression was not restricted to β cells (24). This receptor is essential for LPS signaling. Evidence has shown that TLR4 is an important LPS receptor. Rat insulinoma cell line INS-1 is insulin secreting β cell. It faithfully mimics the function of normal pancreatic islet β cells. Our study showed that there was an expression of TLR4 on INS-1 cells, suggesting that during severe infection, sepsis and other stress conditions, LPS maybe act on pancreatic β cells via its receptor TLR4, cause oxidative stress and result in glucose metabolism disorders. It also confirmed that in a certain

period of time, TLR4 mRNA and protein expression up-regulated by the stimulation of LPS. So as the TLR4 ligand, LPS combined with the TLR4 on INS-1 cells and increased the expression of its receptor. Our data indicated that LPS may act directly on the β cells and this may help to interpret in vitro and in vivo experiments which lead to the suggestion that LPS acts through cytokine production.

Previous study (8) demonstrated that mild portal endotoxaemia of rats with low-dose LPS (0.42ng/min) infusion for 4 weeks caused subacute hepatic inflammation and impaired pancreatic insulin secretion, the first- and second-phase insulin secretions in hyperglycaemic clamp were significantly decreased in LPS-treated rats. In a recent study (10) with the BRIN BD11 rat clonal cell line, the authors showed decreased insulin secretion upon culturing in the presence of LPS. The authors also observed a reversal of the effect on insulin secretion upon removal of LPS. It implicated that endotoxaemia is a potential risk factor to link pancreatic β-cell dysfunction. In this study we employed a cellular model in which pancreatic β-cells INS-1 were exposed to different LPS concentration. Preliminary results showed that INS-1 cells viability decreased obviously resulting from treatment with LPS at the concentration of higher than 0.1mg/L, and it was concentration dependent.

In another study with type 2 diabetic patients (7), steady-state low-dose insulin infusion during a short 4h period led to decreased TLR expression while maintaining the patient's glycemia. We supposed that anti-TLR4 could be one of the key points to prevent hyperglycemia under stressful situations. Our study in which LPS stimulated cells were pretreated with anti-TLR4 antibody for 1h, demonstrated a partial recovery of cell viability. It suggested that LPS inhibited pancreatic β-cells in a certain concentration and within a certain time, the effect may be or partly be mediated by TLR4 on the cell surface. In addition, TLR4 (NP_612564.1, 130 a.a.-201 a.a.) partial recombinant protein with a 26kD N-terminal GST tag acted as antigen to the anti-TLR4 antibody used in this study. And the up-regulation of TLR4 mRNA induced by LPS could be partly blocked by anti-TLR4 antibody. It indicated that TLR4 antibody could block the response to LPS at the transcription level. LPS could activate the TLR4, and TLR4 protein may have self-regulation in its transcription.

In pre-terminal sepsis, profound hypoglycemia may occur because of increased tissue uptake of glucose and the failure of hepatic glucose production (11). Impaired glucose homeostasis is often seen in patients with severe bacterial infection, and hypoglycemia is often a pre-terminal finding in sepsis. The pathophysiological mechanism underlying hypoglycemia is unknown and cannot be investigated in clinical sepsis because it has to be corrected instantaneously. Our observations demonstrated that in the early short period of time, TLR4 expression increased in INS-1 cells under a certain concentration of LPS, while the cell viability was unaffected. But with time, TLR4 expression is not increased, and the difference was no statistically significant as compared with the control groups, cell viability decreased. Our data were somewhat different from the study by Dr. Garay-Malpartida et al (6) who observed an increase of TLR4 expression in β-cells exposed for 48h to LPS. It may be related to different concentrations of LPS. We used much higher concentrations of LPS. So the data with lower receptor expression on INS-1 cells which were stimulated by longer duration of action indicated that TLR4 expression maybe associate with the sepsis severity. It could explain why stress hyperglycemia occurs in the early stage of sepsis, hypoglycemia in late sepsis. Regulation of TLR4 expression may be one of the mechanisms. Recently, several reports have shown reduced gene expression for TLR receptors in monocytes of patients according to their state of sepsis: lowest TLR gene expression in septic shock, followed by severe sepsis and sepsis (17-19).

LPS/TLR4 signaling has been intensively studied in the past few years. TLR4 signaling pathways consist of a myeloid differentiation primary response gene 88 (MyD88) -dependent pathway that is common to all TLRs, and a MyD88-independent pathway that is peculiar to the TLR4 signaling pathway. That is, TLR4 stimulated by LPS activates nuclear transcription factor (NF-κb) via Mal and MyD88, and can also trigger interferon regulatory factor 3 (IRF3) activation via Tram and Trif, regulates the downstream inflammatory factors and protein synthesis (15). The signal transduction pathway of LPS in pancreatic β-cells mediated by TLR4 is unclear, further experiments are needed to clarify the mechanism.

Our data indicated that LPS may act directly on the β cells via TLR4 on the β-cell membrane, LPS increased TLR4 expression in an early short

period of time. The study also demonstrated that LPS inhibited the β- cell viability in a concentration dependent manner. It could be one of the mechanisms that hyperglycemia occurs in the early stage of sepsis, hypoglycemia in late sepsis. Anti-TLR4 antibody could inhibit the decrease of cell viability and up-regulation of TLR4 induced by LPS. Early control of endotoxemia may prevent LPS induced β- cell damage.

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