Cell. Mol. Biol. 58 (Supp): OL1654-OL1659
Published on March 23, 2012
DOI 10.1170/192

Cellular & Molecular Biology
http://www.cellmolbiol.com

ROS-MEDIATED LIPOPOLYSACCHARIDE-INDUCED APOPTOSIS IN INS-1 CELLS BY MODULATION OF BCL-2 AND BAX

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Abstract

Overproduction of reactive oxygen species (ROS) or exhaustion of antioxidants may cause oxidative stress which is a major factor of defective insulin secretion and increases apoptosis of pancreatic β-cells in diabetes. So there comes a consideration of whether antioxidant strategies can be used to protect deterioration of the β-cells. In this study, we explored the mechanism of oxidative stress mediated lipopolysaccharide (LPS) induced apoptosis in insulin secreting (INS-1) cells from a rat pancreatic β-cell line. ROS was monitored by using intracellular ROS capture dihydroethidium (DHE) and dihydrorhodamine123 (DHR123). Apoptosis rate was measured by flow cytometry (FCM). The pro-apoptotic gene Bax and anti-apoptotic gene Bcl-2 were analysed by Western blot and RT-PCR. The results demonstrate that LPS-stimulated INS-1cells manifest intensified intracellular fluorescence in both dose- and time- dependent manners. Apoptosis rate of LPS-stimulated INS-1cells is significantly increased by FCM, with a significant increase in Bax/Bcl-2 ratio revealed by Western blot and RT-PCR. Furthermore, α-lipoic acid (α-LA) inhibits LPS-induced apoptosis, but can not restore the function of glucose stimulated insulin secretion (GSIS) in INS-1 cells.

Key words: LPS, apoptosis, ROS, antioxidant, β-cells.

Article information

Received on February 19, 2012
Accepted on February 28, 2012

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INTRODUCTION

Stress-induced hyperglycemia is a common problem in patients admitted to hospital with septic shock or bacterial sepsis, even when glucose homeostasis has previously been normal (12). The causes of stress hyperglycemia include the presence of excessive anti-insulin hormones, such as glucagon, growth hormone, catecholamine and glucocorticoid, and high levels of cytokine, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (15). Lipopolysaccharide (LPS), a component of the outer cell membrane of gram-negative bacteria, is a principal mediator in the pathophysiology of gram-negative bacterial sepsis (24). However, whether LPS can cause hyperglycemia by directly injuring pancreatic β-cells remains unknown.

Our previous observations have revealed the existence of LPS receptor-toll like receptor 4 (TLR4) in INS-1 cells, which viability was decreased by the stimulation of LPS. Furthermore, this effect of LPS was inhibited by treatment with α-lipoic acid (α-LA), which has an important effect of protecting cells from oxidative stress-induced injury (7).

Therefore, we speculate that the effect of LPS on cell viability may be mediated by apoptosis of INS-1 cells. In this study, we test the involvement of Bcl-2 and Bax in LPS-induced apoptosis and the effect of α-LA on apoptosis and function of glucose stimulated insulin secretion (GSIS) in INS-1 cells.

MATERIALS AND METHODS

Reagents

DMEM, fetal calf serum (FCS) and β-mercaptoethanol were purchased from Gibco Company (Gibco, NY, USA). LPS from Escherichia coli 055:B5, α-LA, DHE and DHR123 were purchased from Sigma Company (Sigma-Aldrich, MO, USA). The antibodies against Bcl-2 and Bax were purchased from Santa Cruz Company (Santa Cruz, CA, USA). Trizol reagent and AnnexinV-FITC apoptosis detection kit were purchased from Invitrogen Company (Invitrogen, NY, USA). Rat insulin radioimmunoassay kit was purchased from Linco Company (Linco Research, MO, USA). BCA protein assay kit was purchased from Bio-Rad Company (Bio-Rad Laboratories, CA, USA). All other chemicals were of ultrapure grade.

Cell culture

The rat insulinoma cell line INS-1 cells were kindly provided by Ruijin Hospital, Shanghai Jiaotong University School of Medicine. INS-1 cells were cultured in RPMI 1640 medium containing 10%FCS, 10mmol/L HEPES, 2mmol/L L-glutamine, 1mmol/L Na-pyruvate, 50μmol/L β-mercaptoethanol, 100units/mL of penicillin, and 100μg/mL streptomycin at 37°C and 5%CO2.

Bioimaging of intracellular ROS levels

ROS production was assessed by DHE probe. The INS-1 cells were grouped as follows:(1) CON group (blank control group), (2) LPS 0.1 group (treated with 0.1μg/mL LPS), (3) LPS 1 group (treated with 1μg/mL LPS), (4) LPS 10 group (treated with 10μg/mL LPS), (5) LA+LPS group (pretreated with 1mmol/L α-LA for 1 h, then treated with 1μg/mL LPS). INS-1 cells were incubated in the presence of 10μmol/L DHE for 1 h at 37℃ after the treatment with indicated concentrations of LPS for 48 h, respectively, and then were washed three times with PBS. The fluorescence

of INS-1 cells was observed by fluorescent microscopy (excitation 520 nm, emission 610 nm).

Flow-cytometric assessment of intracellular ROS levels

DHR123 was used as ROS capture, and the cells were incubated together with 5μmol/L DHR123 in culture medium for 1 h. Blank controls were set, in which DHR123 incubation was omitted. DHR123 is a kind of nonfluorescent compound that can freely permeate cells and interact with intracellular oxidants to form fluorescence compound rhodamine123 (Rh123) which is maintained there. After DHR123 incubation, INS-1 cells were trypsinized and harvested, then washed twice with phosphate-buffered saline (PBS) and directly collected before an immediate detection of mean fluorescence intensity (MFI) of Rh123 by flow cytometry. The results were expressed by relative light units (RLU) per 10^5 cells.

Flow-cytometric assessment of apoptosis

INS-1 cells were plated at a density of 3×10^5 cells per well in a 12-well plate and were grouped as follows: (1) CON group (blank control group), (2) LPS group (treated with 1μg/mL LPS), and (3) LA+LPS group (pretreated with 1mmol/L α-LA for 1 h, then treated with 1μg/mL LPS). The apoptosis of INS-1 cells was assessed by measuring membrane redistribution of phosphatidylserine with an AnnexinV-FITC apoptosis detection kit. According to the manufacturer's protocol, after treatment, cells were collected and washed twice with PBS, followed by being resuspended in 500μL of staining solution containing FITC-conjugated annexinV antibody (5μL) and propidium iodide (PI 5μL of 250μg/mL stock solution). After 30 minutes incubation at 4℃ in the dark, cells were analyzed by flowcytometry. Annexin V-positive and PI-negative cells were defined as apoptotic cells, while Annexin V-positive and PI-positive cells as necrosis cells.

RT-PCR and Real-time RT-PCR analysis

According to the manufacturer's instruction, total RNA was isolated from INS-1 cells by Trizol reagent. A total of 200ng RNA was reversely transcribed to cDNA. The resulting cDNA was stored at -20℃ until amplification. RT-PCR was conducted for the determination of Bax mRNA and Bcl-2 mRNA, with GAPDH mRNA serving as internal control. The sequences of the primers used are available in Table 1. Total reaction volume was 20 μL, and 30 cycles of PCR were performed at 95℃ for 5 min, then 95℃ for 30 s, 58℃ for 30 s, 72℃ for 30 s, and 72℃ for 7 min. All RT-PCR products were subjected to 2% agarose gel eletrophoresis and photographed.

Real time RT-PCR was performed using the SYBR Green PCR Master Mix and ABI Prism 7000 Sequence Detection System.

Western blot analysis

INS-1 cells were cultured and treated as described above, and then lysed with ice-cooled lysis buffer. After the determination of protein content, Western blot was performed as described previously (17). Individual immunoblots were probed with mouse anti-Bcl-2 and anti-Bax monoclonal antibodies. Target protein levels were quantified relative to levels of control protein, mouse anti-β-actin monoclonal antibody. The autoradiographies were scanned and semi-quantitatively quantified.

Table 1. Primers Sequences

BAX	Sense	5'-CTGACGGCAACTTCAACTGGG-3'
	Antisens	5'-CTTCCAGATGGTGAGCGAGG-3'
BCL-2	Sense	5'-GGTGGACAACATCGCCCTGTG-3'
	Antisens	5'-ATAGCTGATTCGACGTTTTGC-3'
Caspase-3	Sense	5'-CACTGGAATGTCAGCTCGCA-3'
	Antisens	5'-TCAGGGCCATGAATGTCTCTC-3'
Caspase-9	Sense	5'-CTGAGTATTTCTCTGTGTTCCA-3'
	Antisens	5'-CATGTCACTGTTGCCC-3'
GAPDH	Sense	5'-GGCACAGTCAAGGCTGAGAATG-3'
	Antisens	5'-ATGGTGGTGAAGACGCCAGTA-3'

GSIS assay

INS-1 cells were preincubated for 1 h in Krebs-Ringer bicarbonate buffer containing 3.3mmol/L glucose, then were incubated for 1 h in Krebs-Ringer bicarbonate buffer containing basal (3.3mmol/L) or stimulatory (16.7mmol/L) concentrations of glucose. After the static incubation, the supernatants were obtained and frozen at -70℃ for subsequent determination of insulin concentration. The insulin levels were measured using rat insulin radioimmunoassay according to the manufacturer's protocol. Intracellular protein concentration was determined using a BCA protein assay kit. Insulin concentration was adjusted for the intracelluar protein content.

Statistical analysis

Each experiment was carried out in triplicate and more than three independent experiments were performed. Results were expressed as means ± standard deviation (SD) and analyzed with SPSS 11.5 software. Groups were compared using analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

RESULTS

Effects of LPS on intracellular ROS production in INS-1 cells measured by fluorescent microscopy

After the cultured INS-1 cells were exposed to LPS at the concentration of 0.1, 1 or 10μg/mL for 48 h, the intracellular fluorescence was intensified as compared with blank control group (Fig. 1A).

The intracellular ROS levels measured by flow cytometry

Exposure of the cells to LPS at the concentration of 1 and 10μg/mL for 24 h induced a significant increase in ROS production compared with control (p <0.05), while ROS production was significantly decreased by pretreatment with 1μg/mL α-LA (Fig. 1B). Exposure of the INS-1 to LPS with concentration of 1 and 10μg/mL for 24, 48, 72h induced a significant increase in ROS production compared with control (p <0.05) (Fig. 1C). The effect was dose- and time- dependent. The ROS production of INS-1 cultured with 10μg/mL LPS for 72 h was almost 3-fold to that of control group.

FCM detection of Apoptosis Rate

FCM revealed that exposure of INS-1 cells to 1μg/mL LPS for 48 h induced a marked increase in apoptosis rate to 15.03 ± 3.26%. Pretreatment with α-LA decreased the

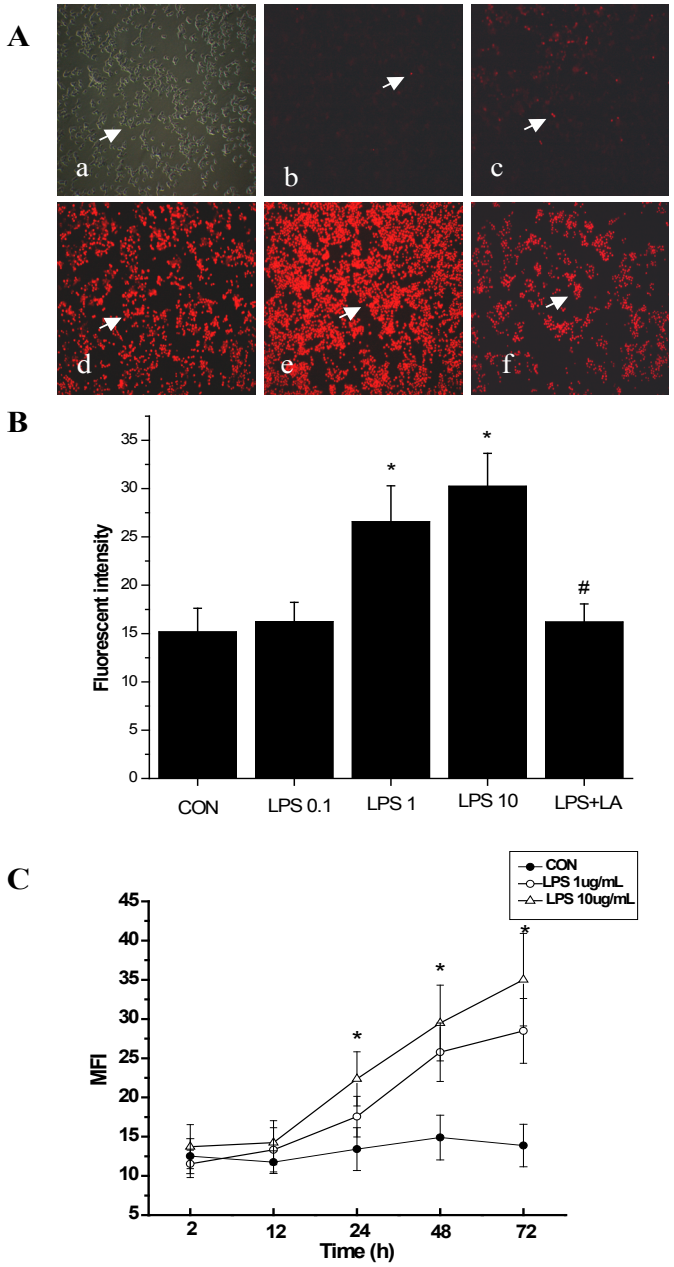

Figure 1. ROS is increased in INS-1 cells treated with LPS. (A) ROS of INS-1 detected with probe DHE by fluorescent microscopy ×100 (a) INS-1 cells observed by optical microscopy (b) control (c) treated with LPS 0.1μg/mL for 48 h (d) treated with LPS 1μg/mL for 48 h (e) treated with LPS 10μg/mL for 48 h (f) pretreatment with 1μg/mL α-LA before treated with LPS 1μg/mL for 48 h. (B) DCF MFI of INS-1 cultured in indicated concentrations of LPS for 24 h. The effect of LPS on INS-1 cells was dose-dependent. DCF MFI of INS-1 was decreased by pretreatment with 1μg/mL α-LA. (C) DCF MFI of INS-1 cultured in 1μg/mL LPS for indicated time. The effect of LPS on INS-1 cells was time-dependent. Values are means±SD of more than three individual experiments. **p* <0.05 vs. Control, #*p* <0.05 vs. LPS 1 group.

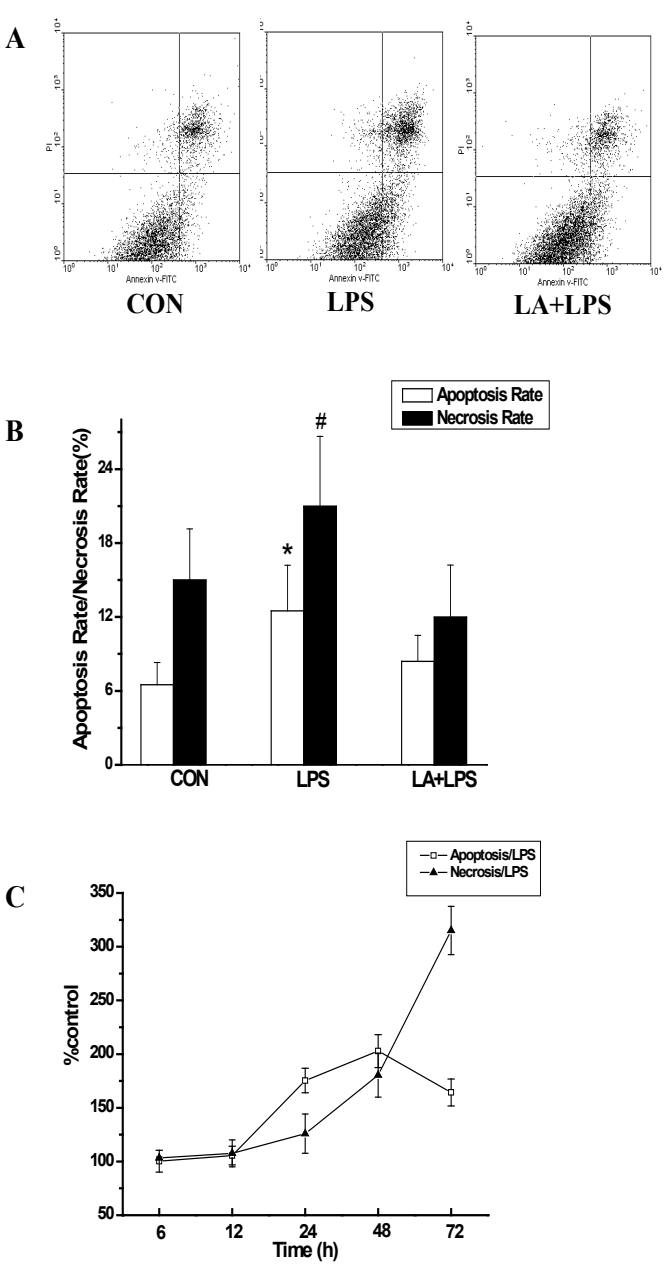

Figure 2. LPS induces pancreatic β-cell apoptosis. (A) Graphical spectrum of apoptosis rate of three groups as detected by FCM. INS-1 were treated with LPS or pretreatment with α-LA followed by flow cytometry using AnnexinV-FITC dye assays to evaluate the cell apoptosis and necrosis. (B) Treatment with 1μg/mL LPS for 48h induced a significant increase in apoptosis or necrosis rate, the white column represents apoptosis rate induced by LPS, while the black column represets necrosis rate. (C) Time-course of LPS-induced apoptosis and necrosis. INS-1 cells were cultured in 1μg/mL LPS for indicated time. Values are means ± SD of more than three individual Experiments. **p* < 0.05 vs. control, #*p* < 0.05 vs. control.

LPS-induced cell apoptosis rate to 8.70 ± 1.53% (*p* <0.05 vs. LPS treated). Furthermore, the necrosis rate induced by 1μg/mL LPS for 48 h was inreased to 1.4 fold, and α-LA also shows protective effect (Fig. 2A, B). Maximal value of apoptosis was obtained after 48 h of incubation of LPS. Necrosis occurred after apoptosis, with maximal value obtained after 72 h of incubation (Fig. 2C). The time course of apoptosis correlated with the reduction of cell viability as measured by the MTT assay we have conducted previously (7).

Effect of LPS-induced apoptosis on Bcl-2 and Bax

Since the ratio of Bax/Bcl-2 was a known mediator of cell apoptosis, we investigated the influence of LPS treatment on the ratio of Bax/Bcl-2 by both RT-PCR and Western blot assays.

Incubation of cells for 6 h with 1μg/mL LPS slightly raised Bax mRNA levels and reduced Bcl-2 mRNA levels. However, the ratio of Bax/Bcl-2 mRNA was increased 30.1% compared to controls as measured by both RT-PCR and Real time RT-PCR (Fig. 3A).

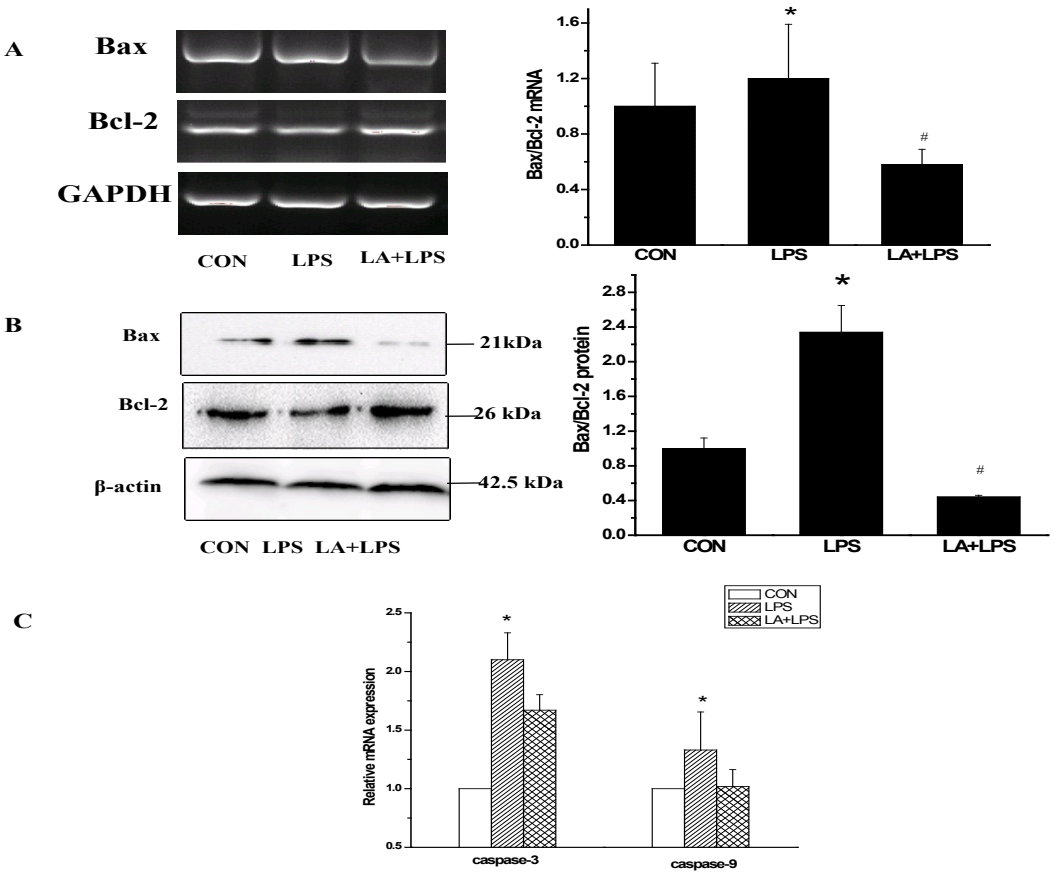

Figure 3. (A) Effect of LPS and α-LA on Bax/Bcl-2 ratio in INS-1 cells. Incubation of cells with 1μg/mL LPS for 6 h significantly increased Bax/Bcl-2 mRNA ratio, which was prevented by pretreatment with 1μg/mL α-LA as measured by RT-PCR. Left: RT-PCR. Right: Histogram of the result of Real Time RT-PCR. (B) Similar effect of LPS and α-LA on Bax/Bcl-2 protein ratio in INS-1 cell. (C) Histogram of the result of mRNA expression levels of caspase-3 and caspase-9. Values are means ± SD of more than three individual experiments. *p <0.05 vs. control, $^{\#}p$ < 0.05

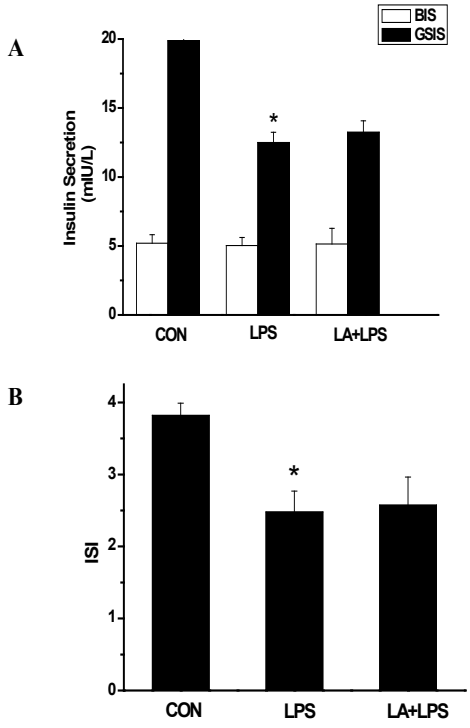

Figure 4. LPS induces decrease in insulin secretion and α-LA fails to reverse the inhibited GSIS. (A) Treatment with 1μg/mL LPS for 48h induced a significant decrease in GSIS. The white column represents BIS induced by 3.3mmol/L glucose. The black column represets GSIS induced by 16.7mmol/L glucose. (B) The ISI was derived as follows: GSIS/BIS. Values are means ± SD of more than three individual experiments. *p <0.05 vs. control.

Incubation of cells for 48 h with 1μg/mL LPS dramatically raised Bax protein levels to 131.2% and reduced Bcl-2 protein levels to 51.5% compared to controls, as was measured by Western blot (Fig. 3B).

Preteatment with α-LA reversed the LPS-induced increase of the ratio by modulation of Bcl-2 and Bax expression in INS-1 cells (p < 0.05 vs. LPS treated) as measured by both RT-PCR and Western blot (Fig. 3A, B).

The relative mRNA expressions of caspase-3 and caspase-9 were shown in Fig 3C. Obviously the mRNA level of caspase-3 and caspase-9 in LPS group were significantly increased compared with the control group. The R was 2.1 and 1.33, respectively (p < 0.05). Preteatment with α-LA reversed the expression of these genes.

Insulin secretion in LPS treated INS-1 cells

To evaluate the involvement of LPS in pancreatic β-cell dysfunction, we conducted glucose stimulated insulin secretion (GSIS) assays. The cells were stimulated with 3.3mmol/L or 16.7mmol/L glucose, with the amount of insulin subsequently secreted measured as basal insulin secretion (BIS) or GSIS, respectively. The insulin secretion index (ISI) was then derived by GSIS/BIS. LPS treatment diminished insulin secretion from INS-1 cells stimulated with 16.7mmol/L glucose relative to controls (p <0.05) (Fig. 4A). However, this negative effect of LPS was not reversed by α-LA treatment (Fig. 4B).

DISCUSSION

LPS is a major component of the cell wall of Gram

negative bacteria implicated in the pathogenesis of bacterial infection. TLR4-MD2-CD14 complex is a receptor of LPS, which has been expressed in many cells in vivo. It is generally believed that LPS activates classical inflammatory cells such as macrophages, lymphocytes and neutrophils by binding TLR4–MD2–CD14 complex in the cells, and leads to the synthesis and release of systemic levels of pro-inflammatory cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 (9, 21). The increase in these cytokines can directly promote apoptotic destruction of the pancreatic β-cells, thus contributing to the decrease in β-cell mass and the impaired insulin secretion observed in patients with type 2 diabetes (3).

The pancreatic β-cells of the islets are the sole source of insulin. Apoptotic destruction of the insulin-producing pancreatic β-cells is involved in the aetiology of both type 1 and type 2 diabetes (27). Oxidative stress is widely accepted as a key mediatory role in the development and progression of diabetes and its complications due to increased production of free radicals and impaired antioxidant defenses (4, 5, 18). Exposure of β-cells to inflammatory cytokines induces the generation of ROS, and their inappropriate activation may lead to the apoptosis of β-cells (11). In a normal cellular environment, ROS is essential to life, while overproduction of ROS or exhaustion of antioxidants is one major factor in the development of diabetes and its complications (8, 22).

The fluorescence methodology, associated with the use of suitable probes, is an excellent approach to measure ROS because of its high sensitivity, simplicity in data collection, and high spatial resolution in microscopic imaging techniques (25, 26). DHE has been used as a fluorescent probe for detecting superoxide radical ($O_2^{-}\cdot$) due to its reported relative specificity for this kind of ROS (1). The product is a red fluorescent compound (probably ethidium). DHR123 is a non-fluorescent molecule that diffuses across cell membranes and is oxidized by ROS (particularly H_2O_2) to the fluorescent rh123 within cells.

However, whether LPS can directly promote apoptotic destruction of the pancreatic β-cells remains unknown. Vives-Pi et al. found TLR4, MD2 and CD14 expressing in endocrine cells of the human pancreatic islets and LPS inhibited glucose-induced insulin release (29). Garay-Malpartida et al. found LPS-induced TLR4 expression in cultured β-cells and LPS led to loss of cell viability (6). Our previous observations also revealed the existence of LPS receptor-toll like receptor 4 (TLR4) in INS-1 cells, which viability was decreased by stimulated with LPS. Now we found the effect of LPS on cell viability was mediated by apoptosis of INS-1 cells by modulation of Bcl-2 and Bax in vitro directly. Furthermore, LPS induced not only apoptosis but also necrosis in INS-1 cells. The development of apoptosis started before necrosis (data not shown), so we speculated that inhibition of apoptosis might further prevent necrosis.

The Bcl-2 protein family is characterized by the ability to modulate cell death. Bcl-2 protein has been shown to enhance cell survival by inhibiting apoptosis induced by cytokines in rat insulin-producing cells (23) and the induction of apoptosis correlates with a reduced Bcl-2 content (16). Bax protein is homologous to Bcl-2 and suppresses the ability of Bcl-2 to block apoptosis, when overexpressed, accelerates the apoptotic death (19). The increase in Bax/Bcl-2 ratio may be important in initiating further signaling cascades leading to apoptosis. Our results demonstrated that the level of the protective Bcl-2 decreased, while Bax increased, resulting in the increase of the Bax/Bcl-2 ratio. These results suggest that LPS disarranges the ratio of Bcl-2 and Bax and therefore leads to apoptosis.

The role of Bax/Bcl-2 ratio in the mechanism of programmed cell death is not yet known. Tran et al. suggested that Bcl-2 protected against apoptosis by inhibiting the generation or action of ROS in β-cells (28). In contrast, Wang et al. showed that ROS were not required for LPS-induced apoptosis (30). Our present results demonstrated a regulation of Bax and Bcl-2 by oxidative processes in INS-1cells, because the antioxidant α-LA prevented the dysregulation of Bcl-2 and Bax induced by LPS both in mRNA and protein level. Recent findings demonstrated that ROS-mediated activation of the TRAF6-ASK1-p38 pathway was crucial for TLR4-mediated mammalian innate immunity (14) and ASK1 was required for oxidative stress-induced apoptotic signal transduction pathway (13). The activation of the apoptotic signaling might also account for the disarrange of the Bax/Bcl-2 ratio. However, further studies should be conducted to evaluate the underlying mechanism.

Recent study (6, 10, 29) showed decreased insulin secretion upon culturing in the presence of LPS with rat clonal cell line. Kiely et al. observed a reversal of the effect on insulin secretion upon removal of LPS (10). Our observation was in accordance with these findings. However, antioxidants α-LA could not restore the function of GSIS.

Our study provides evidence that LPS induces INS-1 cells apoptosis and ROS are involved in this way. Exposure of INS-1 cells to LPS significantly increase intracellular ROS production in both dose- and time- dependent manners. α-LA has the ability to protect INS-1 cells from apoptosis via regulation of the Bax /Bcl-2 ratio but can not restore the function of GSIS in INS-1 cells.

It has been reported that TLR4 directly interacted with NADPH oxidase 4, which was required for LPS-induced ROS generation in HEK 293 cells (20) and ROS might injure the β-cells by penetrating through cell membranes (2). However, the precise intracelluar source from which ROS generates and the relationships among ROS, antioxidants and TLR4 proteins in the LPS-induced pancreatic cells apoptosis remain to be elucidated.

Acknowledgments

This work was supported by grants from the National Nature Science Foundation of China (No. 30872727 and No. 81000354). We are most grateful to the editor for the letters and to the reviewers for their precious comments.

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