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The dysfunction of ATPases due to impaired mitochondrial respiration in phosgene-induced pulmonary edema

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Abstract

Phosgene is a toxic gas that is widely used in modern industry, and its inhalation can cause severe pulmonary edema. There is no effective clinical treatment because the mechanism of phosgene-induced pulmonary edema still remains unclear. Many studies have demonstrated that the Na^+/K^+-ATPase plays a critical role in clearing pulmonary edema and the inhibition of Na^+/K^+-ATPase protein expression has been found in many other pulmonary edema models. In the present study, after the mice were exposed to phosgene, there was serious pulmonary edema, indicating the dysfunction of the ATPases in mice. However, in vitro enzyme study showed that there were increases in the activities of the Na^+/K^+-ATPase and Ca^{2+}-ATPase. Further investigation showed that the ATP content and mitochondrial respiratory control ratio (RCR) in the lungs decreased significantly. The oxidative stress product, malondialdehyde (MDA), increased while the antioxidants (GSH, SOD, and TAC) decreased significantly. These results indicate that mitochondrial respiration is the target of phosgene. The dysfunction of ATPases due to impaired mitochondrial respiration may be a new mechanism of phosgene-induced pulmonary edema.

Keywords: Phosgene; Pulmonary edema; Mitochondrial respiration; Na^+/K^+-ATPase; Oxidative stress; Antioxidant

Phosgene ($COCl_2$), discovered in 1812 by John Davy, is an economical and versatile reagent widely used in organic chemistry. Its economic importance is growing tremendously, and world wide phosgene production exceeds 5 billion pounds [1]. Although phosgene is nearly completely consumed during industrial uses, it is often released when processes fail [2]. Phosgene is a highly reactive toxic gas. Phosgene inhalation can cause severe pulmonary edema and the death rate associated with inhalation is high. Today, no effective clinical treatment exists because the actual mechanism of phosgene-induced pulmonary edema still remains unknown [3].

Accumulating evidence has shown that the Na^+/K^+-ATPase (Na^+ pump) plays a critical role in acute pulmonary edema [4]. The main function of the Na^+/K^+-ATPase is to maintain sodium and potassium ion gradients, which are important for the preservation of normal cellular activities such as volume regulation, the action potential, and secondary active transport [5]. The pulmonary alveolar surface is composed of two morphologically distinct epithelial cells, known as type 1 and type 2. The Na^+/K^+-ATPase has been demonstrated to be expressed on both types of cells [6]. In the alveolar epithelium, the Na^+/K^+-ATPase creates a transepithelial Na^+ gradient crucial to keeping fluid from the pulmonary air space [4,7]. Studies have shown that disabling the Na^+/K^+-ATPase with toxins such as ouabain can lead to severe edema in the lungs [8]. On the other hand, stimulation of Na^+/K^+-ATPase activity, primarily through β-adrenergic stimulation, can significantly improve edema clearance [9]. Overexpression of the Na^+/K^+-ATPase also improves edema clearance in injured lungs [10,11]. Working in

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conjunction with Na^+/K^+-ATPase, Ca^{2+}-ATPase (Ca^{2+} pump) also contributes to the maintenance of ionic gradients and water balance in cells.

The Na^+/K^+ and Ca^{2+} ATPases are adenosine triphosphate (ATP) dependent, meaning that sufficient ATP is necessary to maintain their function. It is estimated that Na^+/K^+-ATPase activity accounts for up to 40% of a cell's energy expenditure [12]. In eukaryotic cells, ATP (about 80%) is mainly produced through mitochondrial respiration [13]. During the process of mitochondrial oxidative phosphorylation, most of the electrons are transported to facilitate O_2 conversion to H_2O, but some of the electrons leak from the electron transport chain, resulting in the formation of superoxide radical ($\cdot O_2^-$), followed by the formation of hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and other forms of reactive oxygen species (ROS) [14]. It is estimated that more than 2% of the oxygen consumed by cells is converted to ROS under physiological conditions [13]. If mitochondrial respiration is disrupted, more electrons leak, leading to oxidative stress. Some reports, including our previous studies, have found that phosgene inhalation can cause oxidative damage [15–17]. Currie demonstrated that a decrease in lung ATP concentration occurred immediately following phosgene exposure, suggesting that the changes in ATP levels were not the result of edema [18]. These results indicated that mitochondrial respiration may be the target of phosgene.

We hypothesize that phosgene inhalation could impair mitochondrial respiration in the lungs such that electrons are transported and produce ROS, in lieu of generating ATP. The resultant deficiency in ATP needed to drive the Na^+/K^+ and Ca^{2+} ATPases leads to pulmonary edema, while the increased ROS induce oxidative stress in the lungs.

Materials and methods

Animals. Adult male Balb/c mice, weighing 20–22 g, were supplied by the Experimental Animal Center of the Fourth Military Medical University. All of the mice were housed in cages (5 mice per cage) at an ambient temperature of 20–25 °C under a 12 h light/dark cycle and allowed free access to food and water. All experiments were carried out with the approval of the local animal use committee. Efforts were made to minimize animal suffering.

Phosgene exposure and sample preparation. Seventy male mice were randomly divided into seven groups: one naive mice group, three control groups, and three phosgene exposure groups, with ten mice in each group. The mice in the phosgene exposure groups were exposed to phosgene with an initial concentration of 595 ppm for 5 min as previously described [19]. The mice in the control groups were exposed to room air for 5 min in the same chamber. At 1, 3, or 5 h after phosgene exposure, the mice' eyes were extirpated to collect blood and then sacrificed by cervical dislocation to collect the lungs.

Wet to dry lung weight ratio analysis. Wet to dry lung weight analysis was performed immediately after lung collection. The specimens were lightly blotted, weighed immediately, and then placed in a heated vacuum chamber at 60 °C for 3 days, until the weight of the remaining tissue stabilized. The wet to dry lung weight ratio of each mouse was calculated.

Na^+/K^+-ATPase and Ca^{2+}-ATPase activity in the lungs. The activities of the Na^+/K^+-ATPase and Ca^{2+}-ATPase were determined as described previously [20]. Briefly, the reaction mixture for the Na^+/K^+-ATPase assay contained 5 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl, and 40 mM Tris–HCl buffer, pH 7.4, in a final volume of 200 μl. The reaction was started by the addition of ATP (Sigma, USA) to a final concentration of 3 mM. The control was assayed under the same conditions with the addition of 1 mM ouabain (Sigma). Ca^{2+}-ATPase (ouabain-insensitive) was assayed under the same conditions. The enzyme reaction was stopped by the addition of 200 μl 10% trichloroacetic acid (TCA, Sigma) to a final concentration of 5%. The enzyme activity was calculated as the difference between the two assays. Released inorganic phosphate (Pi) was measured using the method of Chan [21]. Enzyme specific activity was expressed as μmol Pi released per hour per mg of protein.

Determination of ATP content in the lungs. Lung ATP contents were determined by the luciferin–luciferase method, as previously described by Salzman [22]. Briefly, the lung was weighed exactly before determination and put into 2% TCA solution to make a homogenate. The homogenate was centrifuged at 10,000*g* for 5 min at 4 °C, and the supernatant was diluted in the luciferin–luciferase reagent at 1:5. Bioluminescence measurements were subsequently performed in a SGH-luminometer (Shanghai, China). An ATP standard curve was made and used to calculate the ATP content of the samples, expressed as nanomoles per milligram of protein.

Mitochondria isolation and the mitochondrial respiratory control ratio (RCR). Lung mitochondria was isolated according to the procedure described by Abreu [23]. Lung homogenate was prepared with separate medium (25 mmol/l sucrose, 10 mmol/l Tris, 2 mmol/l EGTA, pH 7.4). The homogenate was centrifuged at 800*g* for 10 min and then the supernatant was centrifuged at 10,000*g* for 10 min. The mitochondrial pellet was resuspended and centrifuged twice at 10,000*g* for 10 min before obtaining a final mitochondrial suspension with separate medium. All procedures were performed at 4 °C. The respiratory function of mitochondria was evaluated by the method of Clark [24]. The oxygen uptake was measured by a dissolved oxygen determinator (Shanghai, China) in 3 ml respiratory medium containing 130 mmol/l sucrose, 50 mmol/l KCl, 5 mmol/l MgCl, 5 mmol/l Hepes, pH 7.2. State 4 respiration was initiated by the addition of 5 mmol/l succinate. To determine the state 3 respiration, 0.1 mmol/l potassium-adenosine diphosphate (ADP) was added to the assay medium. The ratio of the state 3/state 4 respiration rates was used to calculate the respiratory control ratio.

Determination of lipid peroxidation. The lipid peroxidation products in the lung were determined by measuring malondialdehyde (MDA). The method of Yagi [25] was used with some modifications. Briefly, 20 μl of sample was placed in a glass centrifuge tube. 4.0 ml of 1/12 N H_2SO_4 was added and mixed gently. Then, 0.5 ml of 10% phosphotungstic acid (Sigma) was added and mixed. After allowing it to stand at room temperature for 5 min, the mixture was centrifuged at 1600*g* for 10 min. The supernatant was discarded and the sediment mixed with 2.0 ml of 1/12 N H_2SO_4 followed by 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged at 1600*g* for 10 min. The sediment was then suspended in 1.0 ml of distilled water and 1.0 ml of 0.67% (w/v) TBA reagent (Merck, German) was added. The reaction mixture was heated at 95 °C for 60 min. After cooling with tap water, 5.0 ml of *n*-butanol (Kemio, China) was added and the mixture was shaken vigorously. After centrifugation at 1600*g* for 15 min, the *n*-butanol layer was taken for fluorometric measurement at 553 nm with excitation at 515 nm. 1,1,3,3-Tetraetoxypropane (Sigma) was used as the primary standard.

Determination of glutathione (GSH). Fluorometric estimation was used to assay GSH. Briefly, 200 μl of sample was placed in a glass centrifuge tube. Two hundred microliters of 10% TCA was added and mixed, then centrifuged at 5000*g* for 10 min. One hundred microliter supernatant was transferred to another tube and 100 μl formaldehyde reagent (1 volume of formaldehyde mixed with 4 volumes of 0.1 M phosphate buffer (pH 8.3, 5 mM EDTA)) was added. Then, 3.6 ml of 0.1 M phosphate buffer (pH 8.3, 5 mM EDTA) was added and then 200 μl 0.1% *o*-phthalaldehyde (OPT, Sigma) was added and mixed. After being kept at room temperature for 40 min, the mixture was measured with a fluorometric spectrophotometer at 425 nm with excitation at 350 nm. Commercially procured GSH (Sigma) was used

to establish a standard curve. The results were expressed in micromoles GSH per gram protein.

Determination of SOD activity. The total activities of SOD in the lung were determined using the method of Kono [26], with some modifications. Ten microliter of sample or 50 mM phosphate buffer (blank) was added to the reaction system, consisting of 50 mM sodium carbonate buffer (pH 10.2, 0.1 mM EDTA), 24 μM NBT (Sigma) and 0.03% (v/v) Triton X-100 (Sigma). The reaction was initiated by 1 mM hydroxylamine (Tianjin, China) in the 37 °C water bath. After 20 min of incubation, the reaction was terminated by adding 10% TCA. Absorbance measurement was carried out with a spectrophotometer at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of hydroxylamine oxidation in the control by 50%.

Total antioxidant capacity (TAC) assay. The total antioxidant capacities of the lung were measured using a kit purchased from the Nanjing Jianchen Institute (Nanjing, China). The results from this kit did not distinguish between lipid- and water-soluble antioxidants but rather provided an estimate of the total antioxidant capacity.

The total protein concentration in the aforementioned experiments was determined by the Lowry method calibrated with bovine serum albumin.

Statistical analysis. The data were presented as means ± SD. Group differences were evaluated by ANOVA or by Student's *t* test as appropriate. Differences between mean values were considered statistically significant at $p < 0.05$ and are labeled with an asterisk (*).

Results

Increased wet to dry lung weight ratio after phosgene exposure

In our previous study, we successfully established a phosgene-induced pulmonary edema model in mice [19]. The wet to dry lung weight ratio reflected the extent of pulmonary edema. In the present study, after phosgene exposure, the wet to dry lung weight ratio increased. This difference became significant after 3 h, as presented in Fig. 1. These results demonstrate that phosgene exposure successfully induced pulmonary edema in mice, and also indicate that there was decreased clearance of edema in the lungs.

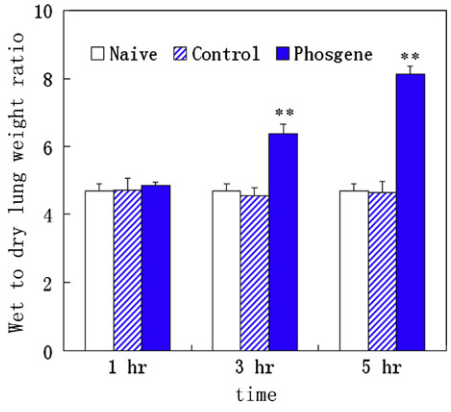

Fig. 1. Wet to dry lung weight ratio in mice. Wet to dry lung weight ratios were determined at 1, 3, and 5 h after phosgene exposure. Naïve, untreated group. Control, control group for phosgene. Phosgene, phosgene exposure group. Data are presented as means ± SD. $n = 10$, $^{**}p < 0.01$ *vs* Control.

Increased activities of Na^+/K^+-ATPase and Ca^{2+}-ATPase in vitro

Accumulating evidence has demonstrated that the dysfunction of the Na^+/K^+-ATPase plays a key role in the decreased clearance of edema. In another pulmonary edema model, the inhibition of expression of the ATPase protein accounts for its dysfunction [27]. In the present study, the ATPase activities were determined by in vitro enzyme assays. After phosgene exposure, the activities of the Na^+/K^+-ATPase and Ca^{2+}-ATPase increased. After 3 h, the activities of both Na^+/K^+-ATPase and Ca^{2+}-ATPase became significantly higher than those in control groups (Fig. 2). These in vitro data indicate that there was no injury or impairment of the ATPase proteins. This result rules out the possibility that ATPases were damaged directly by phosgene.

Decreased concentration of ATP and mitochondrial RCR in the lungs

As ATPases are ATP dependent, ATP availability can affect these enzymes' activities. In the present study, after the phosgene exposure, the concentration of ATP in the lung decreased immediately (Fig. 3A), indicating that the decrease of ATP may be the reason for ATPase dysfunction. In physiological conditions, ATP is produced by mitochondrial respiration. The function of mitochondrial respiration was investigated by measuring the mitochondrial RCR. The mitochondrial RCR represents coupling between electron transport and oxidative phosphorylation. The RCR will decrease if the mitochondria are damaged and fewer electrons are transported for the production of ATP. In the present study, the mitochondrial RCR decreased greatly after phosgene exposure (Fig. 3B). These results demonstrate that mitochondrial respiration was impaired by phosgene, suggesting that mitochondrial respiration impairment may be the reason behind the phosgene-induced ATPase dysfunction that finally results in pulmonary edema.

Oxidative stress in lungs

The decrease in mitochondrial RCR suggests that, due to leakage, more electrons are involved in the generation of ROS, leading to oxidative stress. To investigate the oxidative stress, the product of lipid peroxidation, MDA, was examined. After exposure to phosgene, the MDA content in the lungs increased greatly. Three hours later, it became markedly higher than that in control group (Fig. 4A). It is known that there are some important antioxidants in the body, such as GSH and SOD, which help prevent oxidative injury. In the present study, the GSH content and SOD activity decreased dramatically after phosgene exposure (Fig. 4B and C). The TAC also decreased significantly (Fig. 4D). These results suggest that phosgene exposure induced oxidative stress in the lungs.

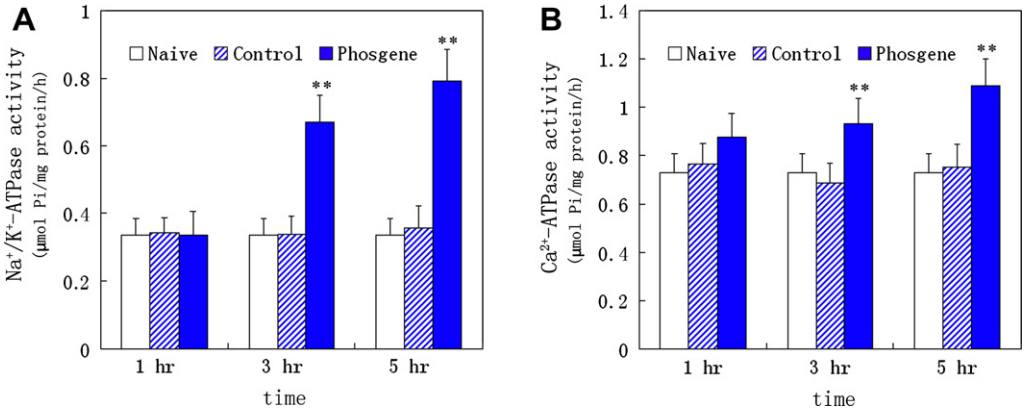

Fig. 2. Activities of Na^+/K^+-ATPase and Ca^{2+}-ATPase in vitro. Activities of Na^+/K^+-ATPase and Ca^{2+}-ATPase in lungs were determined at 1, 3, and 5 h after phosgene exposure. Naïve, untreated group. Control, control group for phosgene. Phosgene, phosgene exposure group. Data are presented as means ± SD. $n = 10$, $^{**}p < 0.01$ *vs* Control.

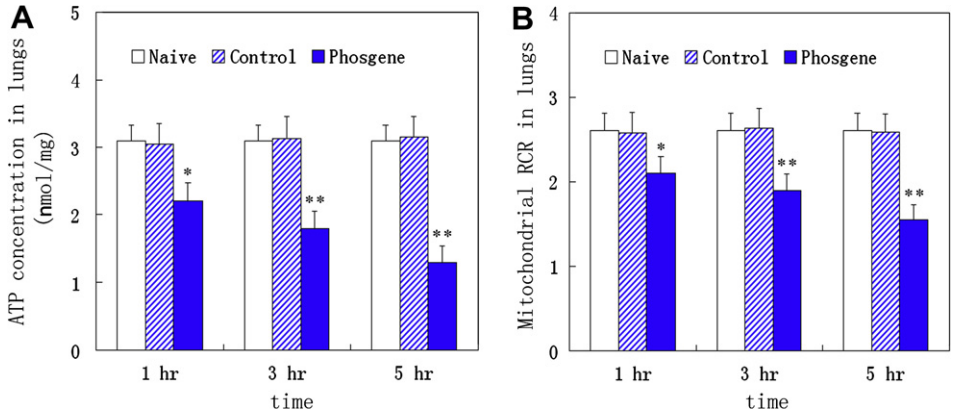

Fig. 3. ATP concentration and mitochondrial RCR in lungs. The concentrations of ATP (A) and the mitochondrial RCR (B) in lungs were determined at 1, 3, and 5 h after phosgene exposure. Naïve, untreated group. Control, control group for phosgene. Phosgene, phosgene exposure group. Data are presented as means ± SD. $n = 10$, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* Control.

Discussion

In many other pulmonary edema models, the lung's ability to clear edema is decreased. Further studies have shown that this is mainly due to dysfunction of the Na^+/K^+-ATPase (reviewed by Sznajder [27]). There are two factors that may result in the dysfunction of the Na^+/K^+-ATPase. One is the inhibition of expression or impairment of Na^+/K^+-ATPase protein, such as in hypoxia-induced edema [9]. The other is the lack of the power, ATP, to drive the ATPase. Currie [18] measured the ATP concentration in rat lung tissue before and during phosgene-induced pulmonary edema. The data showed that a decreased ATP level preceded the onset of edema or increase in lung weight following phosgene exposure. In the present study, the severe pulmonary edema demonstrated the decreased clearance in the lung, suggesting dysfunction of the Na^+/K^+-ATPase. However, after extraction from the lungs in mice exposed to phosgene, the ATPase activities in vitro were much higher than those in the control group. As sufficient ATP was added to the enzyme reaction system, the increased ATPases activities showed that there was no inhibition of ATPase protein expression, indicating the lack of ATP may be the reason for dysfunction of the Na^+/K^+-ATPase. This hypothesis was confirmed by the immediate decrease in the concentration of ATP in lungs exposed to phosgene (Fig. 3A).

ATP is produced in mitochondria through the respiratory chain. Further results (Fig. 3B) showed that there was a decrease in the mitochondrial RCR, indicating that phosgene impaired mitochondrial respiration, resulting in decreased ATP production and finally leading to dysfunction of the ATPases. In physiological conditions, mitochondrial respiratory is also the main source of ROS [28]. In pathological conditions, more ROS are generated due to the disruption of mitochondrial respiration, resulting in oxidative stress [29]. Some reports and our previous studies have demonstrated the occurrence of oxidative stress after phosgene exposure [15,30,31]. In the present study, the oxidative stress product (MDA) increased and

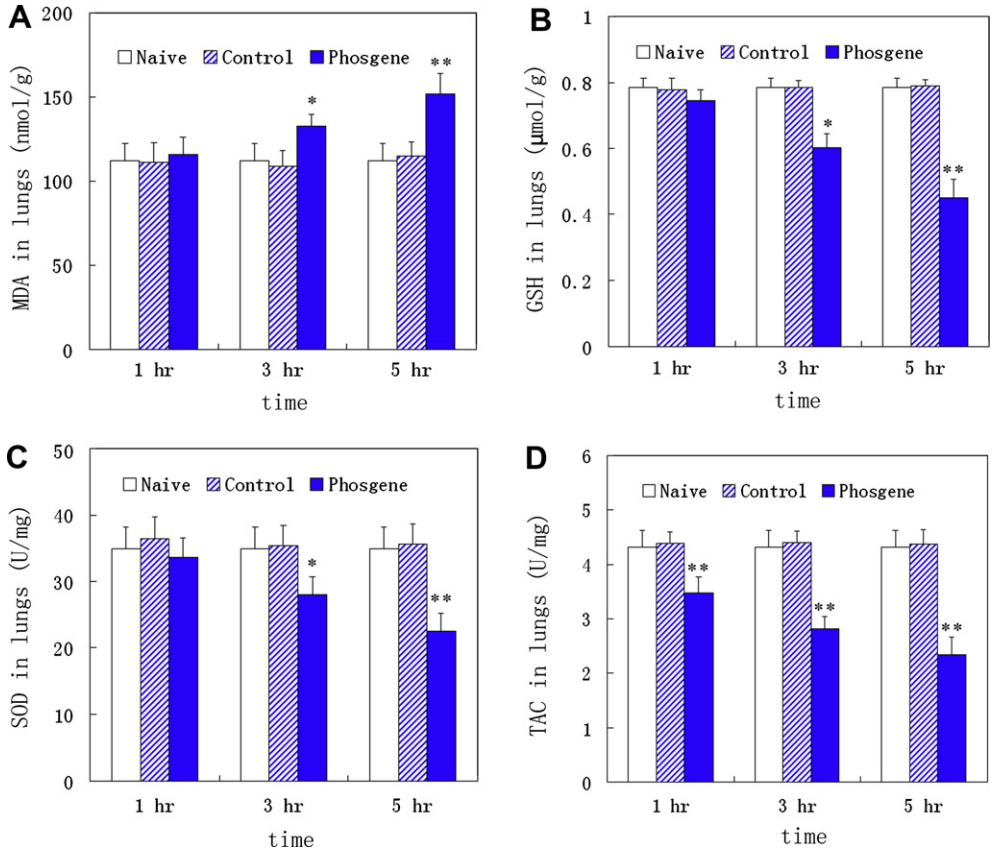

Fig. 4. MDA, GSH, SOD, and TAC in lungs. The content of MDA (A), GSH (B), and activities of SOD (C), TAC (D) in lungs were determined at 1, 3, and 5 h after phosgene exposure. Naïve, untreated group. Control, control group for phosgene. Phosgene, phosgene exposure group. Data are presented as means ± SD. $n = 10$, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* Control.

some key antioxidants (GSH, SOD, and TAC) decreased significantly, demonstrating the phosgene-induced oxidative stress in the lungs. The occurrence of oxidative stress also confirms the impairment of mitochondrial RCR induced by phosgene.

In summary, many studies have demonstrated that the Na^+/K^+-ATPase plays a critical role in clearing pulmonary edema and the inhibition of Na^+/K^+-ATPase protein expression has been found in many other pulmonary edema models. Little is known about the role of the Na^+/K^+-ATPase in phosgene-induced pulmonary edema. We found that after phosgene exposure, even the decreased clearance of edema demonstrated the dysfunction of Na^+/K^+-ATPase, the in vitro enzyme study showed that there was increase in the activity of the Na^+/K^+-ATPase. Further investigation showed that the ATP content and mitochondrial RCR in the lungs decreased significantly. These results suggest that mitochondrial respiration is the target of phosgene. The disruption of mitochondrial respiration results in the production of less ATP as well as more ROS. Insufficient ATP leading to dysfunction of the ATPases contributes to edema, while more ROS induce oxidative stress in the lungs. This may be a new mechanism of phosgene-induced pulmonary edema. The details should be the subject of future study.

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