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Original Contribution

Role of sestrin2 in the regulation of proinflammatory signaling in macrophages

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ABSTRACT

Sestrins (Sesns) are conserved antioxidant proteins that accumulate in cells in response to various stresses. However, the regulatory roles of Sesn2 in the immune system and in inflammatory responses remain obscure. In the present study, we investigated whether Sesn2 regulates Toll like receptor (TLR)-mediated inflammatory signaling and sought to identify the molecular mechanism responsible. In cells expressing Sesn2, it was found that Sesn2 almost completely inhibited lipopolysaccharide (LPS)-induced NO release and iNOS expression. A gene knockdown experiment confirmed the role of Sesn2 in LPS-activated RAW264.7 cells. Consistently, proinflammatory cytokine (e.g., TNF-α, IL-6, and IL-1β) release and expression were inhibited in Sesn2-expressing cells. Furthermore, Sesn2 prevented LPS-elicited cell death and ROS production via inhibition of NADPH oxidase. NF-κB and AP-1 are redox-sensitive transcription factors that regulate the expressions of diverse inflammatory genes. Surprisingly, Sesn2 specifically inhibited AP-1 luciferase activity and its DNA binding, but not those of NF-κB. AP-1 inhibition by Sesn2 was found to be due to a lack of JNK, p38, and c-Jun phosphorylation. Next, we investigated whether Sesn2 protects galactosamine (Gal)/LPS-induced liver injury in mice infected with a recombinant adenovirus Sesn2 (Ad-Sesn2). Ad-Sesn2 present less severe hepatic injury as supported by decreases in the ALT, AST, and hepatocyte degeneration. Moreover, Ad-Sesn2 attenuated Gal/LPS-induced proinflammatory gene expression in mice. The study shows that Sesn2 inhibits TLR-induced proinflammatory signaling and protects cells by inhibiting JNK- or p38-mediated c-Jun phosphorylation.

Introduction

Reactive oxygen species (ROS) are essential mediators of normal cell physiology and are generated from the metabolism of molecular oxygen, mainly in mitochondria [1,2]. ROS can serve as intracellular signaling molecules and are involved in regulation of diverse biological processes. Moreover, hydrogen peroxide (H_2O_2) is a major ROS in terms of cell signaling regulation [3]. However, oxidative stress occurs when the balance between the ROS production and the antioxidant defense mechanism is skewed in favor of ROS production, and the excessive ROS then causes direct damage to macromolecules, such as lipids, nucleic acids, and proteins [4]. Furthermore, it is well known that oxidative damage is associated with various human diseases, such as cancer, diabetes, hepatitis, and cardiovascular disease.

The peroxiredoxins (Prxs) constitute a family of thiol-dependent peroxidases that scavenge H_2O_2 and alkyl hydroperoxides. In addition to antioxidant activities, they are also associated with diverse cellular functions, such as proliferation, differentiation, and apoptosis [5,6]. Furthermore, the six mammalian Prx family members exhibit different tissue and organelle distributions [6]. The Prxs are classified into three subgroups, designated 2-Cys Prx, atypical 2-Cys Prx, and 1-Cys Prx. The 2-Cys Prx exists as homodimers and contains two conserved cysteine residues for peroxide reduction. Under highly oxidizing conditions, Prxs lose peroxidase activity due to the over-oxidation of cysteine to sulfinic acid (Cys-SO_2H) or sulfonic acid (Cys-SO_3H) [5] in the active site. However, sufinylated Prxs are reactivated by sulfiredoxin (Srx), which reduces sulfinylated Prxs via an ATP-dependent mechanism [7–9].

It was recently proposed that sestrins (Sesns) also have cysteine sulfinyl reductase activity and inhibit ROS production by regenerating

Abbreviations: Ad, adenovirus; ATF, activating transcription factor; BMDM, bone marrow-derived macrophage; bZIP, basic region leucine zipper; ERK1/2, extracellular-regulated protein kinases 1 and 2; Gal, D-galactosamine; IκB, inhibitory κB; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; NO, nitric oxide; NOX, NADPH oxidase; Prx, peroxiredoxin; RNS, reactive nitrogen species; ROS, reactive oxygen species; Sesns, sestrins; Sesn2, sestrin-2; Srx, sulfiredoxin; TLR, Toll-like receptor

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overoxidized Prxs, despite the fact that Sesns share no structural similarity with Srx. Sestrin2 (Sesn2; also known as Hi95) is responsive to various stress, such as hypoxia, DNA damage, and oxidative stress [10]. It is reported that the expression of Sesn2 is regulated via a p53- [10], Nrf2- [11], and HIF-1α- [12] dependent manner. Moreover, Sesn2 induces autophagy by inhibiting mTOR signaling via an AMPK activation, which results in more efficient elimination of ROS-producing damaged mitochondria [13–15].

Toll like receptors (TLRs), mammalian homologues to the *Drosophila* Toll, compose a family of transmembrane proteins that function in immunity and development. TLRs are ubiquitously expressed pattern recognition receptors, which are central to inflammatory response in a broad range of species. Furthermore, it is becoming apparent that a link exists between oxidative stress and TLR signaling. TLR4 activation by lipopolysaccharide (LPS) is capable of inducing ROS and NF-κB activation due to a direct interaction between TLR4 and NADPH oxidase (NOX) [16]. Moreover, Prx2 is critical for the regulation of LPS-induced inflammatory gene expression and for the activation of MAPKs and NF-κB through NOX and ROS signaling [17]. However, the involvement of Sesn2 in the regulations of inflammatory responses and ROS generation in macrophages is poorly understood.

In the present study, we sought to determine whether Sesn2 protects proinflammatory and intracellular ROS signaling in TLR ligand-activated macrophages. We established RAW264.7 macrophages stably expressing Sesn2 to identify the molecular mechanism involved. In addition, we investigated whether Sesn2 prohibits LPS-induced acute hepatitis in mice infected with a recombinant adenovirus Sesn2.

Materials and methods

Materials

Antibodies against iNOS, PARP1/2, and IκBα were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Sesn2 antibody was obtained from Proteintech (Chicago, IL). Phospho-ERK1/2, ERK1/2, phospho-p38, p38, phospho-JNK1/2, JNK1/2, phospho-c-Jun, c-Jun, phospho-c-Fos, and c-Fos antibodies were obtained from Cell Signaling (Danvers, MA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies were purchased from Invitrogen (Carlsbad, CA). SB203580 and SP600125 were purchased from Calbiochem (Billerica, MA). LPS (*Escherichia coli* 055:B5), peptidoglycan, poly(I:C), loxoribine, flagellin, ODN 1826, dimethyl sulfoxide (DMSO), sodium nitrite, galactosamine (Gal), and β-actin antibody were from Sigma Chemicals (St. Louis, MO).

Cell culture

RAW264.7 cells (a murine macrophage cell line) were purchased from ATCC (The American Type Culture Collection, Rockville, MD). Cells were plated at 1×10^5 per well in six-well plates, and used when 70–80% confluent. Cells were maintained in DMEM containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. LPS (0.3–1 μg/ml) or peptidoglycan (30 μg/ml), poly(I:C) (50 μg/ml), loxoribine (500 μM), flagellin (1 μg/ml), and ODN 1826 (1 μM) were added to cells and incubated at 37 °C for the indicated times. Cells were then washed twice with ice-cold PBS before sample preparation.

Animals

The protocols for the animal studies were approved by the Animal Care and Use Committee of Chosun University. Male ICR mice (6 weeks old) were obtained from Oriental Bio (Sungnam, Korea) and acclimatized for 1 week. Mice ($N=5$/group) were housed at 20 ± 2 °C with 12 h light/dark cycles and a relative humidity of $50 \pm 5\%$ under filtered, pathogen-free air, with food (Purina, Korea) and water available ad libitum.

Adenovirus preparation

For the generation of an adenoviral Sesn2 construct murine Sesn2 ORF was amplified by using attB-fused specific primers, and then inserted into pDONR™221 entry plasmid by BP recombination reaction (Invitrogen, Carlsbad, CA). The recombinant adenovirus was constructed and generated by using pAD/CMV/V5-DEST gateway plasmid according to the manufacturer's instructions (Invitrogen). The DNA sequences of recombinant adenovirus were verified by sequencing using the ABI7700 DNA cycle sequencer. Recombinant adenovirus for *in vivo* study was further purified by $CsCl_2$ density gradient centrifugation. Virus titer was calculated from $TCID_{50}$ method for *in vitro* study or optical intensity of 260 nm for *in vivo* study. Adenovirus which expresses LacZ (Ad-LacZ) was used as an infection control.

Gal/LPS-induced hepatitis

Acute hepatitis was induced by i.p. injection with 5 μg/kg LPS (Sigma-Aldrich) plus 700 mg/kg Gal (Sigma-Aldrich) and euthanized 8 h posttreatment. Adenovirus particles (1×10^9 pfu) suspended in PBS were injected in the tail vein 48 h prior to Gal/LPS injection.

Blood chemistry

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using Spectrum, an automatic blood chemistry analyzer (Abbott Laboratories, Abbott Park, IL).

Histopathology

Samples from liver were separated and fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (3–4 μm), and then stained with hematoxylin and eosin (H&E) for general histopathology. After that the histopathological profiles of each sample were observed under a light microscope (Nikon, Tokyo, Japan).

Establishment of a stable cell line expressing Sesn2

pCMV-SESN2 construct was generated as previously described [18]. RAW264.7 cells were transfected with the plasmid pCMV-Tag3A (MOCK) or pCMV-SESN2 using Lipofectamine 2000, according to the manufacturer's instructions (Life Technologies). One day after transfection, cells were transferred to fresh DMEM medium containing 800 μg/ml G418 (Invitrogen) (the medium was replaced every 3 days). Two weeks later, trypsin-EDTA was added to plates and colonies of G418-resistant cells were isolated under an inverted light microscope and grown further. Sesn2 expression was confirmed by Western blotting using c-myc antibody.

Bone marrow-derived macrophage (BMDM) culture and differentiation

For the BMDM cultures, bone marrow was isolated from the femurs and tibias of mice and cultured in minimum essential medium alpha (α-MEM) supplemented with 10% FBS. The cells were plated and cultured overnight in the presence of macrophage colony-stimulating factor (M-CSF, 10 ng/ml) (Peprotech, Rocky Hills, NJ). The nonadherent cells were collected and cultured for