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Hydrogen sulfide attenuates oxidative stress-induced NLRP3 inflammasome activation via S-sulfhydrating c-Jun at Cys269 in macrophages

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ABSTRACT

Oxidative stress and inflammation are closely related to cardiovascular diseases. Although hydrogen sulfide (H_2S) has been shown to have powerful anti-oxidative and anti-inflammatory properties, its role in macrophage inflammation was poorly understood. The aim of this study was to investigate the role of H_2S in the regulation of macrophage NLRP3 inflammasome activation. We reported here that H_2S attenuated hydrogen peroxide (H_2O_2)-induced NLRP3 inflammasome activation, which led to caspase-1 activation and IL-1β production in macrophages. Moreover, H_2S exerted its protective effects by lowering the generation of mitochondrial reactive oxygen species (mtROS). Mechanistically, S-sulfhydration of c-Jun by H_2S enhanced its transcriptional activity on SIRT3 and p62, which contributed to the decrease of mtROS production. S-sulfhydration sites are investigated by site directed mutagenesis. Findings showed that S-sulfhydrated c-Jun exerted its protective influences via a c-Jun Cys269-dependent manner. Moreover, the protective effects of H_2S were absent in macrophage from SIRT3 knockout mice. In conclusion, these results demonstrate that H_2S attenuates oxidative stress-induced mtROS production and NLRP3 inflammasome activation via S-sulfhydrating c-Jun at cysteine 269 in macrophages.

1. Introduction

Cardiovascular diseases and their consequences are the most serious health problems worldwide [1]. In general, oxidative stress and inflammation are closely related to cardiovascular diseases, including atherosclerosis, ischemia-reperfusion injury, cardiomyopathy, and heart failure [2,3]. Moreover, the concept of oxidative stress and chronic inflammation as part of the pathophysiology of cardiovascular diseases has been accepted [4]. The excessive increase in reactive oxygen species (ROS) generation is pivotal in the progression of cardiovascular diseases [5,6]. Monocytes/macrophages are involved in the pathogenesis of atherosclerosis, aortic aneurysm, and heart diseases [7–11]. Additionally, monocytes/macrophages have been found to be diagnostic, prognostic, or therapeutic targets in cardiovascular diseases [7,12]. Macrophage infiltration and oxidative stress play a vital role in the progression of cardiovascular diseases [4,13–16].

The NLRP3 inflammasome is composed of the NLRP3(NACHT, LRR and PYD domains-containing protein 3), the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and caspase-1 [17]. The activation of caspase-1 by NLRP3 inflammasome contributes to the processing and secretion of the proinflammatory cytokines IL-1β and IL-18, which contribute to the so-called sterile inflammation response [18,19]. To date, several studies have indicated that NLRP3 inflammasome is activated through three possible pathways, including reactive oxygen species (ROS), intracellular potassium (K^+) concentration, and the disruption of the lysosomal membrane [20,21]. Macrophage NLRP3 inflammasome activation has been suggested to be involved in cardiovascular diseases [22–28].

Abbreviations: ANOVA, analysis of variance; BMM, bone marrow-derived macrophages; BSA, bovine serum albumin; CVD, cardiovascular diseases; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulphoxide; DHE, dihydroethidium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H_2S, Hydrogen sulfide; H_2O_2, Hydrogen peroxide; HRP, horseradish peroxidase; IL-1β, interleukin-1β; M-CSF, macrophage colony-stimulating factor; MMTS, methyl methanethiosulfonate; mtROS, mitochondrial reactive oxygen species; NLRP3, NLR family pyrin domain containing 3; PBS, phosphate buffered solution; p62, sequestosome 1; PMA, 4β-phorbol-12-myristate-13-acetate; PVDF, polyvinylidene fluoride; RIPA, radio immunoprecipitation assay; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SIRT3, Sirtuin 3

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Hydrogen sulfide (H_2S) is a gaseous transmitter and plays important roles in several diseases [29,30]. H_2S is a biologically active gas that is synthesized naturally by three enzymes, cystathionine γ-lyase (CSE), cystathionine β-synthetase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) [31]. In addition, H_2S acts as an endogenous scavenger for reactive oxygen species [32–34]. Furthermore, H_2S physiologically modifies protein cysteines to form S-sulfhydration, which appears to be a physiologic posttranslational modification for proteins [29,35]. Our previously studies have demonstrated that H_2S has powerful antioxidative and anti-inflammatory properties in several pathological process, including myocardial fibrosis, endothelial dysfunction, diabetes-accelerated atherosclerosis, myocardial hypertrophy, and testicular dysfunction [36–40].

In this study, exogenous treatment with H_2O_2, a ROS-generating agent, was used to mimic oxidative stress in macrophages. We demonstrated that H_2S attenuated H_2O_2-induced NLRP3 inflammasome activation in macrophages THP-1. H_2O_2-induced NLRP3 inflammasome activation led to caspase-1 activation and IL-1β production. We further demonstrated that NLRP3 bound to ROS-generated mitochondria in macrophages exposed to H_2O_2. However, pretreatment with H_2S reduced these above effects of H_2O_2 on macrophages. In addition, we found that H_2S enhanced c-Jun transcriptional activity directly via S-sulfhydration of c-Jun protein at cysteine 269. The increased c-Jun transcriptional activity contributed to the increasing expression of SIRT3 and p62, which exhibited remarkable antioxidant and anti-inflammatory effects in macrophages. This study deepens our understanding of the protective effects of H_2S via c-Jun S-sulfhydration in macrophages.

2. Materials and methods

2.1. Cell culture and treatment

THP-1 human monocyte cells obtained from Cell Bank at the Chinese Academy of Sciences were cultured in 25 mM HEPES-buffered RPMI 1640 (Life Technologies, Rockville, MD, USA) containing 10% FBS (Life Technologies, Rockville, MD, USA) and 50 μM β-mercaptoethanol (Life Technologies, Rockville, MD, USA). THP-1 cells were differentiated into macrophages by incubation with 5 ng/mL 4β-phorbol-12-myristate-13-acetate (PMA; Biyuntian, Hangzhou, China) for 24 h. RPMI 1640 complete medium was replaced before addition of H_2S donor, NaHS (100 μM; Sigma-Aldrich, St Louis, MO, USA). After 4 h treatment, cells were incubated in freshly prepared medium containing H_2O_2 (250 μM; Sigma-Aldrich, St Louis, MO, USA) for a further 4 h. Mitochondria-targeted antioxidant Mito-TEMPO was purchased from Abcam (ab144644, Cambridge, MA, USA). Cathepsin B inhibitor CA-074Me was purchased from Bachem (4027913, Bubendorf, Switzerland). $P2X_7$ receptor inhibitor A438079 was purchased from Selleck Chemicals (S7705, Shanghai, China). For cell experiments, these reagents were dissolved in dimethyl sulfoxide (DMSO) and were used at the desired concentration (indicated in figure legends).

2.2. Transient transfection and dual-luciferase reporter assay

Human c-Jun (Gene ID: 3725) cDNA was purchased from GENEWIZ, Inc. (Suzhou, China). Human c-Jun cDNA was cloned into pcDNA3.1 by GENEWIZ. Single mutation at cysteine-99 (C99A), cysteine-269 (C269A), or cysteine-320 (C320A) in c-Jun was conducted by GENEWIZ. pAP1-TA-luc vector is a luciferase reporter construct with multiple AP-1 (TGACTAA) response elements and purchased from Biyuntian (Hangzhou, China). The response elements of c-Jun in SIRT3 and p62 genes promoter were predicted via using JASPAR databases (Supplementary data 1). The wild type and deletion of the c-Jun response element in the SIRT3 and p62 promoter were purchased from GENEWIZ, Inc. (Suzhou, China). SIRT3-WT (−500−−1), $SIRT3_{\Delta-337--325}$, p62-WT (−500−−1), and $p62_{\Delta-389--377}$ were subcloned into luciferase reporter vector pGL4 (Promega, Madison, WI, USA) by GENEWIZ. pGL4.74 (Promega, Madison, WI, USA) vector was also purchased from Promega. After incubation with 5 ng/mL PMA for 24 h, THP-1 macrophages were transfected with indicated plasmids by using Lipofectamine 3000 reagent (Invitrogen) according to manufacturer's recommendations and the method as previously described [37]. Cells were cultured for 24 h after transfection. The luciferase activity was measured by the dual luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Data were normalized by the activity of Renilla luciferase. Mcrophages were transfected with NLRP3 siRNA (sense 5′-GUUUGACUAUCUGUUCUdTdT-3′ [41]; GenePharma, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.3. Western blotting

RIPA lysis buffer (Biyuntian, Hangzhou, China) containing protease inhibitor cocktail (ThermoFisher, Waltham, MA, USA) was used to obtain whole-cell lysates from macrophages. Protein concentrations were quantified using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded into SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were incubated with rabbit antibodies to caspase-1 (#3866, CST, Danvers, MA, USA), IL-1β (#ab45692, Abcam, Cambridge, MA, USA), CBS (#ab135626, Abcam), CSE (#ab151769, Abcam), NLRP3 (#13158, CST), SIRT3 (#2627, CST), and GAPDH (#AP0063, Bioworld, Nanjing, China) as well as mouse antibodies to caspase-1 p20 (#AG-20B-0048, AdipoGen, San Diego, CA, USA), 3-MST (#sc-374,326, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and p62 (#MB9009, Bioworld). All blots were overlaid with appropriate HRP-conjugated secondary antibodies and visualized by enhanced chemiluminescence substrate (Bio-Rad, Hercules, CA, USA).

2.4. Immunofluorescent staining and confocal microscopy

THP-1 monocyte cells were seeded on glass slides (NEST, Wuxi, China) and incubated with 5 ng/mL PMA for 24 h. Then, the cells were treated as described above. After the treatment, the cells were washed twice with sterile PBS and fixed with ice-cold methanol, permeabilized with 0.01% Triton X-100 and blocked in 3% BSA. The cells were then incubated overnight with primary antibodies, including anti-NLRP3 (#AG-20B-0014, AdipoGen, San Diego, CA, USA), anti-caspase-1 (#3866, CST), and anti-c-Jun (#9165, CST). For experiments that used mitotracker, cells were stained with 5 μM MitoTracker Deep Red FM (#M22426, Life Technologies) for 20 min prior to methanol fixation. Secondary fluorescent antibodies (Alexa-488, or -594; Life Technologies) were added for 1 h and DAPI (#sc-24,941, Santa Cruz) was used for nuclear counterstaining. Samples were imaged through confocal microscope (Zeiss LSM 410, Oberkochen, Germany) and quantified using Image-Pro Plus analysis software.

2.5. Measurement of ROS and mtROS formation

Superoxide production in cells was detected by dihydroethidium (DHE; Vigorous, Beijing, China) assay as the manufacturer's instructions. Briefly, macrophages were treated as described above, after which the cells were washed twice with PBS and incubated with 5 μM of DHE for 30 min and washed twice with PBS. Fluorescence was measured with a Nikon TE2000 Inverted Microscope and quantified using Image-Pro Plus analysis software. Mitochondrial ROS were measured using MitoSOX (#M36008, Molecular Probes, Carlsbad, CA, USA). Briefly, macrophages were treated as above described, after which the cells were washed twice with PBS and loaded with 5 μM of MitoSOX and 100 nM MitoTracker Green FM (#M7514, Molecular Probes) for 20 min. Fluorescence was measured through confocal microscope (Zeiss