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Cortistatin protects myocardium from endoplasmic reticulum stress induced apoptosis during sepsis

Bo Zhang [a], Yue Liu [a], Jin-Sheng Zhang [b], Xiao-Hui Zhang [a], Wen-Jia Chen [a], Xin-Hua Yin [a,*], Yong-Fen Qi [b,c,**]

[a] Department of Cardiology, First Affiliated Hospital of Harbin Medical University, Harbin 150001, China
[b] Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Peking University Health Science Center, Beijing 100191, China
[c] Laboratory of Cardiovascular Bioactive Molecule, School of Basic Medical Sciences, Peking University, Beijing 100191, China

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ABSTRACT

Sepsis and septic shock are common entities encountered in intensive care units. Myocardial depression is a well-recognized manifestation of organ dysfunction in sepsis, and myocardial apoptosis is a key step for this progression, which may contribute to cardiac contractile dysfunction. Increasing evidence suggested the anti-inflammatory role of cortistatin (CST) during lethal endotoxemia. However, the direct protective effect of CST on myocardial is still not clear. Here, we aimed to study whether CST can directly protect myocardial from apoptosis. To test that, we used cecal ligation and puncture (CLP) induced sepsis rat model. CST (175 μg/kg, intraperitoneal administration) was injected every 24 h before the model induction for 3 days. Electron microscopy, TUNEL staining, caspase-3 expression, and the Bcl-2/Bax ratio were used to measure myocardial apoptosis. In addition, the protein levels of endoplasmic reticulum stress (ERS) markers were overexpressed in sepsis. To further test whether CST can directly protect myocardial apoptosis from ERS, we compared dithiothreitol (DTT) induced cardiomyocyte (CM) ERS with or without CST in vitro. We found that CST strongly attenuated lipopolysaccharide (LPS) and DTT induced CM ERS. Blocking GHS-R1a, one of CST's receptors expressed by CMs, completely abrogated CST's protective effect. Finally, CST's protective effect was associated with the decrease of ERS both in vivo and in vitro. In conclusion, our results for the first time showed the previously unexpected role of CST to directly protect myocardial from apoptosis through inhibiting ERS and partly through GHS-R1a.

1. Introduction

Sepsis has high incidence and mortality rates around the world. Myocardial dysfunction is a recognized manifestation of sepsis and septic shock, with myocardial depression occurring in almost 40–50% of patients (Rudiger and Singer, 2007). Patients with septic shock display ventricular dilation, decreased myocardial ejection fraction and contractility (Niederbichler et al., 2006; Vieillard-Baron et al., 2008). Various mechanisms have been proposed for this myocardial dysfunction, including excessive cardiac inflammation (Merx and Weber, 2007), mitochondrial dysfunction (Suliman et al., 2004), cardiac cell death by apoptosis or necrosis (Sharma, 2007). Apoptosis is known to cause myocardial depression in disease states such as chronic heart failure and I/R injury (Buerke et al., 1995). And the role of apoptosis in myocardial depression during sepsis has been increasingly emphasized.

Various pathophysiological stimuli, such as cellular redox alteration, glucose deprivation, aberrant regulation of intracellular Ca^{2+}, and viral infection, may cause the accumulation of unfolded proteins in the ER lumen (Kaufman, 2002). Protein markers of endoplasmic reticulum stress (ERS) have been monitored in myocardia of mouse treated with LPS (Ceylan-Isik et al., 2010). In the early stages of the ERS response, ER can help cells manage stress and enhance the chances of survival (Boyce and Yuan, 2006). However, if ER stimuli are prolonged and/or overwhelmed the capacity of UPR, a maladaptive ER overload response (EOR) occurs. EOR is associated with the transcriptional induction of C/EBP homologous protein (CHOP), cleavage of the ER-resident procaspase-12 to active caspase-12, and eventual programmed cell death through the activation of caspase-9 and caspase-3 (Glembotski, 2008). Another abundant chaperone molecule is GRP94, a member of the

* Corresponding author. Department of Cardiology, First Affiliated Hospital of Harbin Medical University, Harbin 150001, China. Tel.: +86 451 85555063; fax: +86 451 86620586.
E-mail address: harbin0910@yahoo.com (X.-H. Yin)
** Corresponding author. Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Peking University Health Science Center, Beijing 100191, China. Tel.: +86 10 82805627; fax: +86 10 82805627.
E-mail address: yongfenqi@yahoo.com (Y.-F. Qi).

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heart shock protein 90 (HSP90) family, which plays a role in more advanced intermediates or incompletely assembled protein (Argon and Simen, 1999).

Cortistatin (CST), a peptide discovered in 1996, was named after its predominantly cortical expression and ability to depress cortical activity (de Lecea et al., 1996). CST contains a FWKT tetramer crucial for SST receptors (SSTRs) binding. Thus, CST has high affinity for all SSTR subtypes (Siehler et al., 1998). A recent report showed that CST binds to GHS-R1a (Deghenghi et al., 2001), the ghrelin receptor, and mas-related gene X_2 ($MrgX_2$) (Robas et al., 2003). CST and its receptors are widely distributed in all types of tissues and organs in the body, including the heart, arterial blood vessels, and immune system (Dalm et al., 2003). Recently, a report indicated that GHS-R1a participates in inhibiting ERS and ERS-mediated apoptosis (Zhang et al., 2013). CST protects against endotoxin-induced lethality and prevents septic shock-associated histopathology, including inflammatory cell infiltration and multi-organ intravascular disseminated coagulation (Gonzalez-Rey et al., 2006). Recent studies showed that the CST levels are elevated in the plasma of coronary heart disease (CHD) patients (Tang and Liu, 1996; Tian et al., 2009), and CST attenuates vascular calcification, whose effects are mediated by GHS-R1a rather than SSTRs or MrgX2 (Liu et al., 2010). These findings suggested that CST has a potential role in the cardiovascular system. However, little is known about the function of CST in sepsis-induced myocardium injury. Therefore, this study was conducted to determine CST has an essential function in sepsis-elicited myocardium apoptosis by inhibiting ERS.

2. Materials and methods

2.1. Animals and reagents

Male Sprague–Dawley (SD) rats (160 ± 10 g) were provided by the Animal Department, Peking University Health Science Center (Beijing, China). All animal care and experimental protocols were in compliance with the Animal Management Rule of the People's Republic of China (Ministry of Health, P. R. China, document no. 55, 2001), and approved by the Animal Care Committee of Peking University Health Science Center.

Rat CST-14 was obtained from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). Rabbit polyclonal anti-GRP94 (ab13509), anti-caspase12 (ab18766), and anti-CHOP (ab10444) antibodies were obtained from Abcam Inc. (Cambridge, MA, USA). Anti-beta-actin and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Trizol agent was purchased from Gibco (Gaithersburg, MD, USA). dNTPs were obtained from Clontech (Palo Alto, CA, USA). M-MuLV reverse transcriptase, Taq DNA polymerase, RNasin, and oligo (dT) 15 primer were purchased from Promega (Madison, WI, USA). All the sequences of oligonucleotide primers were synthesized by Invitrogen (CA, USA). Sodium dodecyl sulfate (SDS), LPS, dithiothreitol (DTT), and GHS-R1a inhibitor D-GHRP-6 were purchased from Sigma (St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade.

2.2. Animal model of polymicrobial sepsis

Male SD rats were housed under standard conditions (room temperature, 20 ± 1 °C; humidity 60 ± 10%; lights from 6 AM to 6 PM), and freely given standard rodent chow and water. The polymicrobial septic model of rats with cecal ligation and puncture (CLP) was obtained as described (Dalm et al., 2004). Under complete anesthetization with pentobarbital sodium (45 mg/kg, intraperitoneal administration), a 3 cm ventral midline incision was made in the rats. The cecum was exposed and ligated just below the ileocecal valve using a 3-0 silk suture to avoid intestinal obstruction. The cecum was punctured twice with a 16-gauge needle, and returned to the abdominal cavity. The incision was then closed. Each rat received normal saline (4 mL/100 g) by subcutaneous injection immediately after CLP. Sham-operated animals underwent the same surgical procedure, except the cecum was neither ligated nor punctured.

Table 1
The schematic diagram of the protocol.

Control group		**3 days**			**18 hours**
	↑	↑	↑	↑	↑
	Injected with saline			Sham operation	Measured
Sepsis group		**3 days**			**18 hours**
	↑	↑	↑	↑	↑
	Injected with saline			CLP	Measured
CST + sepsis group		**3 days**			**18 hours**
	↑	↑	↑	↑	↑
	Injected with CST (175 μg/kg)			CLP	Measured

Rats were divided into the control (vehicle + sham) group, vehicle + sepsis group, and CST + sepsis group. The schematic diagram of the protocol was shown (Table 1). At 18 h after CLP or sham operation (the late, hypodynamic stage of sepsis), rats were deeply anesthetized using pentobarbital sodium (45 mg/kg, intraperitoneal administration). A catheter filled with heparin saline (500 U/mL) was inserted into the right carotid artery, and further inserted into the left ventricle to measure MABP and left ventricular (LV) pressure. Heart rate, maximal LV pressure development ($LVdp/dt_{max}$), LV endsystolic pressure, and LV end-diastolic pressure (LVEDP) were recorded using a Powerlab (4S, Australia).

2.3. Preparation of primary neonatal cardiomyocytes (CMs) and LPS and DTT treatment

Neonatal rat CMs were isolated from 1- to 3-day-old SD rats. In brief, the excised hearts were washed in Hanks balanced salt solution (HBSS; Ca^{2+} and Mg^{2+} free). The ventricular tissues were minced fine scissors in HBSS containing trypsin (0.05%) and collagenase (0.055%), and digested at 37 °C. The cells were isolated through multiple 10 min rounds of tissue digestion. After each incubation, the supernatant was added to an equal volume of DMEM containing 10% fetal bovine serum. The total cell suspension was centrifuged at 1000 rpm for 10 min. Supernatants were discarded and cell pellets were resuspended in DMEM containing 10% fetal bovine serum. The cells were plated in plastic culture dishes, and incubated for 90 min. The CMs were harvested and seeded on to 60 mm culture dishes at density of 10^5 cells/cm^2. The cells were divided into groups and treated as follows: (1) the control group, which was cultured in culture medium; (2) the LPS group, which was cultured in DMEM that contained LPS (100 ng/mL) for 24 h; (3) the DTT group, which was incubated with DTT (2 mmol/L) for 3 h; (4) the CST + LPS group and the CST + DTT group, which was incubated with CST (10^{-7} mol/L) for 30 min, then the medium was removed, added LPS (100 ng/mL) for 24 h or DTT (2 mmol/L) for 3 h; (5) the D-GHRP-6 + CST + LPS group and the D-GHRP-6 + CST + DTT group, which was incubated with D-GHRP-6 10^{-6} mol/L for 30 min, then added CST 10^{-7} mol/L for 30 min, then the medium was removed, added LPS (100 ng/mL) for 24 h or DTT (2 mmol/L) for 3 h. After incubation, the experiment was terminated, and the cells were analyzed.

2.4. Electron microscopy

Electron microscopy was used to determine the presence of apoptosis. Cells were seeded in six-well plates, treated as indicated, fixed in 2.5% glutaraldehyde in Hank's modified salt solution, postfixed in 1% OsO4 in 0.1 mol/L cacodylate buffer, scraped off, and serially dehydrated using ethanol. Dehydration was completed in