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SO_2 inhalation modulates the expression of pro-inflammatory and pro-apoptotic genes in rat heart and lung

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ABSTRACT

SO_2 is a common air pollutant, and human exposure to SO_2 has become increasingly widespread due to the combustion of fossil fuels. The epidemiological studies have linked SO_2 exposure not only with many respiratory responses, but also with cardiovascular diseases. Also, its possible toxicity has been implicated by determining oxidative stress, DNA damage and membrane channel alteration in rat heart and lung. However, its detailed mechanisms remain unclear. In the present study, rats were treated with 7, 14 and 28 mg/m^3 SO_2 for 6 h/day for 7 days, and the mRNA levels of TNF-α, IL-1β, iNOS, ICAM-1, Bax and Bcl-2 and subsequent insults were determined in the heart and lung. The results indicate that SO_2 inhalation markedly elevated TNF-α and IL-1β mRNA levels and secretions, enhanced iNOS and ICAM-1 mRNA levels and the ratio of Bax/Bcl-2 in a concentration-dependent manner, and induced occurrence of apoptosis. This suggests that SO_2 inhalation induced an inflammatory response and subsequent insults via modulating pro-inflammatory and pro-apoptotic genes in the heart and lung, which contributed to the increased risk of respiratory and cardiovascular diseases.

1. Introduction

As a ubiquitous air pollutant, gaseous SO_2 releases into the atmosphere from the combustion and processing of sulfur-containing fossil fuels. The long-term effect of exposure to low-concentration SO_2 presents in the community air is similar with the short-term effect of exposure to high-concentration SO_2 in many industrial settings and chemical industries. In the past decade, accumulating epidemiological studies suggest that SO_2 pollution in atmospheric environment was associated with the risk and mortality due to respiratory tract diseases, lung cancer and cardiovascular diseases [1–3]. It is reported that SO_2 was a systemic toxic agent, and caused DNA damage and oxidative damage in various organs of mice and rats, especially in the lung and heart [4]. SO_2 inhalation thickened the mucous layer of the respiratory tract, affected the blood pressure of rats [5], and its derivatives enhanced sodium and L-type calcium currents in rat ventricular myocytes and increased intracellular Ca^{2+} [6]. The information implicates that SO_2 inhalation might contribute to the increased risk of respiratory and cardiovascular disease. However, its molecular mechanisms remain elusive.

Inflammation and subsequent apoptosis are now recognized as common mechanisms of various diseases. Inflammation is initiated by production and release of pro-inflammatory cytokines. The typical pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), and interleukin 1β (IL-1β), form an important part of the inflammatory response. TNF-α enhances the permeability of endothelial cells where it also induces expression of adhesion molecules and prothrombotic activities [7]. IL-1β is produced in peripheral tissue and increases the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes to sites of infection. These cytokines initiate immune responses and activate other pro-inflammatory genes expression such as intercellular adhesion molecule 1 (ICAM-1) and inducible nitric oxide synthase (iNOS), which play a key role in regulating leukocyte recruitment [8]. Expression and/or activation of ICAM-1 contribute to the progress of leukocytes recruiting to sites of inflammation [9]. Moreover, expression of iNOS may exacerbate organ inflammatory injury via mediating NO release, further increase circulating leukocytes to adhere to the endothelium, eventually contribute to both necrosis and apoptosis [10].

Apoptosis, a type of cell death that represents the culmination of naturally occurring, is also known as a protective reaction to multiple types of cellular damage such as inflammation. Among the occurrence of apoptosis, more attention is paid to the activation of the mitochondrial pathway, which is thought to be the primary apoptotic pathway associated with oxidative stress in the heart and lung [11,12]. The mitochondrial death pathway is controlled by members of the Bcl-2 family, particularly, pro-apoptotic Bax and anti-apoptotic Bcl-2. The ability of Bax to promote apoptosis is critically dependent on the ratio of Bax to Bcl-2. When Bax is in excess and predominated, Bax homodimers are formed, Bax and

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Bcl-2 translocate to the mitochondrial and initiate mitochondrial dysfunction leading to apoptosis [13].

However, limited data are available on the mechanisms for SO_2 exposure-induced inflammatory responses and the occurrence of apoptosis in rat heart and lung. In this study, we treated rats with 7, 14 and 28 mg/m^3 SO_2 for 6 h/day for 7 days, and determined the expression of pro-inflammatory genes (TNF-α, IL-1β, iNOS and ICAM-1) and pro-apoptotic genes (Bax and Bcl-2), the secretion levels of TNF-α and IL-1β, and pathological characteristics of apoptosis in rat heart and lung.

2. Materials and methods

2.1. Animals and treatment protocols

Male Wistar rats (weighing 260–290 g), supplied by Center of Experimental Animal of Hebei Province were divided randomly into four equal groups, each containing six rats. The rats were housed in metallic cages under standard conditions. Three groups were exposed to 7, 14 and 28 mg/m^3 SO_2 in 1 m^3 exposure chambers for 6 h/day for 7 days, respectively; while the control group was exposed to filtered air in another 1 m^3 chamber using the same schedule [4]. The SO_2 concentrations were measured every 60 min by pararosaniline hydrochloride spectrophotometry [14].

Rats were killed by decapitation, 18 h after the last exposure. The hippocampus was immediately separated after the brain was removed, and stored in prelabeled freezing tubes for being quickly frozen in liquid nitrogen and following stored at −80 °C. The study involving experimental animals was conducted in accordance with national and institutional guidelines for the protection of animal welfare.

2.2. Hematoxylin and eosin (H&E) staining

The tissue was rapidly removed, washed for several times with 0.1-M phosphate buffer saline (PBS, pH 7.4), fixed in 10% formalin for 24 h at room temperature, dehydrated by graded ethanol and embedded in paraffin. Sections (5–6-μm-thick) were deparaffinised with xylene, stained with haematoxylin and eosin, and observed by light microscopy.

2.3. Real-time RT-PCR

Total RNA was isolated from less than 100 mg of tissue by using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was quantified by determination of optical density at 260 nm. First-strand cDNA was synthesized according to the manufacturer's instruction of reverse transcription kit (TOYOBO, Japan). The cDNA product was stored at −20 °C until use.

Each 20 μl PCR reaction contained 1 μl cDNA, 2 μl PCR buffer, 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 500 nM each primer, 200 nM TaqMan probe and 1 U/20 μl Taq DNA polymerase. The sequences of primer and probes used were as follows: TNF-α (NM_012675), sense: 5′-GCCGATTTGCCATTTCATACC-3′, antisense: 5′-GGACTCCGTGATGTCTAAGTAG-3′, TaqMan probe: 5′-FAM-AGTCAGCCTCCTCTCCGCCATCAAG-TAMRA-3′; IL-1β (NM_031512), sense: 5′-GCCTCAAGGGGAAGAATCTATACC-3′, antisense: 5′-GGGAACTGTGCAGACTCAAACT-3′, TaqMan probe: 5′-FAM-TGATGAAAGACGGCACACCCACCCT-TAMRA-3′; iNOS (NM_012967), sense: 5′-CAGAAGCAGAATGTGACCATCAT-3′, antisense: 5′-CGGAGGGACCAGCCAAATC-3′, TaqMan probe: 5′-FAM-ACCACCACACAGCCTCAGAGTCCTT -TAMRA-3′; ICAM-1 (NM_052799), sense: 5′-TTCAACCCGTGCCAGGC-3′, antisense: 5′-GTTCGTCTTTCATCCAGTTAGTCT-3′, TaqMan probe: 5′-FAM-TCTGCTCCTGGTCCTGGTCGCCG-TAMRA-3′; Bax (NM_017059), sense: 5′-CCAAGAAGCTGAGCGAGTGTCTC-3′, antisense: 5′-AGTTGCCATCAGCAAACATGTCA-3′, TaqMan probe: 5′-FAM-CCACCCGGAAGAAGACCTCTCGGGG-TAMRA-3′; Bcl-2 (NM_016993), sense: 5′-GGAGCGTCAACAGGGAGATG-3′, antisense: 5′- GATGCCGGTTCAGGTACTCAG-3′, TaqMan probe: 5′-FAM-TCCACAGAGCGATGTTGTCCACCA-TAMRA-3′; and β-actin (NM_017008), sense: 5′-GCCCTAGACTTCGAGCAAGAG-3′, antisense:5′-AGCACTGTGTTGGCATAGAGGT-3′, TaqMan probe: 5′-FAM-CCACTGCCGCATCCTCTTCCTCCCT-TAMRA-3′. Each treatment had six samples and each PCR reaction was carried out in duplicate. Reactions were run on a Rotor-Gene 3000 Real-Time Cycler (Corbett Research, Australia). Cycling conditions were as follows: 3 min at 95 °C, 55 cycles of 20 s at 94 °C, 20 s at 58 °C and 20 s at 72 °C. Fluorescence data were acquired at the 72 °C step. The threshold cycle (Ct) was calculated by the Rotor-gene 6.0 software to indicate significant fluorescence signals above noise during the early cycles of amplification. Quantification of the samples by the software was calculated from Ct by interpolation from the standard curve to yield copy numbers for the target samples. The relative quantification of the expression of the target genes was measured using β-actin mRNA as an internal control.

2.4. ELISA

The tissue was weighed and homogenized in PBS and centrifuged at 3000 rpm for 10 min. Supernatants were removed and assayed by ELISA using TNF-α and IL-1β kits (Westang, China), according to the procedure described by the manufacturer.

2.5. TUNEL staining

Apoptosis was determined by TUNEL assay using the in situ cell death detection kit (Roche, Germany), following the manufacturer's instructions. Briefly, 50 μl of DAB solution was applied to each section following TUNEL incubation, and sections were counterstained with hematoxylin. The slides were observed by light microscopy (Olympus BX51, Japan).

2.6. Statistics and analysis

Results were expressed as mean ± SE. The data were analyzed using one-way ANOVA (Origin 7.0 software) for significant comparison between-group, and differences were considered significant when $P < 0.05$, $P < 0.01$, $P < 0.001$.

3. Results

3.1. Morphological changes

With the increase of SO_2 inhalation concentration, the levels of inflammatory cells infiltration in the myocardium heightened gradually. (Fig. 1B–D). Similarly, SO_2 inhalation at different concentrations significantly increased the lung injuries, which were characterized by massive infiltration of inflammatory cells, alveolar edema, alveolar-capillary wall thickening (Fig. 1F–H).

3.2. Effects of SO_2 inhalation on pro-inflammatory gene expression and secretion in the heart and lung

SO_2 inhalation at 14 and 28 mg/m^3 caused statistically significant increases of TNF-α and IL-1β mRNA levels in the heart and lung in a concentration-dependent manner (Fig. 2A–D), whereas the levels did not increase significantly in both heart and lung treated with lower concentration (7 mg/m^3). To determine whether SO_2 inhalation caused inflammatory processes and the elevation of TNF-α and IL-1β secretion contributed to the process, we further analyzed the

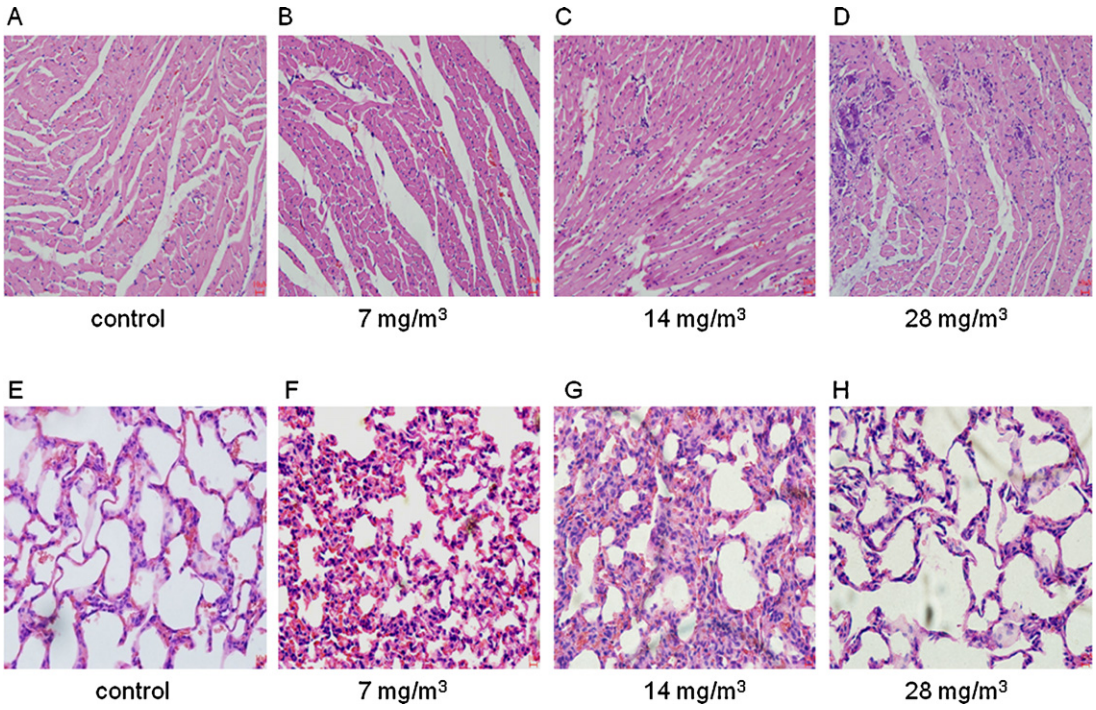

Fig. 1. HE staining of morphological characteristics of sections in the heart of rats from control (A), SO_2 inhalation 7 (B), 14 (C) and 28 mg/m^3 (D) group, and in the lung of rats from control (E), SO_2 inhalation 7 (F), 14 (G) and 28 mg/m^3 (H) group. 400× magnification. Bar = 10 μm.

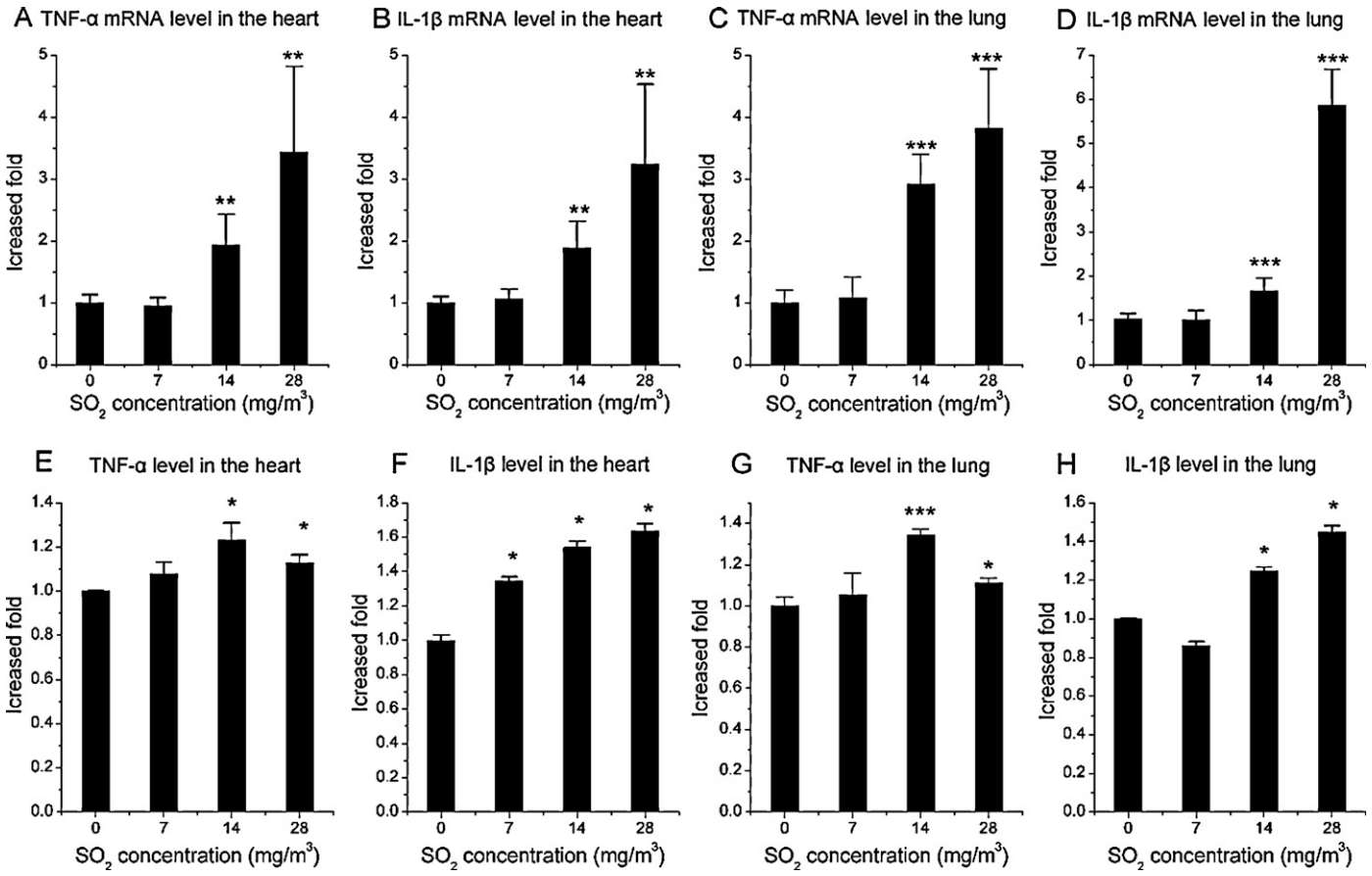

Fig. 2. Effects of SO_2 inhalation on TNF-α and IL-1β mRNA levels (A–D) and secretion (E–H) in the heart and lung of rats. Value in each treated group was expressed as a fold increase compared to mean value in vehicle control group, which has been ascribed as an arbitrary value of 1. Data were expressed as means ± SE ($n = 6$); $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control.

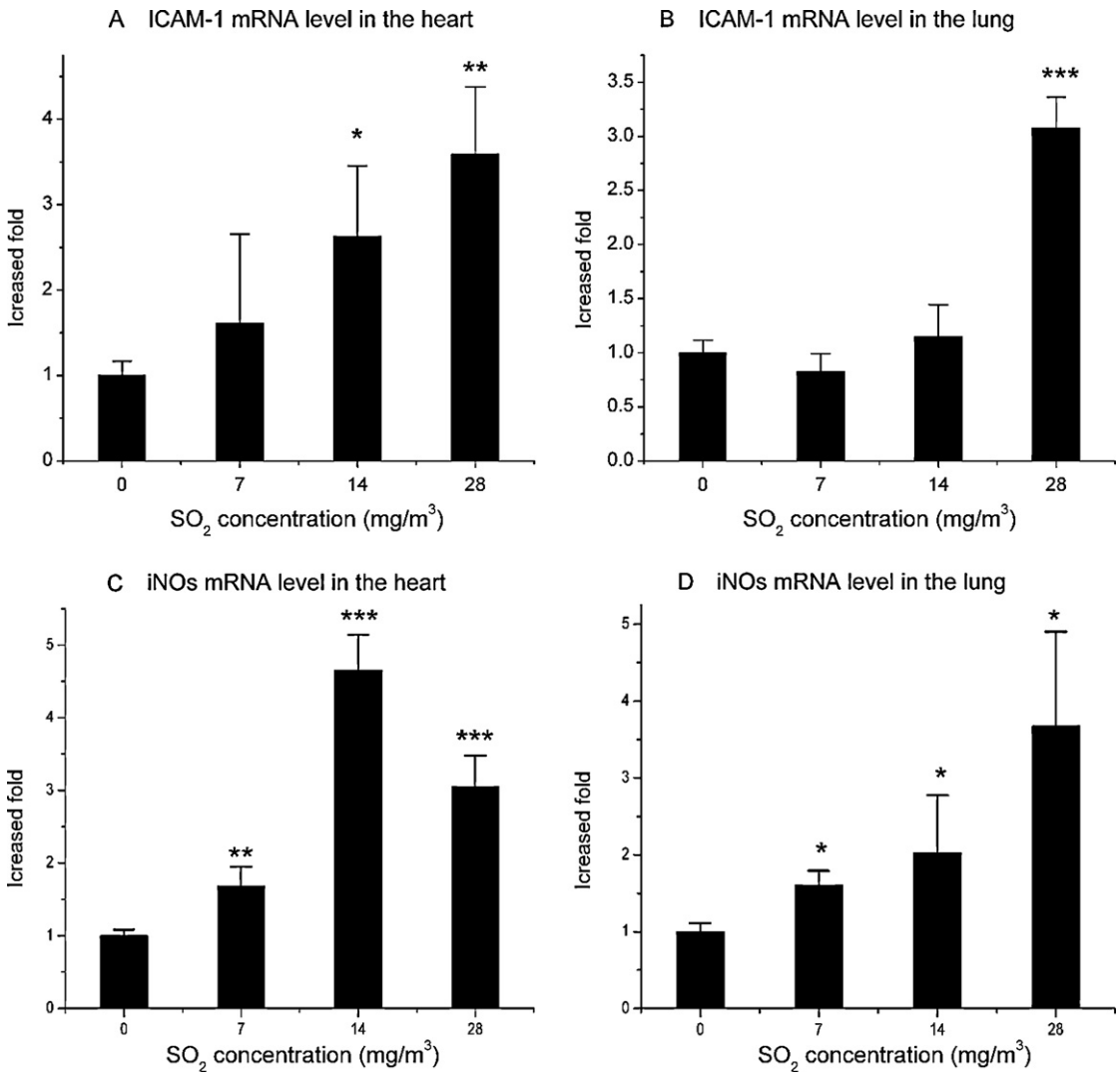

Fig. 3. Effects of SO_2 inhalation on ICAM-1 and iNOS mRNA expression in rat heart (A) and lung (B). Value in each treated group was expressed as a fold increase compared to mean value in vehicle control group, which has been ascribed as an arbitrary value of 1. Data were expressed as means ± SE ($n = 6$); $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control.

levels of TNF-α and IL-1β secretion after exposure to SO_2 at different concentrations. As shown in Fig. 2E–H, in agreement with the mRNA levels of TNF-α and IL-1β, SO_2 inhalation at higher concentrations significantly increased the levels of TNF-α and IL-1β secretion in the heart and lung.

In the heart (Fig. 3A), SO_2 inhalation at low concentration did not statistically increase ICAM-1 mRNA level; whereas, SO_2 exposure at higher concentrations significantly enhanced the expression (2.62-fold of control for 14 mg/m^3, $P < 0.05$, $n = 6$; 3.59-fold of control for 28 mg/m^3, $P < 0.01$, $n = 6$) In the lung (Fig. 3B), ICAM-1 mRNA level remained unchanged after SO_2 inhalation at lower concentrations (7 and 14 mg/m^3), except for a significant increase at the highest concentration (3.08-fold of control for 28 mg/m^3, $P < 0.001$, $n = 6$).

iNOS mRNA expression in the heart significantly elevated after exposure to SO_2 at lower concentrations (1.69-fold of control for 7 mg/m^3, $P < 0.01$, $n = 6$; 4.65-fold of control for 14 mg/m^3, $P < 0.001$, $n = 6$); the effect was still obvious at high concentration but slightly weaker than it after 14 mg/m^3 SO_2 inhalation (3.05-fold of control for 28 mg/m^3, $P < 0.001$, $n = 6$) (Fig. 3C). For the lung, iNOS mRNA level significantly increased after SO_2 exposure (Fig. 3D), and reached 1.60-, 2.02- and 3.67-fold of control at 7, 14 and 28 mg/m^3, respectively ($P < 0.05$, $n = 6$).

3.3. Effects of SO_2 inhalation on pro-apoptotic gene expression in the heart and lung

SO_2 inhalation changed the mitochondria-targeted Bax mRNA and ratio of Bax/Bcl-2 mRNA both in the heart and lung from rats. In the heart, Bax mRNA level significantly increased after SO_2 exposure at higher concentrations with a concentration-dependent property (3.53-fold of control for 14 mg/m^3, $P < 0.05$, $n = 6$; 3.84-fold of control for 28 mg/m^3, $P < 0.05$, $n = 6$); however, no statistical difference was observed after 7 mg/m^3 exposure (Fig. 4A). Not as expected, the slight enhancement of Bcl-2 mRNA expression after SO_2 inhalation occurred at all concentrations tested(1.17-, 1.09- and 1.04-fold of control at 7, 14 and 28 mg/m^3) (Fig. 4B). Following this, we calculated the ratio of Bax/Bcl-2 mRNA, and confirmed that the ratio statistically augmented at higher concentrations (5.05-fold of control for 14 mg/m^3, $P < 0.01$, $n = 6$; 5.61-fold of control for 28 mg/m^3, $P < 0.01$, $n = 6$); whereas the value did not significantly change after the exposure of lower SO_2 concentration (7 mg/m^3) (Fig. 4C).

SO_2 inhalation at higher concentrations (14, 28 mg/m^3) significantly increased Bax mRNA level in the lung (3.62-fold of control for 14 mg/m^3, $P < 0.05$, $n = 6$; 5.23-fold of control for 28 mg/m^3, $P < 0.001$, $n = 6$), the decrease was observed after 7 mg/m^3 inhala-

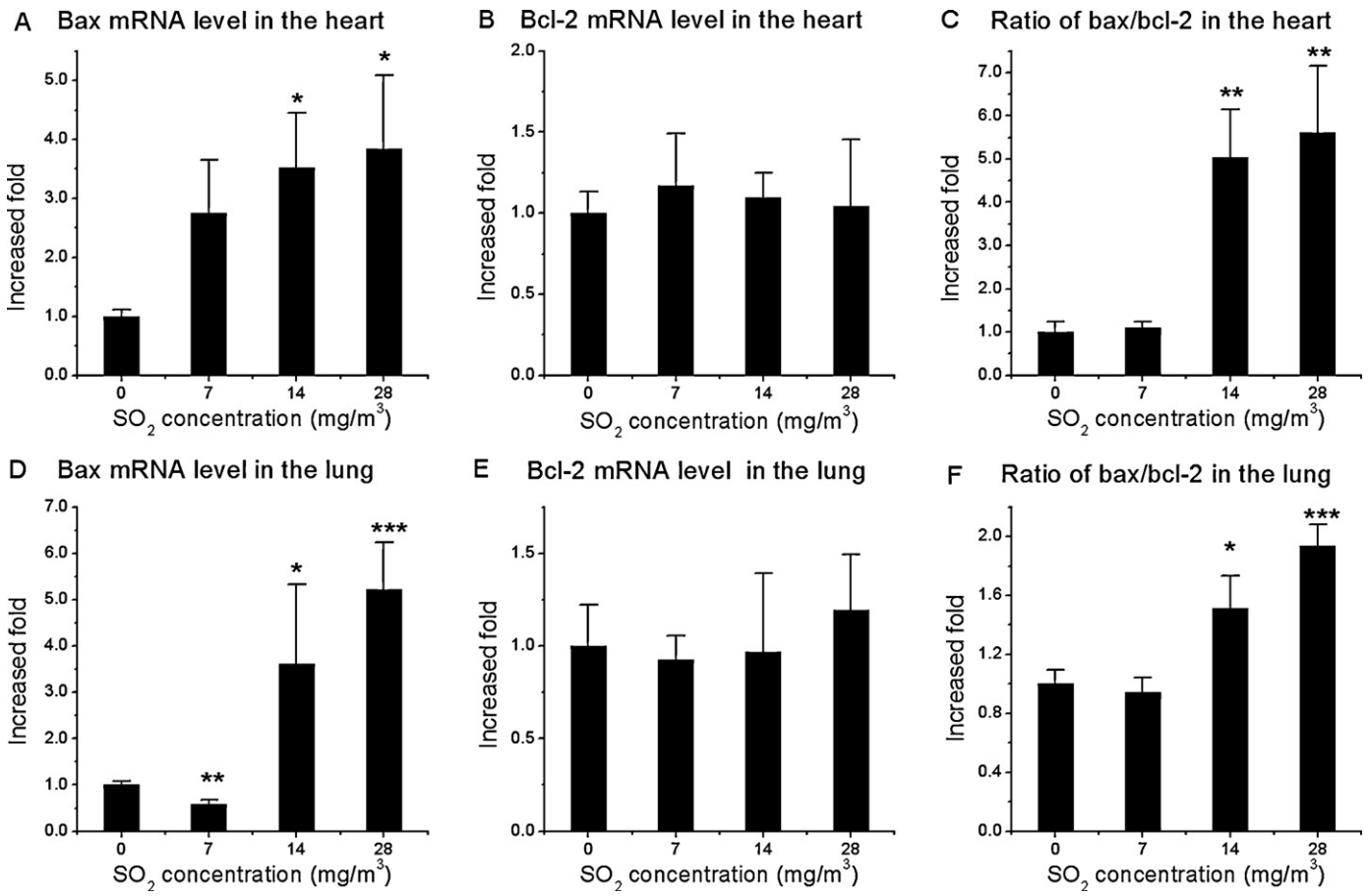

Fig. 4. Effects of SO_2 inhalation on Bax and Bcl-2 mRNA levels and the ratio of Bax to Bcl-2 in the heart (A–C) and lung (D–F) of rats. Value in each treated group was expressed as a fold increase compared to mean value in vehicle control group, which has been ascribed as an arbitrary value of 1. Data were expressed as means ± SE ($n = 6$); * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs* control.

tion (0.59-fold of control for 7 mg/m^3, $P < 0.01$, $n = 6$). (Fig. 4D). However, Bcl-2 mRNA expression remained unchanged statistically after exposure at all concentrations tested (Fig. 4E). Whereas, the ratio of Bax to Bcl-2 elevated with the increase of exposure concentration, and statistical differences occurred at higher concentrations (1.52-fold of control for 14 mg/m^3, $P < 0.05$, $n = 6$; 1.94-fold of control for 28 mg/m^3, $P < 0.001$, $n = 6$) (Fig. 4F).

3.4. SO_2 inhalation-induced apoptosis in the heart and lung

TUNEL staining results in both the heart (Fig. 5A–D) and lung (Fig. 5E–H) indicate that TUNEL positive cells were increased with exposure concentration. Compared to control group, the statistical increase of the number of TUNEL positive cells occurred at 14 and 28 mg/m^3 exposure.

4. Discussion

In this study, SO_2 at 7–28 mg/m^3 (2.5–10 ppm) levels, some 5–20-fold greater than the typical urban concentration (0.5 ppm), were used to examine the responses of rat tissues. Here, typical urban concentration (0.5 ppm) was referenced basing two considerations: first, the secondary National Ambient Air Quality Standards and China's Ambient Air Quality Standard Grade III for SO_2 is 0.5 ppm over a 3-h period [15,16]; second, the ambient concentration of SO_2 in an occupational setting recommended by the National Institute for Occupational Safety and Health is 0.5–20.0 mg/m^3, approximately 0.35–14 ppm [17]. Although the experiments may be viewed as beyond the normal atmosphere encountered in the human environment, three important points must be taken into account. First, the animals were subjected to regular periods of extended exposure, with relief periods between protocols (i.e., 6 h/day, for 7 days, with 18 h between exposures). This may provide a corollary to individuals exposed to the gas in an occupational setting. Second, the rat is an obligate nose breather and approximately 95% of the inhaled SO_2 is trapped in the nasal passages. The actual concentrations of gas reaching the lung may therefore have been significantly lower than that in the exposure chamber. Third, real time and mean concentrations in the upper and lower airways were higher in humans, when compared with rats using a comparative simulation of SO_2 gas transport in airway models [18]. Studies by other investigators have confirmed this basic finding that the majority of subjects respond to SO_2 concentrations of 5 ppm or higher, whereas only an occasional sensitive individual responds to 1 ppm SO_2 [19–22]. Therefore, the lowest concentration used in this study was just above the olfactory detection threshold in humans [23], whereas the highest and intermediate concentrations corresponded to the peak values reported in the working environment [24].

SO_2 is a common air pollutant, and inhaled SO_2 can easily be hydrated in the respiratory tract to produce sulfurous acid which directly causes reactive oxygen species (ROS) production, such as H_2O_2 and superoxide, subsequently elicit inflammatory reactions by oxidative stress in the lung [4]. As a result of pulmonary inflammation after SO_2 inhalation, pro-inflammatory cytokines from the lung release into the blood circulation, and contact with cardiac tissue may present almost instantly inflammatory responses. In the initiation and development of inflammation-related lung and heart

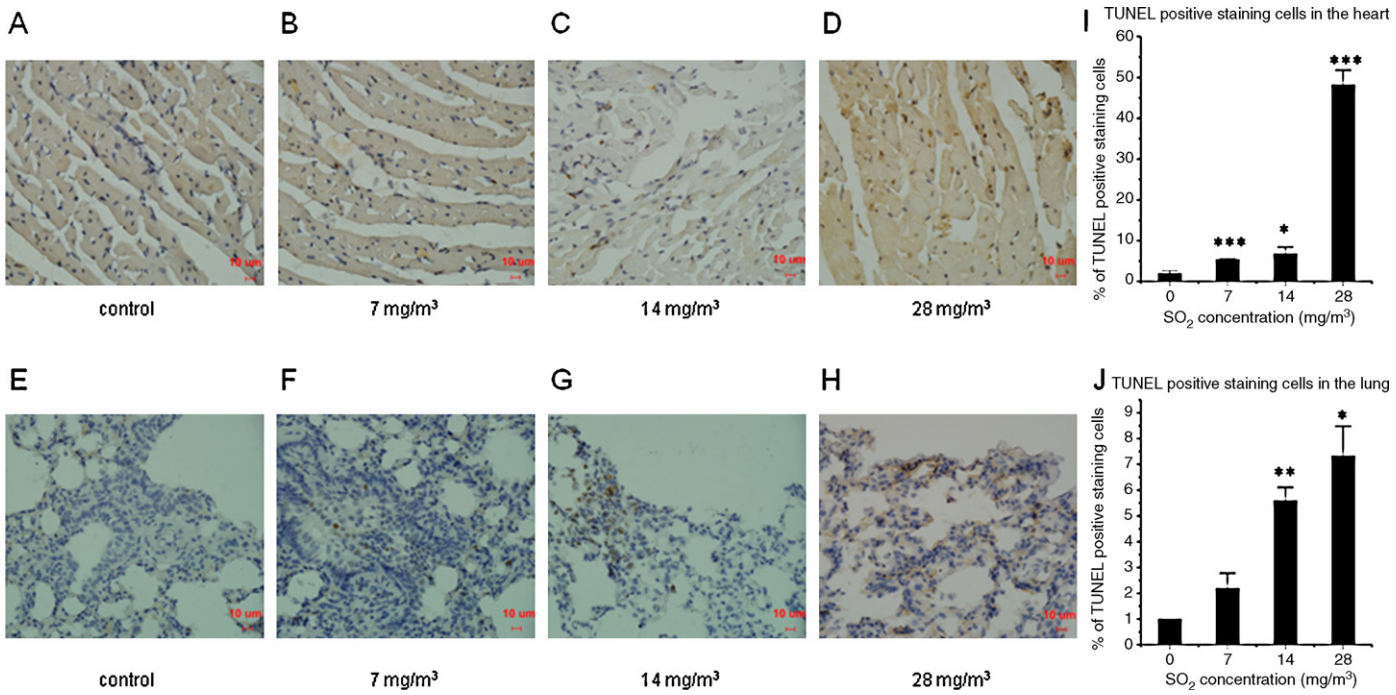

Fig. 5. TUNEL staining in rat heart (A–D) and lung (E–H) before and after SO_2 exposure at different concentrations. 400× magnification. Bar = 10 μm. Average percentages of TUNEL positive staining neuron in rat heart (I) and lung (J) before and after exposure to SO_2 at various concentrations. Data were expressed as means ± SE ($n = 6$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control.

dysfunction, the pro-inflammatory cytokines TNF-α and IL-1β play key roles [25]. Our present studies reveal that TNF-α and IL-1β mRNA levels and secretion increased in rat heart and lung after SO_2 exposure, indicating that the mechanism of inflammatory damage following SO_2 inhalation might be initiated by the release of the pulmonary and cardiac pro-inflammatory cytokines.

Pro-inflammatory cytokines can not only trigger inflammation in tissues, but can also stimulate other pro-inflammatory genes expression. Pro-inflammatory genes, ICAM-1 and iNOS, which regulate leukocyte recruitment, play key roles in inflammatory events. ICAM-1 contributes to the inflammatory process by facilitating leukocyte adhesion to the endothelium followed by extravasation of activated leukocytes to the sites of inflammation [26]. Also, in an inflammatory milieu, pro-inflammatory enzymes iNOS via synthesized excessive nitric oxide (NO) in pathological conditions exacerbate organ damage just because of the effects of NO production to maintain organ blood flow and increase circulating leukocytes to adhere to the sites of inflammation [27]. Therefore, ICAM-1 and iNOS expression are thought to participate in the deleterious effects of inflammation. Our results clearly indicate that the mRNA levels of ICAM-1 and iNOS markedly elevated in the heart and lung after SO_2 exposure at different concentrations, suggesting that the occurrence and development of cardiovascular diseases via inflammatory mechanism following SO_2 inhalation, which was certificated by the observed morphological changes from current HE staining.

Pro-inflammatory cytokines usually stimulate apoptosis through induced mitochondrial dysfunction and this process is accompanied by enhancing synthesis of reactive oxygen species, disrupting the mitochondrial membrane potential, and inducing the activation of caspases. Mitochondrial signaling pathway as a major apoptotic mechanism is tightly regulated by the anti- and pro-apoptotic members of the Bcl-2 family which are mandatory for the initiation of apoptosis [11]. Translocation of pro-apoptotic member Bax to the mitochondria and enhancement of mitochondrial permeability lead to dissipation of the mitochondrial inner transmembrane potential and release of cytochrome c from mitochondria into the cytosol. The released cytochrome c and Apaf-1 from the mitochondria, together with caspase-9, form an apoptosome to activate caspase-3 and the subsequent caspase cascade towards cell apoptosis [28]. Anti-apoptotic gene Bcl-2 may promote cell survival by interfering with the activation of the cytochrome c/Apaf-1 pathway through stabilization of the mitochondrial membrane. Therefore, the balance between the expression of Bax and Bcl-2 may be important in the increased rate of apoptosis [29]. In this study, SO_2 inhalation up-regulated the pro-apoptotic mediator Bax and the ratio of Bax/Bcl-2 in rat heart and lung, but not Bcl-2, implicating potential for translocation of Bax to the mitochondria and subsequent mitochondrial permeabilization.

5. Conclusion

SO_2 inhalation markedly elevated TNF-α, IL-1β, iNOS and ICAM-1 expression, enhanced the ratio of Bax/Bcl-2, and induced the occurrence of apoptosis. This suggests that SO_2 inhalation induced an inflammatory response and subsequent insults via modulation of pro-inflammatory and pro-apoptotic genes in the heart and lung, which might contribute to the increased risk of respiratory and cardiovascular diseases.

Acknowledgments

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