Original Paper

SIRT1 Regulates the Human Alveolar Epithelial A549 Cell Apoptosis Induced by *Pseudomonas Aeruginosa* Lipopolysaccharide

Xiaoli Liu^{a,b} Kuiqing Shao^c Tieying Sun^{b,a}

^aGraduate School of Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing;

^bBeijing Hospital Ministry of health of respiratory and critical care department, Beijing

^cDepartment of Urinary Surgery, Xiyuan Hospital of China Academy of Chinese Medical Sciences, Beijing

Key Words

LPS • SIRT1 • Apoptosis • ROS

Abstract

Background: Sirtuin1 (SIRT1) is an NAD⁺-dependent deacetylase that plays an inhibitory role in cell apoptosis, which is associated with p53 deacetylation. Lipopolysaccharide (LPS) is a key virulence factor produced by *Pseudomonas aeruginosa* and plays an important role in mediating the interactions between the bacterium and its host. However, the effect of SIRT1 in the regulation of LPS-induced human alveolar epithelial A549 cells apoptosis is unknown.

Methods: Cell viability, apoptosis and reactive oxygen species (ROS) production were first examined in A549 cells that were treated with LPS. Relative cell signaling pathways were further explored by western blot analysis. **Results:** Exposure of A549 cells to LPS decreased cell viability in a concentration- and time- dependent manner. LPS stimulated cell apoptosis and ROS production while inhibiting the expression of SIRT1 in A549 cells. Activation of SIRT1 by exposure to resveratrol significantly reversed the effects of LPS on A549 cells. In contrast, inhibition of SIRT1 by nicotinamide had the opposite effects enhancing cell apoptosis and ROS production. **Conclusion:** SIRT1 plays an important role in regulating the human alveolar epithelial A549 cell apoptosis process induced by LPS.

Copyright © 2013 S. Karger AG, Basel

Tieying Sun

Beijing Hospital Ministry of health of respiratory and critical care department
No.1, Dahua Road, Dong Dan, Beijing 100730 (China)
E-Mail suntieying3@hotmail.com

Introduction

Pseudomonas aeruginosa, an increasingly prevalent opportunistic and virulent pathogen, is the most common Gram-negative bacterium and produces many virulence factors, including exotoxins (LPS) and enzymes, involved in infectious diseases. [1]. *P. aeruginosa* accounts for 18.1% of nosocomial pneumonia [2], 9.3% of ventilator-associated pneumonia [3] and 0.9% to 1.9% of the community-acquired pneumonia requiring hospitalization [4-6]. Lipopolysaccharide (LPS) is a key factor in the virulence produced by *P. aeruginosa* and plays an important role in mediating the interactions between the bacterium and its host. Pulmonary alveolar type II epithelial cells, located in the corner of the alveoli, possess many types of actions including metabolic, secretory, progenitor, and immunologic functions, to maintain the normal function of alveoli. [7-8].

Apoptosis, a type of programmed cell death, is triggered by either intrinsic or extrinsic factors [9-10]. The extrinsic pathway is related to the stimulation of the transmembrane death receptors, including Fas, TNF-R1, and Apo2/Apo3, while the intrinsic pathway is regulated by signaling factors released from the mitochondria. Blocking or delaying cell death is a major mechanism, by which the pathogens promote intracellular survival and replication of the bacterium [11-12]. LPS can result in the apoptosis of human alveolar epithelial A549 cells [13]. ROS, an apoptotic factors can induce oxidative stress, which can result in cell apoptosis [14-15].

Sirtuin1 (SIRT1) is an NAD⁺-dependent class III protein deacetylase and belongs to the silent information regulator (Sir2) family [16]. SIRT1 plays important roles in regulating cell apoptosis or cell survival, endocrine signaling, cell differentiation, metabolism, caloric restriction (CR) and chromatin remodeling [17-18]. SIRT1 is considered a type of apoptosis inhibitor because of its action in deacetylating p53 [19], Ku70 [20], the forkhead transcription factor [21-22] and NF- κ B [23].

However, there has been no report about the possible roles of SIRT1 in the regulation of the human alveolar epithelial A549 cell apoptosis induced by *P. aeruginosa* LPS. In this study, we examined the effect of SIRT1 on the regulation of LPS-induced A549 cell apoptosis.

Materials and Methods

Cell culture and treatment

A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM) F-12 culture medium (Hyclone) supplemented with 10% heat-inactivated fetal calf serum, 100U/ml penicillin, and streptomycin in 25-cm² culture flask at 37°C in a humidified atmosphere with 5% CO₂. LPS (Sigma) was dissolved in PBS. Nicotinamide (NAM), N-acetyl-L-cysteine (NAC) and resveratrol (dissolved in dimethyl sulfoxide, DMSO) (all from Sigma) were pretreated for 1 h before LPS treatment. The concentration of DMSO in the medium never exceeded 0.1% to avoid the toxicity of this solvent to the A549 cells.

Assay of cell viability

Cell viability was determined using a colorimetric, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. Briefly, A549 cells (5×10⁴ cells per well) were seeded in 96-well tissue culture plates. At 70–80% confluence, the cells were incubated for 16 h in serum-free DMEM F-12 medium. After drug treatment, the A549 cells were cultured in fresh medium containing 0.5 mg/ml MTT for a further 4 h. The blue formazan products in the A549 cells were dissolved in DMSO and spectrophotometrically measured at a wavelength of 550 nm.

Quantification of intracellular ROS

Intracellular ROS levels were quantified to determine the oxidative stress to the A549 cells in response to LPS stimulation. A549 cells (1×10⁵ cells per well) were cultured in 6-well tissue culture plates. At 70–80% confluence, the cells were incubated for 16 h in serum-free DMEM F-12 medium. After LPS treatment, the cells were treated with dichlorofluorescein diacetate, a ROS-sensitive dye, and incubated for forty minutes

at 37°C in a humidified and dark atmosphere. A549 cells were harvested and suspended in 1× phosphate-buffered saline (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 quantified using flow cytometry (Becton–Dickinson).

Quantification of apoptotic cells

The proportion of apoptotic A549 cells was measured using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences, USA) according to the manufacturer's protocol. Briefly, A549 cells (1×10⁵ cells per well) were cultured in six-well tissue culture plates. At 70–80% confluence, the cells were incubated for 16 h in serum-free DMEM F-12 medium. After drug treatment, the cells were harvested into a 5 ml culture tube. The cells were washed twice with cold PBS (0.14 M NaCl, 2.6 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄) and then resuspended in 1×binding buffer at a concentration of 1×10⁶ cells/ml. The solution (100 µl, 1×10⁵ cells) was transferred to a 5ml culture tube. FITC-AnnexinV (5 µl) and propidium iodide (5 µl) were added and the cells were gently vortexed and incubated at room temperature for 15 min in the dark. Then 400 µl of 1×binding buffer was added to each tube and the samples were analyzed using flow cytometry within 1 hr.

Hoechst 33258 staining

A549 cells (1×10⁵ cells per well) were cultured in six-well tissue culture plates and at 70–80% confluence, the cells were incubated for 16 h in serum-free DMEM F-12 medium. After drug treatment, the medium was removed, and the cells were rinsed once with cold PBS and then fixed with 4% formaldehyde in PBS for 15 min (37°C). The cells were washed three times with PBS, and the nuclei were then stained with Hoechst 33258 (10 µg/ml) for 5 min before being washed three times with PBS and dried.

Protein preparation and western blot

Proteins were extracted from the A549 cells in RIPA buffer (1% TritonX-100, 150 mmol/L NaCl, 5 mmol/L EDTA, and 10mmol/L Tris-HCl (pH 7.0)) supplemented with a protease inhibitor cocktail and subjected to centrifugation at 12,000 g for 20 min. Total protein (10–50 µg per lane) was electrophoresed and separated on a 10% SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), which was soaked in 8% milk in Tris-buffered saline Tween (TBST, pH 7.6). The membrane was incubated overnight with a rabbit SIRT1 polyclonal antibody (Millipore, Billerica, MA, USA) at a dilution of 1:3000, a rabbit p53 polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:1000, a rabbit Bax polyclonal antibody (Cell Signaling Technology) at a dilution of 1:1000 or a rabbit Bcl-2 polyclonal antibody (Cell Signaling Technology) on a rotating platform at 4°C. Subsequently, the membrane was rinsed in TBST (pH 7.6) and incubated with horseradish peroxidase-conjugated IgG antibodies diluted in TBST (1:5000) for 2 h on a rotating platform at 37°C. Bands were visualized using a horseradish peroxidase developer, and background-subtracted signals were quantified on a laser densitometer (Bio-Rad, Hercules, CA, USA). A β-actin antibody (Santa Cruz Biotechnology) was used to normalize the signal obtained for the total protein extracts. The protein bands were quantified using a PhosphorImager and ImageQuant (Amersham Biosciences) software analysis.

Immunofluorescence

A549 cells were cultured on 6-well chamber slides and fixed with 4% formaldehyde for 10 minutes at -20°C. All slides were washed in PBS three times for 5 minutes per wash. The slides were incubated with 3% BSA (prepared in PBS) for 10 minutes and washed in PBS three times again for 5 minutes per wash. The slides were then incubated with a polyclonal antibody against SIRT1 (1:100 diluted in PBS with 1% BSA) overnight at 4°C. After three washes in PBS, the slides were incubated with FITC-conjugated anti-rabbit IgG (1:100 diluted in PBS with 1% BSA) for 60 minutes at room temperature. After three washes in PBS, the slides were incubated with Hoechst 33258 (10 µg/ml) for 5 min. The slides were washed again and dried before the mounting agent was added and the coverslips were placed on top. The slides were finally examined using a fluorescence microscope.

Statistical analysis

Data are expressed as the means ± SE. Multiple comparisons were evaluated by ANOVA followed by Turkey's multiple-comparison procedure with P<0.05 being considered significant.

Fig. 1. A549 cell viability is affected by LPS in a dose- and time-dependent manner. A549 cells were exposed to 0.1, 1, or 10 $\mu\text{g}/\text{ml}$ LPS for 48 h (A) or to 10 $\mu\text{g}/\text{ml}$ LPS for 12, 24, or 48 h (B). Cell viability was measured using a spectrophotometer at

550 nm. Exposing A549 cells to LPS can affect cell viability in a dose- and time-dependent manner. Data represent the means $\pm$ SEM, $n=6$ independent experiments. * $p<0.05$, ** $p<0.01$ versus control.

Results

A549 cell viability was affected by LPS in a dose- and time-dependent manner

A549 cells were exposed to 0.1, 1, and 10 $\mu\text{g}/\text{ml}$ LPS for 48 h or to 10 $\mu\text{g}/\text{ml}$ LPS for 12, 24, and 48 h. A549 cell viability was decreased when the cells were treated with 10 $\mu\text{g}/\text{ml}$ LPS for 48 h (Fig. 1A) or 10 $\mu\text{g}/\text{ml}$ LPS for 24 h and 48 h (Fig. 1B). LPS at a concentration of 0.1 or 1 $\mu\text{g}/\text{ml}$ did not impact cell viability while, at a concentration of 10 $\mu\text{g}/\text{ml}$, cell viability decreased by 15%. In addition, the study showed that LPS at a concentration of 10 $\mu\text{g}/\text{ml}$ decreased cell viability by 10% and 15% at 24 h and 48 h, respectively.

SIRT1 was detected in the nuclei of A549 cells, and SIRT1 expression in A549 cells was decreased by LPS

SIRT1 is a type of nucleoprotein that is widely expressed in tissues. In our study, we detected SIRT1 in the nuclei of A549 cells using immunofluorescence (Fig. 2C). To estimate the effects of LPS on SIRT1 protein expression, A549 cells were treated with 0.1, 1, or 10 $\mu\text{g}/\text{ml}$ LPS for 48 h or with 10 $\mu\text{g}/\text{ml}$ LPS for 12, 24, or 48 h. Western blot analysis showed that LPS treatment significantly reduced the level of SIRT1 expression in a dose-dependent manner (Fig. 2A). At doses of 1 and 10 $\mu\text{g}/\text{ml}$, the decreases in SIRT1 protein expression were 39% and 52%, respectively. When the A549 cells were treated with 10 $\mu\text{g}/\text{ml}$ LPS for 48 h, SIRT1 protein expression was decreased by 53% (Fig. 2B).

SIRT1, p53, and Bcl-2 regulate the A549 cell apoptosis induced by LPS

Apoptosis of A549 cells was investigated in this study. Using an Annexin V and PI kit, the rate of cell apoptosis was determined to be significantly increased in the A549 cells after LPS exposure (Fig. 3A). Cell apoptosis was also detected using Hoechst 33342 staining. The results show apoptotic cells in the LPS-induced A549 cells (Fig. 3B), which suggests that the LPS induced A549 cell apoptosis. Moreover, we found that LPS elevated ROS generation in the A549 cells in a time-dependent manner (Fig. 3C). We then investigated the molecular mechanisms involved in the A549 cell apoptosis induced by LPS. Through a 48-h LPS exposure, we found that LPS decreased SIRT1 protein expression and stimulated p53 protein expression and p53 protein acetylation. Bcl2 is also a key factor that regulates apoptosis, and a decreased level of Bcl2 was found in the A549 cells treated with LPS (Fig. 3D).

A549 cells apoptosis was attenuated by Res

Resveratrol (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. To determine whether Res influenced LPS-induced A549 cell apoptosis, the cells were divided into four groups, including a DMSO group (control), a Res group, a LPS

Fig. 2. SIRT1 is detected in the nuclei of A549 cells, and SIRT1 expression is decreased by LPS in A549 cells. A549 cells were treated with 0.1, 1, or 10 $\mu\text{g/ml}$ LPS for 48 h (A) or 10 $\mu\text{g/ml}$ LPS for 6, 12, 24, or 48 h (B). Western blot illustrating that SIRT1 protein expression was decreased by LPS in the A549 cells in a dose-dependent manner (A). SIRT1 protein expression in the A549 cells decreased significantly by 48 h (B). Immunofluorescence shows that SIRT1 protein is detectable in the A549 cell nuclei. Data represent the means $\pm$ SEM, $n=3$ independent experiments. * $p<0.05$, ** $p<0.01$ versus control.

Fig. 3. SIRT1, p53, and Bcl-2 mediate the A549 cell apoptosis induced by LPS. A549 cells were treated with 10 $\mu\text{g/ml}$ LPS for 48 h. The apoptosis of the A549 cells induced by LPS was examined using an Annexin V and PI kit (A) and as illustrated by PI and Hoechst 33258 double staining (B). A549 cells were treated with 10 $\mu\text{g/ml}$ LPS for 12, 24, or 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 (C). Western blotting illustrating that LPS decreases SIRT1 protein expression, stimulates the level of acetylated p53 and decreases the level of BCL2 (D). Data represent the means $\pm$ SEM, $n=3$ independent experiments. * $p<0.05$, ** $p<0.01$ versus control.

treating plus DMSO group (DMSO+LPS), and a LPS-supplemented Res group (Res+LPS). First, A549 cell viability was examined when the cells were treated with Res (20 μM). The results

Fig. 4. A549 cell apoptosis is attenuated by Res through the SIRT1-mediated deacetylation of p53. A549 cells were exposed to 20 μ M Res for 48 h. Cell viability was measured using a spectrophotometer at 550 nm. Exposure of A549 cells to Res does not affect cell viability (A). Western blot illustrating that SIRT1 protein expression was increased by Res in the A549 cells (B). Using an Annexin V and PI kit, we demonstrated that Res alleviates LPS-induced A549 cell apoptosis (C). Res decreases ROS generation in A549 cells (D). The effects of Res on the activation of the SIRT1 pathways were rescued by mediating the p53 deacetylation in the A549 cells (E). Data represent the means $\pm$ SEM, n=3 independent experiments. *p<0.05, **p<0.01 versus control.

suggested that exposing A549 cells to Res did not affect cell viability (Fig. 4A). Our findings demonstrate that Res induces SIRT1 protein overexpression in A549 cells (Fig. 4B). We next investigated the role of Res in the apoptosis induced by LPS in A549 cells. Using Annexin V and PI kit, the data show that the A549 cell apoptosis induced by LPS was alleviated by Res (Fig. 4C) and that ROS generation was decreased by Res in the A549 cells (Fig. 4D). The effects of Res on the activation of the SIRT1 pathways were rescued by mediating p53 deacetylation in the A549 cells (Fig. 4E).

The A549 cell apoptosis induced by LPS was aggravated by NAM

To further determine the role of SIRT1 in regulating the human alveolar epithelial A549 cell apoptosis induced by LPS, we chose NAM as an inhibitor of SIRT1. Our findings demonstrate that SIRT1 protein expression in the A549 cells is decreased by NAM in a dose-dependent manner (Fig. 5A). Using Annexin V and PI kit, it was demonstrated that A549 cell apoptosis is aggravated by NAM (Fig. 5B), while ROS generation is increased (Fig. 5D). The effects of LPS on inhibiting SIRT1 pathways were aggravated by NAM in the A549 cells (Fig. 5C).

In response to NAC, ROS generation is decreased in A549 cells, and A549 cell apoptosis was alleviated

The A549 cells were pretreated with 5mM NAC (an antioxidant) for 1 h before LPS treatment. The data indicate that ROS generation was decreased in the A549 cells (Fig. 6B) and that cell apoptosis was alleviated by NAC (Fig. 6C). In addition, western blotting demonstrated that NAC restored SIRT1 expression during LPS treatment (Fig. 6A).

Fig. 5. NAM aggravates LPS-induced A549 cell apoptosis by inhibiting the SIRT1 pathway. A549 cells were treated with 2.5, 5, or 10 mM NAM for 48 h. Western blot illustrating that SIRT1 protein expression was decreased in the A549 cells in a dose-dependent manner (A). Using Annexin V and PI kit, it was demonstrated that the A549 cell apoptosis induced by LPS is aggravated by NAM (B). NAM increases ROS generation in A549 cells (D). The effect of LPS on the inhibition of the SIRT1 pathways is aggravated by NAM in the A549 cells (C). Data represent the means $\pm$ SEM, $n=3$ independent experiments. * $p<0.05$, ** $p<0.01$ versus control.

Fig. 6. NAC can decrease ROS generation and alleviate LPS-induced A549 cell apoptosis. A549 cells were pretreated with 5 mM NAC for 1 h before LPS treatment. The data illustrate that NAC decreases ROS generation in the A549 cells (B) and alleviates LPS-induced A549 cells apoptosis (C). The western blotting shows that the SIRT1 pathway is remitted by NAC (A). Data represent the means $\pm$ SEM, $n=3$ independent experiments. * $p<0.05$, ** $p<0.01$ versus control.

Discussion

Pseudomonas aeruginosa is one of major pathogens of hospital-acquired pneumonia which often occurs in the elderly and immunocompromised patients. LPS is one of the

Fig. 7. Summary. LPS can induce A549 cell apoptosis and increase ROS generation in A549 cells. SIRT1 plays an important role in regulating the human alveolar epithelial A549 cell apoptosis process induced by LPS through p53 deacetylation.

virulence traits of *P. aeruginosa*. This study elucidated the role of SIRT1 in regulating of *P. aeruginosa*'s LPS-induced human alveolar epithelial A549 cell apoptosis.

The results show that LPS influences A549 cell viability in a dose- and time-dependent manner. It is possible that LPS has a deleterious effect on human alveolar epithelial cell viability, which may be associated with lung injury.

No studies of the relationship between SIRT1 and *P. aeruginosa* pneumonia-induced lung injury have been reported. The present study demonstrated that treatment with LPS significantly reduces SIRT1 expression in A549 cells in a dose-dependent manner. Moreover, SIRT1 expression decreased significantly by 48 h.

This study's findings suggest that LPS can induce A549 cell apoptosis, which is consistent with previous studies [13, 24-26]. Therefore, we hypothesize that the possible mechanisms of *P. aeruginosa*-induced lung damage are related to the alveolar type II epithelial cells apoptosis induced by the LPS. ROS is a major apoptotic factor that can induce cell apoptosis. Previous studies have reported that LPS facilitates the generation of reactive oxygen species in lungs, resulting in lung damage [27-28]. Our study also indicates that exposing of A549 cells to LPS increases intracellular ROS generation in a time-dependent manner. NAC is a well-known antioxidant [29], and pre-treating A549 cells with 5mM NAC for 1 h before LPS exposure resulted in a significant decreased in ROS generation and apoptosis induced by LPS in A549 cells. In addition, western blotting indicated that the SIRT1 pathway was activated after NAC pre-treatment. ROS are crucial apoptotic factors that can cause oxidative stress and subsequent cell apoptosis [14-15]. Thus, augmentation of intracellular ROS might be one of the major factors contributing to the LPS-induced apoptosis in the A549 cells.

SIRT1 is involved in many cellular functions, such as regulating lipid metabolism, controlling cell survival, stress resistance, apoptosis, gene silencing, and energy homeostasis [30-31]. P53, a well-known gene that controls cell proliferation and apoptosis, is known as a Sirtuin substrate. SIRT1 can deacetylate p53 and ameliorate its ability to activate its downstream target genes, such as Bax, for apoptosis [19, 32]. Our findings demonstrated that SIRT1 expression decreased during the process of LPS-induced A549 cell apoptosis, but that p53 and acetylated p53 expression increased, indicating that SIRT1 plays a crucial inhibitory role during the process of A549 cell apoptosis induced by LPS.

SIRT1 can inhibit apoptosis in various cells through p53 deacetylation [33-35]. Therefore decrease in SIRT1 expression induced by LPS might be associated with lung injury. Resveratrol is a well-known activator of SIRT1. Our study demonstrates that treating A549 cells with Res does not affect A549 cell viability but increases SIRT1 expression. In addition, cell apoptosis is reversed, and levels of intracellular ROS are decreased. We hypothesize that the protective effect of Res against LPS-induced A549 cell apoptosis might be related to SIRT1 activation and to the subsequent deacetylation of p53. Our data show that LPS exposure dramatically increased p53 acetylation in A549 cells but that this effect is attenuated by a combined treatment with LPS and Res. These results indicate that Res protected the A549 cells from LPS-induced apoptosis through SIRT1-modulated p53-mediated apoptosis. To further test the above hypothesis, we also utilized NAM as a SIRT1 inhibitor to examine

the role of SIRT1 regulation in LPS-induced A549 cell apoptosis. The results demonstrate that treating A549 cells with NAM decreased SIRT1 protein expression in the A549 cells in a dose-dependent manner. The A549 cell apoptosis induced by LPS was aggravated by NAM, and ROS generation increased in the A549 cells. The effects of LPS on SIRT1 pathways inhibition were aggravated by NAM in the A549 cells.

In summary, our findings suggest that A549 cells apoptosis is induced by LPS. SIRT1 plays a crucial role in the apoptosis induced by LPS through p53 deacetylation. Res protects against LPS-induced A549 cell apoptosis by activating of SIRT1. However NAM aggravates the A549 cell apoptosis induced by LPS. Furthermore, pre-treating the A549 cells with NAC decreased ROS generation, alleviated the LPS-induced A549 cell apoptosis and activated the SIRT1 pathway. SIRT1 activators or antioxidants could be a promising therapeutic intervention for *P. aeruginosa* infections.

Acknowledgements

We would like to thank the Beijing Hospital Ministry of Health Institute of Geriatric Medicine Biochemical Laboratory for providing us with excellent facilities for our experimental studies. This work was supported by grants from projects supported by the National Natural Science Foundation of China (30872719).

References

- 1 Sadikot RT, Blackwell TS, Christman JW, Prince AS: Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med* 2005;171:1209-1223.
- 2 Gaynes R, Edwards JR, National Nosocomial Infections Surveillance System: Overview of nosocomial infections caused by gram-negative bacilli. *Clin Infect Dis* 2005;41:848-854.
- 3 Rello J, Ollendorf DA, Oster G, Vera-Llonch M, Bellm L, Redman R, Kollef MH, VAP Outcomes Scientific Advisory Group: Epidemiology and outcomes of ventilator-associated pneumonia in a large US database. *Chest* 2002;122:2115-2121.
- 4 Fang GD, Fine M, Orloff J, Arisumi D, Yu VL, Kapoor W, Grayston JT, Wang SP, Kohler R, Muder RR: New and emerging etiologies for community-acquired pneumonia with implications for therapy. A prospective multicenter study of 359 cases. *Medicine (Baltimore)* 1990;69:307-316.
- 5 Blanquer J, Blanquer R, Borrás R, Nauffal D, Morales P, Menéndez R, Subías I, Herrero L, Redón J, Pascual J: Aetiology of community acquired pneumonia in Valencia, Spain: a multicentre prospective study. *Thorax* 1991;46:508-511.
- 6 Neill AM, Martin IR, Weir R, Anderson R, Chereshsky A, Epton MJ, Jackson R, Schousboe M, Frampton C, Hutton S, Chambers ST: Town GI Community acquired pneumonia: aetiology and usefulness of severity criteria on admission. *Thorax* 1996;51:1010-1016.
- 7 Crapo JD, Barry BE, Gehr P, Bachofen M, Weibel ER: Cell number and cell characteristics of the normal human lung. *Am Rev Respir Dis* 1982;126:332-337.
- 8 Herzog EL, Brody AR, Colby TV, Mason R, Williams MC: Knowns and unknowns of the alveolus. *Proc Am Thorac Soc* 2008;5:778-782.
- 9 Chen TG, Chen TL, Chang HC, Tai YT, Cherng YG, Chang YT, Chen RM: Oxidized low-density lipoprotein induces apoptotic insults to mouse cerebral endothelial cells via a Bax-mitochondria-caspase protease pathway. *Toxicol Appl Pharmacol* 2007;219:42-53.
- 10 Ferrari R, Ceconi C, Campo G, Cangiari E, Cavazza C, Secchiero P, Tavazzi L: Mechanisms of remodelling: a question of life (stem cell production) and death (myocyte apoptosis). *Circ J* 2009;73:1973-1982.
- 11 Arnoult D, Carneiro L, Tattoli I, Girardin SE: The role of mitochondria in cellular defense against microbial infection. *Semin Immunol* 2009;21:223-232.
- 12 Rudel T, Kepp O, Kozjak-Pavlovic V: Interactions between bacterial pathogens and mitochondrial cell death pathways. *Nat Rev Microbiol* 2010;8:693-705.
- 13 Chuang CY, Chen TL, Cherng YG, Tai YT, Chen TG, Chen RM: Lipopolysaccharide induces apoptotic insults to human alveolar epithelial A549 cells through reactive oxygen species-mediated activation of an intrinsic mitochondrion-dependent pathway. *Arch Toxicol* 2011;85:209-218.

- 14 Tai YT, Chergn YG, Chang CC, Hwang YP, Chen JT, Chen RM: Pretreatment with low nitric oxide protects osteoblasts from high nitric oxide-induced apoptotic insults through regulation of c-Jun N-terminal kinase/c-Jun-mediated Bcl-2 gene expression and protein translocation. *J Orthop Res* 2007;25:625-635.
- 15 Pellegrini M, Baldari CT, Baldari: Apoptosis and oxidative stress-related diseases: the p66Shc connection. *Curr Mol Med* 2009;9:392-398.
- 16 Imai S, Armstrong CM, Kaerberlein M, Guarente L: Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 2000;403:795-800.
- 17 Yang T, Fu M, Pestell R, Sauve AA: SIRT1 and endocrine signaling. *Trends Endocrinol Metab* 2006;17:186-191.
- 18 Michan S, Sinclair D: Sinclair, Sirtuins in mammals: insights into their biological function. *Biochem J* 2007;404:1-13.
- 19 Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L, Gu W: Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* 2001;107:137-148.
- 20 Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA: Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 2004;305:390-392.
- 21 Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME: Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004;303:2011-2015.
- 22 Motta MC, Divecha N, Lemieux M, Kamel C, Chen D, Gu W, Bultsma Y, McBurney M, Guarente L: Mammalian SIRT1 represses forkhead transcription factors. *Cell* 2004;116:551-563.
- 23 Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW: Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J* 2004;23:2369-2380.
- 24 Fujita M, Kuwano K, Kunitake R, Hagimoto N, Miyazaki H, Kaneko Y, Kawasaki M, Maeyama T, Hara N: Endothelial cell apoptosis in lipopolysaccharide-induced lung injury in mice. *Int Arch Allergy Immunol* 1998;117:202-208.
- 25 Ma X, Xu D, Ai Y, Ming G, Zhao S: Fas inhibition attenuates lipopolysaccharide-induced apoptosis and cytokine release of rat type II alveolar epithelial cells. *Mol Biol Rep* 2010;37:3051-3056.
- 26 Song Y, Shi Y, Harken AH, Meng X: Induction of type II alveolar epithelial cells apoptosis in mouse by lipopolysaccharide does not require TNF-alpha. *Chin Med J (Engl)* 2003;116:625-629.
- 27 Chian CF, Chiang CH, Yuan-Jung C, Chuang CH, Liu SL, Yi-Han J, Zhang H, Ryu JH: Apocynin Attenuates Lipopolysaccharide-Induced Lung Injury in an Isolated and Perfused Rat Lung Model. *Shock* 2012;38:196-202.
- 28 Boots AW, Gerloff K, Bartholomé R, van Berlo D, Ledermann K, Haenen GR, Bast A, van Schooten FJ, Albrecht C, Schins RP: Neutrophils augment LPS-mediated pro-inflammatory signaling in human lung epithelial cells. *Biochim Biophys Acta* 2012;1823:1151-1162.
- 29 Chen RM, Chou MW, Ueng TH: Induction of cytochrome P450 1A1 in human hepatoma HepG2 cells by 6-nitrochrysene. *Toxicol Lett* 2000;117:69-77.
- 30 Yamamoto H, Schoonjans K, Auwerx J: Auwerx, Sirtuin functions in health and disease. *Mol Endocrinol* 2007;21:1745-1755.
- 31 Kong XX, Wang R, Liu XJ, Zhu LL, Shao D, Chang YS, Fang FD: Function of SIRT1 in physiology. *Biochemistry (Mosc)* 2009;74:703-708.
- 32 Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA: hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 2001;107:149-159.
- 33 Gu J, Song ZP, Gui DM, Hu W, Chen YG, Zhang DD: Resveratrol Attenuates Doxorubicin-Induced Cardiomyocyte Apoptosis in Lymphoma Nude Mice by Heme Oxygenase-1 Induction. *Cardiovasc Toxicol* 2012;12:341-349.
- 34 Kim DH, Jung YJ, Lee JE, Lee AS, Kang KP, Lee S, Park SK, Han MK, Lee SY, Ramkumar KM, Sung MJ, Kim W: SIRT1 activation by resveratrol ameliorates cisplatin-induced renal injury through deacetylation of p53. *Am J Physiol Renal Physiol* 2011;301:F427-435.
- 35 Lee JH, Song MY, Song EK, Kim EK, Moon WS, Han MK, Park JW, Kwon KB, Park BH: Overexpression of SIRT1 protects pancreatic beta-cells against cytokine toxicity by suppressing the nuclear factor-kappaB signaling pathway. *Diabetes* 2009;58:344-351.