Upregulated Expression of Secretory Leukocyte Protease Inhibitor in Lung by Inhalation of High Concentration of Sulfur Dioxide

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To the Editor: Sulfur dioxide (SO_2) is one of the main air pollutants, which is formed when sulfur-containing fuel is burned.[1] In some special situation, people may be exposed to high concentration of SO_2, such as an accident of SO_2 tank leakage, mine blast, smoke of gunpowder in a war, and smoke of volcanic eruption, which may be harmful to the persons who are exposed to it. Many studies have indicated that SO_2 exposure increases morbidity and mortality. SO_2 can not only produce a variety of adverse pulmonary effects, such as bronchitis and airway hyperresponsiveness,[2] but also have harmful effects on other systems and organs. It is associated with increased risk of acute myocardial infarction, and inhalation of SO_2 can cause injury in brain including stroke. The expressions of oncogenes and tumor suppressor genes in lung and liver of rat were affected by exposure to SO_2 and benzo(a)pyrene. The expressions of apoptosis-related genes can be augmented and the apoptosis of the cells was induced in liver, lung, and brain of rats exposed to SO_2.

Secretory leukocyte protease inhibitor (SLPI) is a glycoprotein with a molecular weight of about 11,700.[3] SLPI is present in human mucus secretions and tissues and produced primarily in the epithelial cells lining the respiratory, digestive, and reproductive tracts.[4] Its major physiological function is to inhibit serine proteases, including cathepsin and tryptase, and to protect tissues from excessive protease digestion at the sites of inflammation *in vivo*.[5] It has antibacterial and antifungal properties *in vitro* and has been shown to prevent viral infection. SLPI inhibits the expression of inflammatory cytokines such as tumor necrosis factor (TNF)-a, interleukin (IL)-8, and IL-6 via translocation from cytoplasm to nucleus and binding to nuclear factor-kappa B binding sites.

Although SLPI is well characterized at both the gene and protein levels, little is known about the regulation of SLPI expression in the lung. In this study, we investigated whether the expression of SLPI both at mRNA and protein levels in the lungs of rats is influenced by inhalation of high concentration of SO_2.

Animals experiments was approved by the Ethics Committee of General Hospital of Shenyang Military Command. An exposure chamber of SO_2 was designed. The device consisted of SO_2 source, air pump, intake port, SO_2 chamber, and SO_2 detector and some connective tubes and valves. The SO_2 was diluted with fresh air at the intake port of the chamber to yield the desired SO_2 concentrations. The desired SO_2 was delivered to animals via a tube positioned at the upper level of the chamber and distributed homogeneously via a fan in each chamber. The concentration of SO_2 was determined in real-time manner by a SO_2 sensor (JSA5- SO_2 sensor, Shenzhen Ji-shun-an Technology Co., Ltd., China). The concentration of SO_2 in the chamber was adjusted by opening and closing a valve between intake port and SO_2 chamber according to the quantitative value of the SO_2 sensor.

The Sprague-Dawly rats were divided randomly into two groups with 10 rats in each group. The rats in SO_2 exposed group were placed into the exposure chamber described above and exposed to 6×10^{-4} SO_2 for 2 h each day for consecutive 7 days. The rats in control group were exposed to filtered air in another identical chamber for the same period of time. When not being treated, all of the rats had free access to food and water *ad libitum*. After the SO_2 exposure was finished, the rats of both groups were sacrificed and the sera and bronchoalveolar lavage fluid (BALF) were harvested and restored at −20°C until detection. The lungs were removed and divided into three parts for reverse transcription-polymerase chain reaction (RT-PCR), Western blot, and histological analysis.

Total RNA was isolated from <100 mg of lung tissue using TRIzol reagent (Invitrogen Life Technologies, USA) according to the manufacturer's instructions. Quantitative RT-PCR (QRT-PCR) was performed using a sequence detection system (ABI PRISM 7000; Applied Biosystems, Life Technologies, Grand Island, NY, USA), and all reaction components were purchased from the same source (SYBR PrimeScript RT-PCR Kit obtained from TaKaRa Biotechnology). The standard experiments were carried out based on the instructions of the provider. All reactions were performed

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in triplicate. The mRNA expressions of the indicated genes were normalized relative to β-actin mRNA. The primers were designed as follows: forward 5'-CCTCTATGCCAACACAGTGC-3' and reverse 5'-GTACTCCTGCTTGCTGATCC-3' for β-actin, with annealing temperature of 60°C and 211 bp; and forward, 5'-TTGTGAGGGTATGTGTGGGAA-3', reverse, 5'-CCTTAGGGGCTTGATGAATG-3' for *SLPI*, with annealing temperature of 60°C and 238 bp.

Western blot analysis was performed as follows: the proteins were separated by SDS-polyacrylamide gel electrophoresis (10% separating, 5% stacking) and transferred to polyvinylidene difluoride membranes (Millipore, USA). After the membranes were blocked in 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20, immunostaining was performed with rabbit polyclonal antibodies specific for rat SLPI at a concentration of 1:200 at 4°C overnight. Anti-rabbit secondary antibody at a concentration of 1:5000 was added to membranes and incubated for 45 min at 37°C. The protein signal was amplified and visualized via chemiluminescence using the ECL Western blotting detection system and Hyperfilm ECL autoradiography film (Amersham Pharmacia Biotech Inc., USA). To verify equal protein loading and transfer, membranes were stripped with stripping buffer and reprobed with anti-β-actin antibody. Images were quantified using the LabWorks v3.0.2 image scanning and analysis software (Gel-Pro-Analyzer, Media Cybernetics Inc., USA).

The levels of SLPI in BALF were measured by ELISA kit according to the manufacturer's instruction. The concentrations of SLPI were calculated by generating a standard curve using standard proteins and analyzed using CurveExpert 1.3 software (People Tec., Inc., Hyams Development, Huntsville, AL, USA). The activities of cathepsin K in BALF and serum were measured by ELISA kit according to the manufacturer's instruction. The activity of cathepsin K was expressed as fluorescence intensity. The partial left lung was fixed in 4% formaldehyde. The fixed tissue samples were dehydrated in graded ethanol, embedded in paraffin. Each paraffin block was cut into 5-μm-thick slices. Then, these slices were dewaxed in xylene, rehydrated in gradient alcohols, and rinsed with distilled water. Each section placed on glass slide was stained with hematoxylin and eosin and double-blindly evaluated under microscope for histological changes by a professional pathologist.

All values were expressed as mean ± standard deviation (SD) and were compared using the unpaired Student's *t*-test. A $P < 0.05$ was considered statistically significant.

The QRT-PCR showed that the expressions of SLPI in control and SO_2 groups were 1.01 ± 0.21 and 1.75 ± 0.07, respectively, with significant difference ($t = 7.316$, $P < 0.01$). To clarify whether SLPI expression at protein level is influenced by inhalation of high concentration of SO_2, the protein was extracted from the lungs of both control and SO_2 groups, and Western blot analysis showed that the gray values for control and SO_2 groups were 1.11 ± 0.35 and 1.55 ± 0.41, respectively, with significant difference ($t = 2.672$, $P < 0.05$; Figure 1a and 1b). To demonstrate if the SLPI in BALF is changed, the ELISA showed that the SLPI levels in BALF of control and SO_2 groups were 8.93 ± 0.90 and 11.14 ± 2.25 pg/ml, respectively, with significant difference ($t = 2.404$, $P < 0.05$). Next, we investigated if increased SLPI can inhibit some enzymes. Cathepsin K was selected as an indicator of SLPI activity. Cathepsin K activities in both serum and BALF were detected. The results showed that serum cathepsin K activities in SO_2 and control groups were 31597 ± 6116 and 31602 ± 5894, respectively, without significant difference ($t = -0.002$, $P > 0.05$). The BALF cathepsin K activities in SO_2 and control groups were 836.50 ± 299.21 and 3836.00 ± 3187.45, respectively, with significant difference ($t = -0.295$, $P < 0.05$).

In histopathological examination, no abnormality in lungs and tracheas was observed in the control group. The pathological changes, such as chronic bronchitis, local alveolar hemorrhage, and lymphocytes infiltration, were observed in SO_2 group [Figure 1c and 1d].

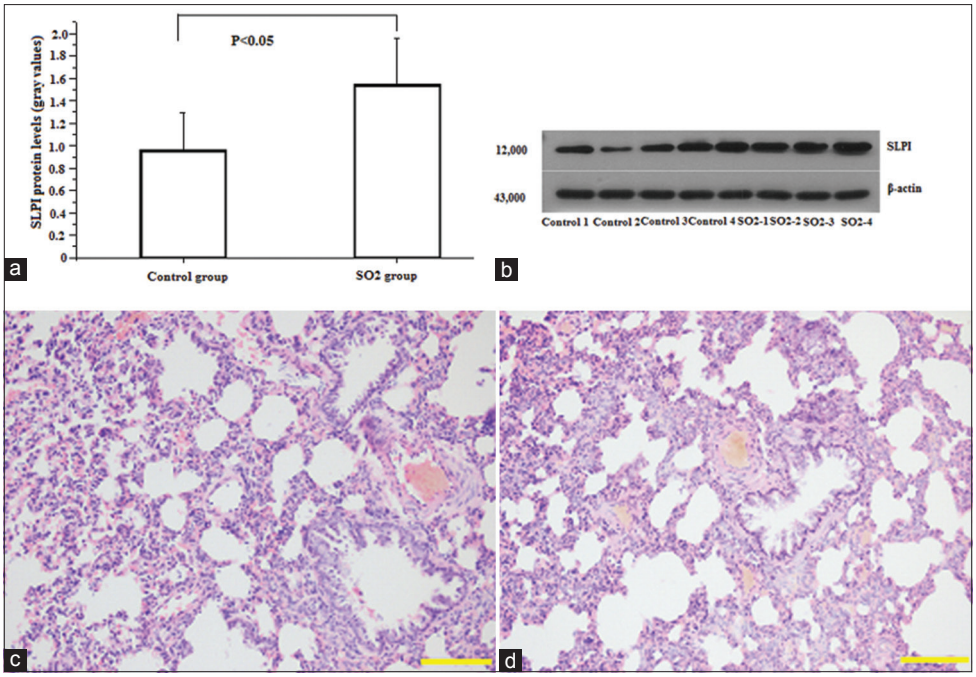

Figure 1: Effects of high concentration of SO_2 on the SLPI expression in lung of rats (a and b). The gray values of SLPI protein level in control and SO_2 groups. β-actin as the internal control. The histopathological test (HE staining) showed that no abnormality in lungs and tracheas was observed in control group (c), and the pathological changes, such as chronic bronchitis, local alveolar hemorrhage, and lymphocytes infiltration, were observed in SO_2 group (d). Scale bar = 100 μm. SO_2: Sulfur dioxide; SLPI: Secretory leukocyte protease inhibitor.

Air pollution has been paid special attention by the public because it gives rise to many health and environmental problems. SO_2 is one of the most frequently exposed air pollutants. Many evidences have demonstrated that some respiratory-tract and cardiopulmonary diseases were caused by exposure to SO_2. The researchers have shown inflammatory cell infiltration, mucus cell increase, and mucus hypersecretion in the airways of SO_2 exposed rats. However, to the best of our knowledge, little studies focused on the effect of SO_2 on the expression and function of SLPI. The present study demonstrated that the expression of SLPI was upregulated both at mRNA and protein levels in the lung tissue of SO_2 group. Because SLPI is produced by epithelial cells of lining the respiratory, digestive, and reproductive tracts, we investigated whether SLPI in BALF is also influenced by inhalation of high concentration of SO_2. Interestingly, SLPI in BALF was also elevated in SO_2 group. SLPI has antiprotease activity and plays an important role in neutralizing enzymes such as neutrophil elastase to prevent excessive tissue damage during inflammation.[5] For this reason, we investigated if the cathepsin K activity of BALF can be inhibited by SLPI. The result showed that the cathepsin K activity in BALF of SO_2 group was significantly lower than that of control group. However, the serum cathepsin K activity in SO_2 group was not influenced, which suggested that the inhibitory effect of SLPI to cathepsin K activity was limited to the airway and lung, instead of the whole body. It was well documented that SLPI could inhibit cathepsin G activity,[5] but our result indirectly showed that SLPI could also inhibit cathepsin K activity. As for the mechanism underlying that SLPI expression and secretion were upregulated by inhalation of high concentration of SO_2, we speculated that there were two possibilities. One was that SO_2 acted directly on the epithelial cells of respiratory tract and induced the expression and secretion of SLPI. Another was that SO_2 caused inflammation in airway and lung, and the expression and secretion of SLPI were induced by the inflammation. It was reported that the expression of SLPI could be increased by proinflammatory stimuli such as TNF-a and IL-1b. This was supported by the histopathological results in this study, which showed the lymphocytes infiltration in the lung of SO_2 group. The further studies are needed to elucidate the detail mechanism.

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Conflicts of interest

There are no conflicts of interest.

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