Carbon Monoxide Activates Autophagy via Mitochondrial Reactive Oxygen Species Formation

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Autophagy, an autodigestive process that degrades cellular organelles and protein, plays an important role in maintaining cellular homeostasis during environmental stress. Carbon monoxide (CO), a toxic gas and candidate therapeutic molecule, confers cytoprotection in animal models of acute lung injury. The mechanisms underlying CO-dependent lung cell protection and the role of autophagy in this process remain unclear. Here, we demonstrate that CO exposure time-dependently increased the expression and activation of the autophagic protein, microtubule-associated protein–1 light chain-3B (LC3B) in mouse lung, and in cultured human alveolar (A549) or human bronchial epithelial cells. Furthermore, CO increased autophagosome formation in epithelial cells by electron microscopy and green fluorescent protein (GFP)-LC3 puncta assays. Recent studies indicate that reactive oxygen species (ROS) play an important role in the activation of autophagy. CO up-regulated mitochondria-dependent generation of ROS in epithelial cells, as assayed by MitoSOX fluorescence. Furthermore, CO-dependent induction of LC3B expression was inhibited by N-acetyl-L-cysteine and the mitochondria-targeting antioxidant, Mito-TEMPO. These data suggest that CO promotes the autophagic process through mitochondrial ROS generation. We investigated the relationships between autophagic proteins and CO-dependent cytoprotection using a model of hyperoxic stress. CO protected against hyperoxia-induced cell death, and inhibited hyperoxia-associated ROS production. The ability of CO to protect against hyperoxia-induced cell death and caspase-3 activation was compromised in epithelial cells infected with LC3B-small interfering (si)RNA, indicating a role for autophagic proteins. These studies uncover a new mechanism for the protective action of CO, in support of potential therapeutic application of this gas.

Keywords: apoptosis; autophagy; carbon monoxide; epithelial cells; hyperoxia

Carbon monoxide (CO), a low–molecular weight diatomic gas, can induce clinical toxicity and even death at high ambient concentrations (1). The adverse effects of CO in humans are associated with hypoxemia as the result of competitive binding of CO with the heme iron centers of the oxygen carrier protein hemoglobin (2, 3). The cellular toxicity of CO may involve complex formation and inhibition of respiratory chain enzymes (i.e., cytochrome *c*: oxidase) (4, 5). In contrast to these toxic mechanisms, recent studies over the last decade have revealed cyto- and tissue-protective effects of CO when applied at low ambient concentrations in several models of lung or vascular injury, including acute lung injury (i.e., endotoxin shock, hyperoxia, mechanical ventilation), and ischemia–reperfusion injury (6–12). The protective effects of CO in these models depend on anti-inflammatory, antiapoptotic, and/or antiproliferative activities of this gas (13). Several signaling pathways have been implicated in CO-dependent cytoprotection, including the p38 mitogen activated protein kinase (MAPK) and NF-κB pathways (antiapoptotic, anti-inflammatory effects), soluble guanylate cyclase, and cyclin-dependent protein kinase $p21^{Waf1/Cip1}$ (antiproliferative effects) (6, 7, 13–16).

CLINICAL RELEVANCE

Carbon monoxide (CO) has been implicated as a potential inhalation therapeutic for acute lung injury. This study shows that CO can induce autophagy, a homeostatic process, in lung epithelial cells through a reactive oxygen species–dependent mechanism. These findings suggest a novel key regulatory step by which CO confers potential therapeutic effects in lung disease.

The protective effects of CO on apoptotic cell death have now been characterized in several cell types, including epithelial and endothelial cells (16–18). On the other hand, currently nothing is known of the effects of CO on the autophagic pathway, a cellular homeostatic pathway associated with programmed cell death mechanisms (19).

Autophagy represents a regulated pathway for the turnover of cellular organelles and long-lived proteins, which can promote survival under various stress conditions, including nutrient starvation and hypoxia (20, 21). During autophagy, double-membraned autophagic vacuoles (AVs) or autophagosomes surround cytosolic organelles (e.g., endoplasmic reticulum, mitochondria) or proteins, and subsequently fuse with lysosomes, where the engulfed components are degraded by hydrolases (22). To date, a number of genes critical for the regulation of autophagy (*Atg*) have been identified in mammals, each with distinct roles in the regulation of the autophagic pathway (23). The conversion of microtubule-associated protein–1 light chain 3B (LC3B) from LC3B-I (free form) to LC3B-II (phosphatidylethanolamine-conjugated form) is a major step in autophagosome formation (24, 25).

In this study, we show that low-dose CO exposure induces biochemical and morphological markers of autophagy *in vivo* and in cultured epithelial cells. The induction of autophagic markers by CO was dependent on the increased generation of mitochondria-derived reactive oxygen species (ROS). The cytoprotective effect of CO against high oxygen stress was compromised by genetic inhibition of the autophagic protein, LC3B. These results identify autophagy as a fundamental cellular process that can be affected by CO exposure, and thus impact the understanding of the therapeutic potential of CO.

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MATERIALS AND METHODS

Chemicals and Reagents

N-acetyl-L-cysteine (NAC) was from Sigma-Aldrich (St. Louis, MO). Mito-TEMPO ((2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride, monohydrate) was from Enzo Life Sciences (Plymouth, PA). Enhanced chemiluminescence reagent was from Thermo Scientific (Rockford, IL). Green fluorescent protein (GFP)-LC3 expression plasmid was a gift of Dr. Noboru Mizushima (Tokyo Medical and Dental University). Small interfering (si)RNA specific for LC3B and control (si)RNA were from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were from Sigma-Aldrich.

Cell Culture and CO Exposure

Human alveolar adenocarcinoma epithelial cells, A549, and human bronchial epithelial (HBE) cells were from ATCC (Manassas, VA). A549 cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS and antibiotics (Invitrogen, Carlsbad, CA). The HBE cells were grown in Airway Epithelial Cell Basal Medium supplemented with the Bronchial Epithelial Cell Growth Kit (ATCC). Cell cultures were maintained in humidified incubators (5% CO_2, 95% air) at 37°C. Cells grown to 60–80% confluence were exposed to 250 ppm CO in air containing 5% CO_2 in exposure chambers, as previously described (6). For hyperoxia/CO treatments, the A549 cells were grown to 95% confluence, changed to fresh medium, and transferred to an incubator chamber (Billups-Rothenberg, San Diego, CA) containing a hyperoxic atmosphere (95% O_2 and 5% CO_2) in the absence or presence of CO (250 ppm) (18). Control cells were cultured in standard tissue culture conditions (95% room air, 5% CO_2).

Animals and *In Vivo* CO Exposure

All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care. The Animal Research Committee of Brigham and Women's Hospital approved all protocols. C57Bl/6 mice (8–10 wk old; Charles River, Germantown, NY) were exposed to air or air containing CO (250 ppm; AirGas, Boston, MA) in modular exposure chambers as described (6).

Western Blot Analysis

Whole-cell extracts were prepared for Western analysis, as previously described (18). The anti-LC3B and anti–β-actin were from Sigma, and the anti–caspase-3, cleaved form, was from Cell Signaling Technology (Danvers, MA).

Cell Viability Assay

Cell viability was determined by crystal violet staining (26).

Lactate Dehydrogenase Release Assay

Lactate dehydrogenase (LDH) release was measured using a commercially available assay (Cytotoxicity Detection Kit; Roche Molecular Biochemicals, Indianapolis, IN). After gentle agitation, 200 μl of medium were removed at various times to be used for the assay. The samples were incubated (30 min) with buffer containing nicotinamide adenine dinucleotide (NAD^+), lactate, and tetrazolium. LDH converts lactate to pyruvate, generating NAD^+ reduced. The NAD^+ reduced then reduces tetrazolium (yellow) to formazan (red), which was detected by absorbance at 490 nm.

Transient Transfection

A549 cells were seeded at 1×10^5 cells per well in 12-well dishes. After 24 hours, each well was approximately 80–90% confluent. The media were changed to opti-MEM media (Invitrogen) for 3 hours. GFP-LC3B expression plasmid (2 μg) or LC3B siRNA (40 nmol/ml) was incubated with Lipofectamine LTX (Invitrogen) for 1 hour and then added to each well. After 6–8 hours, the media were aspirated and complete growth media were replaced in each well. Exposure to CO and/or hyperoxia was initiated 24 hours after transfection.

Transmission Electron Microscopy and Imaging

Electron microscopy and confocal microscopy were performed as previously described (27).

Mitochondrial ROS Measurement by FACS

Cells were grown to 80% confluence. MitoSOX (Invitrogen) was added to cell cultures at a final concentration of 5 μM. After 30-minute incubation, the cells were washed three times with PBS and then exposed to CO for the indicated intervals. MitoSOX fluorescence was detected by flow cytometry.

Statistical Analysis

Results are expressed as means (±SE) from at least three independent experiments. Differences in measured variables between experimental and control groups were assessed by using the Kruskal-Wallis test. Statistically significant differences were accepted at a P value less than 0.05.

RESULTS

CO Exposure Increases LC3B Expression and Activation *In Vivo*

To examine the potential of CO to induce the autophagic pathway *in vivo*, C57BL/6 mice were exposed to CO (250 ppm) for up to 72 hours. Lung tissue homogenates were harvested at 24 and 72 hours, and immunoblotted using an antibody against autophagic protein LC3B. CO inhalation induced time-dependent increases of total LC3B expression and increased the accumulation of its active form (LC3B-II) in mice (Figure 1A). Quantification

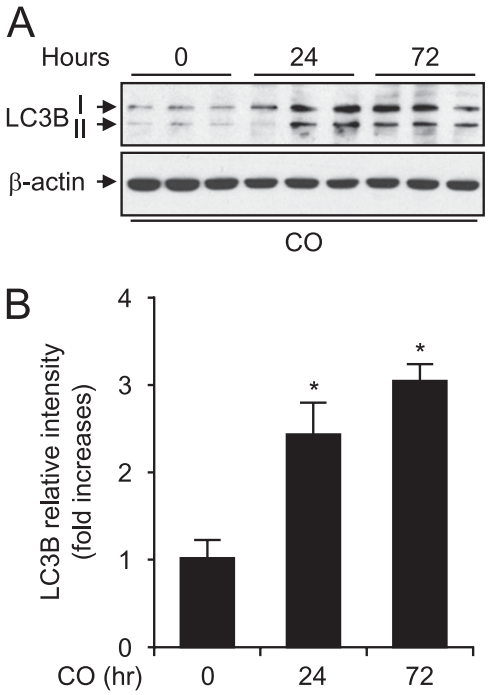

Figure 1. Autophagic markers are increased in lung tissues of carbon monoxide (CO)–exposed C57BL/6 mice. C57BL/6 mice were exposed to 250 ppm CO for 0 (control), 24, and 72 hours. Lung tissues were harvested at the end of each experiment. (*A*) Western blot analysis of microtubule-associated protein–1 light chain 3B (LC3B)–I/–II expression in lung tissue from C57BL/6 mice. Total LC3B expression was normalized to β-actin. (*B*) The quantification of LC3B protein levels from each group ($n = 6$) was measured using ImageJ Software. β-actin was used as the standard. Data represent mean (±SD). *$P < 0.05$ compared with room air.

of Western immunoblots revealed that the total expression of LC3B increased two- to threefold in the mouse lung during 24–72 hours of continuous CO exposure (Figure 1B). These results identify the activation of autophagic protein LC3B as an inducible response to CO exposure in the lung.

CO Increases Autophagic Markers in Cultured Human Epithelial Cells

We examined the expression of autophagic markers in human epithelial cells during CO exposure (250 ppm) *in vitro*. CO exposure induced time-dependent increases of total LC3B expression and the accumulation of its active form (LC3B-II) in A549 cells, with an apparent maximum at 24 hours (Figure 2A). However, CO did not affect the expression of autophagic proteins, Atg5, Beclin 1, and Atg7, in these cells (data not shown). CO exposure (250 ppm, 24 h) induced the formation of GFP-LC3 puncta, a gold-standard marker of autophagosome formation (20), relative to air exposure alone, in GFP-LC3–transfected A549 cells (Figure 2B). Quantification of these results revealed a three- to fourfold increase in the percentage of cells with GFP puncta after CO exposure relative to air exposure (Figure 2B, *right panel*).

We also examined the response in primary HBE cells. CO exposure (250 ppm) induced time-dependent increases of total LC3B expression and the accumulation of both LC3B-I and LC3B-II in HBE cells, with an apparent maximum at 24 hours (Figure 2C). Exposure to high oxygen concentrations (hyperoxia; 95% O_2, 0–24 h) elicited a comparable response in HBE cells with similar kinetics (Figure 2D). During extended exposures to CO (250 ppm), the accumulation of LC3B-II (active form) was sustained for up to 72 hours (Figure 2E). On the other hand, although hyperoxia acutely induced LC3B conversion, the response decayed in a time-dependent fashion with extended exposure up to 72 hours. In HBE cells treated with hyperoxia in the presence of 250 ppm CO, the accumulation of LC3B-II was sustained, with an apparent maximum at 72 hours continuous exposure (Figure 2E).

We also investigated morphological indices of autophagy in CO-treated epithelial cells. CO exposure resulted in increased formation of immature and degradative AVs after 24-hour exposure in A549 epithelial cells, as detected by transmission electron microscopy (Figure 3A). Quantification of electron micrographs revealed approximately threefold increases in total autophagosome number in CO-exposed A549 cells relative to cells exposed to room air (Figure 3B).

CO-Induced Epithelial Cell Autophagy Is Dependent on Mitochondrial ROS Production

Recent studies suggest that CO exposure can modulate intracellular ROS production originating from membrane and mitochondrial sources (18, 28–30). To examine the role of ROS in the regulation of autophagy by CO, we first measured the effect of CO on mitochondria-derived ROS generation in epithelial cells. MitoSOX Red is a fluorogenic dye developed and validated for highly selective detection of superoxide anion in the mitochondria of live cells, which can be detected by flow cytometry (31, 32). FACS analysis demonstrated an increase of the fluorescence intensity of MitoSOX Red in A549 epithelial cells after 2-hour exposure to 250 ppm CO (Figures 4A and 4B). Similar augmentation of mitochondrial ROS production was observed in HBE cells after 2-hour exposure to 250 ppm CO (Figure 4C). Rotenone, an inhibitor of mitochondrial complex I, which is known to promote mitochondrial ROS production, produced a strong activation of the response (Figure 4C). The ROS production in HBE cells that was stimulated by CO treatment was inhibited by cotreatment with the general antioxidant, NAC, and by the mitochondria-targeted triphenylphosphonium-conjugated antioxidant, Mito-TEMPO (Figure 4D). Hyperoxia treatment (>95% O_2) caused

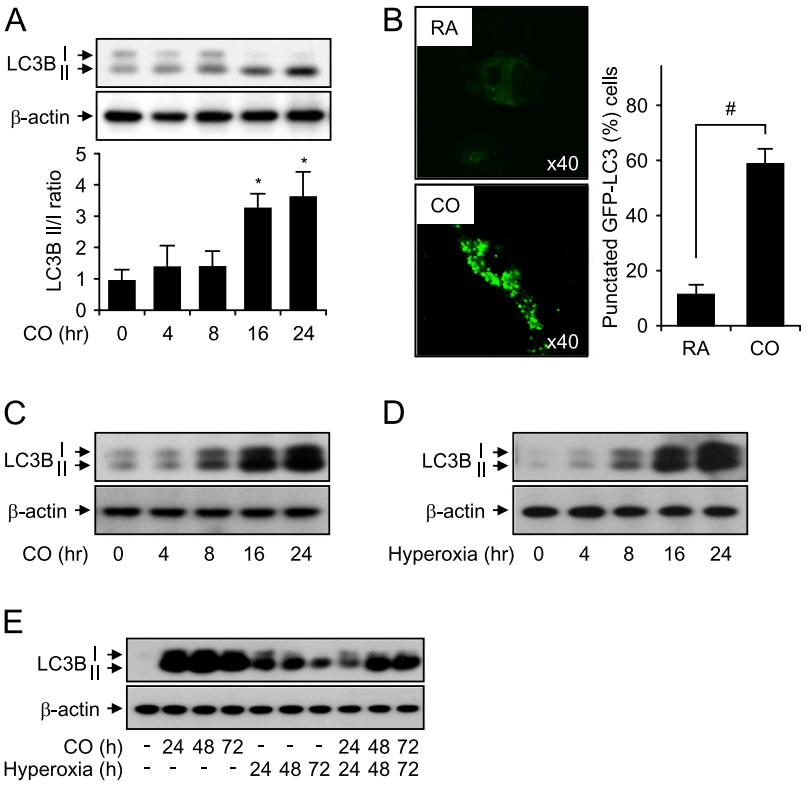

Figure 2. CO increases autophagy in cultured human epithelial cells *in vitro*. (*A*) A549 human alveolar epithelial cells were exposed to 250 ppm CO for 0–24 hours and evaluated for time-dependent expression of LC3B-I and -II by Western blot analysis; β-actin served as the standard. (*B*) A549 transfected with green fluorescent protein (GFP)-LC3 were exposed to 250 ppm CO or room air for 24 hours. Cells were visualized by confocal laser scanning microscopy. The percentage of cells exhibiting punctuated GFP-LC3 fluorescence was calculated relative to all GFP-positive cells. Data are presented as average (±SE) of three independent experiments. $^{*}P < 0.05$, $^{\#}P < 0.01$. (*C–E*) Human bronchial epithelial (HBE) cells were exposed to (*C*) CO alone (250 ppm) for 0–24 hours, (*D*) hyperoxia alone (>95% O_2) for 0–24 hours, or (*E*) combination treatments (CO and/or hyperoxia) for 0–72 hours and evaluated for time-dependent expression of LC3B-I and -II by Western blot analysis; β-actin served as the standard.

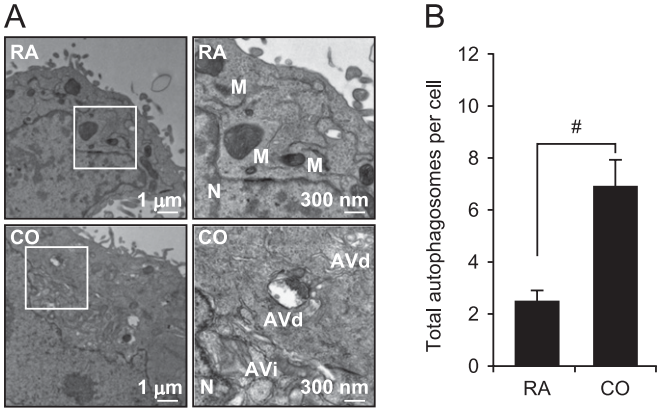

Figure 3. CO increases autophagosome formation in human epithelial cells *in vitro*. (*A*) Electron microscopic (EM) analysis of A549 cells after 24 hours of 250 ppm CO exposure. We indicate the presence of immature autophagic vacuoles (AVis) or degradative AVs (AVds). (*B*) Quantification of autophagosomes (AVi + AVd) per cell in A549 cells exposed to CO or room air. Data represent mean (±SD) (n = 15 EM images per condition); $^{\#}P < 0.01$.

an elevation of mitochondrial ROS production (Figure 4E). On the other hand, 250 ppm CO, when applied during hyperoxia exposure, resulted in a net reduction of ROS production (Figure 4E). Quantification of these results (Figures 4F–4H) indicated that CO exposure alone augments mitochondrial ROS production in epithelial cells that is reversible by general and mitochondria-specific antioxidants, whereas CO diminishes the stimulatory effects of high oxygen exposure.

We further hypothesized that the induction of autophagic protein gene expression by CO treatment is dependent on the increased generation of mitochondrial ROS induced by CO. Consistent with this hypothesis, treatment of A549 cells with NAC (5 mM) inhibited the activation of LC3B induced by CO exposure (250 ppm, 24 h) (Figure 5A). Furthermore, treatment of A549 with Mito-TEMPO (100 μM) also inhibited LC3B activation induced by 24-hour CO exposure compared with cells exposed to CO in the absence of the antioxidant (Figure 5B). Similar attenuation of the CO-induced LC3B response by both NAC and Mito-TEMPO were observed in HBE cells (Figure 5C). Hyperoxia-induced cell death, as measured by LDH release, was inhibited by both antioxidant compounds, NAC and Mito-TEMPO whereas treatment with these antioxidants alone did not affect LDH release (Figure 5D). These results indicate that CO increases the expression and activation of LC3B through augmented ROS generation of mitochondrial origin. Hyperoxia-induced cytotoxicity, however, is ultimately dependent on excessive ROS production, and is also reversed by antioxidant treatment.

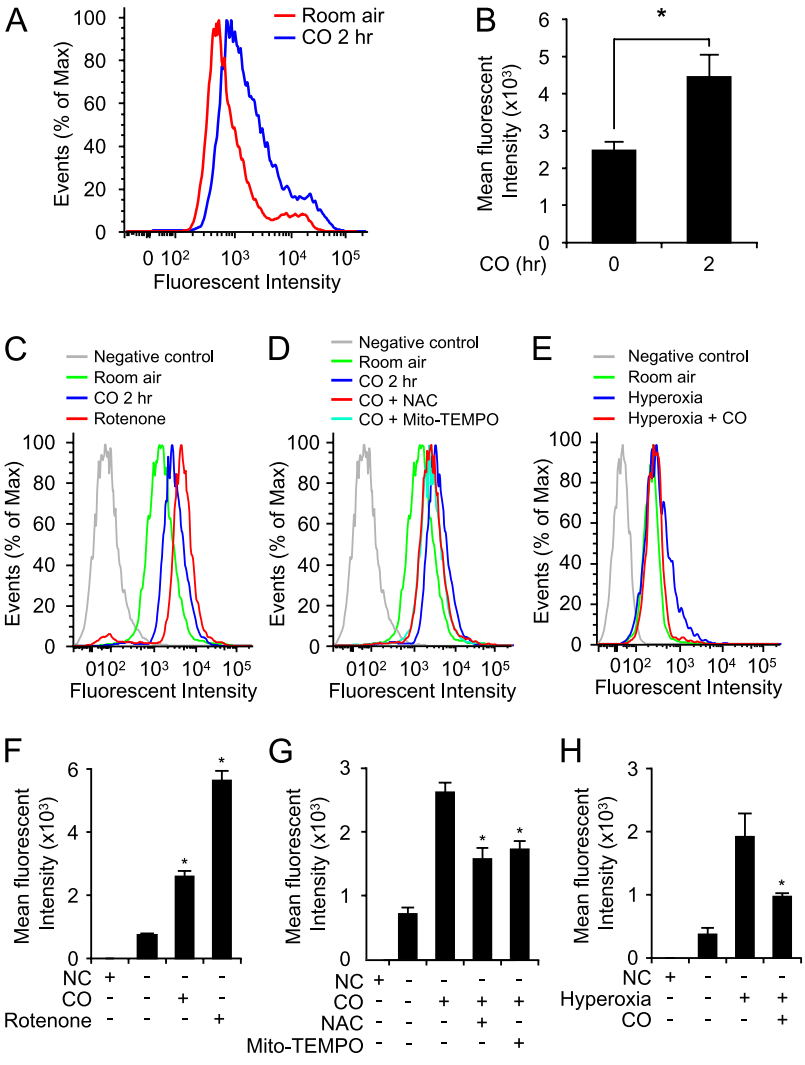

Figure 4. CO modulates mitochondrial reactive oxygen species (ROS) generation. A549 cells (*A* and *B*) or HBE cells (*C–H*) were exposed to 250 ppm CO or room air (Control), in the absence or presence of antioxidants, N-acetyl-L-cysteine (NAC) or Mito-TEMPO (*D*), or in the presence of high oxygen tension (>95% O_2) (*E*) (stained with MitoSOX Red and analyzed by flow cytometry). Rotenone was used as a positive control for mitochondrial ROS production (*C* and *F*). Cells left unstained served as an additional negative control (NC). Representative histograms of flow cytometry experiments demonstrate increase in fluorescent intensity of MitoSOX Red (5 μM) after CO treatment for 2 hours (*A* and *C*), which is reversible by antioxidant treatment (*D*). Hyperoxia increases MitoSOX Red signal, which is diminished in the presence of 250 ppm CO (*E*). Quantification data (*B* and *F–H*) are presented as average (±SE) of three independent experiments; $^{*}P < 0.05$.

CO-Mediated Protection against Hyperoxia-Induced Epithelial Cell Death Was Dependent on Autophagic Protein, LC3B

Our previous studies showed that CO protects against caspase-3–dependent apoptosis during hyperoxic exposure in endothelial cells (18). To further explore the mechanisms by which CO can mitigate hyperoxia-induced apoptosis, we evaluated the role of CO-induced LC3B in hyperoxia-induced cell death.

Infection of epithelial cells (A549 or HBE) with LC3B-specific siRNA further decreased cell viability during hyperoxic exposure relative to control-infected cells. Stimulation with 250 ppm CO during hyperoxia improved cell viability in control siRNA–infected epithelial cells. However, the protection afforded by CO against hyperoxia-induced cell death was compromised in LC3B-siRNA–infected A549 and HBE cells relative to control siRNA–infected cells. These data suggest a cytoprotective role of the autophagic protein LC3B in human epithelial cells during hyperoxia (Figures 6A and 6C), and also indicate a role for LC3B in the cytoprotection afforded by CO. Interestingly, the LC3B-specific siRNA–infected epithelial cells also displayed increased activation of hyperoxia-induced caspase-3 cleavage compared with control siRNA–infected cells (Figures 6B and 6D). The protection afforded by CO against hyperoxia-induced activation of caspase-3 was compromised in LC3B-siRNA–infected A549 and HBE cells (Figures 6B and 6D).

DISCUSSION

The process of macroautophagy can extend cell survival under conditions of starvation or nutrient depletion by recycling metabolic precursors from damaged organelles or denatured proteins (21, 22). Furthermore, autophagy is now known to play regulatory roles in innate and adaptive immunity, and may directly assist in the clearance of pathogens (33). The function of autophagy in stress is not always clear, and has been linked to both pro- and antiapoptotic mechanisms. For example, in cigarette smoke exposure, autophagy may promote, rather than inhibit, the progression of apoptosis (27). In this study, we show for the first time, that autophagy, a cellular homeostatic program, can be regulated by CO exposure in epithelial cell culture. We show that CO induces biochemical and morphological markers of autophagy, including the enhanced expression of autophagic proteins, and the formation of autophagosomes. Given that CO can provide pleiotropic cyto- and tissue-protective effects at low concentration, these observations add the modulation of autophagy and/or autophagic proteins as additional candidate mechanism(s) by which CO can protect cellular function and preserve homeostasis under stress.

A role for autophagic regulator proteins in CO-dependent cytoprotection is indicated by studies showing that genetic interference of LC3B abolishes the cytoprotective effects of CO in hyperoxic lung cell death in epithelial cells. LC3B is a major regulator of autophagosome formation, and is the sole *atg* protein retained by mature autophagosomes. We cannot exclude the possibility that nonautophagic functions of LC3B may contribute to the apparent cytoprotection, including possible effects on mRNA translation, microtubule organization, or interaction with signaling mechanisms (34, 35). Recently, we have shown that LC3B can modulate the apoptotic program in epithelial cells challenged with cigarette smoke extract (27).

The mechanism(s) by which CO can modulate the autophagic program remain incompletely clear. The current study is the first to show that autophagy can be modulated in epithelial cells by CO, through an ROS-dependent signaling component. Several other recent studies have implicated intracellular ROS and/or redox signaling mechanisms in the regulation of the autophagy. For example, starvation, a well known activator of autophagy, was associated with increased intracellular ROS production in CHO and HeLa cells (36). Treatment of CHO cells with antioxidant (i.e., NAC) or catalase reduced starvation–induced autophagosome formation. The authors identified autophagic protein,

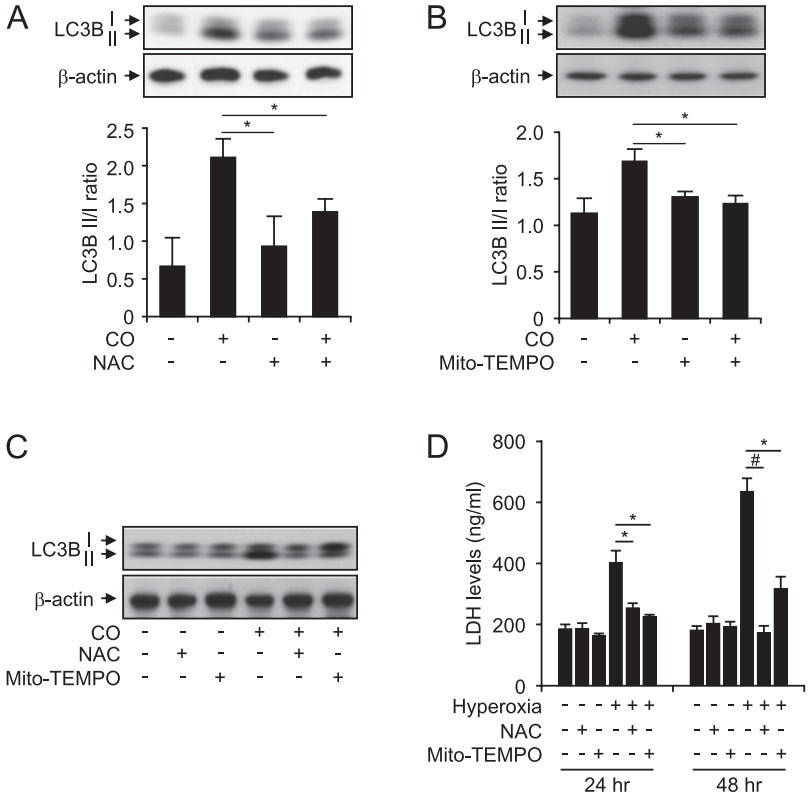

Figure 5. CO-induced LC3B is dependent on ROS production in A549 cells. (*A*) A549 cells were pretreated with 5 mM NAC for 30 minutes followed by CO treatment (250 ppm) for 24 hours. LC3B protein levels were determined by Western blotting. (*B*) A549 cells were treated with Mito-TEMPO (100 μM) for 24 hours in the presence of CO (250 ppm) or room air. (*C*) HBE cells were pretreated with 5 mM NAC for 30 minutes followed by CO treatment (250 ppm) for 24 hours, or were treated with Mito-TEMPO (100 μM) for 24 hours in the presence of 250 ppm CO or room air; β-actin was used as the standard (*A–C*). (*D*) HBE cells were treated with 5 mM NAC or Mito-TEMPO (100 μM) for 24 or 48 hours. Lactate dehydrogenase (LDH) levels were detected in cell culture supernatants by colorimetric assay (*see* MATERIALS AND METHODS). Data are presented as average (±SE) of three independent experiments. $^{*}P < 0.05$, $^{\#}P < 0.01$ (*A–B* and *D*).

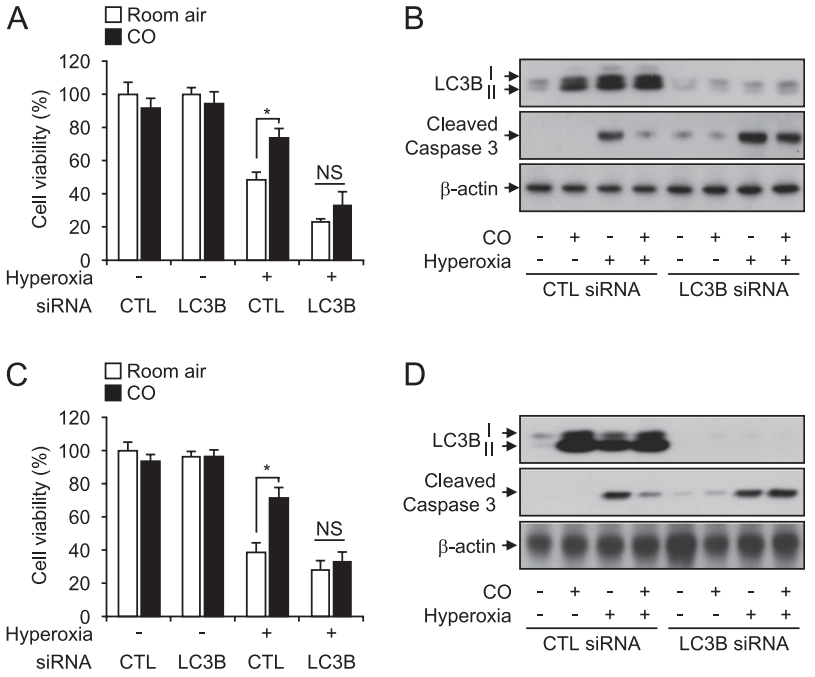

Figure 6. CO protection against hyperoxia-induced cell death in human epithelial cells requires autophagic protein, LC3B. A549 cells (*A* and *B*) or HBE cells (*C* and *D*) were transfected with small interfering (si)RNA of LC3B for 12 hours and recovered in fresh medium containing 10% FBS. After 24 hours, cells were cultured in serum-containing medium and further incubated with hyperoxia (95% O_2) in the presence (250 ppm) or absence of CO. (*A* and *C*) After 72 hours, cell viability was measured by the crystal violet staining method. (*B* and *D*) After 72 hours, the protein levels of cleaved caspase-3, LC3B, and β-actin were determined by Western blot analysis. Data shown in (*A*) represent the mean (±SD) ($n \geqslant 3$); *$P < 0.05$ versus hyperoxia alone.

Atg4, as a potential target for ROS, and proposed a redox regulation mechanism for the activity of this protein (36).

Currently, intracellular ROS are regarded not only as a potential source of toxicity under pathophysiological states, but also as integral components of signaling pathways at physiological levels. In hyperoxia, the excess production of ROS leads not only to the activation of autophagic markers, as shown in this study, but also to epithelial cell killing. In contrast, nontoxic levels of CO (250 ppm) generate sufficient levels of ROS, as shown by MitoSOX staining, to activate autophagic process without cell killing. Consistently, antioxidant compounds can both reduce overt toxicity by hyperoxia, as shown by LDH assays, as well as inhibit LC3B expression induced by exposure to CO alone.

Intracellular ROS generation can occur at several sites, including reduced NAD phosphate (NADPH) oxidase, xanthine oxidase, and mitochondrial respiratory chain complexes I and III (37). CO exposure has previously been reported to modulate intracellular ROS generation (18, 28–30) at membrane and/or mitochondrial sources. The down-regulation of plasma membrane–dependent ROS production by CO has been implicated in antiapoptotic (18), antiproliferative (29), and anti-inflammatory effects (28, 30) of CO through inhibition of NADPH oxidase activity and consequent effects on cell signaling. For example, down-regulation of NADPH oxidase–dependent ROS production by CO inhibited Toll-like receptor–dependent trafficking to the lipid raft after LPS stimulation (28). Furthermore, the up-regulation of mitochondrial ROS by CO, through deregulated electron flow through the respiratory chain, has also been implicated in antiproliferative (29) and anti-inflammatory (30) effects of CO. For example, up-regulation of peroxisome proliferator–activated receptor–γ by CO-dependent ROS production led to down-regulation of Egr-1 and downstream inflammatory responses in macrophages (30).

In this study, we show that scavenging of mitochondrial ROS with Mito-TEMPO, a triphenylphosphonium-conjugated antioxidant that localizes to the mitochondria (38), inhibits the activation of LC3B by CO, indicating a role for mitochondrial-derived ROS in the activation of autophagy by CO. Recent literature has identified the preferential mitochondrial localization and ROS scavenging effect of Mito-TEMPO (38, 39). Although mitochondria-derived ROS are thought to arise primarily from respiratory activity, we cannot completely exclude the potential contribution of ROS from other cytosolic sources, such as nonphagocytic NADPH oxidase isoforms, in the signaling effects of CO.

Although both hyperoxia and CO alone can increase cellular ROS, the simultaneous application of CO results in a net protective effect, associated with the stabilization of LC3B. Interestingly, the stimulatory effects of hyperoxia treatment as a known activator of mitochondrial ROS production were diminished by the simultaneous inclusion of CO, suggesting that CO antagonizes ROS production under high oxygen tension. Thus, although both treatments appear to stimulate LC3B conversion, hyperoxia leads to a faster degradation of LC3B, probably due to a higher degree of oxidative stress than that induced by CO alone.

Recent studies also implicate ROS-dependent signaling in the stimulation of mitochondrial biogenesis by CO through redox-dependent activation of nuclear respiratory factor 1, which, together with nuclear respiratory factor 2 and peroxisome proliferator–activated receptor γ coactivator–1α, stimulate the transcription of mitochondrial transcription factor A, a key regulator of mitochondrial DNA replication (40, 41). These studies, taken together with our identification of autophagy as an inducible response to CO exposure, indicate that CO can potentially accelerate the turnover of mitochondria by stimulating both mitochondrial degradation and biosynthesis pathways. However, we cannot exclude the possibility that the role of LC3B in CO-dependent cytoprotection involves signaling effects of LC3B independent of autophagic process.

These studies lend further support to the potential therapeutic application of CO, which has been demonstrated in numerous *in vitro* and *in vivo* models of acute lung injury (6–12). Although the clinical application of CO in humans for diseases of the lung remains unrealized, the feasibility of low-dose CO application in humans and nonhuman primates has now been tested (42–44). A recent study shows potential therapeutic effects of CO in human subjects with chronic obstructive pulmonary disease (44). The further examination of therapeutic efficacy of CO in human acute

lung injury/acute respiratory distress syndrome awaits approved clinical trials.

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