Postexposure Administration of a β_2 -Agonist Decreases Chlorine-Induced Airway Hyperreactivity in Mice

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Exposure to chlorine (Cl_2) damages airway and alveolar epithelia, resulting in acute lung injury and reactive airway dysfunction syndrome. We evaluated the efficacy and mechanisms by which arformoterol, a long-term β_2 -agonist, administered after exposure, mitigated the extent of this injury. Exposure of C57BL/6 mice to 400 ppm Cl_2 for 30 minutes increased respiratory system resistance and airway responsiveness to aerosolized methacholine (assessed by FlexiVent) up to 6 days after exposure, and decreased Na^+ -dependent alveolar fluid clearance (AFC). Inducible Nitric Oxide Synthase (iNOS) knockout mice developed similar degrees of airway hyperreactivity as wild-type controls after Cl_2 exposure, indicating that reactive intermediates from iNOS do not contribute to Cl_2 -induced airway dysfunction in our model. Intranasal administration of arformoterol mitigated the Cl_2 effects on airway reactivity and AFC, presumably by increasing lung cyclic AMP level. Arformoterol did not modify the inflammatory responses, as evidenced by the number of inflammatory cells and concentrations of IL-6 and TNF- α in the bronchoalveolar lavage. NF- κB activity (assessed by p65 Western blots and electrophoretic mobility shift assay) remained at control levels up to 24 hours after Cl_2 exposure. Our results provide mechanistic insight into the effectiveness of long-term β_2 -agonists in reversing Cl_2 -induced reactive airway dysfunction syndrome and injury to distal lung epithelial cells.

Keywords: alveolar fluid clearance; cAMP; iNOS; NF- κB ; lung injury

Chlorine (Cl_2) is a highly irritating and reactive gas produced in large quantities throughout the world. Its extensive industrial and domestic usage (in the form of bleach) poses significant concerns, with potential occupational and environmental hazards. Even casual exposure to Cl_2 exacerbates the clinical outcome of a number of pulmonary diseases, including asthma and chronic obstructive pulmonary disease (1, 2). In addition, spillage of Cl_2 in the environment during transportation and industrial accidents or acts of terrorism may result in significant morbidity and mortality to humans (3–5).

The primary target of inhaled Cl_2 is the respiratory system: exposure of animals or humans to high concentrations of Cl_2 results in acute lung injury, which may progress to acute respiratory failure (4, 6, 7). Theoretical calculations show that Cl_2

CLINICAL RELEVANCE

We demonstrate that postexposure administration of a long-term β_2 -agonist (arformoterol tartrate) in mice exposed to chlorine (Cl_2), in concentrations likely to be encountered during industrial accidents or deliberate release of Cl_2 , mitigates airway hyperreactivity and the decrease of alveolar fluid clearance across distal lung spaces by restoration of lung cAMP levels depleted by exposure to Cl_2 .

molecules react with low-molecular weight oxidant scavengers (ascorbate, urate, and reduced glutathione) present in the lung epithelial lining fluid (8). Once these antioxidants are depleted, Cl_2 converts to hypochlorous acid (HOCl) and reacts with biomolecules in the epithelial lining fluid. The main targets of HOCl and its conjugate base (hypochlorite) are free functional groups of proteins and amino acids, resulting in post-translational modifications (oxidation, chlorination), which may alter protein function (9–11) and the generation of secondary intermediates (chloramines), with considerable reactivity of their own (8). Previously, we demonstrated that exposure of rats and mice to Cl_2 (400 ppm for 30 min) leads to extensive injury to distal lung compartments, resulting in increased alveolar permeability, decreased ability of alveolar epithelial cells to actively transport sodium ions and clear fluid, and damage to the pulmonary surfactant system (6, 9, 10). In addition, airway hyperresponsiveness, termed as reactive airway dysfunction syndrome (RADS), after Cl_2 inhalation has also been reported, and asthma-like symptoms can persist for long periods after Cl_2 exposure (7, 12–14).

Presently, the reactive intermediates responsible for lung injury after the cessation of Cl_2 exposure have not been identified. Furthermore, there are no effective countermeasures, which, when administered after exposure, may diminish Cl_2 -induced injury to the alveolar and airway epithelia. Here, we demonstrate that postexposure administration of a long-term β_2 -agonist (arformoterol tartrate) in mice exposed to Cl_2 , in concentrations likely to be encountered during industrial accidents or deliberate release of Cl_2 (2), mitigates airway hyperreactivity and the decrease of alveolar fluid clearance (AFC) across distal lung spaces by restoration of lung cyclic AMP (cAMP) levels, depleted by exposure to Cl_2 .

MATERIALS AND METHODS

Animals

C57BL/6 male mice (8 wk old; 20 gm body weight) were purchased from Charles River Laboratories (Wilmington, MA). Inducible nitric oxide synthase (iNOS) knockout mice (iNOS^{-/-}; strain B6.129P2-Nos2^{tm1Lau/J}) were purchased from Jackson Laboratory (Bar Harbor, Maine). All experimental procedures involving animals were approved by the University of Alabama at Birmingham institutional animal care and use committee.

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Exposure of Mice to Cl₂ Gas

Wild-type and iNOS^{-/-} mice were exposed to Cl₂ gas (400 ppm) in a cylindrical glass chamber for 30 minutes, as previously described (6, 9). At the end of the exposure, they were returned to room air. Mice were anesthetized with 3.5% isoflurane and arformoterol (10 ng in 0.1 ml saline/20 g body weight; brovana; provided by Sepracor, Inc., Marlborough, MA) was instilled drop-wise in the external nares at 10 minutes and every 24 hours after exposure. In some cases, the instillate contained a mixture of arformoterol and the selective β_2 adrenergic antagonist, butoxamine (100 μ g/20 g body weights; Sigma-Aldrich, St. Louis, MO).

Respiratory System Mechanics

Mice were mechanically ventilated and challenged with increasing concentrations of methacholine, as described previously (15). Briefly, mice were anesthetized with diazepam (17.5 mg/kg) and ketamine (450 mg/kg), intubated, connected to a ventilator (FlexiVent; SCIREQ, Montreal, PQ, Canada), and ventilated at a rate of 160 breaths per minute at a tidal volume of 0.2 ml, with a positive end-expiratory pressure of 3 cm H₂O. Total respiratory system resistance and elastance were recorded continuously, as previously described (15). Baseline was set via deep inhalation. Increasing concentrations of methacholine chloride (0–50 mg/ml; Sigma-Aldrich) were administered via aerosolization within an administration time of 10 seconds. Airway responsiveness was recorded every 15 seconds for 3 minutes after each aerosol challenge. Broadband perturbation was used, and impedance was analyzed via constant phase model.

Lung Histology

Section from the parahilar regions of the lung were prepared as previously described (16), and stained with hematoxylin and eosin.

Lung cAMP Measurement

cAMP concentrations were measured using a cAMP Enzyme Immunoassay kit (Assay Designs, Ann Arbor, MI) in lung homogenates according to the producer's protocol.

AFC

Na⁺-dependent AFC of an isotonic solution instilled intratracheally into the lungs of anesthetized and ventilated mice was measured at 1 hour after Cl₂ exposure, as described previously (17, 18).

Bronchoalveolar Lavage

Mice were lavaged as previously described (18).

Western Blot and Electrophoretic Mobility Shift Assay for NF- κ B

Nuclear extracts from lung tissues were prepared as previously described (19), with minor modifications (*see the online supplement for details*).

Cytokine Measurements

Mice were killed and lavaged as previously described (20). IL-6 and TNF- α were measured in the total lung homogenate and the cell-free bronchoalveolar lavage (BAL) fluid of separate animals using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN) and the appropriate antibodies.

Statistical Analysis

Data were analyzed using Origin 7.0 (Northampton, MA). Results are reported as group means ($\pm$ SE). A one-way ANOVA was used to determine differences among group means. Differences at a *P* value less than 0.05 were considered significant.

RESULTS

Cl₂-Induced Increases of Respiratory System Resistance and Elastance Are Mitigated by Arformoterol

For measurements of airway reactivity, mice were mechanically ventilated and challenged with nebulized methacholine

(0–50 mg/ml) while ventilated with a FlexiVent. As shown in Figures 1A–1C, mice exposed to Cl₂ had significantly higher respiratory system resistance both before and after challenge with methacholine even at 6 days after Cl₂ exposure. Intranasal instillation of arformoterol (but not of saline) significantly mitigated the Cl₂-induced increase of resistance (Figures 1A–1C; Table 1), whereas it had little effect on the resistance of control air-breathing mice (*see* Figure E1 in the online supplement). Cl₂ exposure also significantly increased elastance, and arformoterol mitigated the increase of elastance as well, especially after 6 days (Figures 1D–1F; Table 1).

Histological Evaluation of Cl₂-Induced Lung Injury and Effect of Arformoterol

Lung sections of parahilar regions were examined from control air-breathing mice and saline- or arformoterol-treated, Cl₂-exposed mice after 24 hours. As shown in Figures 2A–2C, Cl₂ exposure caused significant airway injury, especially in the epithelial cells, ranging from cilia disappearance and epithelium sloughing to complete detachment. In the alveolar compartment, increased numbers of inflammatory cells were noted in a number of alveolar sections; however, no overt morphological injury was noted (Figures 2D–2F), despite the presence of considerable functional impairment (*see* subsequent description). Treatment of mice with arformoterol after Cl₂ exposure did not alter the extent of morphological injury in either compartment.

Arformoterol Increased cAMP in the Lungs of Cl₂-Exposed Mice

We have reported that there is a transient decrease of amiloride-sensitive AFC across the distal lung spaces after Cl₂ exposure, indicating functional inhibition of epithelial sodium channels in alveolar epithelial cells (9). Because activation of myosin light-chain kinase causes direct airway smooth muscle contraction, and both myosin light-chain kinase and epithelial sodium channels are regulated by cAMP and protein kinase A (PKA) (21, 22), we hypothesized that Cl₂ exposure decreases cellular cAMP content and/or PKA activity, which impairs airway smooth muscle relaxation. As shown in Figure 3A, Cl₂ exposure significantly decreased lung cAMP concentrations at 1, 4, and 24 hours after exposure. In addition, instillation of arformoterol significantly replenished the decreased cAMP concentration at 1, 4, and 24 hours, which correlated with the previously mentioned mitigation of increased respiratory system resistance and elastance after instillation of arformoterol at 1 and 24 hours after Cl₂ exposure (Figure 1).

In another set of experiments, we treated mice with arformoterol immediately after Cl₂ exposure, and measured their AFC 1 hour later. As shown in Figure 3B, compared with the value of 28.2 ($\pm$ 0.9)% (percent of instilled volume per 30 min; *n* = 18) in air-breathing control mice, Cl₂ exposure significantly decreased the AFC to 15.6 ($\pm$ 1.5)% (*P* < 0.05 compared with air control; *n* = 7). Although arformoterol treatment did not change the AFC (26.3 $\pm$ 0.6%; *n* = 5) in air-breathing mice, mice instilled with arformoterol after Cl₂ exposure had near-normal AFC values of 26.1 ($\pm$ 2.1)% (*n* = 10), most likely caused by an increase of cAMP in distal lung spaces.

Cl₂-Induced Lung Injury and Inflammation Is Not Mitigated by Arformoterol

As shown in Figures 4A–4C, Cl₂ exposure caused injury and inflammation in the respiratory system, as indicated by increased protein concentration, as well as increased number of inflammatory cells in the BAL. Protein increased from 0.13

Figure 1. Chlorine (Cl₂) exposure increases respiratory resistance and elastance, which are mitigated by arformoterol (Arfor). (A–C) Cl₂ significantly increased both baseline resistance and airway responsiveness to methacholine at 1 hour, 24 hours, and 6 days after exposure as compared with that of the air-breathing, saline control animals. Arfor significantly decreased resistance at the corresponding time points after exposure. (D–F) Cl₂ exposure significantly increased elastance as compared with air-breathing, saline control mice. Arfor decreased elastance at 6 days after exposure. Squares, Cl₂/saline ($n = 10$); circles, Cl₂/Arfor ($n = 10$); triangles, air/saline ($n = 6$). Mice were exposed to 400 ppm Cl₂ for 30 minutes, then intranasally administered either saline or Arfor as described in MATERIALS AND METHODS (* $P < 0.05$, compared with Cl₂/Arfor group; # $P < 0.05$, compared with saline control). Values are means ($\pm$ SEM) for the indicated number of mice. E, elastance; R, resistance.

(± 0.01) mg/ml in air-breathing control mice to $0.29 (\pm 0.05)$ mg/ml ($P < 0.05$; $n = 6$) at 30 minutes after Cl₂ exposure, and remained higher than normal after 6 days (0.20 ± 0.01 mg/ml; $n = 6$). Right after exposure, detached epithelial cells could be

seen in the BAL (data not shown), as previously reported (6, 12). However, the increase in the number of inflammatory cells in the BAL did not occur until 2 hours after exposure. At 24 hours after exposure, inflammatory cell numbers were significantly

TABLE 1. AIRWAY RESISTANCE AND ELASTANCE AFTER CHLORINE EXPOSURE AND ADMINISTRATION OF ARFORMOTEROL

Time after Exposure/Treatment	R (cm H ₂ O/ml/s)		E (cm H ₂ O/ml)	
	Basal	Peak	Basal	Peak
1 h				
Air/saline	1.08 $\pm$ 0.10	7.40 $\pm$ 1.51	48.74 $\pm$ 4.85	125.63 $\pm$ 14.98
Cl ₂ /saline	2.98 $\pm$ 0.66*	15.50 $\pm$ 2.36*	118.08 $\pm$ 13.93*	363.02 $\pm$ 54.99*
Cl ₂ /arformoterol	1.79 $\pm$ 0.19	7.95 $\pm$ 1.47†	92.49 $\pm$ 29.58	188.08 $\pm$ 65.06
24 h				
Air/saline	1.04 $\pm$ 0.06	3.8 $\pm$ 0.6	45.11 $\pm$ 3	78.32 $\pm$ 9.85
Cl ₂ /saline	2.29 $\pm$ 0.43*	17.75 $\pm$ 2.03*	88.76 $\pm$ 20.78	291.01 $\pm$ 86.54*
Cl ₂ /arformoterol	1.98 $\pm$ 0.28	10.06 $\pm$ 1.23†	69.14 $\pm$ 8.36	162.53 $\pm$ 10.84
6 d				
Air/saline	0.90 $\pm$ 0.07	2.67 $\pm$ 0.71	44.05 $\pm$ 5.42	91.30 $\pm$ 29.42
Cl ₂ /saline	5.26 $\pm$ 1.51*	15.81 $\pm$ 3.80*	135.66 $\pm$ 36.89	239.29 $\pm$ 43.91
Cl ₂ /arformoterol	2.08 $\pm$ 0.58	11.41 $\pm$ 2.33	65.01 $\pm$ 6.36	228.51 $\pm$ 37.08

Definition of abbreviations: Cl₂, chlorine; E, elastance; R, resistance.

"Basal" indicates values at 0 mg/ml methacholine; "Peak" indicates values at 50 mg/ml methacholine. Values are expressed as means $\pm$ SEM; n values are shown in Figure 1.

* $P < 0.05$ as compared with corresponding air/saline control.

† $P < 0.05$ as compared with corresponding Cl₂/saline group.

Figure 2. Lung histology after Cl₂ exposure and treatment of Arfor: Airway cross-sections of mice breathing air (A), or exposed to Cl₂ treated with saline (B) or Arfor (C), and killed at 24 hours after exposure. Significant injury to both groups of Cl₂-exposed mice is present. Cross-sections of alveolar compartments from mice breathing air (D), or exposed to Cl₂ treated with saline (E) or Arfor (F), and killed at 24 hours after Cl₂. Sections from Cl₂-exposed mice show increased granularity, but no overt injury. Sections are representatives of three control, five Cl₂/saline-, and six Cl₂/Arfor-treated mice. Stain used was hematoxylin and eosin. Scale bars, 50 μ m.

increased for all three major cells types (lymphocytes, neutrophils, and macrophages), with the increase of neutrophils being the most prominent (from $2.5 \pm 0.3\%$ to $53.4 \pm 3.1\%$; $n = 5$). No significant changes in either IL-6 or TNF- α concentrations were observed in total lung homogenates up to 24 hours after Cl₂ exposure (data not shown). In contrast, levels of both IL-6 and TNF- α were significantly elevated in the BAL at 2 hours after exposure. However, at 24 hours after exposure, IL-6 and TNF- α returned to near control levels (Figures 5A and 5B). After Cl₂, arformoterol administration did not prevent the increase of protein and cell counts in the BAL. Although there was a decrease of IL-6 and TNF- α in the BAL after arformoterol treatment at 2 hours after Cl₂ exposure, these differences were not statistically significant. Surprisingly, no significant changes of NF- κ B p65 expression (data not shown) or NF- κ B nuclear binding (Figure 5C and Figure E3) were observed in lung homogenate at 30 minutes, 2 hours, or 24 hours after Cl₂ exposure.

Lack of iNOS Did Not Prevent the Onset of Airway Hyperreactivity Induced by Cl₂

It has been reported that administration of 1,400W, a specific inhibitor of iNOS, completely abrogated the Cl₂-induced increase of airway methacholine responsiveness in 5 minutes of 400 ppm Cl₂-exposed A/J mice (12), suggesting that alterations in airway reactivity by Cl₂ are mediated at least in part by reactive intermediates generated by iNOS. However, the fact

that we did not detect increased NF- κ B activity, a major regulator of iNOS, led us to re-examine the possible contribution of iNOS in Cl₂-induced airway hyperreactivity. Therefore, we exposed iNOS^{-/-} mice to 400 ppm Cl₂ for 30 minutes, and measured their airway reactivity after 24 hours. As shown in Figure 6, iNOS^{-/-} mice exhibited significantly higher basal resistance and responsiveness to methacholine at 24 hours after Cl₂ exposure as compared with air breathing control iNOS^{-/-} mice. Both basal resistance and responsiveness to methacholine were similar to those of the wild-type mice at 24 hours after Cl₂ exposure (Figure 1B).

DISCUSSION

Our present study demonstrates that exposure of C57BL/6 mice to 400 ppm Cl₂ for 30 minutes damages airways, resulting in epithelial cell injury and detachment, and functional changes, including increased baseline respiratory system resistance and elastance, and airway hyperresponsiveness to methacholine. Although, morphologically, no significant damage was observed in the alveolar compartment, functional injury occurred as expressed by increased permeability of the distal lung to plasma proteins and diminished ability of lung epithelial cells to clear alveolar fluid, secondary to active Na⁺ transport. Previously, we have shown significant injury to the alveolar epithelium of rabbits exposed to hyperoxia before the onset of overt

Figure 3. Changes in lung cAMP levels in mice exposed to Cl₂ and instilled with saline or Arfor. (A) Lung cAMP concentration in total lung homogenate was measured by ELISA at the indicated time points after Cl₂ exposure with or without treatment with Arfor (* $P < 0.05$, compared with corresponding Cl₂-exposed group; # $P < 0.05$, compared with control; $n = 4-8$ for each group). (B) Alveolar fluid clearance (AFC) was measured in control air-breathing, saline-treated mice; air-breathing mice treated with Arfor for 1 hour; mice exposed to 400 ppm Cl₂ for 30 minutes, then returned to room air and treated with vehicle (saline) for 1 hour; or mice exposed to Cl₂, and then returned to room air and treated with Arfor for 1 hour (* $P < 0.05$, compared with Cl₂/saline; # $P < 0.05$, compared with control; $n = 18, 5, 7$, and 10 for air/saline, air/Arfor, Cl₂/saline, and Cl₂/Arfor groups, respectively).

Figure 4. Cl₂ exposure induced lung injury and inflammation. (A) Protein concentration in bronchoalveolar lavage (BAL) fluid at different time points after Cl₂ exposure and treatment with Arfor. (B) Total live cell counts and (C) cell differentials for lymphocytes, macrophages, and neutrophils in BAL fluid at different time points after Cl₂ exposure and treatment with Arfor (**P* < 0.05, compared with control; *n* = 6 from two independent experiments for each time point).

morphological injury, indicating that functional measurements are more sensitive indices of injury (23, 24). In addition, iNOS^{-/-} mice exposed to Cl₂ developed similar increases of airway reactivity after exposure to Cl₂, indicating that reactive intermediates from iNOS did not contribute to the onset of airway hyperreactivity in the current experimental conditions. These functional changes were accompanied by decreased levels of lung cAMP and the onset of inflammation, as shown by higher levels

of TNF- α and IL-6 and the number of inflammatory cells in the BAL fluid. NF- κ B activity in lung tissues, assessed by p65 levels and electrophoretic mobility shift assay, remained unchanged. Intranasal administration of arformoterol after Cl₂ exposure decreased airway hyperreactivity, presumably by replenishing cellular cAMP and dilating airway smooth muscle. This protective effect was most prominent at early phases (2–24 h) after Cl₂ exposure.

Figure 5. Cytokine levels and lung NF- κ B activity of Cl₂-exposed mice. (A) IL-6 and (B) TNF- α in BAL fluid measured by ELISA at different time points after Cl₂ exposure and treatment with Arfor (**P* < 0.05, compared with control; *n* = 6 from two independent experiments for each time point). (C) Nuclear extract from lung homogenate of control, Cl₂-exposed, and Cl₂-exposed mice treated with Arfor were subjected to electrophoretic mobility shift assay at 30 minutes, 2 hours, and 24 hours after Cl₂ exposure. Mice with intranasally instilled LPS (2 μ g/g body weight) for 24 hours served as positive control. Densitometric analysis of the gel is shown in Figures E3. Results are gel images of typical experiments that were repeated at least twice. Con, control.

Figure 6. Airway hyperreactivity of inducible nitric oxide synthase (iNOS)^{-/-} mice exposed to Cl₂. iNOS^{-/-} mice were exposed to 400 ppm Cl₂ for 30 minutes; respiratory system resistance was measured at 24 hours after exposure. Air-breathing iNOS^{-/-} mice served as control. Values of respiratory resistance of wild-type (iNOS^{+/+}) mice at 24 hours after Cl₂ exposure are also shown for comparison (**P* < 0.05, compared with control; *n* = 8 for each group of knockout mice; *n* = 10 for wild-type mice).

Our results are generally consistent with those from the studies of Martin and colleagues (12), who showed that exposure of mice to Cl₂ resulted in increased airway reactivity, with the following important caveats: our data indicate that exposure to Cl₂ increased both baseline resistance and airway responsiveness to methacholine, and these changes persist up to 6 days after exposure. In contrast, Martin and colleagues (12) reported no change in baseline resistance. This is an important issue, as an increase in basal airway resistance will cause a significant increase in the work of the respiratory system. Another difference is that, whereas Martin and colleagues reported that elastance responsiveness to methacholine was much affected, and that of the resistance was not significantly changed at any time points after Cl₂ exposure (12, 25), we found a time-dependent increase in both resistance and elastance responsiveness, with resistance being affected more than elastance. The major difference in the experimental design between these two studies is the duration of the exposure (5 min versus 30 min). Thus, our results suggest that longer exposures to Cl₂ will cause more severe injury. One probable explanation is that longer duration of Cl₂ exposure resulted in a more extensive shedding of epithelial cells, which would allow more extensive exposure of afferent nerve endings and subsequent release of neuropeptides. This would result in increased bronchoconstriction, mucus secretion, and endothelial leakage, leading to edema of airway wall and extravasation of plasma into airway lumen (26).

In the present study, we report, for the first time, a transient but significant decrease of lung cAMP concentration after exposure of mice to Cl₂. Previously, we found a transient decrease of AFC in C57BL/6 mice after 30 minutes of 400 ppm Cl₂ exposure; the time course of the change of AFC correlated well with the time course of the decrease and recovery of cAMP (19). The extent to which a decrease of total lung cAMP contributes to the development of RADS is unknown. However, our results show that administration of arformoterol replenishes lung cAMP after Cl₂ exposure and mitigates RADS. This provides the rational basis for the use of β_2 -agonists for the treatment of Cl₂-induced lung injury.

The mechanisms by which Cl₂ decreases lung cAMP are not clear. Decreased levels of endogenous catecholamines, down-

regulation of β_2 receptors and/or changes of their binding affinity, changes in the coupling of β_2 receptors with G proteins, or decreased adenylate cyclase activity could contribute to a decrease of cellular cAMP content. Previous studies have shown that reactive species decrease β_2 adrenergic receptor signaling. For example, rat lung β_2 adrenergic receptor density was decreased by lipid peroxidation (27). Ozone, another common inhalant oxidant, has been reported to increase the pD₂ value of the β_2 adrenergic receptors of guinea pig tracheal smooth muscle (28), and affect G protein coupling or downstream pathways in guinea pig lung without influencing receptor density or sensitivity (28). Hypochlorite, the major hydrolysis product of Cl₂, inhibits the Gs-coupled adenylate cyclase of the β_1 -adrenoreceptor in the heart (29), and damages G protein level in cat esophageal smooth muscle. In addition, G protein receptor kinase-2, a mediator of desensitization of β_2 -adrenoreceptor, is significantly up-regulated in the lungs of mice after challenge with ovalbumin (15, 30). Finally, there is also the possibility that the decrease of lung cAMP is caused by the damage of circulating catecholamine, due to its direct reaction with HOCl (31), as our results showed that arformoterol, an exogenous agonist, could replenish lung cAMP after Cl₂ exposure.

Cl₂-induced lung injury initiates a series of events leading to inflammation. However, the relationship between inflammation and the initiation and exacerbation of airway hyperresponsiveness is in question, with recent work suggesting that airway hyperreactivity is dissociated from cellular inflammation (32). Surprisingly, no significant changes of NF- κ B activation or IL-6 and TNF- α concentration were observed in the total lung homogenate of Cl₂-exposed mice in our studies, although transient increases in IL-6 and TNF- α were observed in the BAL fluid. These data are in agreement with those of previous reports showing that oxidants, including chloramines, specifically inhibit NF- κ B pathway via stabilization of I κ B (33, 34). It is also possible that NF- κ B was activated only in epithelial cells, which are the critical cell type in the transduction of proinflammatory signals (33, 35). However, we were unable to collect enough detached cells to measure NF- κ B activity. On the other hand, our results show a clear difference from the ovalbumin-induced murine asthma model, in which NF- κ B DNA binding is significantly increased in total lung homogenate (36).

One major regulation target of NF- κ B is iNOS; thus, the lack of NF- κ B activation raised the question as to the role that iNOS played in Cl₂-induced airway hyperreactivity, which has been under debate. Although Martin and colleagues clearly showed that inhibition of iNOS by 1,400W caused attenuation of Cl₂-induced airway changes in methacholine responsiveness in a 5-minute exposure A/J mouse model (12), in another report, iNOS^{-/-} mice were actually more susceptible to ozone-induced lung injury (37), and equally susceptible to hyperoxia (38), as compared with wild-type control animals. In our experiment of iNOS^{-/-} mice exposed to 400 ppm Cl₂ for 30 minutes, both baseline resistance and airway responsiveness to methacholine were increased to a similar extent as those in the Cl₂-exposed wild-type mice. It is not known if different exposure time, which may lead to different injury patterns, caused the loss of protective effect by inhibition of iNOS.

In summary, we have shown that acute exposure to 400 ppm Cl₂ for 30 minutes resulted in increased airway reactivity in mice that lasts up to 6 days after exposure. The underlying mechanisms include a transient decrease of cellular cAMP and a persistent neutrophilic inflammation. Arformoterol could significantly decrease airway hyperreactivity by replenishing cellular cAMP and directly relaxing airway smooth muscle, and therefore provides a valid treatment for the Cl₂-induced RADS. Its potential anti-inflammatory effect necessitates further exploration.

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