BBA - Molecular Basis of Disease 1864 (2018) 307–316

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

The therapeutic value of protein (de)nitrosylation in experimental septic shock

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ARTICLE INFO

Keywords:
Denitrosylation
Nitrosylation
Sepsis
Vascular dysfunction
Organ damage

ABSTRACT

Cardiovascular dysfunction and organ damage are hallmarks of sepsis and septic shock. Protein *S*-nitrosylation by nitric oxide has been described as an important modifier of protein function. We studied whether protein nitrosylation/denitrosylation would impact positively in hemodynamic parameters of septic rats. Polymicrobial sepsis was induced by cecal ligation and puncture. Female Wistar rats were treated with increasing doses of DTNB [5,5′-dithio-bis-(2-nitrobenzoic acid)] 30 min before or 4 or 12 h after sepsis induction. Twenty-four hours after surgery the following data was obtained: aorta response to phenylephrine, mean arterial pressure, vascular reactivity to phenylephrine, biochemical markers of organ damage, survival and aorta protein nitrosylation profile. Sepsis substantially decreases blood pressure and the response of aorta rings and of blood pressure to phenylephrine, as well as increased plasma levels of organ damage markers, mortality of 60% and *S*-nitrosylation of aorta proteins increased during sepsis. Treatment with DTNB 12 h after septic shock induction reversed the loss of response of aorta rings and blood pressure to vasoconstrictors, reduced organ damage and protein nitrosylation and increased survival to 80%. Increases in protein *S*-nitrosylation are related to cardiovascular dysfunction and multiple organ injury during sepsis. Treatment of rats with DTNB reduced the excessive protein *S*-nitrosylation, including that in calcium-dependent potassium channels (BK_{Ca}), reversed the cardiovascular dysfunction, improved markers of organ dysfunction and glycemic profile and substantially reduced mortality. Since all these beneficial consequences were attained even if DTNB was administered after septic shock onset, protein (de)nitrosylation may be a suitable target for sepsis treatment.

1. Introduction

The high mortality rate caused by sepsis and septic shock remains a challenge. Sepsis is defined as a clinical syndrome associated to a dysregulated response against infection accompanied by relevant changes in the cardiovascular, neuronal, autonomic, hormonal, bioenergetics, metabolic, clotting and immune aspects of the host that may cause tissue damage ending in multiple organ failure [1–4].

Nitric oxide (NO) overproduction during sepsis has been pointed out as a key element responsible for the cardiovascular dysfunction, characterized by a severe hypotension refractory to volume replacement and vasoconstrictor therapy [5,6]. In both physiology and during sepsis, NO exerts its effects through soluble guanylate cyclase and potassium channel activation [7–9].

In addition, NO effects also can be carried out by posttranslational protein modification, *S*-nitrosylation. This modification involves the reaction of NO with protein thiol groups (sulfhydryls) of cysteine residues, producing *S*-nitrosothiols (SNO). Importantly, *S*-nitrosylation is reversible. SNO may act as endogenous reservoirs of NO and thereby increase its bioavailability [10,11]. *S*-nitrosylation can change protein function, stability, location and hence is responsible for a broad spectrum of cell signaling effects [10]. A growing body of research indicates that SNO have an essential role in many physiological processes [11–13] but dysregulated *S*-nitrosylation may compromise cell function and cause diseases [14–16].

Increases in *S*-nitrosylated protein content in vascular tissue is associated with hyporeactivity to vasoconstrictors in vessels exposed to NO donors [17,18]. Furthermore, previous work from our laboratory has shown that the oxidation of sulfhydryls prevented hyporesponsiveness to phenylephrine in rat aorta rings incubated with NO donors [19]. Additionally, it was shown that the nitrosative stress during endotoxic shock increases *S*-nitrosylated proteins which are associated

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http://dx.doi.org/10.1016/j.bbadis.2017.10.029
Received 17 January 2017; Received in revised form 16 September 2017; Accepted 26 October 2017
Available online 28 October 2017
0925-4439/

with low blood pressure, tissue injury, renal dysfunction and higher mortality, thus contributing to the pathogenesis of endotoxic shock [20–22]. Although SNO play an important role in the pathophysiology associated with endotoxemia, their precise role in the cardiovascular dysfunction during sepsis remains unclear.

In the present report, it was investigated the role of protein *S*-nitrosylation/denitrosylation in the cardiovascular dysfunction and organ damage during septic shock. To better understand the role of *S*-nitrosylation/denitrosylation in sepsis, we used an experimental model of sepsis/septic shock, namely the cecal ligation and puncture method and evaluated the effects of treating rats with DTNB [5,5′-dithio-bis-(2-nitrobenzoic acid)], a sulfhydryl-oxidizing agent.

2. Methods and materials

2.1. Animals

All animal care and experimental procedures were approved by the University Institutional Ethics Committee (Protocol PP-0790) and are in accordance with CONCEA (National Council for the Control of Animal Experimentation, Brazil) and National Institutes of Health (NIH, USA) guidelines. Experiments were performed in female Wistar rats weighing 200–250 g. Animals were housed (5 per cage) in a temperature and light controlled room (22 ± 2 °C; 12 h light/dark cycle) with free access to water and food. Animals from different cages were randomized to form the experimental groups. At the end of the experiment, animals were euthanized with anesthetics overdose (ketamine and xylazine).

2.2. Cecal ligation and puncture

Sepsis was induced by cecal ligation and puncture (CLP) as previously described [23]. Briefly, the animals were sedated intraperitoneally (i.p.) with xylazine (5 mg/kg). At the same time, analgesia was induced with tramadol hydrochloride (10 mg/kg). Then the animals were accommodated in a breathing chamber for induction of anesthesia by oxygen-isoflurane (5%) inhalation. Anesthesia was maintained by oxygen-isoflurane (3%) delivered by a face mask. Body temperature was maintained at 37 ± 1 °C by means of a heating pad. After laparotomy, the cecum was ligated distal to the ileocecal valve with surgical thread, and a transfixing puncture with a 14-gauge needle was made. Then, a small amount of cecal content was squeezed through the puncture. All animals received 30 mL/kg of sterile warm Dulbecco's phosphate-buffered saline (PBS; in mM, 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4 and 8.1 $NaHPO_4$; pH 7.4) subcutaneously (s.c.). Animals were kept in a heated environment to recover from anesthesia (60 to 120 min) and were given tramadol hydrochloride (5 mg/kg, s.c.) every 12 h for analgesia.

2.3. Lactate, glucose, creatinine, urea, ALT, AST and CK-MB determination

Plasma levels of lactate, creatinine, urea, alanine aminotransferase (ALT), aspartate transaminase (AST) and creatine kinase-MB (CK-MB) were determined using commercial kits (Labtest Diagnostica S/A, Lagoa Santa, MG, Brazil). To obtain the plasma fraction, blood was collected in tubes containing EDTA (final concentration of 2 mg/mL of blood), centrifuged at 1200 × *g* for 8 min and the plasma was stored at − 80 °C until the biochemical tests were performed. Glucose was determined by a glucometer using a drop of the blood (Accu-Chek Active®, Roche, Mannheim, Germany).

2.4. Mean arterial pressure measurement

Rats were anesthetized with ketamine and xylazine (90 and 15 mg/kg, respectively, supplemented at 45 to 60 min intervals) and body temperature was maintained at 37 °C by means of a heating pad. Heparinized PE-20 and PE-50 polyethylene catheters were inserted into the tail vein for drug injections and into the right carotid artery for recording of mean arterial pressure (MAP), respectively. Animals breathed spontaneously via a tracheal cannula. Blood pressure data were recorded with a catheter pressure transducer (Mikro-Tip, Millar Instruments, Inc., Houston, TX, USA) coupled to a Powerlab 8/30 (AD Instruments Pty Ltd., Castle Hill, Australia). Results were expressed as mean ± SEM of the peak changes in MAP (as mm Hg) relative to baseline, and recorded following administration of a given compound.

2.5. Rat thoracic aortic rings

Twenty-four hours after CLP surgery, rats were euthanized by anesthesia overdose. The thoracic aorta was removed, cleaned of perivascular tissues, cut in rings of 3–4 mm width and placed under a tension of 2 g in standard organ baths (2 ml) filled with nutritive Krebs-Ringer solution (pH 7.4; composition in mM: 118 NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.1 $MgSO_4$; 0.9 KH_2PO_4; 25 $NaHCO_3$; 0.03 EDTA; and 11 D-glucose), warmed at 37 °C and constantly bubbled with 95% O_2/5% CO_2. Aortic rings were connected to isometric force transducers (ADI Instruments, Dunedin, New Zealand) to measure tension. After 60 min of stabilization, the viability was tested using KCl (120 mM). Cumulative concentration–response curves for phenylephrine (10^{-10}–10^{-4} mol/L) were obtained.

2.6. Biotin switch assay

The biotin switch method to detect *S*-nitrosylation was performed with the *S*-nitrosylated protein detection assay kit (Cayman Chemical, Ann Arbor, MI, USA) modified from the method of Jaffrey et al. [24]. For this, the aortas were immersed in liquid nitrogen, pulverized and treated as recommended by the manufacturer. Biotin-labeled proteins were analyzed by Western blot and detected by avidin-linked fluorescein in a Typhoon FLA 9000 scanner (GE Healthcare Life Sciences, SP, Brazil) using excitation wavelength at 490 nm. The fluorescence of the labeled proteins was quantified using NIH ImageJ 1.50i imaging software (NIH, Bethesda, MD, USA).

2.7. NOx (nitrite + nitrate) assay

NOx levels were evaluated by measuring the plasma concentration of NO end products, nitrate and nitrite. Briefly, zinc sulfate-deproteinized plasma samples were subjected to nitrate conversion. Nitrate was converted to nitrite using *Escherichia coli* nitrate reductase for 3 h at 37 °C. Samples were centrifuged to remove bacteria, and 100 μL of each sample was mixed with Griess reagent (1% sulfanilamide in 10% phosphoric acid/0.1% naphthylethylenediamine in Milli-Q water) in a 96-well plate and read at 540 nm in a plate reader. Standard curves of nitrite and nitrate (0–150 μM) were run simultaneously. As under these conditions nitrate conversion was always > 90%, no corrections were made. Values are expressed as μM NOx (nitrate + nitrite).

2.8. Immunofluorescence assays

Thoracic aortas were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 M, pH 7.4), immersed in sucrose (20% in PBS) for 3 h and frozen in Tissue Tek (Sakura Finetek Inc., Torrance, CA, USA). Cryostat sections (8 μm thick) were obtained (Leica CM 1850 UV Biosystems Leica, Wetzlar, Germany) and placed on gelatinized slides. The sections were blocked with 5% fetal bovine serum for 1 h. Then, slices were incubated overnight at 4 °C with rabbit polyclonal antibody anti-SNO (1:200 dilution, Sigma-Aldrich, St. Louis, MO, USA) and goat polyclonal antibody anti-BK_{Ca} [1:200 dilution, Santa Cruz Biotechnology, CA, USA]. Subsequently, slices were washed three times with PBS and incubated with Alexa Fluor 488- (1:700, dilution) and 594- (1:800 dilution) conjugated secondary antibodies (Invitrogen Life Technologies, Carlsbad, CA, USA) for 1 h at room