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Activation of mTOR/IκB-α/NF-κB pathway contributes to LPS-induced hypotension and inflammation in rats

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ABSTRACT

Mammalian target of rapamycin (mTOR), a serine/threonine kinase plays an important role in various pathophysiological processes including cancer, metabolic diseases, and inflammation. Although mTOR participates in Toll-like receptor 4 signalling in different cell types, the role of this enzyme in sepsis pathogenesis and its effects on hypotension and inflammation in endotoxemic rats remains unclear. In this study we investigated the effects of mTOR inhibition on lipopolysaccharide (LPS)-induced changes on expressions and/or activities of ribosomal protein S6 (rpS6), an mTOR substrate, nuclear factor-κB (NF-κB) p65, inhibitor κB (IκB)-α, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)−2 with production of nitric oxide, peroxynitrite, prostacyclin, and tumor necrosis factor (TNF)-α and activity of myeloperoxidase (MPO), which results in hypotension and inflammation. Injection of LPS (10 mg/kg, i.p.) to male Wistar rats decreased blood pressure and increased heart rate that were associated with elevated nitrotyrosine, 6-keto-$PGF_{1\alpha}$, and TNF-α levels and MPO activity, and increased expressions and/or activities of rpS6, NF-κB p65, iNOS, and COX-2 and decreased expression of IκB-α in renal, cardiac, and vascular tissues. LPS also increased serum and tissue nitrite levels. Rapamycin (1 mg/kg, i.p.) given one h after injection of LPS reversed these effects of LPS. These data suggest that the activation of mTOR/IκB-α/NF-κB pathway associated with vasodilator and proinflammatory mediator formation contributes to LPS-induced hypotension and inflammation.

1. Introduction

Septic shock is characterized by cardiovascular changes including cardiac dysfunction and vascular hyporeactivity leading to life-threatening refractory hypotension (Levy et al., 2003; Tunctan et al., 2012). It is associated with exacerbated systemic inflammation with elevated production of proinflammatory cytokines and reactive oxygen/nitrogen species (Hollenberg, 2009; Levy et al., 2010; Martin et al., 2003). The pathophysiology of septic shock caused by Gram-negative bacteria has been focused on the lipopolysaccharide (LPS), which is an outer membrane component and well-characterized pathogen-associated molecular pattern (Kang-Birken and Dipiro, 2008; Maeshima and Fernandez, 2013). The host defence response to LPS includes activation of nuclear factor-κB (NF-κB) that has been implicated in the release of immune-related cytotoxic factors such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)−2, and proinflammatory mediators such as tumor necrosis factor (TNF)-α and interleukin (IL)−6 (Ajmone-Cat et al., 2003; Choi et al., 2009). Although this inflammatory response is essential for eradication of infectious agents, their dysregulated and prolonged production can lead to tissue damage, multiple organ failure, and ultimately death (Cauwels, 2007). Excessive nitric oxide (NO) produced during septic shock reacts with superoxide ions to form peroxynitrite, an oxidant and nitrating molecule, which represents an NO-dependent pathogenic mechanism in shock and inflammatory diseases (Tunctan et al., 2012). In addition to NO, enhanced production of COX-2-derived vasodilator prostanoids such as prostacyclin (PGI_2) and prostaglandine E_2 have also been shown to contribute to hypotension and related organ damage associated with decreased survival in animals and humans with sepsis (Tsiotou et al., 2005).

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Mammalian target of rapamycin (mTOR), a serine/threonine kinase is a member of phosphatidylinositol kinase family. It acts as a central controller of cell growth, survival and metabolism by regulating protein synthesis and other cellular processes implicated in many diseases such as cancer, neurodegenerative diseases, and inflammation (Dazert and Hall, 2011; Hay and Sonenberg, 2004). mTOR also acts as a central regulator of immune responses and is involved in differential regulation of pro- and anti-inflammatory cytokine levels, which are produced by various immune cells including neutrophils, natural killer cells, T cells, and macrophages (Delgoffe and Powell, 2009; Mills and Jameson, 2009; Ohtani et al., 2008; Thomson et al., 2009; Turnquist et al., 2010; Weichhart et al., 2008; Weichhart and Saemann, 2009). mTOR regulates proinflammatory immune responses through modulating the transcriptional factors including NF-κB (Schmitz et al., 2008; Weichhart et al., 2008). Although mTOR has shown to be involved in metabolic and inflammatory diseases, there has been no previous attempt to examine the effects of mTOR inhibition on the LPS-induced hypotension and inflammation. Therefore, the present study was conducted to determine whether changes in expressions and activities of signalling molecules including ribosomal protein S6 (rpS6), NF-κB p65, inhibitor of κB (IκB)-α, iNOS, and COX-2 and production of NO, peroxynitrite, PGI_2, and TNF-α in addition to myeloperoxidase (MPO) activity, participate in mTOR inhibition of hypotension and inflammation in LPS-treated rats.

2. Materials and methods

2.1. Chemicals and materials

Rapamycin was obtained from Gold Biotechnology (St. Louis, MO, USA), and LPS (*Escherichia coli* LPS, O111:B4) from Sigma Chemical Company (St. Louis, MO, USA). Rat nitrotyrosine, TNF-α, and 6-keto-$PGF_{1\alpha}$ enzyme-linked immunosorbent assay (ELISA) kits were purchased from Hycult Biotech (Netherland), eBioscience Company (San Diego, CA, USA), and Cayman Chemical (Ann Arbor, MI, USA), respectively. rpS6 and phosphorylated rpS6 (Cell Signalling Technology, Danvers, MA, USA), NF-κB p65, phosphorylated NF-κB p65, IκB-α, phosphorylated IκB-α, and COX-2 (Santa Cruz Biotechnology, Texas, USA), and iNOS (BD Transduction Lab., San Jose, CA, USA) were purchased. Secondary antibodies (sheep anti-mouse IgG-horseradish peroxidase and goat anti-rabbit IgG-horseradish peroxidase) and ECL Prime Western Blotting Detection Reagents were also obtained from Amersham Life Sciences (Cleveland, OH, USA).

2.2. Animals

All procedures were carried out in male Wistar rats (200–300 g; n=32) (Research Center of Experimental Animals, Mersin University, Mersin, Turkey) according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were maintained under controlled conditions of 12:12 h light-dark cycle and were fed with standard rat chow. This protocol was approved by the Ethics Committee of Mersin University School of Medicine.

2.3. Experimental protocol

Rats were randomly divided into control (n=8), LPS (n=8), rapamycin (n=8), and LPS+rapamycin (n=8) groups. The endotoxic shock was induced as previously described by Tunctan et al. (2005). In the control and rapamycin groups, rats received saline (4 ml/kg, i.p.), Animals in LPS and LPS+rapamycin groups were rats received LPS (10 mg/kg, i.p.; sublethal dose) at time 0. In the rapamycin and LPS +rapamycin groups, rapamycin (1 mg/kg, i.p.) was administrated to rats 1 h after injection of saline or LPS. Mean arterial pressure (MAP) and heart rate (HR) of all animals were measured by using a tail-cuff device (MAY 9610 Indirect Blood Pressure Recorder System, Commat Ltd., Ankara, Turkey) at time 0 and 1, 2, 3, and 4 h. After termination of experiments, rats were euthanized and blood samples, kidneys, hearts, thoracic aortas, and superior mesenteric arteries were collected. Sera were obtained from blood samples after centrifugation at 23,910×*g* for 15 min at 4 °C and stored at −20 °C for measurement of nitrite levels. Tissues were rapidly frozen in liquid nitrogen and stored at −80 °C. Frozen tissues were pulverised into powder in liquid nitrogen and homogenized in 1–2 ml of an ice-cold 20 mM HEPES buffer (pH 7.5) containing 20 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 2 mM ethylenediaminetetraacetic acid, 20 mM sodium fluoride, 10 mM benzamidine, 1 mM dithiothreitol, 20 mM leupeptin, and 10 mM aprotinin. Homogenates were centrifuged at 23,910×*g* for 10 min at 4 °C followed by sonication for 15 s on ice with 50 μl ice-cold Tris (50 mM, pH 8.0) and KCl (0.5 M). The samples were centrifuged at 23,910×*g* for 15 min at 4 °C and then supernatants were obtained and stored at −80 °C until the measurement of β-actin, rpS6, IκB-α, NF-κB p65, iNOS, and COX-2 protein levels and/or activities and nitrite, nitrotyrosine, 6-keto-$PGF_{1\alpha}$, and TNF-α protein levels in addition to MPO activity. Total protein concentration in the supernatants was determined by the Coomassie blue method using bovine serum albumin (BSA) as a standard (Bradford, 1976).

2.4. Immunoblotting

Immunoblotting for rpS6, phosphorylated rpS6, IκB-α, phosphorylated IκB-α, NF-κB p65, phosphorylated NF-κB p65, iNOS, COX-2, and β-actin proteins were performed according to the method described previously by Tunctan et al. (2013a and 2013b). Briefly, equal amounts of protein (75–130 μg), were subjected to 10% SDS-polyacrylamide gel electrophoresis and separated proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBST) buffer (mmol/l: Tris-HCl 25 [pH 7.4], NaCl 137, KCl 27% and 0.05% Tween 20) at room temperature for 1 h and incubated with specific primary antibodies in TBST including 5% BSA at 1:500-1:20.000 dilutions overnight at 4 °C followed by incubation with their horseradish peroxidase-conjugated secondary antibodies in TBST including 0.1% BSA at 1:1.000 for 1 h at room temperature. The immunoreactive proteins were detected using ECL Prime Western blotting detection reagent by enhanced chemiluminescence. Densitometric analysis was performed with Image J 1.42r (National Institutes of Health, USA) software. β-actin was used to normalize the expression of proteins in each sample.

2.5. Measurement of nitrotyrosine, PGI_2, and TNF-α levels

Nitrotyrosine, PGI_2, and TNF-α levels in tissue samples were measured by ELISA according to the manufacturer's instructions in the specific ELISA kits.

2.6. Measurement of nitrite levels

Nitrite (a stable product of NO) levels in sera and tissue samples were measured using the diazotization method based on the Griess reaction, as an index for NOS-derived NO production (Green et al., 1982; Tunctan et al., 2005). Briefly, samples (25 μl) were pipetted into 96-well microtiter plates, and an equal volume of Griess reagent (1% sulphanilamide and 0.1% N-1-naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) was added to each well. After incubation for 10 min at room temperature, absorbance was measured at 550 nm with a microplate reader. Standard curves were also constructed using sodium nitrite concentrations ranging from 0.25 to 100 μM.