Chlorine Gas Exposure Causes Systemic Endothelial Dysfunction by Inhibiting Endothelial Nitric Oxide Synthase–Dependent Signaling

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Chlorine gas (Cl_2) exposure during accidents or in the military setting results primarily in injury to the lungs. However, the potential for Cl_2 exposure to promote injury to the systemic vasculature leading to compromised vascular function has not been studied. We hypothesized that Cl_2 promotes extrapulmonary endothelial dysfunction characterized by a loss of endothelial nitric oxide synthase (eNOS)-derived signaling. Male Sprague Dawley rats were exposed to Cl_2 for 30 minutes, and eNOS-dependent vasodilation of aorta as a function of Cl_2 dose (0–400 ppm) and time after exposure (0–48 h) were determined. Exposure to Cl_2 (250–400 ppm) significantly inhibited eNOS-dependent vasodilation (stimulated by acetycholine) at 24 to 48 hours after exposure without affecting constriction responses to phenylephrine or vasodilation responses to an NO donor, suggesting decreased NO formation. Consistent with this hypothesis, eNOS protein expression was significantly decreased ($\sim$ 60%) in aorta isolated from Cl_2–exposed versus air-exposed rats. Moreover, inducible nitric oxide synthase (iNOS) mRNA was up-regulated in circulating leukocytes and aorta isolated 24 hours after Cl_2 exposure, suggesting stimulation of inflammation in the systemic vasculature. Despite decreased eNOS expression and activity, no changes in mean arterial blood pressure were observed. However, injection of 1400W, a selective inhibitor of iNOS, increased mean arterial blood pressure only in Cl_2–exposed animals, suggesting that iNOS-derived NO compensates for decreased eNOS-derived NO. These results highlight the potential for Cl_2 exposure to promote postexposure systemic endothelial dysfunction via disruption of vascular NO homeostasis mechanisms.

Keywords: endothelium; nitric oxide; inflammation; inhaled reactive oxidants

Chlorine gas (Cl_2) is used extensively in a wide variety of manufacturing processes and ranks among the leading chemicals transported by rail in close proximity to major population centers. As such, Cl_2–induced toxicity is a concern and is exemplified by many cases worldwide of accidental exposure secondary to train derailment (1–3). This, coupled with the continual use of Cl_2 in military warfare and exposure in the home (secondary to mishandling of bleach) (4–6), has led to recent interest into a more detailed understanding of the mechanisms of Cl_2–induced toxicity (7).

CLINICAL RELEVANCE

Data presented in this study shows that chlorine gas toxicity comprises not only lung injury, but systemic vascular endothelial injury characterized by loss of endothelial nitric oxide synthase derived nitric oxide bioactivity. In addition, chlorine gas increased systemic inflammation characterized by induction of inducible nitric oxide synthase. Taken together these data demonstrate the potential for vascular inflammation and dysfunction in post chlorine gas toxicity with disruption of nitric oxide homeostasis as a central mechanism.

The lungs are primary targets of Cl_2 toxicity, with the initial injury that occurs during exposure being dependent on the dose of Cl_2 and length of exposure. This initial injury is thought to be mediated largely through direct reactions of Cl_2 with biomolecules and via secondary (to Cl_2 hydrolysis) generation of hypochlorous acid (HOCl). We have demonstrated that exposure of rats and mice to Cl_2 leads to extensive injury to airway and alveolar lung epithelia, decreased surfactant function, decreased ability of alveolar epithelial cells to actively transport sodium ions and clear fluid, and decreased levels of ascorbate and a decreased ratio of glutathione to oxidized glutathione in BAL and lung tissues (8–10). These inflammatory responses continue to induce injury after cessation of Cl_2 exposure, culminating in acute lung injury, adult respiratory distress syndrome, and reactive airway syndrome (9, 11–17). The precise mechanism of this post-Cl_2 exposure injury remains unclear and important to address because this aspect of Cl_2–induced injury is the primary goal for therapies. Recent studies suggest that post-Cl_2–induced acute lung injury is mediated by inflammation and the reactivity of a variety of reactive oxygen, reactive nitrogen, and reactive chlorine species (9, 15).

Less is known on the potential for Cl_2 to induce injury to extrapulmonary tissues and specifically to the extrapulmonary/systemic vasculature. Recent studies have demonstrated a role for dysfunction in vascular endothelial nitric oxide synthase (eNOS) signaling in mediating increased susceptibility to cardiovascular disease in response to environmental exposure to inhaled species that can promote oxidative tissue injury (e.g., cigarette smoke, diesel, or ozone) (18–21). Nitric oxide produced from eNOS plays a central role in vascular homeostasis mechanisms, including regulating vessel tone and cellular respiration, inhibiting smooth muscle proliferation, and maintaining an antithrombotic and antiinflammatory luminal surface (22, 23). Therefore, dysfunction in eNOS-derived NO signaling

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predisposes the vasculature to the development of inflammatory disease, and its functional assessment is now considered a key parameter in the diagnosis of cardiovascular disease (24, 25). The mechanisms by which inhaled reactive oxidant species promote extrapulmonary injury remain unclear. Because of their reactivity, Cl_2 and HOCl react with biomolecules in the epithelial lining fluid or cell surface (26). Thus, injury to extrapulmonary tissues suggests production of secondary intermediates that are diffusible and longer lived. The potential for exposure to Cl_2 acutely to promote vascular endothelial dysfunction has not been explored. Cl_2 is an interesting example because endogenously reactive chlorine species (including HOCl and Cl_2) formed during inflammation have been closely linked with the development of atherosclerosis through multiple mechanisms, including eNOS inhibition (27–38). In this study, we present data showing that exposure of rats to Cl_2 under conditions that result in lung injury similar to that seen with humans during accidental or deliberate release of Cl_2 into the atmosphere (9, 39–41) causes a postexposure injury to the extrapulmonary vasculature that is mediated via inhibition of eNOS.

MATERIALS AND METHODS

Detailed materials and methods are provided in the online supplement.

Unless stated otherwise, all reagents and antibodies were purchased from Sigma (St. Louis, MO) and AbCam (Cambridge, MA), respectively, except Mahma/NONOate (MNO), which was purchased from Axxora Platform (San Diego, CA). Male Sprague Dawley rats (200–300 g) were purchased from Harlan (Indianapolis, IN) and kept on 12-hour light/dark cycles with access to standard chow and water *ad libitum* before and after chlorine exposure. 1400W was purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA).

Rat Exposure to Cl_2

Whole body exposure of rats to different doses of Cl_2 was performed as previously described (9). Two rats were exposed in the same chamber at any one time. All exposures were performed between 08:00 and 09:00 and were 30 minutes in length and followed by return to room air. Age-matched controls included rats exposed to air only. All experiments involving animals were conducted according to protocols approved by the University of Alabama Institutional Animal Care and Use Committee.

Aortic Vessel Studies

At the indicated times after Cl_2 exposure, aortas were collected, and responses to the indicated vasoconstrictive and vasoactive stimuli were determined in vessel bioassay chambers (Radnoti, Monrovia, CA) as previously described (42). All vessel bioassay studies were performed in vessels pretreated with indomethacin (5 μM) and in Krebs Henseleit buffer perfused with 21%O_2, 5%CO_2 balanced with N_2.

NOS Expression

Expression of eNOS, inducible NOS (iNOS), or neuronal NOS (nNOS) mRNA in the aorta or iNOS in circulating leukocytes was assessed by real-time PCR as previously described (43). Western blotting and immunofluoresence staining and quantitation of eNOS protein were determined as described in the online supplement.

Nitrite Measurement

Plasma nitrite was measured as previously described (42).

Measurement of Cytokines

IFN-γ, IL-1β, and TNF-α were measured using a sandwich immunoassay kit (K11014A-4; Meso Scale Discovery, Gaithersburg, MD). Detection limits for IFN-γ, IL-1β, and TNF-α were 9.77, 15.7, and 5.78 pg/ml, respectively.

Blood Pressure Measurements

Hemodynamic parameters were measured noninvasively using a specialized differential pressure transducer tail cuff (Kent Scientific, Torrington, CT). Rats were acclimated to the blood pressure measuring apparatus following manufacturer recommendations to obtain steady baseline values. Upon reaching stable baseline readings, rats were exposed to Cl_2. Hemodynamic parameters were measured at different times after exposure as indicated.

RESULTS

Effects of Cl_2 on Aortic Responses to Phenylephrine and Acetycholine

To test the hypothesis that chlorine exposure inhibits systemic vascular eNOS signaling, aorta were collected from rats at different times (6, 24, and 48 h) after Cl_2 exposure (400 ppm, 30 min), and vasoconstrictive responses to phenylephrine (PE) and vasodilatory responses to acetycholine (Ach) were determined. Figure 1A shows similar PE dose-dependent vasoconstriction in aorta isolated from Cl_2–exposed animals at various times after exposure as compared with air-exposed control animals (time = 0 h). However, Ach-dependent dilation was significantly inhibited at 24 and 48 hours after Cl_2 exposure (Figure 1B). Next, the effects on aorta responses to PE and Ach 24 hours after exposure of rats to different doses of Cl_2 were determined. Figure 1C shows that PE-induced vasoconstriction was not affected at any Cl_2 dose used. However, as shown by increases in the EC_{50}, Ach-induced vasodilation was significantly inhibited by preexposure to 250 or 400 ppm Cl_2 but not to 100 ppm Cl_2 (Figure 1D). Based on these data, further experiments were performed on aorta isolated from rats 24 hours after exposure to Cl_2 (400 ppm, 30 min). These Cl_2 exposure conditions result in significant and sustained hypoxemia and hypercapnia as well as depletion of lung ascorbate, which mimic injury observed in human incidences of Cl_2 exposure (39).

To evaluate the potential mechanisms for compromised eNOS-dependent signaling in the aorta, we hypothesized that formation of reactive oxygen species (e.g., superoxide anion or lipid radicals) was increased. These reactive oxygen species would rapidly scavenge NO and inhibit subsequent relaxation analogous to previously reported mechanisms of endothelial dysfunction (44–46). To test this hypothesis, aorta responses to the NO donors MNO or sodium nitroprusside (SNP) were assessed. Figure 2 shows MNO- and SNP-induced vasodilation in a dose-dependent manner to similar extents in aorta isolated from air- or Cl_2–exposed rats, indicating that increased ROS did not mediate altered eNOS-dependent vasodilation. Because these NO donors are endothelial-independent vasodilators, these data also indicate that dysfunctional Ach-induced vasodilation is mediated via reactions upstream of NO-dependent activation of soluble guanylate cyclase and do not involve dysfunctional smooth muscle responses.

Cl_2 Exposure Decreases eNOS Protein Expression and Markers of eNOS Activity

We next tested if decreased Ach-dependent vasodilation was attributed to altered NOS expression. Figure 3A shows that there was a significant increase in eNOS mRNA levels in aorta from Cl_2–exposed versus control rats. However, protein expression was decreased by approximately 40 to 60%, as determined by immunofluorescence or Western blotting (Figures 3B, 3C, and 4). This decrease in eNOS was not due to a loss of the endothelial monolayer indicated by similar levels of von Willebrand factor (vWF) expression in both experimental groups (Figures 4C and 4D). Figure 3C shows that plasma levels of nitrite, a selective marker of eNOS activity, were also decreased in Cl_2–exposed rats.

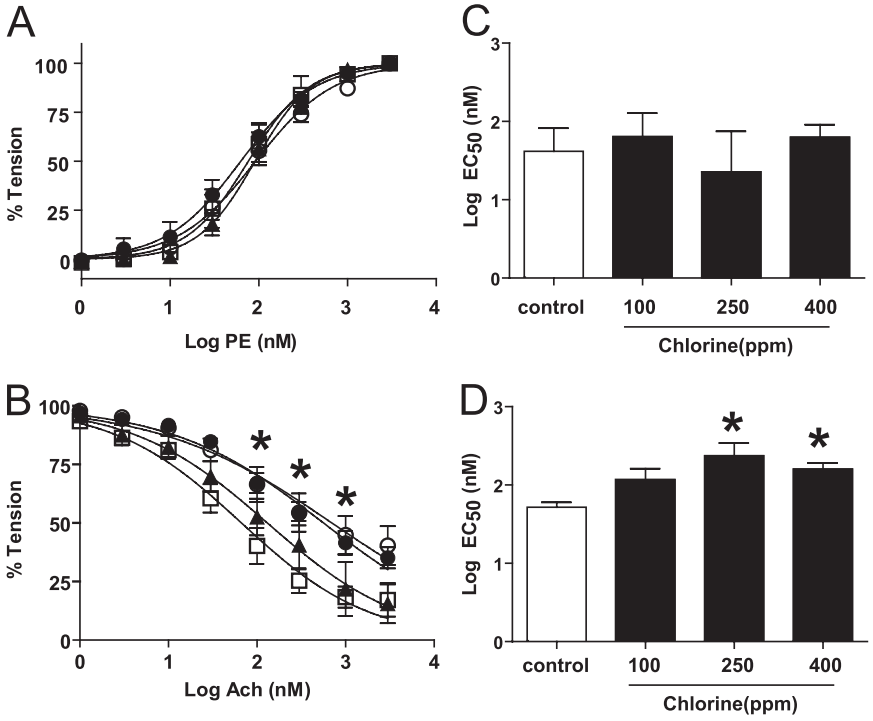

Figure 1. Chlorine gas (Cl_2) exposure inhibits aortic vasodilation in response to acetylcholine. Rats were exposed to air (*open squares*) or Cl_2 (400 ppm, 30 min). Aorta were isolated at 6 (*closed triangles*), 24 (*closed circles*), or 48 hours (*open circles*) thereafter, and vasoconstriction and vasodilation responses to phenylephrine (PE) (*A*) or acetycholine (Ach) (*B*), respectively, were determined. Data are means ± SEM for cumulative dose-dependent changes in tension. *$P < 0.05$ by two-way repeated measures ANOVA with Bonferroni post-test for 24 and 48 hours after Cl_2 exposure relative to control (n = 3–12). (*C* and *D*) EC_{50} values for PE-dependent vasoconstriction and Ach-dependent vasodilation, respectively, in aorta isolated from rats 24 hours after exposure to different doses of Cl_2 (0–400 ppm) for 30 minutes. Data show mean ± SEM. *$P < 0.05$ by one-way ANOVA with Bonferroni post-test relative to control (n = 5–11).

Effect of Cl_2 on Systemic Hemodynamics

Many studies have shown that inhibited eNOS-dependent vasodilation results in increased blood pressure. The effects of Cl_2 exposure on parameters that regulate hemodynamics were therefore measured by tail cuff plethysmography. No change in MAP was observed in rats measured up to 48 hours after Cl_2 exposure (Figure 5). Similarly, no change in heart rate or tail blood flow was observed (not shown). These data suggest that Cl_2 exposure affects additional pathways that counter the loss of eNOS-dependent function, which maintains vascular tone. Previous studies have shown that Cl_2 exposure increases iNOS expression in the lung (15), although this is likely dependent on exposure conditions because a recent study using a longer-duration but lower Cl_2 dose failed to observe changes in iNOS levels in the lung (17). iNOS is induced during inflammation and when elevated in the circulation/systemic vasculature is associated with hypotension. We tested if Cl_2 exposure increases iNOS in extrapulmonary tissues and whether iNOS-derived NO may compensate for the loss of eNOS-derived NO in controlling blood pressure. Figure 6A shows that in aorta and circulating leukocytes, iNOS mRNA is significantly elevated 24 hours after Cl_2 exposure, with no changes in nNOS expression in the aorta. iNOS protein expression in the aorta was localized in the endothelial layer and outer medial layers of the vessel wall (Figures 6B and 6C). To test if increased systemic iNOS plays a role in hemodynamics, rats were exposed to air or Cl_2 (400 ppm, 30 min). MAP was measured 24 hours thereafter, and then saline or the iNOS-specific inhibitor 1400W was administered. MAP was again measured 18 to 24 hours after 1400W addition (i.e., 42–48 h after Cl_2 exposure). Figure 6D shows the significant increase in MAP after 1400W addition to Cl_2–exposed animals compared with saline or 1400W addition to air-exposed rats. To evaluate if iNOS in the aorta contributed to vasomotor tone, the effects of 1400W on isolated aorta responses to PE and Ach were determined and compared with the effects of a general NOS inhibitor, L-NMMA. Figure 6E shows that L-NMMA potentiated PE-dependent constriction of aorta isolated from control and Cl_2–exposed rats, whereas 1400W had no effect. Similarly, Figure 6F shows that L-NMMA inhibited Ach-induced vasodilation in all groups, whereas 1400W had no effect relative to respective controls.

Role for TNF-α in Mediating Cl_2–dependent Down-regulation of Aortic eNOS

TNF-α has been shown to down-regulate vascular eNOS expression and was proposed therefore as a potential mediator in transducing the effects of inhaled toxicants to the systemic vasculature (47). Therefore, TNF-α levels in the circulation were

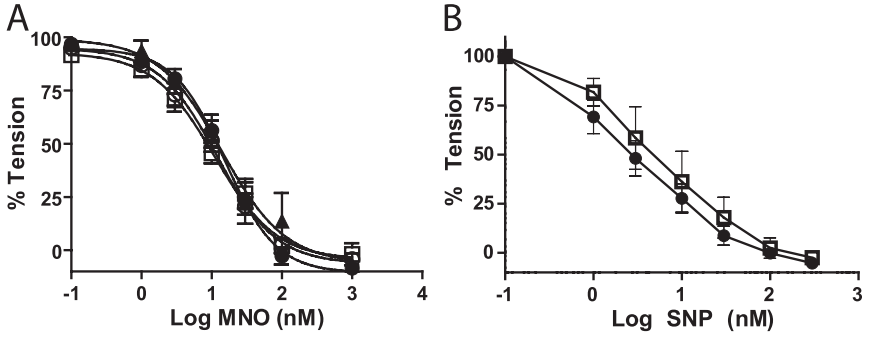

Figure 2. Cl_2 exposure does not affect Mahma/NONOate (MNO)- or sodium nitroprusside (SNP)-induced vasodilation of aorta. Rats were exposed to air (*open squares*) or Cl_2 (400 ppm, 30 min). Aorta were isolated at 6 (*closed triangles*), 24 (*closed circles*), or 48 hours (*open circles*) thereafter, and vasodilation responses to the NO donors MNO (*A*) or SNP (*B*) were determined. Data show mean ± SEM. No significant differences were observed by two-way repeated measures ANOVA with Bonferroni post-test (n = 3–8).

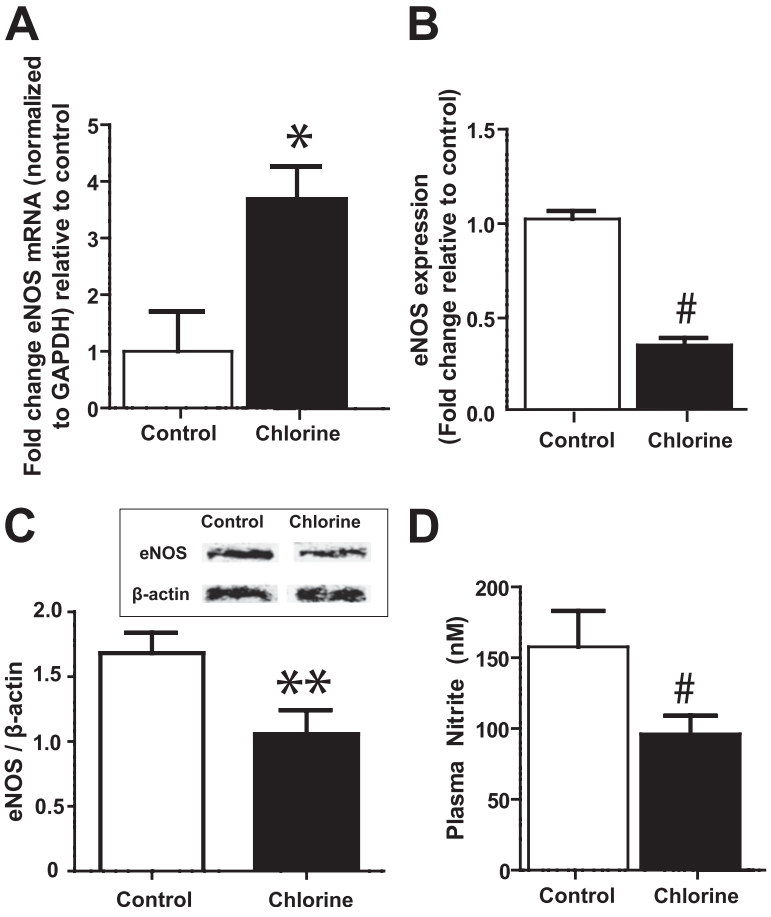

Figure 3. Effects of Cl_2 on aortic endothelial nitric oxide synthase (eNOS) expression eNOS mRNA (*A*) or protein (*B* and *C*) were determined in aorta isolated from rats 24 hours after exposure to air or Cl_2 (400 ppm, 30 min). eNOS protein levels were determined by immunoflouresence (*B*) or by Western blotting (C; *inset* shows representative Western blots). Data show mean ± SEM (n = 3–4). $^{**}P < 0.05$, $^{*}P < 0.03$, and $^{\#}P < 0.001$ relative to control. (*D*) Plasma nitrite concentrations in 24 hours after air or Cl_2 exposure. Data show mean ± SEM (n = 5–7). $^{\#}P < 0.05$ relative to control.

measured at 6 and 24 hours after Cl_2 exposure and were below detection limits at both times (not shown). Moreover, no changes in the levels of IFN-γ or IL-1β were detected (not shown).

DISCUSSION

Cl_2 toxicity is characterized by an initial injury to the lungs that progresses over days and months after cessation of the exposure leading to reactive airway dysfunction syndrome (15, 48). The focus of mechanisms for post-Cl_2 exposure toxicity has been therefore on the pulmonary compartment, with the potential for Cl_2 to cause injury to extrapulmonary vasculature receiving no

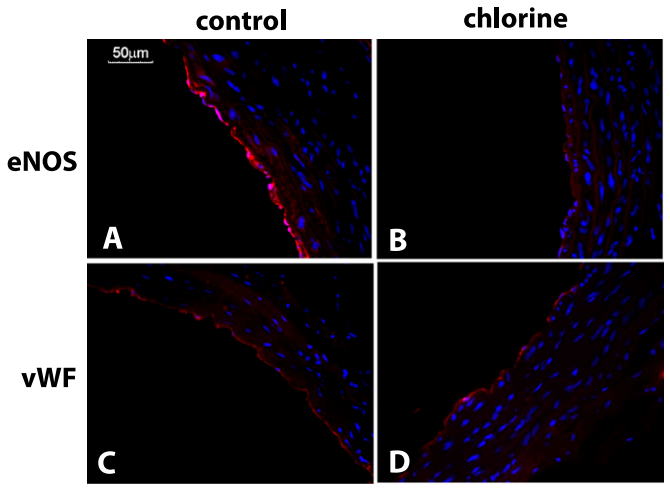

Figure 4. Cl_2 decreases eNOS protein expression. Representative immunofluoresence images for eNOS (*A* and *B*) or von Willebrand factor (vWF) (*C* and *D*) staining from aorta collected 24 hours after exposure to air (*A* and *C*) or Cl_2 (400 ppm, 30 min) (*B* and *D*). *Red* = eNOS or vWF as indicated; *blue* = Hoechst staining for nuclei.

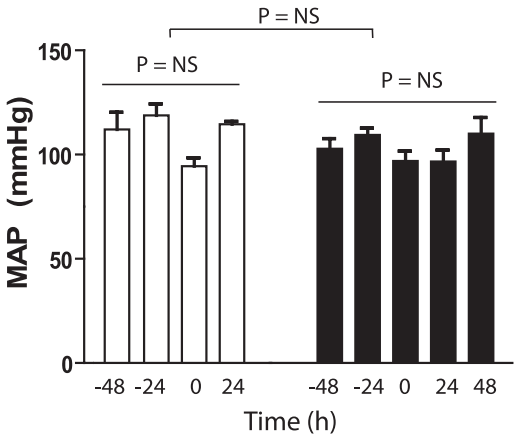

Figure 5. Effects of Cl_2 on blood pressure. Rats were acclimatized to tail-cuff blood pressure measurement protocols and then exposed to air (*open squares*) or Cl_2 (*closed squares*; 400 ppm, 30 min), and blood pressure measured again at 24 and 48 hours thereafter. −48 h and −24 h indicate measurements during acclimatization and indicate stable blood pressures before Cl_2 exposure. Time 0 indicates measurements (30–60 min before Cl_2 exposure). No significant differences between mean arterial blood pressure (MAP) as a function of time in air or Cl_2 groups (by one-way ANOVA) or between groups (by two-way repeated measures ANOVA) were observed. Data are mean ± SEM (n = 2–4).

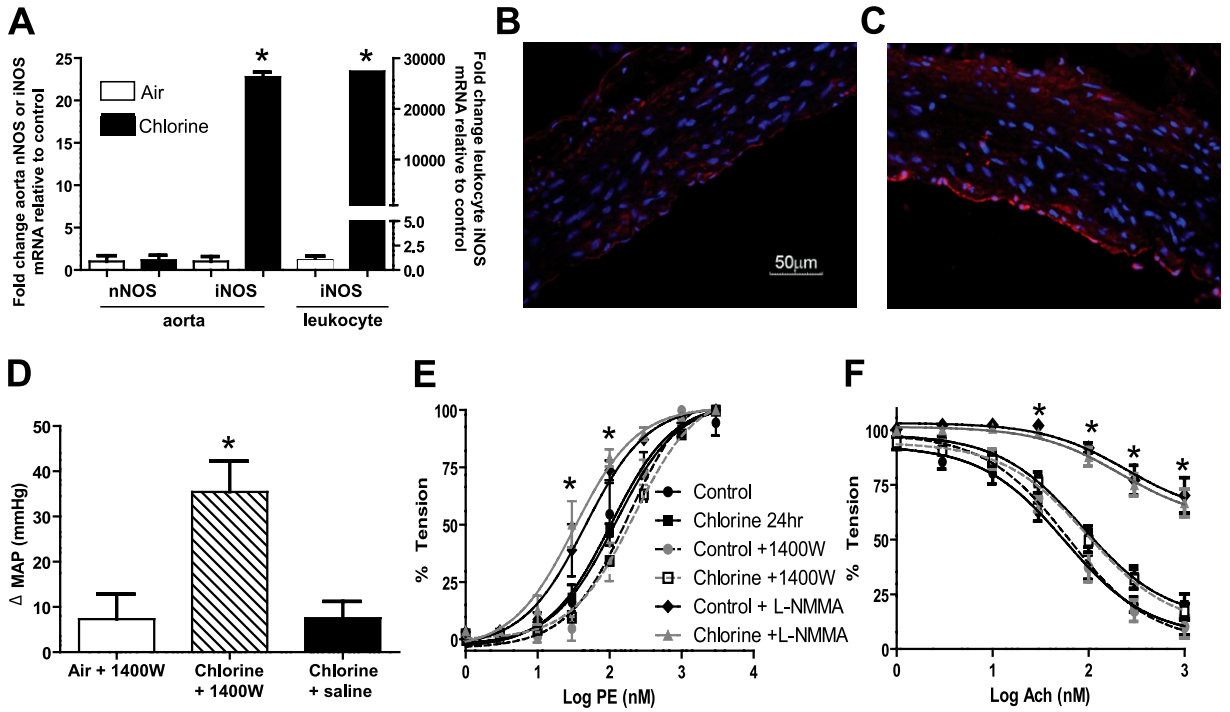

Figure 6. Role of systemic inducible nitric oxide synthase (iNOS) induction in post-Cl_2–induced changes in MAP. (*A*) Rats were exposed to air or Cl_2 (400 ppm, 30 min), and 24 hours thereafter neuronal nitric oxide synthase (nNOS) or iNOS mRNA expression from aorta or circulating leukocytes was determined. mRNA levels are expressed relative to air controls and after normalization to GAPDH. Right-hand *y* axis is for leukocyte mRNA levels. Data are mean ± SEM (n = 2–3). *$P < 0.001$ by *t* test relative to respective air control. (*B* and *C*) Representative immunofluoresence images of iNOS (*red*) staining in aorta from control or 24 hours after Cl_2 exposure, respectively. (*D*) Rats were exposed to air or Cl_2 (400 ppm, 30 min). MAP was measured 24 hours thereafter, and saline or 1400W (10 mg/kg) was added by intraperitoneal injection. After a further 18 to 24 hours, MAP was measured. Data show changes in MAP (post 1400W – pre 1400W administration) and are mean ± SEM (n = 2–4). *$P < 0.05$ relative to air + 1400W or chlorine + saline by one-way ANOVA with Tukey post-test. (*E* and *F*) Effects of 1400W (10 μM) or L-NMMA (100 μM) on PE-induced vasoconstriction or Ach-induced vasodilation of aorta isolated from control of Cl_2–exposed rats, respectively. Data show mean ± SEM (n = 3–5). *$P < 0.05$ for control versus control + L-NMMA or Cl_2 versus Cl_2 + L-NMMA by two-way ANOVA with Bonferroni post-test.

attention. Underlying this premise is the general paradigm of environmental exposure to inhaled reactive oxidant gases being associated with acute and chronic cardiovascular inflammatory disease. We found that Cl_2 promoted injury distal to the lung compartment that was characterized by postexposure decreases in expression and function of eNOS, which is similar to effects observed with other inhaled reactive oxidants, including ozone (18). An important distinction between Cl_2 exposure and these other irritants is the exposure regimen. In the latter, exposures are intermittent, typically over longer time periods (days to weeks) and at relatively lower doses compared with Cl_2, which is significantly shorter (min) in duration but occurs at higher doses, at least in industrial accident and military exposure situations. Data presented herein suggest that short-term (30 min) exposure to Cl_2 (250–400 ppm) is sufficient to promote dysfunction in systemic eNOS over at least 48 hours after exposure. These results further support the model that dysfunction in the eNOS-signaling cascade is a common mechanism underlying how inhaled reactive oxidant species, independent of reactivity, promote systemic vascular toxicity.

Current therapeutics for Cl_2–induced injury are symptomatic and focus on acute injury primarily associated with lung function. Our data suggest that systemic endothelial dysfunction should also be considered. Many studies have documented that a loss of eNOS signaling predisposes the vasculature to a hypertensive, proinflammatory, and procoagulant state, raising the question of whether Cl_2 exposure has similar effects. Despite decreased eNOS, no changes in MAP were observed in rats after Cl_2 exposure. eNOS-derived NO is one of many factors that work in balance to control vascular tone, and we reasoned therefore that concomitant decreased vasoconstrictor or increased vasodilator activity was also induced by Cl_2. Administration of the iNOS-specific inhibitor 1400W increased MAP only in Cl_2–exposed animals, suggesting that increased vasodilator activity from iNOS was countering decreased eNOS-derived NO to maintain blood pressure. Moreover, the lack of effect of 1400W on isolated vessels suggests that iNOS in circulating cells plays an important role in maintaining MAP in the background of eNOS inhibition. The mechanisms by which this could occur are not clear and require further study but could involve direct effects of NO derived from iNOS in circulating cells or iNOS-dependent changes in other vasodilators or vasoconstrictors. For example, iNOS can activate cycocloxygenase-2 (49), which in turn could alter the balance of prostanoid-derived vasodilator/vasoconstrictors. These data suggest that Cl_2 exposure acutely alters the balance of vasoconstrictor and vasodilator mechanisms in the circulation and specifically those related to NO homeostasis. Moreover, given the relatively high prevalence of cardiovascular risk factors in humans, the potential for Cl_2 to cause systemic hypertension and inflammation in these individuals where eNOS signaling is already compromised could be significant. Consistent with this notion, case reports of accidental Cl_2 exposure document a prevalence of delayed hypertension after exposure (2), although earlier reports document no hypertension (50). We hypothesize that Cl_2 exposure disrupts the regulatory mechanisms for controlling

vascular homeostasis, which, on the background of existing vascular injury, may exacerbate endothelial dysfunction and may have implications for treatment of patients with existing cardiovascular problems. Also, the variance of underlying cardiovascular disease may contribute to the variance in hypertension observed in case studies of Cl_2 exposure.

Our data support the model whereby Cl_2 inhalation decreases expression of eNOS protein, which leads to a loss of agonist-induced vasodilation. There were no changes in α1-adrenergic receptor-dependent contraction or in NO-dependent vasodilation *per se*, suggesting that the loss of Ach-induced dilation was solely due to decreased expression of eNOS and less NO being formed in the vessel wall. This is further supported by decreased levels of plasma nitrite, a selective marker for vascular eNOS activity (51). Whether eNOS degradation is increasing or gene transcription/translation is decreasing is not clear. The finding that eNOS protein was decreased despite eNOS mRNA expression being increased suggests that increased protein turnover is occurring. Alternatively, increased eNOS mRNA may represent a compensatory response to decreased protein levels. Irrespective of the molecular mechanism, a key question remaining is how the effects of Cl_2, the direct reactivity of which with biological molecules is limited to the epithelial lining fluid, are transduced to the periphery to mediate down-regulation of eNOS expression in the aorta. This question applies to other inhaled irritants, and one potential common mechanism that may encompass inhaled irritants with different reactivities involves stimulation of inflammation secondary to the initial exposure (21). In this model, initial injury in the lungs activates alveolar macrophages and other inflammatory cells to secrete proinflammatory cytokines that recruit other immune cells to the lung (e.g., neutrophils) and in doing so encompass the so-called "second wave" of inflammation. The possibility remains therefore that increased proinflammatory cytokines in the pulmonary compartment may cross over into the circulation, which in turn may affect systemic endothelial function. Similar principles have been proposed to explain the development of multiorgan failure in patients with primary adult respiratory distress syndrome. One candidate includes TNF-α, which down-regulates eNOS expression in endothelial cell culture and *in vivo* models (47, 52). However, no changes in circulating TNF-α or other proinflammatory cytokines (IFN-γ or IL-1β) were observed. At first glance, this would suggest that Cl_2 does not promote vascular inflammation. However, Cl_2 did increase iNOS expression in circulating leukocytes and vascular tissue. iNOS is an inducible enzyme that is associated with inflammation, and previous studies have shown Cl_2–dependent increases in iNOS in the lung, consistent with proinflammatory effects in this compartment (13). Moreover, no changes in nNOS mRNA suggest specificity toward increases in the iNOS isoform. Using iNOS as an inflammatory marker, our data suggest that vascular inflammation occurs after Cl_2 exposure, albeit without detectable changes in proinflammatory cytokines. We have not measured all cytokines that could contribute to iNOS up-regulation and acknowledge that rapid turnover of cytokines in the circulation, together with the possibility that only small changes in cytokine concentrations may be required to mediate down-regulation of eNOS and up-regulation of iNOS, may have precluded our ability to detect significant changes. Further studies are required to test this hypothesis.

The concept of Cl_2–derived products in modulating eNOS and vascular function is supported by studies that have established the connection between endogenously derived reactive chlorine species (e.g., hypochlorous acid and chloramines) and the development of cardiovascular disease. Specifically, chlorination of biological molecules (lipids, L-arginine) by myeloperoxidase-derived HOCl form products that inhibit eNOS, and recently HOCl-derived advanced glycation end products have been shown to inhibit eNOS expression (28, 29, 32–36, 53, 54). Moreover, advanced glycation end products are activators of iNOS (55), further supporting these species as candidates for transducing extrapulmonary effects of post-Cl_2 exposure. Whether there is an overlap in mechanisms causing vascular eNOS dysfunction by exogenous Cl_2 exposure and endogenously formed reactive chlorine species remains to be determined.

In summary, this study shows that Cl_2 can lead to post-exposure extrapulmonary vascular endothelial dysfunction and inflammation characterized by the loss of eNOS-derived signaling and increased iNOS expression. We hypothesize that this disrupts the normal balance of vascular NO homeostasis, which may lead to acute and chronic cardiovascular events that we propose should be considered when providing medical care for victims of Cl_2 exposure.

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