Estrogen protects cardiomyocytes against lipopolysaccharide by inhibiting autophagy

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Abstract. Autophagy has a significant role in myocardial injury induced by lipopolysaccharide (LPS). Estrogen (E2) has been demonstrated to protect cardiomyocytes against apoptosis; however, it remains to be determined whether it exhibits anti-autophagic effects. The aim of the present study was to investigate whether estrogen-regulated autophagy attenuates cardiomyocyte injury induced by LPS. The cardiomyocytes of neonatal rats were randomized to the control (Con), LPS and estrogen + LPS groups. The LPS group was treated with 1 μg LPS for 24 h and the estrogen + LPS group was treated with 10^{-8} M estrogen 30 min prior to treatment with LPS. Cardiomyocyte autophagy was quantitated by investigating the mRNA and protein level of autophagy-related genes (Atgs). The mRNA expression of Atg5 and Beclin1 were measured by quantitative polymerase chain reaction and the microtubule-associated protein light chain 3 (LC3) protein expression was measured by western blot analysis. To demonstrate the cardiomyocyte protection of estrogen, cell vitality and serum lactate dehydrogenase (LDH) levels were measured following LPS treatment. It was identified that LPS induced cardiomyocyte injury, together with the upregulation of Atg5, Beclin1 mRNA and LC3-II protein. Furthermore, estrogen attenuated the effect of LPS. The present study provides evidence that estrogen has a myocardial protective role against injury induced by LPS by regulating autophagy.

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Introduction

The myocardium is one of the target organs of septic shock (1-2), which is a major cause of mortality. This myocardial injury may occur as a result of the release of proinflammatory cytokines induced by bacterial endotoxin lipopolysaccharide (LPS) (3). Furthermore, it appears that LPS may be responsible for multiple organ failure during septic shock (4). It has also been demonstrated that LPS reduced myocardial function (5). Numerous studies have suggested that LPS-induced myocardial dysfunction is mediated by multiple proinflammatory mediators, including tumor necrosis factor-α (TNF-α), Toll-like receptor 4 (TLR4) and TLR2 (6-8).

Recently, LPS was reported to stimulate cardiomyocyte autophagy (4,9), which may mediate cell death. Autophagy, which has been suggested to be an essential function for cell homeostasis, as well as cell defense and adaptation to an adverse environment, is a type of programmed cell death (10-12). Autophagy has an important role in the heart, and activation of autophagy has been observed in a variety of heart diseases, including cardiac hypertrophy, heart failure and ischemia reperfusion injury. Therefore, it is important to regulate cardiomyocyte autophagy in order to reduce myocardial injury induced by LPS.

It is well established that the incidence of cardiovascular disease is reduced in females prior to menopause, which may be due to estrogen (E2) levels (13-14). Studies have demonstrated that E2 exhibits cardioprotective effects due to the ability to decrease TNF-α levels (15). However, few studies have investigated whether E2 may regulate cardiomyocyte autophagy. Based on these observations, the present study aimed to examine whether E2 may reduce cardiomyocyte injury by regulating autophagy.

Materials and methods

Animal care. All animal experiments were approved by the Animal Research Ethics Committee of the Second Military Medical University (Shanghai, China). The experimental procedures conformed with the guide for the care and use

of laboratory animals published by the US National Institutes of Health.

Cell culture and experimental procedures. Neonatal cardiomyocytes were prepared from the hearts of Sprague-Dawley rats younger than 3 days (16). On the 4th day, the cardiomyocytes were randomized to three groups: The control group (con), where the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% CO_2 and 95% air for 24 h; the LPS group, where the cells were treated with 1 μg/ml LPS for 24 h; and the E2+LPS group, where the cells were treated with 10^{-8} M E2, and then were treated with 1 μg/ml LPS 30 min later.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazonium bromide (MTT) assay. For the MTT assay, 10 μl MTT solution was added to the growing cells and incubated for 4 h. The crystals were then solubilized by adding 100 μl solubilization solution. The absorbance of the purple solution was determined at a wavelength of 450 nm with a microtiter plate reader (Bio-Rad, Hercules, CA, USA).

Lactate dehydrogenase (LDH) assay. LDH release was measured following treatment as a cellular injury index. The culture media was collected for determination of LDH activity using an Hitachi 7020 chemistry analyzer (Hitachi, Ltd., Tokyo, Japan).

Quantitative polymerase chain reaction (qPCR) of Atg5 and Beclin1. Total RNA of cells was isolated using TRIzol reagent and reverse transcribed according to the manufacturer's instructions (Thermo Scientific, Waltham, MA, USA). Dysregulated Atg5 and Beclin1 were validated by qPCR in duplicates using the Mini OPTICON realtime PCR system (Bio-Rad). The annealing temperature of Atg5 and Beclin1 was set at 56˚C. The comparative Ct (threshold cycle) method with arithmetic formulae ($2^{-\Delta\Delta Ct}$) was used to determine the relative quantitation of gene expression of the target and housekeeping genes (β-actin). The following sense and antisense primers were used: Beclin1 (accession number NM_001034117), forward 5'-GGCAGTGGCTCCTATT-3' and reverse 5'-GGCGTGCTGTGCTCTGAAAA-3'; Atg5 (accession number NM_001014250), forward 5'-AGTGGAGGCAACAGAACC-3' and reverse 5'-GACACGAACTGGCACATT-3'.

Western blotting of microtubule-associated protein light chain 3 (LC3). The protein concentration was determined with a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) according the manufacturer's instructions. Equal quantities of protein (40 μg) from the cardiomyocytes were subjected to western blotting analysis to evaluate LC3 expression (the primary rabbit antibody was purchased from Sigma, St. Louis, MO, USA) with an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ, USA). The results are presented as LC3-II/LC3-I.

Statistical analysis. Quantitative data are presented as the mean ± standard error. Statistical significance was determined using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

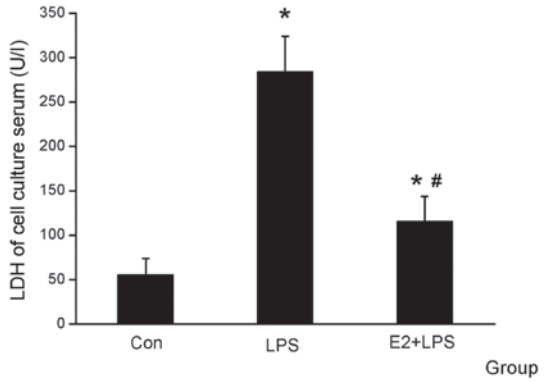

Figure 1. Results of LDH in the cell culture serum (n=6). It was identified that LDH was increased following treatment with LPS (*P<0.05) and was decreased in the E2 + LPS group compared with the LPS group (#P<0.05). LDH, lactate dehydrogenase; LPS, lipopolysaccharide; E2, estrogen; Con, control.

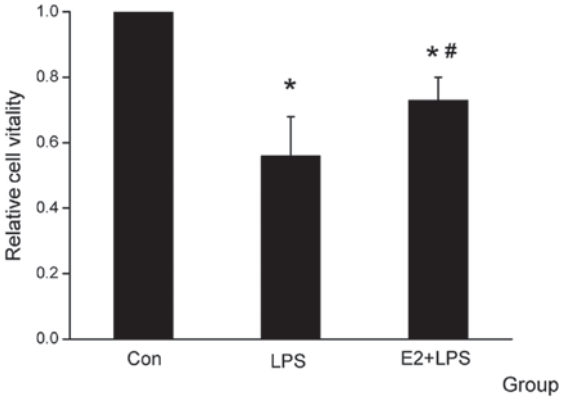

Figure 2. Results of relative cell vitality (n=6). It was identified that cell vitality was decreased following treatment with LPS (*P<0.05), and was increased in the E2 + LPS group compared with the LPS group (#P<0.05). E2, estrogen; LPS, lipopolysaccharide; Con, control.

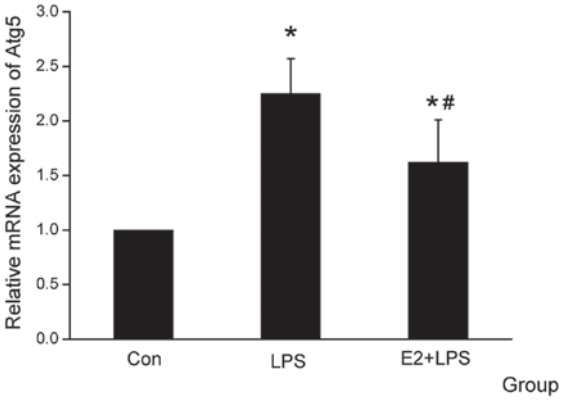

Figure 3. Results of Atg5 expression with quantitative polymerase chain reaction (n=5). Atg5 was upregulated by LPS compared with the Con group (*P<0.05) and was downregulated by E2 compared with the LPS group (#P<0.05). LPS, lipopolysaccharide; E2, estrogen; Con, control.

Results

E2 produces a cardioprotective effect against LPS. The serum was collected for the LDH and MTT assays and was used for

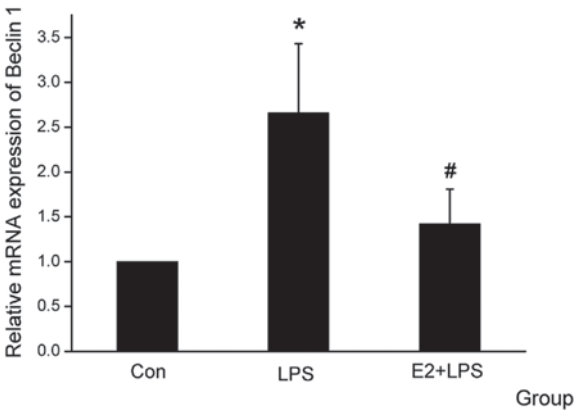

Figure 4. Results of Beclin1 expression with quantitative polymerase chain reaction (n=5). Beclin1 was upregulated by LPS compared with the Con group (*P<0.05), while was downregulated by E2 compared with the LPS group ($^{\#}$P<0.05). E2, estrogen; LPS, lipopolysaccharide; Con, control.

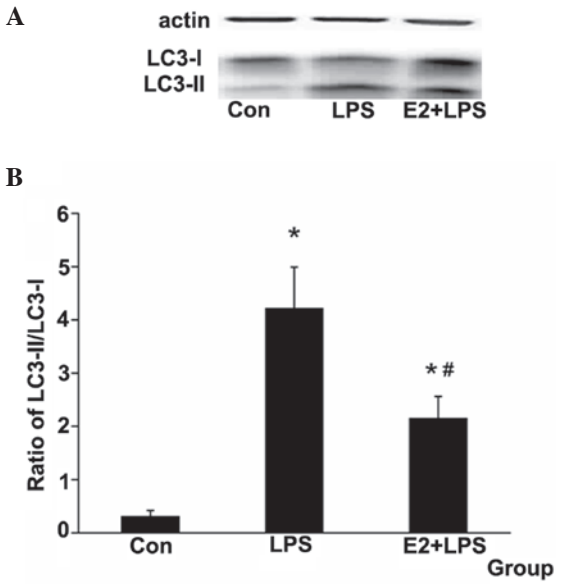

Figure 5. Results of LC3 protein expression with western blotting (n=5). (A) Representative western blot of LC3 from the different groups. (B) The ratio of LC3-II/LC3-I in different groups. It was identified that LC3-II was upregulated by LPS (*P<0.05) and it was attenuated by E2 ($^{\#}$P<0.05). LC3, microtubule-associated protein light chain 3; E2, estrogen; LPS, lipopolysaccharide; Con, control.

comparing cardiomyocyte vitality. It was identified that LDH was higher and the cell vitality was decreased in the LPS group compared with the Con group, and that E2 was able to attenuate the effect of LPS (Figs. 1 and 2).

E2 attenuates Atg5 and Beclin1 mRNA expression level. The Atg family members, particularly Beclin1 and Atg5, have been reported to have an important role in the autophagic cell death pathway (17-18). When the cardiomyocytes were treated with E2, the mRNA expression of Atg5 and Beclin1 was downregulated, which was upregulated by LPS (Figs. 3 and 4).

E2 attenuates the LC3-II protein relative expression level. LC3-II was used as a marker of autophagy (19). The ratio of LC3-II/LC3-I was used to examine the autophagy level in the present study. It was identified that the ratio was increased in the LPS group and decreased in the E2 + LPS group (Fig. 5).

Discussion

LPS, which is a major component of bacterial outer walls, has been demonstrated to be responsible for the multiorgan dysfunction that characterizes septic shock (4). It has been found that LPS is able to stimulate inflammatory mediator production and activate NF-κB (20-21). The myocardium is one of the main target organs of septic shock (2). Therefore, it is clinically beneficial to investigate ways to attenuate the myocardial injury induced by LPS in patients with septic shock. In the present study LPS was used to simulate the heart injury induced by septic shock *in vitro*. According to this model, the present study aimed to investigate novel reagents that may protect the heart against LPS injury. LDH and cell vitality were generally used to evaluate cell injury (16,22). It was found that LPS was harmful to the cardiomyocytes and E2 attenuated the injury induced by LPS.

Apoptosis, necrosis and autophagy occur in cardiomyocytes during cardiac injury (23). The autophagy process is regulated by Atgs, among which Beclin1 is required for the autophagy vesicle nucleation step of autophagy. The autophagosome is formatted through two pathways, the Atg12-Atg5-Atg16 pathway and the Atg4-Atg7-Atg3 pathway. These conjugations lead to the conversion of the soluble form of LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II), which is used as a marker of autophagy (19). In the majority of these studies, the ratio of LC3-II/LC3-I has been used for examining the autophagy level (24).

Apoptosis and necrosis are well established as detrimental processes to the heart (25). However, the effect of autophagy in the heart is controversial (26-27). At low levels, autophagy removed the damaged proteins and organelles, that facilitated myocardial survival during periods of energy deprivation (28). Therefore, low levels of autophagy are beneficial to cardiomyocytes (29). However, excessive levels of autophagy appear to contribute to cardiomyocyte damage (24). Furthermore, accumulation of autophagic vacuoles precedes apoptotic cell death, and autophagy induces cell death when apoptosis is inhibited. For instance, it has been demonstrated that autophagy was marginally increased in the myocardium during the ischemic period, and it was protective for the heart, while during the reperfusion period autophagy was markedly enhanced and was subsequently harmful to the heart (24,27). Furthermore, it has been identified that the inhibition of autophagy is cardioprotective against LPS-induced injury (9). Therefore, moderate regulation of autophagy may aid in attenuating cardiomyocyte injury induced by LPS.

Studies have suggested that E2 has important cardioprotective roles against ischemia-reperfusion (IR) injury (30-31) and that E2 treatment may upregulate the expression of anti-apoptotic genes (32). Recently, several studies demonstrated that Beclin1 was able to downregulate E2ic signaling, suggesting the importance of the interaction between E2 and autophagy (33).

The biological effects of E2 are predominantly mediated via E2 receptors (ERs). The two classic ER isoforms (ERα and ERβ) are expressed in cardiomyocytes, and there appears to be no difference in the distribution or abundance between males

and females (30). ERα is mainly expressed in the ventricles and its activation may result in rapid cardioprotective signaling (34). ERβ is evenly distributed throughout the heart and may not be involved in cardioprotection (35-36). E2 may protect the heart following ischemia-reperfusion by decreasing TNF-α levels (15), which has been associated with cardiomyocyte autophagy induced by LPS. In the present study, cardiomyocyte autophagy was induced by LPS, as is consistent with the results of Yuan *et al* (4). LDH in the serum was increased and the cell vitality was decreased following LPS treatment. This suggested that LPS may be harmful to cardiomyocytes by inducing autophagy. When the cardiomyocytes were treated with E2 1 h prior to LPS, the autophagy level and LDH in the serum were attenuated and the cell vitality was increased. Therefore, E2 may protect cardiomyocytes by attenuating autophagy against LPS, mediated by the ERα subtype receptor.

In conclusion, the results demonstrated that E2 has an important protective role against LPS-induced injury by regulating autophagy. However, further studies are required to investigate the mechanisms underlying the interaction between E2 and the regulation of autophagy.

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