Chlorine Induces the Unfolded Protein Response in Murine Lungs and Skin

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Chlorine (Cl₂) is an important industrial chemical. Accidental full body exposure to Cl₂ poses an environmental, occupational, and public health hazard characterized mainly by injury to the lung, skin, and ocular epithelia. The cellular mechanisms underlying its acute toxicity are incompletely understood. This study examined whether whole body exposure of BALB/c mice to Cl₂ in environmental chambers leads to the up-regulation of the unfolded protein response (UPR) in their lungs and skin. Shaved BALB/c mice were exposed to a sublethal concentration of Cl₂ (400 ppm for 30 min) and returned to room air for 1 or 6 hours and killed. IL-6 and TNF- α were increased significantly at 1 and 6 hours after Cl₂ exposure in the lungs and at 6 hours in the skin. These changes were accompanied by increased UPR signaling (i.e., activation of protein kinase RNA-like endoplasmic reticulum kinase, inositol-requiring enzyme 1 α , and activating transcription factor 6 α) at these time points. The expression of hepcidin, which regulates tissue accumulation and mobilization of iron, was increased in the skin and lungs of Cl₂-exposed mice. The data shown herein indicate for the first time the up-regulation of UPR signaling and hepcidin in the skin and lungs of Cl₂-exposed mice, which persisted when the mice were returned to room air for 6 hours.

Key words: hepcidin; inflammation; unfolded protein response; TNF- α ; IL-6

Chlorine gas (Cl₂) is an important industrial chemical (1). In the United States, Europe, and other parts of the world, several million tons of Cl₂ are produced annually. Its accidental release in the atmosphere may cause significant mortality and morbidity to humans and animals. Its exposure primarily leads to pulmonary edema and to restrictive and obstructive lung diseases. Other clinical symptoms include dyspnea, cough, pneumonitis, cyanosis, nausea, vomiting, and loss of consciousness (2–6).

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CLINICAL RELEVANCE

Exposure to chlorine constitutes a major public threat. We show that exposure to chlorine up-regulates the unfolded protein response signaling and hepcidin in the skin and lungs of Cl₂-exposed mice, which persisted when the mice were returned to room air for 6 hours. Our findings form the rational basis for development of selective agents to ameliorate Cl₂-induced morbidity and mortality

Cl₂ is a strong oxidant. Its exposure rapidly augments reactive oxygen species (ROS) generation and depletes tissue antioxidants such as glutathione and ascorbic acid (7–9). It manifests its toxicity via oxidative damage to lung epithelial cells, leading to egress of plasma proteins from the vascular to the alveolar spaces, production of inflammatory mediators, influxes of neutrophils into lung tissue, and reactive airway disease syndrome (10–13). Systemic injury, characterized by inflammation and inactivation of nitric oxide synthase, has also been reported (14). Postexposure administration of ascorbate and deferoxamine in mice exposed to 600 ppm Cl₂ for 45 minutes decreased mortality, injury to the blood–gas barrier, and lipid peroxidation (2, 9). Similarly, postexposure administration of AEOL10150, a peroxynitrite scavenger, in Cl₂-treated mice decreased airway hyperresponsiveness, inflammation, and 4-hydroxynonenal level, a marker of lipid peroxidation (15).

Many groups have investigated the molecular mechanisms of Cl₂ toxicity in humans and in experimental animals, with the goal of developing novel and molecular targeted antidotes against Cl₂ toxicity. In this regard, cyclic AMP regulating enzyme, type 4 phosphodiesterase (16), NF- κ B, neurokinin 1 receptor (17), nuclear factor (erythroid-derived 2)-like 2 (18, 19), and extracellular signal-regulated kinases-dependent disruption of amiloride-sensitive Na⁺ channels in alveolar Type I and II cells (7) were shown to be involved in the molecular pathogenesis of acute Cl₂ toxicity; inhibitors of these molecular targets afforded some protection against Cl₂-induced pulmonary damage. The resulting inflammatory response plays an important role in the potentiation of Cl₂-induced injury once animals are returned to room air (9, 11, 12, 17).

One important pathway associated with the onset of inflammation is the unfolded protein response (UPR) signaling pathway. UPR signaling contributes to the repair of misfolded proteins and protects against the degradation of unfolded proteins by inducing expression of a number of chaperone proteins after endoplasmic reticulum (ER) stress (20). However, prolonged activation of this pathway underlies pathogenesis of the inflammatory response (21). Because the tissue-damaging effects of Cl₂ are known to be initiated by reactive species (1), we decided to test if Cl₂ exposure affects UPR responses.

UPR signaling is activated through three ER resident proteins (inositol-requiring enzyme 1 α [IRE1 α], protein kinase

RNA-like endoplasmic reticulum kinase [PERK], and activating transcription factor 6 α [ATF6 α]) in a phosphorylation- or proteolysis-dependent manner (see Figure E1 in the online supplement). The activation of these three branches of UPR collectively regulates downstream events, including splicing of Xbp1 mRNA and expression of UPR target genes such as C/EBP homology protein (CHOP), glucose-regulated protein (GRP)94, and GRP78, a UPR regulator and ER chaperone (22). Reactive species are thought to be one of multiple triggers associated with UPR signaling pathways. In this regard, Winter and colleagues showed that bleach, which mainly contains hypochlorous acid, activates a redox-regulated chaperone by oxidative protein unfolding in bacteria (23). This *in vitro* study established the rational basis for further testing possible up-regulation of UPR in the skin and lungs of mice exposed to Cl₂ gas.

We show that whole body exposure of mice to sublethal concentration of Cl₂ (400 ppm for 30 min) induces inflammatory response in the lungs and skin once the mice resume air breathing. In both of these organs, similar but not identical alterations in inflammatory responses were noted, which were associated with the activation of UPR. We report for the first time that UPR signaling-regulated tissue iron metabolism may be disrupted by Cl₂ exposure. These findings help to explain why postexposure administration of deferoxamine, a free iron chelator, in mice exposed to lethal concentrations of Cl₂ decreased mortality and lung injury (2).

MATERIALS AND METHODS

Reagents

The primary antibodies used in this study were Ki67 (sc-15402; Santa Cruz, Dallas, TX), p-PERK (sc-32577; Santa Cruz), PERK (3192 s; Cell Signaling, Danvers, MA), phosphorylated eukaryotic initiation factor- α (p-eIF2 α) (9721; Cell Signaling), eIF2 α (9722; Cell Signaling), CHOP (2895, Cell Signaling), ATF6 α (sc-22799; Santa Cruz), GRP78 (sc-1050; Santa Cruz), GRP94 (2104, Cell Signaling), β -actin (A-5316; Sigma, St. Louis, MO). Primers as described in Table E1 in the online supplement were synthesized by Invitrogen (Grand Island, NY). An In Situ Cell Death Detection kit (Catalog no. 11684795910) was purchased from Roche Diagnostics (Indianapolis, IN).

Animals

BALB/c female mice (20–25 g) were purchased from Charles River Laboratories (Wilmington, MA). All experimental procedures involving animals were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Exposure of Mice to Cl₂

Mice were placed in an environmental chamber and exposed to Cl₂ in air (400 ppm for 30 min) as previously described (2). Before exposure, the fur was removed (24) (see METHODS in the online supplement for more details) so the unprotected skin would be exposed to Cl₂. Immediately after the exposure, mice were returned to room air, where they breathed ambient air for 1 or 6 hours. Food and water were provided *ad libitum*.

Western Blot

Western blots were performed as previously described (25). The integrated density of bands was measured with ImageJ software (<http://rsb.info.nih.gov/ij/>). Statistical analysis was conducted using Excel 2003.

Immunofluorescence

Immunofluorescence staining was performed as described previously (25).

Immunohistochemistry

Immunohistochemistry staining was performed using EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC Kit (ab94710; Abcam, Cambridge, MA) according to the manufacturer's standard protocol.

Measurements of ROS

ROS levels in lung tissues were measured using the chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, c6827; Invitrogen Life Technologies, Grand Island, NY) as described previously (25).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) measurements were performed according to the manufacturer's standard protocol (27).

Figure 1. Cl₂ induces cytokines expression in the skin and lung of BALB/c mice. Real-time PCR shows enhancement in the cutaneous transcript levels of IL-1 β , TNF- α , and IL-6 (A) and enhancement in the pulmonary transcript levels of IL-1 β , TNF- α , and IL-6 (B). * P < 0.05 and ** P < 0.01 when compared with control. Mean $\pm$ SEM (n = 3).

RT-PCR and Real-Time PCR

RT-PCR was performed as described previously (25). The integrated band densities were measured by Image J software. The cycle number for each gene was chosen within an exponential amplification stage instead of an end point. In each sample, band densities for each gene were normalized by a housekeeping gene. Real-time PCR was performed in iQ SYBR Green PCR Master Mix (170-8880; Bio-Rad, Hercules, CA). A comparative $\Delta\Delta C_t$ method was used in the quantification.

Statistical Analysis

Statistical analysis was performed using the Student's *t* test. A *P* value < 0.05 was considered to be statistically significant.

RESULTS

Effects of Cl₂ Exposure on the Expression of Cytokines in the Skin and Lungs

Exposure of mice to 400 ppm Cl₂ for 30 minutes results in arterial hypoxemia; respiratory acidosis; increased levels of albumin, IgG, and IgM in bronchoalveolar lavage fluid; increased bronchoalveolar lavage fluid surfactant surface tension; and significant histological injury to airway and alveolar epithelia (8). Whole body exposure of BALB/c shaved mice to Cl₂ increased proinflammatory cytokine mRNA levels in the skin and lungs (Figure 1). In the skin, transcriptional expression of IL-1 β was significantly increased at 1 hour but returned to its baseline

Figure 2. Cl₂ alters unfolded protein response (UPR) signaling-related proteins in the skin. (A) Western blot analysis of tissue total protein extract. Bars are means $\pm$ SEM. (B) Real-time PCR shows cutaneous transcript levels of the spliced form of X-box binding protein 1 (Xbp1s), C/EBP homology protein (CHOP), and glucose-regulated protein (GRP)78. In A and B, **P* < 0.05 and ***P* < 0.01 when compared with control. Bars represent mean $\pm$ SEM (*n* = 3). (C) Immunofluorescence staining showing tissue localization and expression of CHOP. White arrows indicate the nuclear localization of CHOP. Original magnification: $\times 20$. (D) Pictures show Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in control and Cl₂-exposed skin. Red arrows indicate the TUNEL-positive cells in the epidermis. Original magnification: $\times 40$. (E) Pictures show Ki67 staining in control and Cl₂-exposed skin. Green arrows indicate Ki67 nuclear staining in basal and super-basal layers of epidermis. In C, D, and E, the region above the dotted line represents epidermis, and the region below this line is dermis. Each staining is representative of three independent skin samples. (F) Histology of the skin. Pictures show H&E staining of skin. Original magnification: $\times 40$. Each picture is representative of three independent skin samples. DER = dermis; EPI = epidermis.

Figure 3. Cl₂ alters unfolded protein response (UPR) signaling-related proteins in the lung. (A) Western blot analysis of tissue total protein extract. Bars are means ± SEM (n = 3) for the indicated proteins. (B) Real-time PCR shows pulmonary transcript levels of Xbp1s, CHOP, and GRP78. In A and B, *P < 0.05 and **P < 0.01. Bars represent mean ± SEM. (C) Immunohistochemical staining shows tissue localization and expression of CHOP and activating transcription factor 6α (ATF6α) in bronchial epithelial cells. Original magnification: ×40. (D) Histology of the lung. Pictures show H&E staining of lung. Original magnification: ×40. Each picture is representative of three independent lung samples. (E) Pictures show TUNEL staining in control and chlorine-exposed lung samples. Red arrows indicate the TUNEL-positive cells in the lung. Original magnification: ×40.

value at 6 hours after exposure. The expression of IL-6 mRNA was up-regulated significantly in a time-dependent manner. However, TNF-α was significantly increased only at 6 hours (Figure 1A). In the lung, IL-1β, TNF-α, and IL-6 were highly induced at 1 and 6 hours (Figure 1B). As compared with their respective controls, IL-6 increased 5- and 70-fold in the skin and lungs, respectively, at 6 hours after exposure. The changes in TNF-α and IL-6 are similar to those reported by Song and colleagues in unshaved Balb/c mice exposed to 400 ppm Cl₂ for 30 minutes and returned to room air (12).

Effects of Cl₂ Exposure on UPR Signaling in the Skin and Lung

In the skin, significant increases in the expression of GRP78 and ATF6α p50 protein levels were seen at 1 and 6 hours after exposure. p-PERK/PERK and ATF6α p90 were elevated at 1 hour but returned to control levels at 6 hours, and CHOP protein levels were increased at 6 hours only (Figure 2A and Figure E2A). Similarly, the mRNA levels of GRP78, spliced form of X-box binding protein 1 (Xbp1s) and CHOP, were increased at 6 hours after exposure (Figure 2B). The expression of p-eIF2α protein, which is known to shut down the overall translation

machinery (20), was not altered in the skin (Figure E2A). ATF6α, a transcription factor that regulates multiple UPR target proteins (20), showed enhanced nuclear expression mostly at 6 hours after exposure (Figure E2B) in epidermis keratinocytes by immunofluorescence. Similarly, CHOP, another UPR-related transcription protein, which regulates apoptosis, was significantly enhanced in Cl₂-exposed epidermis at 1 hour and to a larger extent at 6 hours after exposure (Figure 2C).

Sustained activation of CHOP is also known to induce apoptotic cell death (26). Therefore, we assessed whether Cl₂ exposure induces apoptosis in the skin. Whereas TUNEL-positive cells were rare in the skin of control mice, multiple TUNEL-positive cells were seen in the epidermis, particularly in and around the hair follicles, of Cl₂-exposed mice (Figure 2D). In the skin of control mice, Ki67 staining was seen only in a few basal epidermal cells; however, in Cl₂-exposed mice, Ki67 staining was present in the basal layer of their skin (Figure 2E). These results indicate that Cl₂ exposure increased cell proliferation due to cell death or the onset of inflammation; the latter may lead to UPR up-regulation. Consistent with these findings, increased thickness of the epidermis was observed at 6 hours after exposure (Figure 2F).

Figure 4. Cl_2 enhances transcript and protein levels of iron homeostasis-regulating proteins hepcidin and ferroportin. (A) RT-PCR shows cutaneous transcript levels of hepcidin antimicrobial peptide (Hamp) and Slc40a1. 18 s rRNA was used as loading control. Graphs show statistical analysis of normalized band density of each gene when compared with control. Bars represent means $\pm$ SEM ($n = 3$). (B) Immunofluorescence staining shows the expression of ferroportin and hepcidin in the skin. Original magnification: $\times 40$. The region above the dotted line represents epidermis; the region below this line is dermis. (C) RT-PCR shows pulmonary transcript levels of Hamp and Slc40a1. 18 s rRNA was used as loading control. Graphs show statistical analysis of normalized band density of each gene when compared with control. $*P < 0.05$ when compared with control. Bars represent means $\pm$ SEM ($n = 3$). (D) Immunofluorescence staining shows tissue expression of ferroportin and hepcidin in the lung. Original magnification: $\times 20$. White arrows indicate the expression of ferroportin and hepcidin in bronchial epithelia. White dotted line separates the area of bronchi from alveoli. Each picture is representative of three independent lung samples. ALV = alveoli; BR = bronchi; DER = dermis; EPI = epidermis.

In the lung, Cl_2 exposure resulted in significant increases of p-PERK/PERK and p-eIF2 α protein levels at 1 hour, which returned to control levels at 6 hours after exposure (Figure 3A and Figure E3A). Real-time PCR data showed an increase in the lung GRP78 mRNA at 1 hour (Figure 3B). Significant increases in the nuclear localization of CHOP and ATF6 α were observed in bronchial epithelial cells (which encountered higher concentrations of Cl_2 as compared with alveolar epithelial cells) at 6 hours after Cl_2 exposure (Figure 3C). GRP78, a cytoplasmic protein, was also elevated in bronchial epithelial cells (Figure E3B). These findings are consistent with up-regulation of two

branches of the UPR signaling (Figure E1) in the lungs, and more specifically in bronchial epithelial cells, after Cl_2 exposure.

Because UPR signaling is intricately regulated by ROS, we also determined whether Cl_2 exposure enhanced lung ROS levels using an oxidant-sensitive probe (CM-H2DCFDA) that enters cells and remains in their cytoplasm. Increased green fluorescence, indicative of higher levels of ROS, was seen in lung tissues from mice exposed to Cl_2 and returned to room air for 1 and 6 hours (Figure E3C). These results are in agreement with previous findings showing up-regulation of reactive intermediates in alveolar type II cells exposed to Cl_2 and returned to room air for

up to 24 hours (7). Consistent with the effects of Cl_2 on the enhancement of cytokine expression, lung sections of Cl_2 -exposed mice showed increased cellularity and congestion (Figure 3D), confirming the earlier reports (9). We also observed an enhanced presence of TUNEL-positive cells in the lung excised from Cl_2 -exposed animals (Figure 3E), which is consistent with the earlier published observations (27, 28).

Effects of Cl_2 on the Disruption of Iron Metabolism

It has been reported that iron metabolism is under the control of the UPR signaling pathway (29) by altering hepcidin expression. Here, we observed that Cl_2 exposure enhanced transcript levels of hepcidin antimicrobial peptide (Hamp) in the skin and lungs (Figures 4A and 4C). The expression of solute carrier family 40 (iron-regulated transporter), member 1 (Slc40a1) mRNA levels, which codes for a binding receptor of hepcidin (30), was also significantly enhanced at 6 hours in skin and lung of Cl_2 -exposed mice (Figures 4A and 4C). Immunofluorescence studies showed increased ferroportin and hepcidin protein levels in lungs and skin of Cl_2 -exposed mice (Figures 4B and 4D).

DISCUSSION

Cl_2 is known to increase ROS and to deplete tissue antioxidant levels in alveolar type II and airway epithelial cells of the lung tissues (7, 15, 27); thus, the generated ROS are not rapidly detoxified. This provides an opportunity for ROS to act for an extended time period, which seems to be a trigger for early and sustained tissue injury. This is consistent with our observations that Cl_2 exposure induces sustained ROS production in the lung, which could be visualized up to 6 hours after exposure (Figure E3C). Cl_2 is a known pulmonary inflammation-inducing chemical. In this study, we found that whole body Cl_2 gas exposure enhanced inflammation not only in the lung but also in the skin. These inflammatory responses were associated with the enhanced expression of cytokines in the two organs as also confirmed in this study.

Recent studies have focused on defining the intricate relationship between inflammation and UPR signaling (24). Cytokines (e.g., IL-1 β , TNF- α , and IL-6) and ROS generation are known to enhance UPR signaling pathway (21, 31), and both of these agonists of the UPR signaling pathways are induced by Cl_2 . As observed in this study, Cl_2 exposure enhanced the transcript and protein levels of UPR pathway genes (Figure E1), which may be mediated by the enhanced cytokine levels and by ROS generation. However, the observed depletion in the protein level of some of these UPR-regulatory molecules in the lung, particularly at later time points, indicates that Cl_2 induces a protein translational block due to augmented expression of p-eIF2 α , a protein known to manifest a global translation inhibition during the ER stress, and/or induces proteasomal degradation of unfolded or misfolded proteins due to prolonged ROS generation (20).

Hepcidin, a defensin-like peptide, serves as a master regulator of iron homeostasis and innate immunity (29, 32). The molecular pathways regulating hepcidin expression are orchestrated by the complex responses of UPR-regulated XBP-1, IL-6/signal transducer, and activator of transcription 3 and transcription factor cAMP responsive element binding protein 3-like 3 (29, 33, 34). We observed an enhancement in the mRNA level of hepcidin after Cl_2 exposure, which may be due to Cl_2 -induced enhanced production of IL-6 in lung and skin. Under normal physiological conditions, hepcidin triggers the degradation of iron exporter ferroportin to restore the tissue level of iron (35). In this study, enhanced hepcidin was not able to reduce

ferroportin level, suggesting that Cl_2 impairs the iron homeostasis regulatory function of hepcidin. Enhanced levels of ferroportin may lead to the release of tissue-bound iron. It is likely that Cl_2 exposure creates a pool of loosely bound iron that is known to be redox active and initiates free radical-mediated tissue damage (36). In earlier studies, we showed a role of iron in mediating acute Cl_2 toxicity, as administration of the iron chelator; deferoxamine reduced Cl_2 -induced mortality in mice and rats (2, 8, 9).

In summary, these studies reveal that Cl_2 damages skin in addition to lung tissue. The data presented herein indicate for the first time the up-regulation of UPR signaling and hepcidin levels in the skin and lungs of Cl_2 -exposed mice, which persisted when the mice returned to room air for 6 hours.

Author disclosures are available with the text of this article at www.atsjournals.org.

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