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Sirt1 S-nitrosylation induces acetylation of HMGB1 in LPS-activated RAW264.7 cells and endotoxemic mice

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ABSTRACT

Excessive inflammation plays a detrimental role in endotoxemia. A recent study indicated that alarmins such as high mobility group box 1 (HMGB1) have drawn attention as therapeutic targets of sepsis. Post-translational modification (i.e., acetylation of lysine residues) of HMGB1 leads to the release of HMGB1 into the cellular space, operating as a warning signal that induces inflammation. Sirtuin 1 (SIRT1) has been shown to negatively regulate HMGB1 hyperacetylation and its extracellular release in sepsis. Therefore, we hypothesized that the S-nitrosylation (SNO) of SIRT1 may disrupt the ability of SIRT1 to negatively regulate the hyperacetylation of HMGB1. As long as the S-nitrosylation of SIRT1 occurs during septic conditions, it may worsen the situation. We found that the activity of SIRT1 decreased as the SNO-SIRT1 levels increased, resulting in HMGB1 release by LPS in RAW264.7 cells. Both the iNOS inhibitor (1400 W) and silencing iNOS significantly inhibited SNO-SIRT1, allowing increases in SIRT1 activity that decreased the HMGB1 release by LPS. SNAP, a NO donor, significantly increased both SNO-SIRT1 levels and the HMGB1 release that was accompanied by decreased sirt1 activity. However, sirtinol, a Sirt1 inhibitor, by itself decreased Sirt1 activity compared to that of the control, so that it did not affect already increased SNO-SIRT levels by SNAP. Most importantly, in lung tissues of LPS-endotoxic mice, significantly increased levels of SNO-SIRT were found, which was inhibited by 1400 W treatment. Plasma nitrite and HMGB1 levels were significantly higher than those in the sham controls, and the elevated levels were significantly lowered in the presence of 1400 W. We concluded that the S-nitrosylation of Sirt1 under endotoxic conditions may uninhibit the acetylation of HMGB1 and its extracellular release.

1. Introduction

Inducible nitric oxide synthase (iNOS) is one of major mediators of inflammation in various cell types [1]. We and others have previously shown that inhibition of iNOS by pharmacological inhibitors or silencing iNOS ameliorates LPS-mediated endotoxic damages in rodents [2–6]. High-mobility group box 1 (HMGB1), a ubiquitous nonhistone DNA-binding protein, is constitutively expressed in the nucleus of eukaryotic cells and plays important roles in maintaining the nucleosome structure and stability, chromatin remodelling, and regulation of gene transcription [7–9]. In addition, HMGB1 can be released to the extracellular space and acts as a proinflammatory cytokine to induce an inflammatory reaction [10]. There are two types of releasing HMGB; either actively released by activated immune cells or passively released from damaged and necrotic cells [11,12]. The nucleocytoplasmic translocation occurring after posttranslational modification is required for the active release of HMGB1 [13]. As recognized that HMGB1 plays an important role in inflammatory diseases such as sepsis [14,15], targeting HMGB1 may be an important strategy for the treatment of sepsis and other inflammatory diseases.

Several posttranslational modifications, such as phosphorylation, acetylation, and methylation, are involved in the translocation of HMGB1 from the nucleus to the cytoplasm [13,16,17]. Gowing number of study showed that sirtuin 1 (SIRT1), a NAD + -dependent deacylase3, has demonstrated to inhibit extracellular release of HMGB1 by inhibition of acetylation of HMGB1 in septic conditions [18].

On the other hand, protein S-nitrosylation (S-NO) is the covalent attachment of nitric oxide (NO) moiety to reactive cysteine thiols

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and a major mediator of physiological and pathophysiological actions of NO [19]. Given that Sirt1 plays as a negative regulator of HMGB1 release by inhibition of acetylation of HMGB1 in septic situations, it might be great interest to investigate whether S-nitrosylation of Sirt1 happens in endotoxic states due to ample amounts of NO production by LPS in *in vitro* and *in vivo*.

Our study shows that NO (either NO-donor or iNOS-driven NO)-mediated S-nitrosylation of Sirt1, significantly decreases Sirt1 activity that increases release of HMGB1 in LPS-activated RAW 264.7 cells as well as in endotoxic mice lung tissues.

2. Materials and methods

2.1. Materials

HyClone Dulbecco's High Glucose Modified Eagles Medium (DMEM), fetal bovine serum (FBS), antibiotics (penicillin/streptomycin) and HPDP-biotin were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies for iNOS, β-actin and responsible secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibody for SIRT1 was purchased from Cell signaling Technology (Beverly, MA, USA). Anti-HMGB1 was from Abcam (Cambridge, MA, USA). All other chemicals including, LPS (Escherechia coli 0111:B4), SNAP, MMTS and 1400 W were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (4.5 mg/ml), glucose (4.5 mg/ml) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.3. Western blot analysis

The cells were rinsed with ice-cold phosphate-buffered saline and lysed with buffer containing 0.5% SDS, 1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris—Cl (pH 7.5), and protease inhibitors. The protein concentration of each sample was determined using a Bradford assay; absorbance of the mixture at 595 nm was determined with an ELISA plate reader. To detect the secretion of HMGB1 in the supernatants, culture medium samples were briefly centrifuged to remove cellular debris. Same volumes of samples were mixed with 2x loading dye and boiled at 95 °C for 5 min. Culture medium samples and whole cell lysates were subjected to electrophoresis in different percentage polyacrylamide gels, depending on the size of protein of interest. The gels were transferred to polyvinylidene difluoride (PVDF) membranes by semidry electrophoretic transfer at 20 mA for 2 h. The PVDF membranes were blocked for 2 h at room temperature in 5% bovine serum albumin (BSA). And then the membranes were incubated with primary antibodies diluted 1:500 in Tris-buffered saline/Tween 20 (TBS-T) containing 5% BSA overnight at 4 °C and then incubated with the secondary antibody (1:5000 dilution in TBS-T containing 1% BSA) at room temperature for 1 h. After washing three times with TBS-T solution, membranes were incubated with ECL Western blotting detection reagents (Amersham, Piscataway, NJ, USA). The membrane was exposed to Xomat AR films (Eastman Kodak, Rochester, NY, USA). Scanning densitometry was performed using an Image Master® VDS (Pharmacia Biotech Inc., San Francisco, CA, USA).

2.4. NO measurement

NO was measured as its stable oxidative metabolites, nitrite. At the end of the incubation, 100 μl of the culture medium was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1%sulfanilamide in 5% phosphoric acid). The absorbance at 550 nm was measured, and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

2.5. Small interference RNA

Small interfering RNA against mouse iNOS was provided from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and performed using Lipofectamine® RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Briefly, Cells were seeded into 60 mm culture dishes the day before transfection and grown to about 70% confluence. Cells were transfected with iNOS-targeting siRNA. Transfections were allowed to proceed for 24 h. The transfected cells were washed with 4 ml of 1x phosphate buffered saline (PBS, pH 7.4) and then stimulated with 1 μg/mL LPS. Cells were harvested and subjected to Western blot analysis.

2.6. Detection of S-nitrosylated SIRT1

The biotin-switch assay was performed to detect S-nitrosylation of SIRT1. Cultured cells and lung tissue were washed with PBS, and homogenized or lysed in buffer [250 mM N-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid-sodium hydroxide (HEPES-NaOH), pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5% SDS, 1% CHAPS, 0.1 mM neocuproine, 80 μM carmustine, 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail]. After centrifugation, tissue or cell supernatants were mixed with blocking buffer [250 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1 mM neocuproine, 50 mM methyl methanethiosulfonate (MMTS)] (with or without 2.5% SDS for cell lysates and tissue homogenates, respectively) and incubated at 50 °C for 20 min to block free thiol groups. After acetone precipitation, S-nitrosylated thiols were reduced with 20 mM sodium ascorbate, and then biotinylated with N-(6-[biotinamido]hexyl)-3'-(2′-pyridyldithio)-propionamide (HPDP-biotin) (0.4 mM) in the dark for 1 h. After acetone precipitation, streptavidin-agarose beads were added, and gently rotated for 12—18 h at 4 °C. The beads were washed with HEN buffer (25 mM HEPES PH 7.5, 1 mM EDTA, 0.5 M NaCl) containing 0.5% NP-40, and proteins were eluted by incubation with elution buffer (20 mM HEPES pH 7.7, 1 mM EDTA, 100 mM NaCl, 200 mM DTT) for 30 min and subjected to Western blot analysis.

2.7. SIRT1 deacetylase activity assay

SIRT1 deacetylase activity was assayed using a commercial kit (Epigenase Universal SIRT Activity Assay Kit, EpiGentek) according to the manufacturer's instructions. Fluorescence was measured using a fluorometric reader (Infinite® F200, Tecan Group Ltd, Switzerland) with excitation at 535 nm and emission detection at 595 nm.

2.8. Analysis of plasma HMGB1 level

Plasma Concentrations of HMGB1 was determined with an ELISA kit (USCN Life Sciences; Wuhan, Hubei, China) according to the manufacturer's protocol. In brief, the extracted plasma was reacted with the assay reagents in HMGB1 kits and analyzed spectrophotometrically (Infinite® F200, Tecan Group Ltd,

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