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Original Research

Lipopolysaccharide pretreatment inhibits oxidative stress-induced endothelial progenitor cell apoptosis via a TLR4-mediated PI3K/Akt/ NF-κB p65 signaling pathway

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Abstract: Endothelial progenitor cells (EPCs) improve neovascularization and endothelium regeneration. Transplantation with EPCs is a therapeutic strategy for the treatment of ischemic diseases. However, the transplanted EPCs are susceptible to adverse environments such as hypoxia, inflammation and oxidative stress. Oxidative stress-induced apoptosis of transplanted EPCs greatly reduces their therapeutic efficacy. Lipopolysaccharide (LPS) is a highly immunogenic antigen. Recent findings suggest that low dose of LPS pretreatment has protective effect against apoptosis. In this study, the role of LPS in apoptosis of EPCs was investigated. Pretreatment with 1μg/ml LPS prevented oxidative stress-induced EPCs apoptosis and ROS generation, which effects were abolished by TAK-242, a specific TLR4 antagonist. Further investigation of the mechanisms demonstrated that the activation was mediated by TLR4, and that PI3K/Akt/ NF-κB p65 signaling pathway may play a critical role in the process.

Key words: Endothelial progenitor cells; Lipopolysaccharide; Oxidative stress; Toll-like receptors 4.

Introduction

Results from laboratory studies and clinical trials suggest that transplantation with endothelial progenitor cells (EPCs) is a promising strategy for vascular repair and ischemic heart disease (1, 2). However, the primary challenge of EPC transplantation is the low survival rate and high apoptosis rate of EPC cells when exposed to adverse microenvironments such as hypoxia, inflammation and oxidative stress (3, 4). Therefore, it is necessary to develop novel strategies which can promote EPCs survival and protect EPCs against apoptosis so as to improve the efficacy of EPCs transplantation.

Lipopolysaccharide (LPS), an important antigenic component of Gram-negative bacteria, is a critical mediator of endotoxemia, multiple organ dysfunction syndrome and endotoxic shock (5). However, recent studies suggest that pretreatment or preconditioning with an appropriate concentration of LPS may activate intracellular survival mechanisms and attenuate the dysfunctions and injuries in many different organs affected by ischemia or endotoxemia (6-8). Other studies have suggested that a low dose of LPS is cytoprotective to neurons and stem cells (9, 10). However, not much is known about the protective effects of LPS pretreatment against oxidative stress-induced apoptosis in EPCs, and the mechanisms involved.

Toll-like receptors (TLRs) are type-I transmembrane glycoproteins which play important roles in the innate immune system(11). Toll-like receptor 4 (TLR4), the first of the TLRs described, recognizes a diverse range of molecules such as LPS, heat-shock proteins, hyaluronic acid, fibrinogen, β-defensin2, and the fusion protein from respiratory syncytial virus (RSV) (12-15). Recently, it was reported that activation of TLR4 could trigger anti-apoptotic effects and promote cell proliferation (6, 16). The present study was designed to investigate the effect of pretreatment with an appropriate concentration of LPS on activation of the TLR4-signaling pathway in EPCs, and oxidative stress-induced apoptosis in EPCs via down-stream survival pathways.

Materials and Methods

Isolation, cultivation and characterization of EPCs

Human peripheral blood mononuclear cells were isolated from healthy volunteers (who gave written informed consent) using density-gradient centrifugation with Ficoll separating solution (Sedarline Laboratories Ltd., Ontario, Canada). The cells were cultured in EGM-2MV (Lonza Basel, Switzerland) containing 10 % FBS. After 3 days in culture, non-adherent cells were removed by washing with PBS, while adherent cells were maintained in fresh medium for 1-2 weeks. Late outgrowth EPCs were derived from adherent mononuclear cells, and colonies displayed cobblestone morphology. For fluorescent staining, adherent cells were first incubated with 10 mg/mL Dil-acetylated LDL (Molecular Probes, Eugene, OR, USA) at 37 °C for 2 h, and thereafter fixed with 2 % paraformaldehyde for 10 min, followed by incubation with 10 μg/ml FITC labeled UEA-I (Sigma, St Louis MO, USA) at 37 °C for 1 h. The cells were identified under a fluorescence microscope. Double-positive fluorescence was identified as differentiating EPCs.

ROS measurement

Generation of ROS in EPCs was measured using the membrane permeable indicator 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA*)* (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The EPCs were cultured in 6-well plates and either treated with various concentrations of LPS (0.1, 1 and 10 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 12 h, or pretreated with 1 μM TAK-242 (MedChem Express, Princeton, USA) for 1 h with subsequent exposure to different concentrations of LPS, followed by incubation with 150 μM H_2O_2 (Sigma-Aldrich; St. Louis, MO, USA) for 6 h. For measurement of intracellular ROS, the EPCs were incubated with 10 μM H2DCFDA in serum-free EGM-2MV medium at 37˚C for 30 min, and then washed twice with PBS. After trypsinization and re-suspending in serum-free medium, the EPCs were analyzed with flow cytometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA) at excitation and emission wavelengths of 488 and 525 nm, respectively. The production of ROS was determined in terms of mean fluorescence intensity (MFI).

Assay of apoptosis

The apoptosis of cells was assayed using propidium iodide (PI)/Annexin V staining. Following treatment, cells at a density of 1 $\times 10^6$ cells/mL were harvested and washed twice with ice-cold PBS, then stained with FITC-conjugated Annexin V and propidium iodide according to the manufacturer's instructions. The degree of apoptosis of EPCs was assessed using flow cytometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

Western blot analysis

Following treatment as described previously, total protein from EPCs was extracted in a radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF). Protein concentrations were determined using bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China). Cellular proteins were prepared and separated using 10 % SDS-polyacrylamide gel and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5 % non-fat milk and incubated overnight with appropriately diluted primary antibodies before incubation for 1 h with a secondary antibody conjugated with horseradish peroxidase (diluted 1:10,000) at room temperature. The proteins were visualized with enhanced chemiluminescence (Amersham, Haemek, Israel) on a LAS-4000 image reader system (Fujifilm, Tokyo, Japan). To ensure equal loading, protein levels were normalized to that of GAPDH. Anti-Akt, anti-phospho-Akt, anti -NF-κB(p65), anti- phospho-NF-κB(p65), anti-caspase-3, anti-cleaved caspase-3, anti-GAPDH and the secondary anti-rabbit antibody (1:5000 dilution) for Western blot were obtained from Cell Signaling Technology (Beverly, MA, USA), while anti-Bcl-2 and anti-Bax were purchased from Santa Cruz (Santa Cruz, CA, USA).

Assay of NF-κB transcription factor

Following treatment as described previously, nuclear proteins were isolated using Nuclear Extraction Kit (Cayman Chemical, Ann Arbor, MI, USA). The active NF-κB was measured using a specific TransAM NF-κB p65 Transcription Factor Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instruction. Measurements were performed using a Synergy H4 hybrid reader (Biotek, USA) at an absorbance of 450 nm.

Statistical analysis

All experiments were performed at least three times. Values are expressed as mean ± standard error of the mean. One-way ANOVA was used to compare the differences among multiple groups. Values of $p < 0.05$ were considered statistical significant.

Results

Characteristics of EPCs derived from human peripheral blood

The PBMNCs were round in shape, and after 7 days of culture, they exhibited the typical spindle-like shapes of early endothelial progenitor cells (Figures 1A and 1B). After 3 - 14 days of continuous culture, the cells formed cobblestone colonies (Figure 1C), and displayed capacity to take up Dil-Ac-LDL and bind to FITC-UEA-1. The double-positive cells were dividing, late outgrowth EPCs (Figures 1D, 1E and 1F).

Appropriate dose of LPS attenuated oxidative stress-induced generation of ROS in EPCs

To determine the effect of LPS on oxidative changes in EPCs, ROS levels were measured using flow cytometry. As shown in Figures 2A and 2B, exposure of EPCs to 150 μM H_2O_2 for 6 h significantly increased ROS generation ($p < 0.01$). Pretreatment of EPCs with LPS (1 μg/ml for 12 h) markedly reversed H_2O_2-related increase in ROS production ($p < 0.05$). However, pretreatment with 0.1 μg/ml or 10 μg/ml LPS did not alter the ROS production induced by H_2O_2 treatment.

Appropriate dose of LPS protected EPCs against oxidative stress-induced apoptosis

Quantification with FACScan flow cytometric analy-

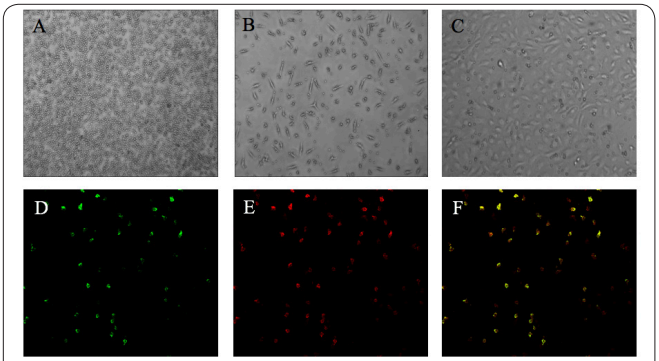

Figure 1. ***Characteristics of EPCs.*** A: PBMNCs (0 day) were round in shape. B: After 7 days, early EPCs exhibited spindle-like shapes. C: Late outgrowth EPCs displayed cobblestone morphology. D: Green color showing FITC-UEA-I positive cells (exciting wavelength = 477 nm). E: Red color of Dil-ac-LDL positive cells (exciting wavelength 543 nm). F: Yellow-colored double-positive cells in the overlay were identified as differentiating late outgrowth EPCs (x400).

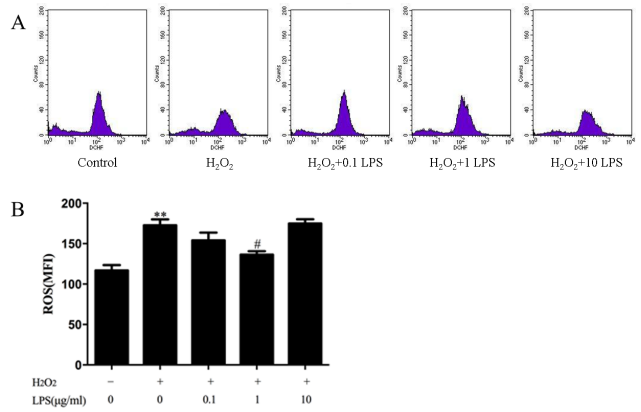

Figure 2. Effect of LPS on ROS generation. After 12 h pretreatment with or without LPS (0.1–10 μg/ml), the EPCs were exposed to H_2O_2 (150 μM) for 6 h and assayed for ROS generation using DCF fluorescence. A: Flow cytometric analysis of cells fluorescently stained with H2DCFDA. B: Histogram showing MFI of each group. Data are presented as mean ± standard error of the mean. (n=3). $^{**}p < 0.01$, compared with control, $\#p < 0.05$, relative to H_2O_2-treated group.

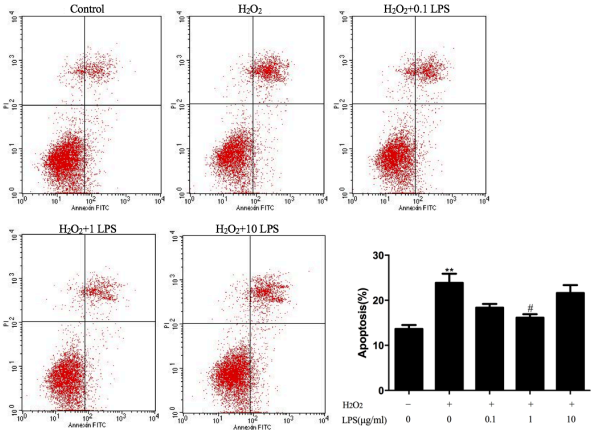

Figure 3. Appropriate dose of LPS protected EPCs against H_2O_2-induced apoptosis. EPCs were pretreated with LPS at doses of 0.1, 1 and 10 μg/ml for 12 h, and subsequently exposed to 150 μM H_2O_2 for 6 h. Flow cytometry was performed to determine apoptosis of EPCs. Data are presented as mean ± standard error of the mean. (n=3). $^{**}p < 0.01$, compared to control; $\#p < 0.05$, relative to H_2O_2-treated group.

sis was used to determine the effect of LPS on apoptosis induced by H_2O_2 in EPCs. As shown in Figure 3, the degree of apoptosis of EPCs was significantly increased in H_2O_2-treated groups, when compared with the control group (23.87+2.024 % vs. 13.64+0.8612 %, $p < 0.01$). However, pre-treatment with LPS (1 μg/ ml) markedly reduced H_2O_2-induced EPCs apoptosis (16.15+0.7772 % vs. 23.87+2.024 %, $p < 0.05$), while LPS at doses of 0.1 μg/ml or 10 μg/ml LPS showed no protective effect against H_2O_2-induced apoptosis.

LPS reduced oxidative stress-induced apoptosis and ROS production in a TLR4-dependant manner in EPCs

Pretreatment with TAK-242 abolished the inhibitory effect of LPS on generation of ROS (Figures 4A and 4B) and also abolished the protective effect of LPS against apoptosis (Figure 4C and 4D). These results indicate that LPS reduced oxidative stress-induced apoptosis and ROS production in a TLR4-dependant manner.

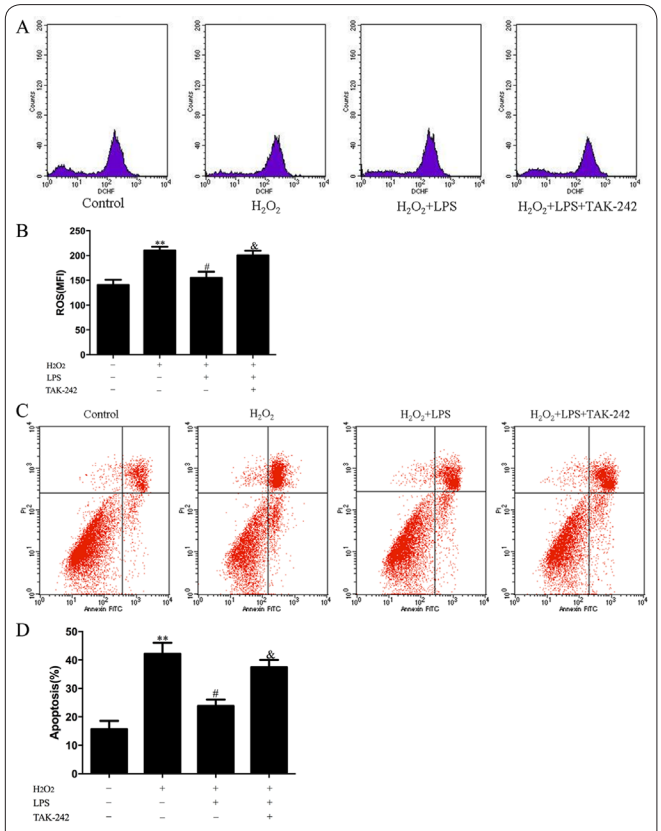

Figure 4. LPS reduced oxidative stress-induced apoptosis and ROS production in a TLR4-dependant manner. EPCs were pretreated with 1 μM TAK-242 for 1 h before LPS treatment (1μg/ml) for 12 h, and finally exposed to 150 μM H_2O_2 for 6 h. ROS generation was determined using DCF fluorescence (A and B). Apoptosis was determined with flow cytometry (C and D). Data are presented as mean ± standard error of the mean. (n = 3). $^{**}p < 0.01$, compared with control; $\#p < 0.05$, compared with H_2O_2-treated group; $p < 0.05$, compared with H_2O_2- and LPS-treated groups.

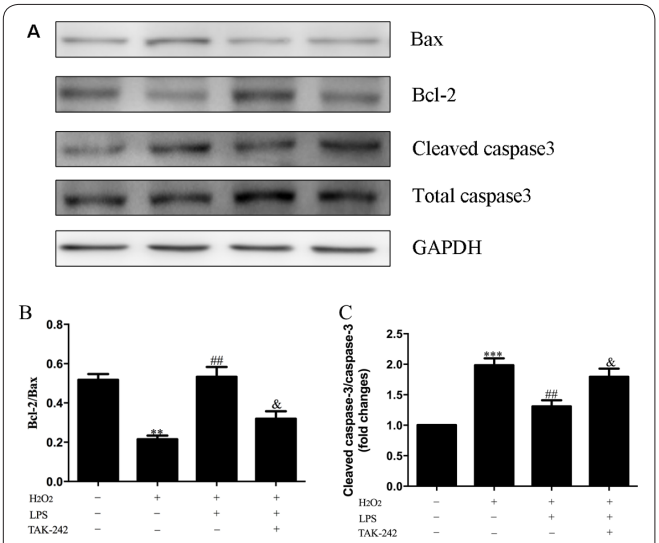

Figure 5. LPS increased the Bcl-2/Bax ratio and inhibited caspase3 activity in oxidative stress-induced apoptosis in EPCs via TLR4. The EPCs were pretreated with 1 μM TAK-242 for 1 h before treatment with LPS (1 μg/ ml) for 12 h, and were then exposed to 150 μM H_2O_2 for 6 h. Western blot analysis was used to determine the protein levels of Bcl-2, Bax, cleaved caspase3 and total caspase3. B: Ratio of Bcl-2/Bax. C: Fold change in cleaved caspase3/total caspase3 ratio. Data are presented as mean ± standard error of the mean. (n=3). $^{**}p < 0.01$, compared with control; $^{***}p < 0.001$, compared with control; $\#\#p < 0.01$, relative to H_2O_2-treated group; $p < 0.05$, relative to H_2O_2- and LPS-treated groups.

LPS increased the Bcl-2/Bax ratio and inhibited caspase3 activity in oxidative stress-induced apoptosis in a TLR4-dependant mechanism in EPCs

The EPCs were pretreated with 1 μM TAK-242 for 1 h before treatment with LPS (1μg/ ml) for 12 h, and were then exposed to 150 μM H_2O_2 for 6 h. Western blot analysis was used to determine the protein levels of Bcl-2, Bax, cleaved caspase3 and total caspase3 (Figure 5A). Pretreatment with 1 μM TAK-242 reduced the Bcl-2/Bax ratio, and increased the expression levels of cleaved caspase3, when compared with H_2O_2 and LPS treatment groups (Figures 5 B and 5C). These results suggest that LPS increased the Bcl-2/Bax ratio and inhibited caspase3 activity in oxidative stress-induced apoptosis in EPCs in a process that might involve TLR4 activation.

LPS pretreatment enhanced phosphorylation of Akt and inhibited activation of NF-κB p65 in oxidative stress-induced apoptosis in a TLR4-dependant mechanism

As shown in Figures 6A and 6B, treatment with LPS (1μg/ ml) significantly stimulated Akt phosphorylation and inhibited NF-κB p65 nuclear translocating activity, while pretreatment with 1 μ M TAK-242 abrogated the activation of Akt and the inhibitory effect of LPS on NF-κB p65 phosphorylation and NF-κB p65 nuclear translocating activity in EPCs exposed to H_2O_2. Taken together, these results indicate that LPS pretreatment enhanced phosphorylation of Akt and inhibited activation of NF-κB p65 in oxidative stress-induced apoptosis via a TLR4-dependant mechanism.

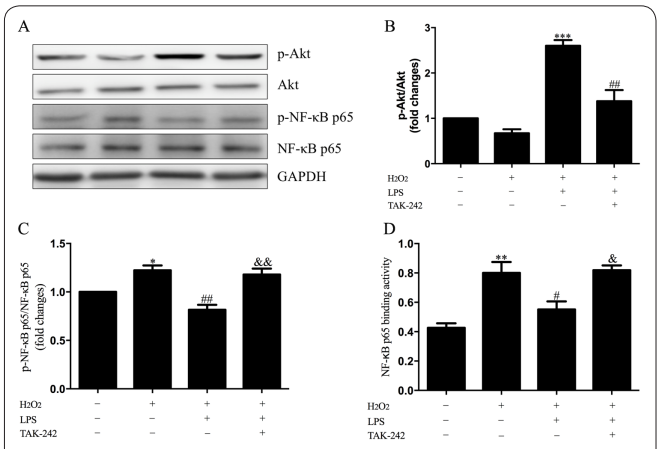

Figure 6. LPS pretreatment enhanced phosphorylation of Akt and inhibited activation of NF-κB p65 in oxidative stress-induced apoptosis in a TLR4-dependant mechanism. The EPCs were pretreated with 1 μM TAK-242 for 1 h before treatment with LPS (1μg/ ml) for 12 h, and were then exposed to 150 μM H_2O_2 for 6 h. Western blot analysis was used to determine the protein levels of Akt, IκBα (A). The fold changes in p-Akt/Akt and p-IκBα/IκBα are shown in B and C, respectively. Figure 6D shows NF-κB p65 transcription factor activity in the various groups. Data are presented as mean ± standard error of the mean (n=3).$^{**}p < 0.01$, compared to control; $^{***}p < 0.001$, compared with control; $^{\#\#}p < 0.01$, relative to H_2O_2- and TAK-242-treated groups; $p < 0.05$, compared to H_2O_2 and LPS-treated groups.

Discussion

As a new strategy for ischemic heart disease, EPC transplantation has gained worldwide acceptance. However, donor EPCs are susceptible to a hostile, oxidative stress and inflammatory microenvironment in the infarct area, resulting in low survival rate and dysfunction of transplanted EPCs, which constitute major challenges to EPC therapy (17, 18). Recently, cell pretreatment or pre-conditioning has been shown to have beneficial effects on stem cell transplantation, and this has attracted more attention and application in cell-based transplantation therapy (19). The findings of the present study indicate that pretreatment with an appropriate dose of LPS protects EPCs from oxidative stress-induced apoptosis and ROS generation, most probably through a mechanism involving the mediation of TLR4 and its downstream effectors PI3K/Akt, NF-κB p65. To the best of our knowledge, this is the first study to describe the roles of LPS pretreatment on oxidative stress-induced apoptosis in EPCs, and the underlying mechanism.

Lipopolysaccharide (LPS) is a cell wall constituent of Gram-negative bacteria and is well known for its roles in systemic inflammation. Previous studies indicated that LPS is recognized trigger for apoptosis and inflammation in multiple cell types (20-22). However, some studies found that appropriate dose of LPS could also activate anti-apoptotic pathways, and that the cyto-protective effect is highly dependent on the concentration of LPS (23). The present study showed that pretreatment with 1 μg/ml LPS for 12 h protected EPCs from oxidative stress-induced apoptosis and also reduced ROS generation, whereas LPS at a dose of 0.1 μg/ml or 10 μg/ml LPS did not exert these effects. Although many studies have reported that LPS pretreatment produced a cyto-protective effect, the molecular mechanism involved was hitherto not fully understood (20-23).

Toll-like receptor 4 (TLR4), an LPS-sensing receptor, plays a pivotal role in cell survival and death. Activation of TLR4 usually triggers release of pro-inflammatory mediators such as interleukin-6 and tumor necrosis factor-α which mediate host damage (14). It has been also reported that activation of TLR4 by low doses of LPS promotes expressions of cell survival genes through suppression of the NF-κB pathway (24, 25). The PI3K/Akt pathway is one of the most crucial cell survival pathways involved in regulating inflammatory responses, cellular activation and apoptosis (26). Recent findings suggest that activation of the PI3K/Akt pathway negatively regulates NF-κB-mediated pro-inflammatory responses (27). A previous study indicated that pretreatment with appropriate dose of LPS protected human dendritic cells or myocytes from apoptosis through PI3K/Akt- and NF-κB-dependent mechanisms via TLR4 (24, 28). In the present study, it was also found that pretreatment with 1μg/ml LPS enhanced phosphorylation of Akt and inhibited activation of NFkB p65 in EPCs treated with H_2O_2, which effects were abrogated by TAK-242, an exogenous synthetic antagonist for TLR4. These findings suggest that a cross-talk may exist between TLR4/ NF-κB and PI3K/Akt signaling pathways in the regulation of apoptosis in EPCs pretreated with low concentration of LPS.

Oxidative stress is one of the major mechanisms

involved in ischemic cardiovascular diseases. It has been recognized as a condition in which excessive ROS or free radicals are generated (29). The ROS, which encompass a range of oxygen-containing species, are important in induction of apoptosis under physiological and pathological conditions (30). Accumulation of ROS and subsequent apoptosis induction ultimately exert a toxic effect on cells (29). Previous studies have demonstrated that oxidative stress-induced apoptosis can significantly reduce the number of EPCs and impair EPC function (31). Transplanted EPCs in the host ischemic environment with high oxidative stress are subjected to injury from apoptosis. Thus, numerous studies have focused on ways to reduce oxidative stress in the transplantation region, and to inhibit the apoptosis of transplanted EPCs (31).

Recently, an experimental evidence of liver protection against ischemia-reperfusion injury showed that low-dose LPS preconditioning prevented hepatocellular apoptosis by inhibiting the activation of caspase-12 and caspase-3 (32). Results from *in vivo* experiments also revealed that LPS preconditioning significantly reduced primary H_2O_2-induced hepatocyte apoptosis. Another study demonstrated that low-dose LPS pretreatment had a significant anti-apoptotic effect on bone marrow mesenchymal stem cells subjected to hypoxia and serum deprivation, through a mechanism probably associated with the ERK signaling pathway (33). Consistent with these previous reports, the present study also found that pretreatment with LPS (1μg/ml) protected EPCs against H_2O_2-induced apoptosis by decreasing ROS production through upregulation of Bcl-2 expression and inhibition of the expressions of cleaved caspase-3 and Bax. However, LPS at a dose of 0.1 μg/ml or 10 μg/ml did not exert similar effects. It was also found that the TLR4 signaling pathway was probably involved in this cyto-protective effect.

The present study has shed new light on the potential role of LPS in protecting EPCs from oxidative stress-induced apoptosis. Pretreatment with appropriate concentration of LPS exerted potent cyto-protective effects against H_2O_2-induced apoptosis in EPCs by inhibiting ROS generation through downregulation of the expression of cleaved caspase-3, and increase in Bcl-2/Bax ratio. The PI3K/Akt/ NF-κB p65 signaling pathway may play a critical role in this progress, since its activation was partially TLR4-dependant. These results provide evidence that LPS preconditioning reduces oxidative stress-mediated EPCs apoptosis. Thus, LPS preconditioning has promising potential as a novel way for refinement of the efficiency of EPC transplantation.

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Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Liang Xia and Guosheng Fu; Meihui Wang, Liang Xia and Guosheng Fu collected and analysed the data; Meihui Wang wrote the text and all authors have read and approved the text prior to publication.

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