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Original Contribution

Overproduction of nitric oxide by endothelial cells and macrophages contributes to mitochondrial oxidative stress in adrenocortical cells and adrenal insufficiency during endotoxemia

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ABSTRACT

We have recently demonstrated that lipopolysaccharide (LPS) causes mitochondrial oxidative stress and dysfunction in adrenal glands, thereby leading to adrenocortical insufficiency. Since nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) leads to mitochondrial damage in various tissues, the present study aims to investigate whether NO contributes to mitochondrial oxidative stress in adrenal cortex and adrenocortical insufficiency during endotoxemia. Systemic administration of LPS increased iNOS expression and NO production in adrenal glands of mice. The specific iNOS inhibitor 1400 W significantly attenuated the LPS-induced mitochondrial superoxide production and dysfunction in adrenal glands, and reversed the LPS-induced adrenocortical hyporesponsiveness to adrenocorticotropic hormone (ACTH). In contrast, administration of the NO donor sodium nitroprusside (SNP) led to mitochondrial oxidative stress and dysfunction in adrenal glands, which resulted in a blunted corticosterone response to ACTH. Using double immunofluorescence staining for iNOS with the vascular endothelial cell marker CD31 or the macrophage marker CD68, we found that increased iNOS expression was found in vascular endothelial cells and macrophages, but not adrenocortical cells in the adrenal gland during endotoxemia. Administration of the hydrogen sulfide (H_2S) donor GYY4137 inhibited NO production and reversed LPS-induced adrenocortical hyporesponsiveness. Our data suggest that overproduction of NO, which is mainly generated by endothelial cells and macrophages during endotoxemia, contributes to mitochondrial oxidative stress in adrenocortical cells and subsequently leads to adrenal insufficiency.

Introduction

Sepsis and septic shock in response to bacterial infections remain a leading cause of death in the intensive care units throughout the world [1,2]. Despite multiple significant therapeutic advances in intensive medicine, mortality associated with sepsis is still unacceptably high [3,4]. A common feature of sepsis is adrenal insufficiency, which is characterized by low basal cortisol levels or low cortisol responses to ACTH stimulation [5]. In addition, the presence of adrenal insufficiency carries relevant prognostic implications in patients with sepsis [5,6]. The inadequate adrenal response to ACTH stimulation is always associated with worse outcomes, including higher mortality rates and prolonged requirement for vasopressor [7,8].

Nitric oxide (NO), an endogenous gas transmitter, has been implicated in the pathogenesis of multiorgan dysfunction during sepsis in animals and humans [9,10]. Basal NO production is associated with constitutive isoforms of NO synthase including neuronal NO synthase (nNOS) and endothelial NO synthase (eNOS) whereas its excessive production during sepsis is mainly due to the inducible NO synthase (iNOS) [11–13]. An increasing body of evidence suggests that overproduction of NO leads to mitochondrial damage in various tissues including skeletal muscle [14], brain [15], and myocardium [16] during sepsis. More recently, we have demonstrated that LPS also

Abbreviations: ACTH, adrenocorticotropic hormone; LPS, lipopolysaccharide; SNP, sodium nitroprusside

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causes mitochondrial oxidative stress and dysfunction in adrenal glands, thereby leading to adrenocortical insufficiency [17]. Thus, it is of interest to know whether NO contributes to mitochondrial oxidative stress in adrenal cortex and adrenocortical insufficiency during endotoxemia.

Hydrogen sulfide (H_2S) is "the third endogenous gaseous signaling transmitter" besides NO and carbon monoxide in mammalian tissues [18,19]. In some tissues, NO and H_2S exert similar biological effects, for instance, both of them induce vasodilation, promote angiogenesis, and protect cardiomyocytes against ischemia–reperfusion [20–22]. Moreover, a number of studies have demonstrated an interaction between NO and H_2S [23,24]. For instance, H_2S suppresses LPS-induced iNOS expression and NO production in macrophages [25]. Whether H_2S affects NO production in adrenal gland remains to be elucidated.

In the present study, we investigated whether NO overproduction contributed to mitochondrial dysfunction in adrenal glands and blunted adrenocortical responsiveness to ACTH during endotoxemia, then identified the source of NO overproduction in adrenal gland, and finally examined the effects of H_2S on NO production and NO-mediated effects within adrenal glands.

Methods

Mouse sodium nitroprusside and GYY4137 treatment

Male ICR mice were obtained from Shanghai SLAC Laboratory Animal Co. (Shanghai, China) and housed at a controlled room temperature with free access to food and water under a natural day/night cycle. All animal protocols were approved by the Ethical Committee of Experimental Animals of Second Military Medical University. Sodium nitroprusside (SNP, Sigma-Aldrich, St. Louis, MO, USA), the NO donor dissolved in sterile saline, was injected into the peritoneal cavity at a dose of 1 mg/kg. GYY4137 (Cayman, Ann Arbor, MI), the slow-releasing H_2S donor dissolved in saline, was injected into the peritoneal cavity immediately before injection of SNP at a dose of 50 mg/kg. MnTBAP (Enzo Life Sciences, Waterloo, Australia), mitochondrial superoxide dismutase 2 (SOD2) mimetic dissolved in saline, was injected into the peritoneal cavity immediately before injection of SNP at a dose of 1 mg/kg. The control group received an equivalent volume of saline.

ACTH stimulation tests

The plasma corticosterone response to exogenous ACTH (Sigma-Aldrich) was determined as described previously [17]. Dexamethasone (Sigma-Aldrich) dissolved in saline, was injected ip at a dose of 5 μg/g body weight at 18:00 PM the night before and at 08:00 AM during the morning of the procedure. Two hours later, mice were anesthetized with intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg). ACTH (30 μg/kg) was infused via a femoral arterial catheter. Arterial samples of 50 μl were obtained immediately before ACTH administration as well as at 15, 60, and 120 min after ACTH administration. Each sample was replaced with an equal volume of saline containing heparin (100 units/ml).

Immunofluorescence analysis

Intact adrenal glands were surgically removed from mice and fixed in buffered formalin prior to processing for paraffin embedding. Paraffin sections (5 μm) were cut, rehydrated, and microwaved in citric acid buffer to retrieve antigens. Serial sections were washed with PBS and incubated with 10% BSA for 1 h. Then, the sections were incubated with primary antibodies against CD68 (ProteinTech Group, Chicago, IL, USA), CD31 (ServiceBio, Wuhan, China), or iNOS(Santa Cruz Biotechnology, Santa Cruz, CA)at a dilution of 1:100 at 4 °C overnight. After washes, sections were incubated with Alexa Fluor 568-conjugated anti-rabbit IgG for CD68 (Invitrogen), Alexa Fluor 546-conjugated anti-goat IgG for CD31 (Invitrogen), or fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG for iNOS (Invitrogen) at a dilution 1:400 at 37 °C for 1 h in the dark. After washing with PBS for 3 times, nuclei were counterstained with 4′6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). The adrenal gland sections were scanned with a 50i Nikon fluorescence microscope (Nikon, Melville, NY).

NO production measurement

Total NO production by liver, lung, kidney, adrenal glands, or primary cultured adrenocortical cells was determined by measuring the concentration of nitrite, a stable metabolite of NO *in vitro*, with a modified Griess (sigma-Aldrich) reaction method [26]. The absorbance was measured at 550 nm using a Bio-Rad (Hercules, CA) microplate reader and nitrite concentration was assessed by reference to the sodium nitrite standard curve.

Primary mouse adrenocortical cell culture

Adrenocortical cells were isolated as previous described [17]. Briefly, the attached fat tissues were removed from adrenal glands, and then the adrenal cortex tissues were carefully separated from the medullas tissue. The cortex tissues were dispersed with 0.1% collagenase (Invitrogen) in a dissociation buffer containing 0.1% bovine serum album (Sigma-Aldrich) at 37 °C on an orbital shaker for three times. Cell pellets were collected and resuspended in DMEM containing 25 mM Hepes, 3.7 g/L $NaHCO_3$, 10% fetal calf serum, 0.01% ampicillin, and 0.01% streptomycin. The cells were seeded in culture dishes and incubated at 37 °C for 1 h to allow attachment of fibroblasts. Supernatants of the viable adrenocortical cells were pooled and seeded in 24-well culture plate (Corning, Inc. Costar Corp., Cambridge, MA) at a density of 1×10^5 cells/well. The viability of isolated cells was assessed by trypan blue exclusion and was about 85%. Cells were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Forty-eight hours later, the media were changed to remove cell debris and unattached cells. Cells were then cultured in basal culture media for another 48 h and treatments were initiated afterward. The purity of primary cultures of adrenocortical cells was assessed by immunofluorescence analysis by using the antibody against cytochrome P450 11β-hydroxylase, a marker of fasciculata cells. It was found that ~90% of the cells presented positive staining (data not shown).

Isolation of mitochondria

Mitochondrial isolation was performed using a mitochondria fractionation kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Briefly, fresh mouse adrenal glands were minced in chilled (4 °C) isolation media which consist of 0.25 M sucrose, 10 mM Tris–HCl buffer, pH 7.4, 1 mM EDTA, and 250 μg/ml BSA). After centrifugation at 600*g* for 10 min, nuclei and cell debris were sedimented. The supernatant was subjected to centrifugation at 10,000*g* for 10 min. The resulting mitochondrial pellets were suspended in the isolation medium for other analysis.

Measurement of mitochondrial superoxide production

The cell-permeable probe, MitoSOX (Molecular Probes, Invitrogen, Carlsbad, CA), specifically accumulates in mitochondria and becomes fluorescent after oxidation by superoxide [27]. Adrenocortical cells or isolated mitochondria were incubated with MitoSOX dissolving in DMSO at a final concentration of 5 μM with DMSO diluted to less than