SIRT1-mediated regulation of oxidative stress induced by *Pseudomonas aeruginosa* lipopolysaccharides in human alveolar epithelial cells

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Received January 10, 2016; Accepted October 19, 2016

DOI: 10.3892/mmr.2016.6045

Abstract. Sirtuin1 (SIRT1) is an NAD⁺-dependent deacetylase that exhibits multiple biological functions, including cell differentiation inhibition, transcription regulation, cell cycle regulation and anti-apoptosis. Lipopolysaccharides (LPS) are crucial virulence factors produced by Pseudomonas aeruginosa and serve an important role in adjusting the interactions between the host and the pathogen. However, the effect of SIRT1 in the regulation of LPS-induced A459 human alveolar epithelial cells (AECs) oxidative stress remains unclear. The cellular reactive oxygen species (ROS) production was examined in A549 cells that were supplemented with LPS. Relative cell signaling pathway proteins were further investigated by western blot analysis. It was identified that LPS downregulated SIRT1 expression, however, upregulated ROS generation, which was associated with the increase of nuclear factor (NF)-kB and acetyl-NF-kB. Activation of SIRT1 by resveratrol significantly reversed the effects of LPS on A549 cells. By contrast, inhibition of SIRT1 by nicotinamide had the opposite effects that enhance cell ROS production. Thus, the results indicated that SIRT1 serves an important role in the regulation of oxidative stress induced by LPS in human AECs.

Key words: NF-κB, acetyl-NF-κB, ROS, nicotinamide

Introduction

Pseudomonas aeruginosa is one of the most prevalent opportunistic gram-negative bacteria that lead to pneumonia in immunocompromised patients through the production of numerous virulence factors, including enzymes and exotoxin, such as lipopolysaccharides (LPS) (1-5). P. aeruginosa accounts for 10-20% of all hospital-acquired infection (6), 18.1% of nosocomial pneumonia (7), 9.3% of ventilator-associated pneumonia (8) and 0.9-1.9% of community-acquired pneumonia requiring hospitalization (9-11). LPS are the most important pathogenic factors produced by P. aeruginosa and serve a major role in the interplay between the host and the pathogen. Type II alveolar epithelial cells (AECs) are major target cells of LPS locating in the alveolar corners, exhibiting various important functions including initiation of immune responses, fluid balance, mucus and surfactant production, and progenitor action to maintain the normal function of the alveoli (12-14).

Sirtuin1 (SIRT1), an NAD⁺-dependent class III protein deacetylase, belongs to the silent information regulator (Sir) family (15) and is suggested to serve diverse roles in gene silencing, stress resistance, apoptosis, senescence, aging and inflammation (16-19). Previous studies have demonstrated that SIRT1 can regulate cellular oxidative stress and its toxicity (20-23). Modulation of the acetylation of the p65 subunit of nuclear factor kB (NF- κ B) in cells or tissues is the major means via which SIRT1 regulates cellular oxidative status (24).

At present, to the best of our knowledge, no reports have focused on the potential roles of SIRT1 in the regulation of oxidative stress induced by *P. aeruginosa* LPS in human AECs. In the current study, the effects of SIRT1 on the regulation of reactive oxidative stress, which is indicated by reactive oxygen species (ROS) generation, was examined in LPS-stimulated A549 cells.

Materials and methods

Cell culture and drug treatment. A549 cells were maintained as previously described (25). Briefly cells were maintained in

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Dulbecco's modified Eagle's medium (DMEM) F-12 culture medium (GE Healthcare Life Sciences, Logan, UT, USA) containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and streptomycin respectively, in 25-cm² culture flasks at 37°C in a humidified atmosphere with 5% CO₂. LPS (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was dissolved in sterilized phosphate-buffered saline (PBS). Cells were pretreated with nicotinamide (NAM) and resveratrol [Res; dissolved in dimethyl sulfoxide (DMSO); all from Sigma-Aldrich; Merck Millipore) for 1 h prior to LPS stimulation. The concentration of DMSO in the medium never exceeded 0.1% to avoid the toxicity of this solvent towards the A549 cells. Cells were divided into four groups, including a DMSO group (control), a Res group, a LPS treatment plus DMSO group (DMSO+LPS), and a LPS-treated Res group (Res+LPS).

Fluorescence staining. A549 cells were cultured on six-well chamber slides and were washed with PBS three times for 5 min per wash, then subsequently incubated with ROS Fluorescent Probe-DHE (Vigorous Biotechnology Beijing Co., Ltd., Beijing, China) in serum-free DMEM F-12 medium for 30 min at 37°C in darkness and fixed in 4% paraformaldehyde for 30 min at room temperature. The slides were washed again and mounted. The slides were finally examined using a fluorescence microscope.

Quantification of intracellular ROS. Intracellular ROS levels were quantified using ROS Fluorescent Probe-Dihydroethidium (DHE) to determine the oxidative stress towards the A549 cells in response to LPS stimulation. Following drug treatment, A549 cells were treated with dichlorofluorescin diacetate, a ROS-sensitive dye, and incubated for 30 min at 37°C in a humidified and dark atmosphere. A549 cells were harvested and suspended in PBS (0.14 M NaCl, 2.6 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄). Relative fluorescence intensities in the A549 cells were analyzed with flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Protein extraction and western blotting analysis. Following treatments, the cells were washed with ice-cold PBS three times. Proteins were extracted from the A549 cells in radioimmunoprecipitation assay buffer [1% Triton X-100, 150 mmol/l NaCl, 5 mmol/l EDTA and 10 mmol/l Tris-HCl (pH 7.0)] containing a protease inhibitor cocktail. Following sonication, cell lysates were subjected to centrifugation at 12,000 x g at 4°C for 15 min and the supernatants were collected as the total protein. Total protein (10-50 μ g/lane) was electrophoresed and separated on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA), which was then soaked in 8% non-fat milk in Tris-buffered saline with 1% Tween-20 (TBST; pH 7.6) at room temperature for 2 h to block non-specific binding sites. The membranes were then incubated at 4°C overnight with a rabbit SIRT1 polyclonal antibody (EMD Millipore; cat. no. 07-131) at a dilution of 1:3,000, a rabbit NF-KB polyclonal antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. sc-7178) at a dilution of 1:1,000 or a rabbit acetyl-NF-KB polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 3045) at a dilution of 1:1,000 on a rotating platform at 4°C. Subsequently, the membrane was rinsed in TBST (pH 7.6) 3 times and incubated with horseradish peroxidase-conjugated IgG antibodies diluted in TBST (1:5,000; Abmart, Inc., Shanghai, China; cat. no. M21003) for 2 h on a rotating platform at room temperature. Bands were visualized using a horseradish peroxidase developer, and background-subtracted signals were quantified on a laser densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A β -actin antibody (Santa Cruz Biotechnology, Inc.) was used to normalize the signal obtained for the total protein extracts. The protein bands were quantified using a PhosphorImager and ImageQuant (GE Healthcare Life Sciences) software analysis.

Statistical analysis. Data are expressed as the mean \pm standard error of the mean. Multiple comparisons were evaluated by one-way analysis of variance followed by Tukey's multiple-comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

SIRT1 expression was reduced and ROS generation was elevated by LPS in A549 cells. In order to investigate the associations between ROS and SIRT1, the effects of LPS on SIRT1 protein expression and the generation of intracellular ROS following LPS treatment were assessed. A549 cells were treated with 10 μ g/ml LPS for 12, 24 or 48 h or with 0.1, 1, or 10 μ g/ml LPS for 48 h. Western blotting indicated that when the A549 cells were treated with 10 μ g/ml LPS for 48 h, SIRT1 protein expression was reduced by 50% (Fig. 1A). LPS significantly reduced the level of SIRT1 expression in a dose-dependent manner. At doses of 1 and 10 μ g/ml, the SIRT1 protein expression was downregulated to 28 and 51% compared with the control group, respectively (Fig. 1B). In addition, the generation of intracellular ROS was investigated. The results demonstrated that LPS elevated the generation of intracellular ROS in a time-dependent manner. At the 48 h time point, the generation of ROS was increased by approximately three times.

SIR1 regulates LPS-induced A549 cell ROS generation associated with the activation of NF- κ B pathway. In order to determine the level of oxidative stress induced by LPS, cells were treated with 10 µg/ml LPS for 48 h. Fluorescence staining illustrated that A549 cells incubated with LPS had increased ROS generation (Fig. 2A). Subsequently, the molecular mechanisms involved in A549 cell oxidative stress induced by LPS were investigated. Through 48-h LPS exposure, it was identified that LPS reduced SIRT1 protein expression and promoted NF- κ B protein expression and NF- κ B protein acetylation (Fig. 2B).

A549 cell oxidative stress is attenuated by Res through the SIRT1-mediated deacetylation of NF- κ B. Res, a natural polyphenol compound widely found in grapes, pine trees, peanuts and other plants and fruits, has comprehensive biochemical and physiological functions, including anti-cancer, anti-inflammatory and anti-oxidant activities, that regulate lipid actions (26-31). Res is widely accepted to activate SIRT1, thus, is frequently adopted as a SIRT1 enhancer. In order to



Figure 1. SIRT1 protein expression was reduced by LPS in the A549 cells. A549 cells were exposed to 10 μ g/ml LPS for (A) 6, 12, 24 or 48 h or (B) 0.1, 1 or 10 μ g/ml LPS for 48 h. Western blotting illustrated that SIRT1 protein expression in the A549 cells was reduced significantly at 48 h (A), and was reduced by LPS in the A549 cells in a dose-dependent manner (B). (C) A549 cells were treated with 10 μ g/ml LPS for 6, 12, 24 and 48 h. Levels of intracellular ROS in the A549 cells were quantified using flow cytometry. ROS generation was increased by LPS in the A549 cells in a time-dependent manner. Data are presented as the mean \pm standard error, n=3 independent experiments. *P<0.05, **P<0.01 vs. control. SIRT1, sirtuin 1; LPS, lipopolysaccharides.

determine the pivotal role of SIRT1 in regulating A549 cell oxidative stress induced by LPS, the cells were divided into four groups, including a DMSO group (control), a Res group, a LPS treatment plus DMSO group (DMSO+LPS), and a LPS-treated Res group (Res+LPS). The results suggested that Res induces SIRT1 protein expression in A549 cells in a dose-dependent manner. At doses of 5 and 20 μ M, the increases in SIRT1 protein expression reached 58% and 91%, respectively (Fig. 3A). According to the results above, 20 μ M was selected as the dose used in the subsequent experiments. It was demonstrated that ROS generation was reduced by Res in the A549 cells (Fig. 3C). The effects of Res on the activation of the SIRT1 pathways were partially reversed by



Figure 2. SIRT1 and NF-κB regulate the A549 cell oxidative stress induced by LPS. (A) A549 cells were treated with 10 μ g/ml LPS for 48 h and were stained with ROS Fluorescent Probe-Dihydroethidium to indicate levels of ROS (magnification, x200). (B) Western blotting illustrated that LPS reduces SIRT1 protein expression and stimulates the level of acetylated NF-κB. Data are presented as the mean ± standard error, n=3 independent experiments. **P<0.01 vs. control. SIRT1, sirtuin 1; NF-κB, nuclear factor κB; ROS, reactive oxygen species; Con, control; LPS, lipopolysaccharides; Ace-NF-κB, acetyl-NF-κB.

LPS-stimulated downregulation of NF- κ B deacetylation in the A549 cells (Fig. 3B).

NAM aggravates LPS-induced A549 cell oxidative stress by inhibiting the SIRT1 pathway. To further determine the role of SIRT1 in regulating the human alveolar epithelial A549 cell oxidative stress induced by LPS, NAM was selected as an inhibitor of SIRT1. The results illustrated that SIRT1 protein expression in the A549 cells was reduced by NAM in a dose-dependent manner. At doses of 5 and 10 mmol/ml, the reductions in SIRT1 protein expression were 27 and 51%, respectively (Fig. 4A). The ROS generation was examined in the A549 cells, indicating that the ROS generation in the A549 cells was increased (Fig. 4C). The effects of LPS on the inhibition of the SIRT1 pathway were aggravated by NAM in the A549 cells (Fig. 4B).

Discussion

P. aeruginosa is a conditional Gram-negative bacterium leading to pneumonia in immunosuppressed patients and is colonized in the lower respiratory tract of patients with primary pulmonary diseases. In addition, ventilated patients are particularly susceptible to developing *P. aeruginosa* pneumonia (32,33). The mortality of ventilator-associated pneumonia due to *P. aeruginosa* has been observed to be significantly higher than that of other pathogens (34). LPS is one of predominant virulence factors produced by *P. aeruginosa*. The current study clarified the role of SIRT1 in regulating oxidative stress induced by *P. aeruginosa* LPS in human alveolar epithelial A549 cells.

Few studies have elucidated the association between SIRT1 and *P. aeruginosa* pneumonia-induced lung injury. The present study demonstrated SIRT1 expression had been significantly reduced in A549 cells that had been treated with



Figure 3. A549 cell oxidative stress is attenuated by Res through the SIRT1-mediated deacetylation of NF-κB. A549 cells were exposed to 0.1, 1, 5 and 20 μ M Res for 48 h. (A) Western blotting illustrated that SIRT1 protein expression was increased by Res in the A549 cells in a dose-dependent manner. (B) The effects of Res on the activation of the SIRT1 pathways were partially reversed by LPS via NF-κB deacetylation in the A549 cells. (C) Res reduces ROS generation in A549 cells. Data are presented as the mean ± standard error, n=3 independent experiments. *P<0.05, **P<0.01 vs. control. Res, resveratrol; SIRT1, sirtuin 1; NF-κB, nuclear factor κB; LPS, lipopolysaccharides; DMSO, dimethyl sulfoxide; Ace-NF-κB, acetyl-NF-κB.



SIRT1 is a NAM adenine dinucleotide (NAD+)-dependent histone deacetylase involved in multiple cellular functions (41,42). Previous studies have elucidated that SIRT1 serves an important role in oxidative stress (43-48). In



Figure 4. NAM aggravates LPS-induced A549 cell oxidative stress by inhibiting the SIRT1 pathway. A549 cells were exposed to 2.5, 5 or 10 mM NAM for 48 h. (A) Western blotting illustrated that SIRT1 protein expression was reduced in the A549 cells in a dose-dependent manner. (B) The effect of LPS on the inhibition of the SIRT1 pathways was aggravated by NAM in the A549 cells. (C) NAM increases ROS generation in A549 cells. Data are presented as the mean \pm standard error, n=3 independent experiments. *P<0.05, **P<0.01 vs. control. NAM, nicotinamide; LPS, lipopolysaccharides; SIRT1, sirtuin 1; ROS, reactive oxygen species; Con, control; DMSO, dimethyl sulfoxide.

addition, SIRT1 participates in adjusting the inflammatory response through modulation of the acetylation status of the p65 subunit of NF- κ B, which is activated by oxidative stress (24). However, it is not currently clear whether SIRT1 is inhibited or activated under oxidative stress. In the current study, SIRT1 expression was reduced during the process of LPS-induced A549 cell oxidative stress, associated with upregulation of NF- κ B and acetylation of NF- κ B. This indicates that SIRT1 serves a crucial inhibitory role during the process of A549 cell oxidative stress induced by LPS through regulating the NF- κ B signaling pathway. Therefore, reductions in SIRT1 expression induced by LPS may be associated with lung injury.

To further verify the pivotal role of SIRT1 in LPS-induced oxidative stress, a SIRT1 activator and inhibitor were used to



Figure 5. Lipopolysaccharides can increase ROS generation in A549 cells. SIRT1 serves an important role in regulating human alveolar epithelial A549 cell oxidative stress induced by lipopolysaccharides through NF- κ B deacetylation. ROS, reactive oxygen species; SIRT1, sirtuin 1; NF- κ B, nuclear factor κ B.

result in functional gain and loss. Res has been previously reported as a widely used activator of SIRT1 (49,50). The current study demonstrates that A549 cell exposure to LPS increases SIRT1 expression. In addition, levels of intracellular ROS were reduced. The current study also hypothesized that the protective effect of Res against LPS-induced A549 cell oxidative stress may be associated with SIRT1 activation and modulation of deacetylation of NF-kB; and this was verified by western blotting analysis of SIRT1, NF-KB and acetylated NF-KB. To further verify the above hypothesis, NAM was utilized as a SIRT1 inhibitor to examine the role of SIRT1 regulation in LPS-induced A549 oxidative stress. The results demonstrated that treatment of A549 cells with NAM reduced SIRT1 protein expression in the A549 cells in a dose-dependent manner. The A549 cell oxidative stress induced by LPS was aggravated by NAM, and ROS generation was increased in the A549 cells. The effects of LPS on SIRT1 pathway inhibition were aggravated by NAM in the A549 cells. Taken together, these results indicate the central and pivotal role of SIRT1 in LPS-induced oxidative stress in A549 cells via modulation of the NF-kB pathway, which maybe a potential translational target for treating P. aeruginosa pneumonia.

In conclusion, the results of the current study suggest that A549 cell oxidative stress is induced by LPS. SIRT1 serves a central role in the oxidative stress induced by LPS through inducing NF- κ B deacetylation. Res defends against LPS-induced A549 cell oxidative stress via activation of SIRT1 and NAM aggravates the A549 cell oxidative stress induced by LPS. SIRT1 activators are suggested as a promising therapeutic interventional target for *P. aeruginosa* infections.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81270495).

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