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SIRT2 ameliorates lipopolysaccharide-induced inflammation in macrophages

Ae Sin Lee [a,1], Yu Jin Jung [a,1], Dal Kim [a], Tung Nguyen-Thanh [a], Kyung Pyo Kang [a,b], Sik Lee [a], Sung Kwang Park [a,b], Won Kim [a,b,*]

[a] Department of Internal Medicine, Chonbuk National University Medical School, Jeonju, Republic of Korea
[b] Research Institute of Clinical Medicine of Chonbuk National University, Chonbuk National University Hospital, Jeonju, Republic of Korea

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ABSTRACT

Introduction: SIRT2 is a NAD(+)-dependent deacetylases and associated with numerous processes such as infection, carcinogenesis, DNA damage and cell cycle regulation. However, the role of SIRT2 in inflammatory process in macrophage remains unclear.

Materials and methods: In the present study, we have evaluated the regulatory effects of SIRT2 in lipopolysaccharide (LPS)-stimulated macrophages isolated from SIRT2 knockout (KO) and wild type (WT) mice or Raw264.7 macrophage cells. As inflammatory parameters, expression of inducible nitric oxide synthase (iNOS), the productions of nitric oxide, reactive oxygen species (ROS) and M1-macrophage-related factors were evaluated. We also examined the effects of SIRT2 on activation of nuclear factor-kappaB (NFκB) signaling.

Results: SIRT2 deficiency inhibits LPS-induced iNOS mRNA and protein expression in bone marrow derived macrophages. SIRT2-siRNA transfection also suppressed LPS-induced iNOS expression in Raw264.7 macrophage cells. Bone marrow derived macrophages isolated from SIRT2 KO mice produced lower nitric oxide and expressed lower levels of M1-macrophage related markers including iNOS and CD86 in response to LPS than WT mice. Decrease of SIRT2 reduced the LPS-induced reactive oxygen species production. Deficiency of SIRT2 resulted in inhibition of NFκB activation through reducing the phosphorylation and degradation of IκBα. The phosphorylation and nuclear translocation of p65 was significantly decreased in SIRT2-deficient macrophages after LPS stimulation.

Discussion: Our data suggested that deficiency of SIRT2 ameliorates iNOS, NO expression and reactive oxygen species production with suppressing LPS-induced activation of NFκB in macrophages.

1. Introduction

Inflammatory process leads to an increase in nitric oxide synthases (NOSs). Expression of inducible NOS (iNOS) is up-regulated in response to inflammatory stimuli and thus, production of nitric oxide (NO) is highly increased [1]. The inflammatory responses are also well characterized by the secretion of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) [2]. Oxygen free radicals are suggested as mediators in lipopolysaccharide (LPS)-mediated inflammatory responses [3]. Reactive oxygen species (ROS) production is increased by inflammatory cytokines and NO in macrophages. Thus, macrophages play critical roles in inflammation. Macrophages have been classified into classically activated macrophages (M1), which is activated by interferon gamma (IFN-γ) and alternatively activated macrophages (M2), which is induced by interleukin (IL)-4 [4]. M1-macrophages express iNOS, TNF-α and CD86, and M2-macrophages express arginase-1, CD206 and FIZZ1 [5].

LPS is one of the potent pro-inflammatory stimulants and exerts its effects by activation of nuclear factor-κB (NF-κB), which is related with expression of many genes related to inflammatory reactions [6]. In inflammatory conditions, p65 also known as RelA, is involved in heterodimer formation, nuclear translocation and activation of NF-κB. Especially, phosphorylation of p65 is critical for NF-κB activity in synthesis of genes. LPS activates upstream mediators of NF-κB activation that requires phosphorylation of p65 in macrophages [7]. Thus, regulation of pro-inflammatory mediators and cytokines including iNOS, NO, phosphorylation of

* Corresponding author at: Department of Internal Medicine, Chonbuk National University Medical School, 634-18, Keum-Am dong, Jeonju 560-180, Republic of Korea. Fax: +82 254 1609.
E-mail address: kwon@jbnu.ac.kr (W. Kim).
[1] Lee A.S. and Jung Y.J. contributed equally in this work.

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p65 in macrophage has been proposed to be a good avenue for the treatment of various inflammatory injury.

SIRT2, a NAD-dependent class I histone deacetylase, is linked to a variety of physiological and pathological conditions such as genomic instability [8], carcinogenesis [9], and cell cycle progression [10]. Recently, it has been demonstrated that SIRT2 is involved in bacterial infection [11], brain microglial cell activation [12] and arthritis [13]. In addition, it has been well known that macrophage has a critical role in host defense process in innate immunity. However, the role of SIRT2 in macrophages during LPS-induced inflammatory process remains to be clarified.

On the basis of above considerations, we have evaluated roles of SIRT2 in LPS-induced inflammatory responses and the molecular basis in macrophages. Our results showed that SIRT2 modulates LPS-mediated inflammatory responses through inhibiting NF-κB activation.

2. Materials and methods

2.1. Chemicals and reagents

LPS was purchased from Sigma–Aldrich (St Louis, MO), AGK2 from Tocris (Bristol, UK), AK-1 from Calbiochem (San Diego, CA). Antibodies against SIRT2 (Abcam, Cambridge, MA), iNOS (BD Transduction Laboratories, San Jose, CA), phospho-p65 (Cell Signaling Technology, Danvers, MA), p65 (EMD Millipore Corporation, Billerica, MA) and β-actin (Sigma–Aldrich) were obtained. HRP-conjugated secondary antibodies were purchased from Enzo Life Science Inc. (Farmingdale, NY). All other reagents were purchased from Sigma–Aldrich (St Louis, MO) unless otherwise indicated.

2.2. Preparation of BMDM cells

Experimental procedures were in agreement with our institutional guidelines on animal care. For the isolation of bone marrow derived macrophage (BMDM) cells, *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ male mice (aged 6 weeks, Jackson Laboratory, Bar Harbor, ME) were killed by cervical dislocation and lower limbs were removed [14]. BMDM cells were flushed from the medullary cavities of tibias and femurs with phosphate buffered saline (PBS) using a 23 G needle. The cell suspension was filtered through a cell strainer (40 μm) to remove debris, followed by centrifugation at 1500 rpm for 5 min. After centrifugation, the supernatant was removed and the cells were suspended in culture medium to give 1.0×10^6 cells/mL.

2.3. Cell culture and treatment

BMDM cells were seeded at a density of 1×10^6 cells/mL as described previously [15,16]. At 18 h after seeding, floating cells were transferred to a new dish with the complete culture media. After incubation for 2 days, cultured BMDM cells that had attached to the dish were removed using cold PBS.

Raw 264.7 macrophage cells (RAW cells; American Type Culture Collection, Manassas, VA) were grown in DMEM-high glucose supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator with 5% CO_2. To evaluate the effects of SIRT2 on iNOS mRNA expression, RAW cells were plated in 6 cm dish (5×10^5 cells/dish) and allowed to grow overnight. siRNA (100 pmoL/L, Dharmacon ON-TARGET plus SMART pool, Dharmacon Inc., CO) and 20 μL Lipofectamine™ 2000 (Invitrogen) were diluted in Opti-MEM (Invitrogen) to a total volume of 2 mL. The diluted siRNA and Lipofectamine™ 2000 were mixed and incubated to generate the transfection mixture at room temperature for 5 min. The cells were washed with Opti-MEM medium, and then the transfection mixture was added to the dish and incubated for 24 h.

2.4. MTT cell viability

The measurement of cell viability of AGK2 or AK-1 was performed using the MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [17]. Briefly, RAW cells were plated at a density of 2×10^3/well in a 96-well plate and incubated at 37 °C for 24 h. The cells were treated with AGK2 (10 and 20 μmol/L), AK-1 (10 and 20 μmol/L) or vehicle alone. After 8 h of incubation at 37 °C, 50 μL of the MTT solution was added to each well and incubated under the same conditions for another 4 h. The absorbance was measured at 490 nm using a spectrofluorometer (VersaMax, Molecular Devices, Sunnyvale, CA).

2.5. Western blotting

For the detection of target proteins, Western blotting was performed as described previously [18,19]. In brief, cells were washed with PBS and lysed with Radio-Immunoprecipitation Assay (RIPA) buffer supplemented with protease and phosphatase inhibitors (Sigma–Aldrich). The cells were sonicated for 5 min for complete cell lysis and microcentrifuged for 15 min 16,000*g*. Samples (20 μg/lane) were loaded onto 10% SDS–polyacrylamide gel and after electrophoresis, separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The PVDF membrane was incubated with 5% non-fat dry milk in TBS (20 mM Tris–HCl (pH 7.6) and 150 mM NaCl) containing 0.1% Tween 20 (v/v) (TBS/Tween) for 1 h before incubation with antibodies against phospho-p65 (dilution 1/500) or iNOS in 5% non-fat milk overnight at 4 °C. Cytoplasmic cell extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Inc., Waltham, MA) and blotted with primary antibody against phosphor-IκBα or IκBα. Anti-β-actin or p65 antibody was used as a loading control.

2.6. Immunocytochemistry of p65

Immunocytochemistry was performed as previously described [20]. BMDM cells from SIRT2 wild and knockout mice were stimulated for 6 h with LPS. After incubation, cells were fixed and further analyzed for nuclear translocation of p65 immune reactions using immune cytochemistry with a primary antibody against NFκB p65 (EMD Millipore Corporation). Stained cells were washed and examined using a laser scanning confocal microscope (Zeiss LSM 510 confocal microscope, Carl Zeiss, Gottingen, Germany). The grading system was used as previously described [20]. In brief, 0 = no immune reaction in cell nucleus; 1 = 1/3 of the cell nucleus demonstrated immune reactions; 2 = 2/3 of the cell nucleus demonstrated immune reactions; 3 = immune reaction all over the cell nucleus and the cytoplasm were visible; 4 = immune reactivity was only located in cell nucleus.

2.7. Nitrite analysis

NO synthesis was spectrophotometrically determined by assaying the culture supernatants for nitrite using Total Nitric oxide and Nitrate/Nitrite Parameter Assay Kit (R&D systems, Minneapolis, MN). Absorbance was measured at 540 nm using a spectrofluorometer (VersaMax, Molecular Devices, Sunnyvale, CA).

2.8. Detection of ROS production

RAW cells were incubated with or without AK-1 for 30 min, followed by stimulation with or without LPS for 4 h. Cells were then