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Research Paper

Celastrol prevents circulatory failure via induction of heme oxygenase-1 and heat shock protein 70 in endotoxemic rats

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ABSTRACT

Ethnopharmacological relevance: Celastrol, a quinone methide extracted from the root of *Tripterygium wilfordii* Hook, possesses anti-oxidant and anti-inflammatory effects. *Tripterygium wilfordii* Hook is officially listed in the Chinese Pharmacopoeia and is used traditionally against rheumatoid arthritis, ankylosing spondylitis, and cancer. Furthermore, the circulatory protective effect of celastrol on an in vivo animal model of sepsis was investigated. *Aim of the study:* Sepsis is a systemic inflammatory disorder that increases tissue oxidative stress and leads to multiple organ injury. We evaluated the beneficial effects of celastrol on multiple organ failure induced by lipopolysaccharide (LPS) in rats.
Materials and methods: Celastrol (0.5 and 1.0 mg/kg, i.v.) was administered to anaesthetized rats 2 h before and 30 min after LPS challenge (10 mg/kg, i.v.). Eight hours later, cardiac and aortic protein expressions related to inflammatory responses, superoxide anion production, and reduced glutathione (GSH) level were measured.
Results: Treatment with celastrol prevented circulatory failure (bradycardia and hypotension) 8 h after LPS challenge. The plasma levels of ALT, LDH, TNF-α, and nitric oxide metabolites increased markedly during sepsis, which significantly reduced after celastrol treatments. Celastrol attenuated iNOS, TNF-α, NF-κB phospho-p65 expression, superoxide anion production, and caspase 3 activity in the cardiovascular system, all of which were markedly elevated after LPS challenge. Furthermore, celastrol induced HO-1 and HSP70 expressions increase in nuclear levels of Nrf2 and HSF-1, respectively, and increase cardiac GSH level 8 h after LPS challenge.
Conclusion: Anti-inflammatory and anti-oxidant effects of celastrol contribute to prevent circulatory failure in sepsis. Induction of HO-1 and HSP70 by celastrol participates in these beneficial effects.
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1. Introduction

Sepsis is a common nosocomial infectious disease that is caused by bacteria or pro-inflammatory factors, and is a serious clinical disorder. The symptoms of sepsis include systemic hypotension, vessel hypoconstriction, multiple organ failure, and septic shock (Cohen, 2002; Merx and Weber, 2007). As a defense against bacteria or other pathogens during sepsis, pro-inflammatory cytokines, e.g., tumor necrosis factor (TNF) and interleukins (IL), are released (Blackwell and Christman, 1996). These pro-inflammatory cytokines usually attract neutrophils and monocytes from the circulatory system to tissues, causing infiltration and damage (Schulte et al., 2013). Lipopolysaccharide (LPS), a type of endotoxin from Gram-negative bacteria cell walls, can induce experimental endotoxemia as a rat model of sepsis (Szabo et al., 1993). LPS binds to the Toll-like receptor 4 (TLR4) of macrophages, leading to the activation of the nuclear factor (NF)-κB-signaling pathway (Schulte et al., 2013), by which the pro-inflammatory factors (e.g., TNF-α), inducible nitric oxide synthase (iNOS) protein, and nitric oxide (NO) are generated and contribute to systemic hypotension, multiple organ failure, and septic shock. Oxidative stress plays an important role in systemic inflammatory responses in sepsis (Guzik et al., 2003). LPS causes the elevation of superoxide anion ($O_2^{\cdot -}$) and other free radical production in various tissues, resulting in multiple organ injury (Berkowitz, 2007). NO reacts with superoxide anion, forming peroxynitrite anion ($ONOO^-$), and further exacerbating tissue damage (Cohen, 2002).

Heme oxygenase-1 (HO-1) is an anti-oxidant and heme-degrading enzyme, and its gene can be regulated by various transcriptional factors such as Nrf2 (Seo et al., 2010). HO-1 can be

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induced within 1–3 h of injection of LPS, which is accompanied by a gradual decline (Barreiro et al., 2002), to protect skeletal muscles via its anti-oxidant effect. We have demonstrated that cardiac HO-1 protein expression is down-regulated at the late stage of sepsis (Lee et al., 2011). It is therefore important to determine how to stabilize and maintain HO-1 expression in sepsis. Furthermore, heat shock protein 70 (HSP70), a stress-responsive protein, has been reported to have a protective effect against tissue damage from sepsis (Bruemmer-Smith et al., 2001). HSP70 can be induced by heat and is used to maintain protein structure and function (Mayer and Bukau, 2005). HSP70 proteins expression is regulated by heat shock factor 1 (HSF1). When HSF1 bind to heat shock element (HSE) of HSP70 DNA, HSP70 mRNA and proteins generation will be up-regulated (Westerheide et al., 2004). By its anti-oxidant effect, HSP70 can prevent LPS-induced apoptosis in aortic endothelial cells and maintain vascular constriction and tension after stress to the aorta (Bernardini et al., 2005). Recently, an in vitro study revealed that HSP70 can inhibit cytokine generation by suppression of NF-κB activation (Afrazi et al., 2012).

Celastrol is a quinone methide isolated from the root extract of *Tripterygium wilfordii* Hook (Chinese: Leigongteng, or "Thunder God Vine"), and acts as an anti-inflammatory and anti-oxidant agent (Kannaiyan et al., 2011). Celastrol has been reported to be a heat shock protein 90 (HSP90) inhibitor and can induce HSP70 protein expression (Zhang et al., 2009). The transcription factor HSF1 for HSP70 expression is repressed by HSP90 (HSP90 Complex). The HSP90 inhibitor can break the HSP90 client protein complex and release HSF1 (Zou et al., 1998). Celastrol also induces the HSF1 activity, and then HSF1 enters nucleus. As the result, HSF1 induces HSP70 mRNA and protein levels (Westerheide et al., 2004; Zhang et al., 2008). Celastrol also inhibits cytokine production (TNF-α and IL-6) by interfering with NF-κB activation in LPS-stimulated microglial cells (Jung et al., 2007). Recently, it has been reported that celastrol can suppress the innate immunity response activated by LPS by inducing HSP70 expression and inhibiting NF-κB pathway activation (Paimela et al., 2011). Furthermore, via induction of HO-1 (HSP32), celastrol inhibits aminoglycoside-induced ototoxicity in hair cells (Francis et al., 2011), hypertension-induced inflammation in rats, and oxidative stress in vascular smooth muscle cells (Yu et al., 2010). Here, we examined the in vivo protective effect of celastrol on multiple organ failure caused by LPS challenge in anaesthetized rats.

2. Materials and methods

2.1. Materials

Celastrol was purchased from Cayman Chemicals (Ann Arbor, MI, USA). LPS from *Escherichia coli* 0127:B8, norepinephrine (NE), phenylephrine (PE), and potassium chloride (KCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enhanced chemiluminescent (ECL), Western Blotting Substrate, and 10 X radio-immunoprecipitation assay (RIPA) lysis buffer were from Millipore Corporate Headquarters (Temecula, CA, USA). Coomassie Protein Assay Reagent was purchased from Thermo Scientific (Rockford, IL, USA)

2.2. Experimental animals

Male Wistar-Kyoto rats (275–300 g) were divided into six groups: (1) control: 1 ml/kg normal saline was given intravenously (i.v.) ($N=3$); (2) celastrol: 1.0 mg/kg, i.v. ($N=5$); (3) LPS: rats treated with *Escherichia coli* LPS 10 mg/kg (i.v. infusion for 10 min) ($N=14$); (4) Celstrol-0.5+LPS: treatment with a low dose of celastrol (0.5 mg/kg) 2 h before LPS challenge (10 mg/kg, i.v. infusion for 10 min) ($N=9$); (5) Celstrol-1.0+LPS: treatment with a high dose of celastrol (1.0 mg/kg) 2 h before LPS challenge (10 mg/kg, i.v. infusion for 10 min) ($N=9$); (6) LPS+Celastrol-1.0: celastrol (1 mg/kg, i.v.) was given 30 min after LPS challenge (10 mg/kg, intravenous infusion for 10 min) ($N=8$). We followed haemodynamic changes (blood pressure and heart rate), hepatic function index (i.e., alanine aminotransferase [ALT]), cell toxicity index (i.e., lactate dehydrogenase [LDH]), and renal function index (i.e., blood urea nitrogen [BUN]) at 0, 1, 2, 6, and 8 h after LPS challenge. All of these biochemical variables were determined using a Fuji DRI-CHEM 3030 analyzer (Fuji Photo Film, Tokyo, Japan). Eight hours after saline or LPS infusion, animals were sacrificed. Hearts, aortas, and other organs were collected immediately for further ex vivo studies.

2.3. Determination of cardiac caspase 3 activity

The hearts were isolated 8 h after LPS challenge and were immediately frozen in liquid nitrogen and stored at −80 °C until processed. Proteins of ventricles were isolated by 1× RIPA buffer. Detection of protein concentration by Coomassie Protein Assay Reagent. The caspase 3 activity in cardiac tissue proteins was determined using a Caspase-3 colorimetric detection kit (Enzo Life Sciences, Farmingdale, NY, USA).

2.4. Vascular reactivity in the thoracic aortas

Thoracic aortas were isolated from rats 8 h after LPS challenge and transferred to cold (4 °C) Kreb's solution (pH 7.4). The preparation of aortic rings and vascular reactivity was performed as described previously (Lee et al., 2014). Myograph (Multi-Wire Myograph System-Model 620M, Danish Myo Technology, Aarhus, Denmark) was used to detect the vascular contraction. Vascular contraction was assessed by the contractile response to PE (1 nM to 10 μM), and vascular tension was expressed as a percentage of the steady-state tension (100%) induced by external 60 mM KCl.

2.5. Plasma nitrite/nitrate determination

Aliquots (30 μl) of thawed plasma taken 8 h after LPS challenge were deproteinated with 100 ml of 95% alcohol for 30 min (4 °C). Serum samples were then centrifuged for 6 min at 12,000 × *g*. The supernatant (6 μl) was injected into a collection chamber containing 5% VCl_3. In this strongly reducing environment, both nitrate and nitrite were converted to NO. A constant stream of helium gas was used to carry the output into an NO analyzer (Sievers 280NOA; Sievers Instruments, Boulder, CO, USA), where the NO reacts with ozone (O_3), resulting in the emission of light. Light emission is proportional to the NO formed. Standard amounts of sodium nitrate (Sigma-Aldrich, St. Louis, MO, USA) were used for calibration.

2.6. Plasma TNF-α determination by ELISA

The plasma levels of TNF-α at 1 and 2 h after LPS challenge were determined using a rat TNF-α ELISA kit (Ready-SET-Go ELISA kit; eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

2.7. Aortic superoxide anion determination

The method used to determine superoxide anion levels was as described previously (Chen et al., 2006; Shih et al., 2008). The thoracic aorta was cut into segments (3–4 mm long) and incubated in 95% O_2/5% CO_2 oxygenated modified Kreb's/HEPES solution (37 °C) for 30 min. Then, the sections of aorta were put into a 96-well plate in which every well was filled with 200 μl of modified Kreb's/HEPES solution and placed in a luminescence measurement system (Hidex, Microplate Luminometer, Turku, Finland). This system injected 250 μM lucigenin (final volume of

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