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Amlodipine inhibits TNF-α production and attenuates cardiac dysfunction induced by lipopolysaccharide involving PI3K/Akt pathway

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ABSTRACT

Calcium channel blockers (CCBs) are widely used in the therapy of cardiovascular diseases. Recent studies have shown that several CCBs exerted distinct anti-inflammatory effect in myocardial dysfunction models. The purpose of the present study was to evaluate therapeutic effect and possible mechanism of action of amlodipine, one of the widely used CCBs, on rat cardiac dysfunction during sepsis induced by lipopolysaccharide (LPS). Pretreatment of the rats with amlodipine (10 or 30 mg/kg, *i.v.*) delayed the fall of mean arterial blood pressure caused by LPS. Amlodipine also significantly inhibited the elevation of plasma tumor necrosis factor α (TNF-α) and decreased levels of inducible nitric oxide synthase (iNOS) in response to LPS challenge. To investigate the mechanism of the action of amlodipine, neonatal rat cardiomyocytes were used as a model. Amlodipine concentration-dependently decreased the release of TNF-α and iNOS protein expression, and suppressed the degradation and phosphorylation of inhibitor of κB-α (IκB-α) in LPS-activated neonatal rat cardiomyocytes. Further studies revealed that amlodipine markedly activated phosphatidylinositiol 3-kinase (PI3K) and Akt, downstream of the PI3K signal cascade. Application of PI3K inhibitors, wortmannin and LY294002 attenuated the depression of TNF-α and iNOS expression by amlodipine in LPS-induced cardiomyocytes. These findings may explain some cardioprotective effects of amlodipine in LPS-mediated sepsis and suggest that the inhibition of TNF-α and iNOS expression by amlodipine is, at least in part, dependent on PI3K/Akt signaling pathway.

1. Introduction

Sepsis and its sequelae represent an important cause of mortality, particularly if cardiovascular dysfunction ensues [1]. Although the pathogenesis of myocardial dysfunction in sepsis is incompletely understood, lipopolysaccharide (LPS), the outer membrane component of Gram-negative bacteria has been recognized as a causative agent in myocardial depression during sepsis [2]. Recent studies have suggested that exposure of mammalian cells to LPS can lead to release of pro-inflammatory cytokines and in turn activate a second level of inflammatory cascades including cytokines, lipid mediators and adhesion molecules [3]. Among them, tumor necrosis factor α (TNF-α) is a major pro-inflammatory cytokine that mediates the signs and symptoms of sepsis and shock [4,5]. Accumulating evidence indicates that myocardial TNF-α is an autocrine contributor to myocardial dysfunction and cardiomyocyte death in sepsis. The production of endotoxin-induced myocardial TNF-α is nearly evenly distributed between cardiomyocyte and resident cardiac macrophage cell types [6,7]. Thus local myocardial TNF-α production as a potential source of TNF-α contributes to myocardial dysfunction *via* direct depression of contractility and induction of myocyte apoptosis [8]. Inflammatory mediator nitric oxide (NO), which is synthesized by NO synthase (NOS), also plays an important role in the development of cardiac dysfunction during sepsis [9]. NOS includes three distinct isoforms, neural NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) [10]. iNOS is a calcium-independent enzyme often induced by LPS, cytokines, ischemia-reperfusion and hypoxia [11]. This induction of iNOS plays an important role in the pathogenesis of cardiac dysfunctions, such as myocarditis, myocardial infarction and septic shock [12].

More and more research data have demonstrated that myocardial P38 mitogen-activated protein kinase (MAPK) pathway and the inhibitor of κB (IκB)-kinase are involved in the regulation of LPS-induced inflammatory cytokines expression [4,8,12,13]. Recent studies have shown that the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway also plays an important role in negatively regulating LPS-induced inflammatory responses [14–16]. The PI3K/Akt pathway imposed a braking mechanism to limit the expression of proinflammatory mediators in LPS-treated monocytes [17]. Similar effects were observed *in vivo*, where inhibition of PI3K enhanced LPS-induced inflammation in endotoxemic mice [18]. The PI3Ks are a conserved family of signal transduction enzymes that are involved in regulating

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cellular survival, inflammatory responses, and apoptosis [19,20]. Once activated, PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate, which allows for recruitment of signaling proteins, including the downstream component, serine-threonine kinase Akt [21]. Recently, transgenic overexpression of Akt has been shown to protect against septic death in the mice infected with the Gram-negative bacterium [15]. Thus, the modulation of inflammation-relevant genes expression in cardiomyocytes may present a useful strategy for the treatment of sepsis-induced cardiac dysfunction.

Calcium channel blockers (CCBs) are important drugs in the treatment of hypertension and heart diseases. Previous studies have demonstrated that several CCBs, such as verapamil, nitrendipine and nilvadipine could limit cardiovascular failure and prolong survival time in different models of septic shock [22,23]. In contrast to CCBs' antihypertensive activity, which is mediated through the calcium channel blockade, up to now the mechanism underlying their inhibition in the expression of inflammation-relevant genes has not been explained [24,25]. Some researches on the possible mechanisms of CCBs' anti-inflammation have been reported recently. Mustafa [26] demonstrated that pretreatment of rats with diltiazem, nifedipine, or verapamil before LPS exposure attenuated iNOS expression in the liver. Furthermore, nifedipine could inhibit the activation of nuclear factor-κB (NF-κB) in LPS-stimulated macrophage cells [27].

As a long-acting CCB, amlodipine is one of the most commonly used drugs for the treatment of hypertension. Amlodipine could improved the recovery of the contractile response in isolated aortic rings from septic shock rats, which suggest that amlodipine may exert beneficial effects on endotoxemia [28].

However, whether amlodipine possesses an anti-inflammatory property on LPS-induced cardiac dysfunction and the possible molecular mechanisms explaining how amlodipine suppresses the inflammatory response in cardiomyocytes are unknown. In the present study, we evaluated the cardiac protection effect of amlodipine on LPS-induced endotoxemic rats, and explained the possible mechanism. We found that amlodipine attenuated LPS-induced cardiac dysfunction, inhibited the expression of TNF-α and iNOS, at least in part, *via* PI3K/Akt pathway in LPS-stimulated rat cardiomyocytes. These results suggest that amlodipine exert beneficial effects during the activation of inflammation, and provide novel insights into the mechanism of reducing the cardiovascular dysfunction by CCBs.

2. Materials and methods

2.1. Reagents

Amlodipine (2-[(2-aminoethoxy)-methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester benzene sulfonate), LPS (*Escherichia coli* serotype 026:B6), 5-bromo-2′-deoxyuridine (BrdU), wortmannin, anti-sarcomeric actin and β-actin antibodies were purchased from Sigma Co. (St Louis, MO, USA). Rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit were purchased from R&D Systems Inc. (Minneapolis, MN, USA). The goat polyclonal anti-iNOS antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The mouse polyclonal antibody specific for phospho-Akt, rabbit polyclonal antibodies against Akt, phospho-IκB-α, IκB-α, and LY294002 were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-PI3K (p85 subunit) antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Supersignal ECL kit was purchased from Pierce Chemical Inc. (Rockford, IL, USA). Superscript III reverse transcriptase was purchased from Invitrogen (Gaithersburg, MD, USA). dNTP mixture, Tag DNA polymerase and RNAase inhibitor were obtained from Takara Bio Inc. (Otsu, Shiga, Japan). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Trizol reagent were obtained from GIBCO-BRL (Carlsbad, California, USA). Dimethyl sulfoxide (DMSO) was used as a solvent. Concentrated amlodipine stock and LY294002 stock were diluted in the medium immediately before use.

2.2. Animals and groups

Male Sprague–Dawley rats (weight 200 to 250 g) were purchased from the Center of Experimental Animals of the Fourth Military Medical University and were housed for six days in standard cages and supplied with laboratory chow and tap water. Laboratory chow was withheld 12 h before the experiments. Animals were randomly divided into four groups. The control group ($n=6$) treated with the vehicle, DMSO diluted in the sterile saline (less than 0.1%, *i.v.*, 0.2 ml). The model group (LPS group) received intravenous injection of 5 mg/kg body weight LPS ($n=6$) [29]. The third group (10 μg/kg amlodipine + LPS group) treated with amlodipine (10 μg/kg, *i.v.*) 30 min before LPS administration ($n=6$). The fourth group (30 μg/kg amlodipine + LPS group) received intravenous injection of amlodipine (30 μg/kg) 30 min prior to LPS administration ($n=6$). All the animal care and handling procedures were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University.

2.3. LPS-induced endotoxemia model and amlodipine treatment

The Sprague–Dawley rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (7.5 mg/kg). The trachea were cannulated to facilitate respiration and environmental temperature was maintained at about 25 °C. The right femoral artery was cannulated and connected to PowerLab/4SP recording system (AD Instruments Co., Australia) by a pressure transducer for the measurement of mean arterial blood pressure (MAP) and heart rate (HR). The left femoral vein was cannulated for the administration of drugs. After the completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 30 min. After recording baseline hemodynamic parameters, animals were given amlodipine (10 or 30 μg/kg), and 30 min later animals received vehicle or LPS (5 mg/kg) in normal saline to induce endotoxemia. The MAP and HR were reassessed at 0.5, 1, 2 and 4 h after vehicle or LPS injection. Prior to and at 0.5, 1, 2 and 4 h after vehicle or LPS administration, 0.5 ml of blood was collected. Any blood withdrawn was immediately replaced by the injection of an equal amount of saline [30]. After the blood samples were centrifuged (6000 *g* for 5 min), the plasma samples were stored at −70 °C. Plasma levels of TNF-α were determined by use of commercial immunoassay kits. At the end of the experiment, animals were sacrificed with an overdose of sodium pentobarbital. Hearts were excised and parts of left ventricular tissue samples were fixed in buffered formalin (10% in phosphate-buffered saline), and then embedded in paraffin for histological examination. The rest tissue samples were stored at −70 °C for the determination of the iNOS protein expression by Western blot assay.

2.4. Histological examination of ventricular tissue

The ventricular tissues from different groups were fixed in buffered formalin for more than 12 h. The fixed tissues were dehydrated in graded ethanol and embedded in paraffin wax. Sections (4 μm thick) were placed on glass slides, and removed paraffin by xylene. Then, the tissue sections were stained with haematoxylin and eosin (H&E) reagent. Examine and photograph with a light microscope (Eclipse 80i, Nikon, Japan).

2.5. Cell culture and stimulation with LPS

Neonatal rat cardiomyocytes were isolated from the ventricles of one to three-day-old Sprague–Dawley rats, essentially according to the procedure described previously [31]. Cells, enriched for