Mitochondrial Respiratory Chain and NAD(P)H Oxidase Are Targets for the Antiproliferative Effect of Carbon Monoxide in Human Airway Smooth Muscle

Carbon monoxide (CO), one of the end products of heme oxygenase activity, inhibits smooth muscle proliferation by decreasing ERK1/2 phosphorylation and cyclin D1 expression, a signaling pathway that is known to be modulated by reactive oxygen species (ROS) in airway smooth muscle cells (ASMCs). Two important sources of ROS involved in cell signaling are the membrane NAD(P)H oxidase and the mitochondrial respiratory chain. Thus, CO could modulate redox signaling in ASMCs by interacting with the heme moiety of NAD(P)H oxidase and/or the respiratory chain is a plausible hypothesis. Here we show that a recently identified carbon monoxide-releasing molecule, [Ru(CO)3Cl2]2 (or CORM-2) 1) inhibits NAD(P)H oxidase cytochrome b558 activity, 2) increases oxidant production by the mitochondria, and 3) inhibits ASMC proliferation and phosphorylation of the ERK1/2 mitogen-activated protein kinase and expression of cyclin D1, two critical pathways involved in muscle proliferation. No such effects were observed with the negative control (Ru(Me2SO)4Cl2), which does not contain CO groups. Because both diphenylene iodonium or apocynin (inhibitors of NAD(P)H oxidase) and rotenone (a molecule that increases mitochondrial ROS production by blocking the respiratory chain) mimicked the effect of CORM-2 on cyclin D1 expression and ASMC proliferation, the antiproliferative effect of CORM-2 is probably related to inhibition of cytochromes on both NAD(P)H oxidase and the respiratory chain. The involvement of increased mitochondrial-derived oxidants is substantiated by the findings showing that the antioxidant N-acetylcysteine partially inhibited the effects of CORM-2. This study provides a new mechanism to explain redox signaling by CO.

HO-1, the limiting step enzyme in heme degradation, is strongly involved in the control of smooth muscle proliferation (1). We have recently reported that bilirubin, one of the products of heme breakdown by HO-1, modulates redox signaling pathways of human ASMCs resulting in inhibition of cell proliferation (2). Other studies have shown that CO, another metabolite of HO activity, could also have an antiproliferative effect on vascular (3–5) and bronchial smooth muscle tissues (6). This effect has also been observed in models in vivo where CO protected against restenosis of carotid arteries following balloon injury (7) and hypoxic pulmonary hypertension (8). The mechanisms by which CO exerts its antiproliferative effects in ASMCs appear to rely on inhibition of ERK1/2 phosphorylation and cyclin D1 expression (6). The involvement of ERK pathway inhibition by CO has been described in other cellular models (9), but the precise mechanism explaining this interaction remains unknown. Interestingly the specific pathway responsible for cyclin D1 expression is sensitive to oxidants (10). In view of these findings, the existence of a potential “CO sensor” that regulates ASMC proliferation cannot be excluded a priori and represents a challenging hypothesis to explore further.

Two important sources of oxidants involved in the control of cell proliferation are the NAD(P)H oxidase (11, 12) and the mitochondrial respiratory chain (13–16). NAD(P)H oxidase is made of an assembly of different proteins: gp91phox and p22phox (which heterodimerize to form the cytochrome b558 and p67phox), p47phox, and Rac1 or -2 subunits. Gp91phox, the catalytic moiety of the oxidase, is a plasma membrane-associated flavohemoprotein containing one flavin-adenine dinucleotide and two hemes that catalyzes the NAD(P)H-dependent reduction of oxygen to form superoxide (17). Activation of NAD(P)H oxidase has been associated with an increased proliferative response (2, 11).

The mitochondrial respiratory chain is composed of different cytochromes accounting for 85–90% of the O2 consumed in the cell. Approximately 1–3% of this O2 is incompletely metabolized and diverted into superoxide anion (18). Inhibition of electron transfer in the mitochondrial respiratory chain is associated with a significant increase in the production of superoxide anion and hydrogen peroxide and a decreased cell proliferation (14, 16). It must be noted that this is in contrast with the mitogenic effect of NAD(P)H oxidase-derived superoxide anion. Although there is no clear explanation for the discrepancy between the effects of mitochondria- and NAD(P)H oxidase-derived ROS on cell proliferation, most evidence suggests that the intensity and subcellular origins of ROS may be crucial in their effects on cell fate.

Because CO has a high affinity for heme groups (for a review, see Ref. 19), we hypothesized that CO could behave as a modulator of redox signals by interacting with heme and inhibiting

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cytchrome b$_{558}$ of the NAD(P)H oxidase and/or cytochromes of the respiratory chain, consequently affecting redox-mediated cell proliferation.

Recently a group of transition metal carbonyls have been characterized as carbon monoxide-releasing molecules (CORMs) to liberate CO in biological systems providing a useful tool in research to examine the mechanism by which CO exerts its pharmacological activities (20–23). Therefore, in this study we investigated whether CO, administered using CORM-2 ([(Ru(CO)$_3$Cl$_2$)$_2$]), could modulate PDGF-induced human ASM cell proliferation and examined whether this effect involves heme-dependent ROS-producing pathways such as the NAD(P)H oxidase and the mitochondrial respiratory chain.

**MATERIALS AND METHODS**

**Reagents—**[Ru(CO)$_3$Cl$_2$]$_2$ (CORM-2) was obtained from Aldrich. Ru$_3$(Me$_2$SO)$_2$Cl$_2$, the negative control for CORM-2, was synthesized as described previously (20). [methyl-$_3$H]Thymidine was purchased from Perbio Sciences, and DAPI was from R&D Systems (Abingdon, UK). Apocynin (acetoxyvanillic acid) was from Acros Organics (Geel, Belgium). DCFH-DA and CM-H$_2$XRos (MitoTracker® Red) were from Molecular Probes (Eugene, OR). Polyclonal anti-p44/42 (phosphorylated and non-phosphorylated) antibody was purchased from New England Biolabs (Ozyme, Saint-Quentin-en-Yvelines, France), monoclonal anti-HO-1 and anti-nitric-oxide synthase type 1 (NOS1) antibodies were from StressGen, and anti-cytochrome D1 antibody was from Santa Cruz Biotechnology (Tebu, Le-Perray-en-Yvelines, France). Culture media, supplements, and fetal calf serum were from Invitrogen. Tissue culture plasticware was supplied by Costar Corp. (Cambridge, MA). Reagents for Western blotting were from Bio-Rad. Other reagents were from Sigma.

**Human Airway Smooth Muscle Cell Isolation and Culture—** Primary cultures of human bronchial smooth muscle were established as already described (2, 24). Briefly human bronchi were obtained from lung resection for cancer of six different patients and dissected from the surrounding parenchyma. Then the epithelium was removed, and bundles of smooth muscle were dissected under binocular microscope. Smooth muscle was cut into 1-mm square pieces, termed explants, and incubated in 6-well plates with Dulbecco’s modified Eagle’s medium (Sigma). 50% fetal calf serum, and antibiotics (100 µg/ml penicillin, 100 µg/ml streptomycin). Cells were subcultured in 24-well plates, with 50 ng/ml PDGF. Fluorescence was measured immediately after the medium was removed, cells were washed twice with phosphate-buffered saline and incubated in 1 ml of the same buffer with and without 300 units/ml superoxide dismutase. Subsequently ferricytochrome c was added at a final concentration of 1 µM in the reaction buffer solution followed by addition of PDGF. After 1 h, the buffer was removed, and absorbance at 550 nm was measured immediately. Superoxide anion production was calculated from the differences in the absorbances between samples with and without superoxide dismutase using an extinction coefficient of 21.1 mM$^{-1}$ cm$^{-1}$ for reduced ferricytochrome c.

ASMC Cytochrome b$_{558}$ Spectra Analysis—The spectrum of cytochrome b$_{558}$ was analyzed as described previously (25). Briefly cells were lysed in phosphate-buffered saline in the presence of 2% Triton X-100 at 4 °C for 10 min. Samples were treated with 10% CORM-2 or Ru$_3$(Me$_2$SO)$_2$Cl$_2$. Then the difference between the reduced and oxidized spectrum was recorded with a dual beam scanning spectrophotometer (Uvikon). The base-line (oxidized) spectrum was measured at 400–600 nm, and then a few grains of sodium dithionite were added to the sample cuvette, and a new spectrum was recorded. The subtraction between spectra was performed automatically.

**Intracellular Superoxide Production by ASMCs: Cytochrome c Reduction Assay—Ferricytochrome c reduction was measured as described previously (25). Briefly cells were cultured in 96-well plates as described previously (2). DCFH-DA (10 µM) was added to the media 30 min before PDGF. Fluorescence was measured immediately after the medium was removed, cells were washed twice with phosphate-buffered saline, and incubated in 1 ml of Hank’s balanced salt solution at a concentration of 2 million/ml. Superoxide production was measured using an extinction coefficient of 21.1 mM$^{-1}$ cm$^{-1}$ for reduced ferricytochrome c.

Extracellular Superoxide Anion Production by ASMCs: Cytochrome c Reduction Assay—Ferricytochrome c reduction was measured as described previously (25). Briefly cells were cultured in 6-well plates. Before addition of PDGF, the medium was replaced with Hank’s balanced salt solution without phenol red and incubated in 1 ml of the same buffer with and without 300 units/ml superoxide dismutase. Subsequent ferricytochrome c was added at a final concentration of 1 µM in the reaction buffer solution followed by addition of PDGF. After 1 h, the buffer was removed, and absorbance at 550 nm was measured immediately. Superoxide anion production was calculated from the differences in the absorbances between samples with and without superoxide dismutase using an extinction coefficient of 21.1 mM$^{-1}$ cm$^{-1}$ for reduced ferricytochrome c.
before PDGF stimulation. NAC (1 mM) was only observed at 100 μM concentration). Cell toxicity of the compound was evident at the medium and trypan blue exclusion test, was evident at the time when proliferation was measured. However, we found no change in HO-1 protein expression after 24-h incubation of cells in the presence of 1 and 10 μM CORM-2 (Fig. 2). We also investigated whether CORM-2 influenced the expression of NOS1 because this enzyme has been shown to modulate ASMC proliferation (31). We found no change in NOS1 protein expression after 24-h incubation of cells in the presence of CORM-2 (data not shown).

Inhibition of NAD(P)H Oxidase by CORM-2 Contributes to the Attenuation of Cell Proliferation—As stated in the Introduction, NAD(P)H oxidase-derived superoxide anions are involved in ASMC proliferation (11, 32). We first confirmed these findings by using DPI (10 μM), an inhibitor of flavin-containing enzymes such as NAD(P)H oxidase (33). As expected, DPI significantly reduced PDGF-induced cell proliferation (Fig. 1). It must be noted, however, that DPI could inhibit all flavo-containing enzymes such as NOS and respiratory chain complex I and could also increase ROS production (34). We think that these effects of DPI are unlikely under our experimental conditions because we have previously shown that the same concentration of DPI (10 μM) used in the present study decreased intracellular ROS production in ASMCs (25). Furthermore if DPI had inhibited complex I, one should expect an increase and not a decrease of intracellular ROS production. Concerning a potential inhibition of NOS by DPI, we measured the nitrite/nitrate concentration in cell culture media after incubation with CORM-2 and found no significant changes in this parameter (data not shown). Moreover the effect of DPI was mimicked by the NAD(P)H oxidase inhibitor apocynin (10 μM) (35), further supporting the involvement of this enzyme in PDGF-induced cell proliferation (Fig. 1).

To further confirm that the effect of CORM-2 was mediated by CO released from the compound, we performed additional experiments in the presence of myoglobin, a CO scavenger. Indeed we observed that myoglobin (10 μM) effectively reversed the antiproliferative effect of CORM-2 (Fig. 1).

Because HO-1 is known to inhibit smooth cell proliferation by itself (2) and because heavy metals can induce HO-1 expression (30), we assessed whether the ruthenium-containing CORM-2 or its negative control could induce HO-1 expression per se at the time when proliferation was measured. Moreover the effect of DPI was mimicked by the NAD(P)H oxidase inhibitors DPI and apocynin (all at 10 μM concentration), or the mitochondrial complex I inhibitor rotenone (at 2 and 0.2 μM concentration) were given 30 min before PDGF stimulation. NAC (1 mM) was given 30 min before CORM-2 or rotenone. Results are expressed as cpm. Each bar represents the mean ± S.E. for four to six experiments. *, significantly different from 1% fetal calf serum (FCS), p < 0.05; #, significantly different from PDGF alone, p < 0.05. DMSO, Me2SO.

FIG. 1. Effect of CORM-2 on ASMC proliferation. Cell proliferation assessed by [3H]thymidine incorporation after 24-h stimulation with 50 ng/ml PDGF. CORM-2, the negative control Ru(Me2SO)4Cl2, the NAD(P)H oxidase inhibitor DPI and apocynin (all at 10 μM concentration), or the mitochondrial complex I inhibitor rotenone (at 2 and 0.2 μM concentration) were given 30 min before PDGF stimulation. NAC (1 mM) was given 30 min before CORM-2 or rotenone. Results are expressed as cpm. Each bar represents the mean ± S.E. for four to six experiments. *, significantly different from 1% fetal calf serum (FCS), p < 0.05; #, significantly different from PDGF alone, p < 0.05. DMSO, Me2SO.

RESULTS

CORM-2 Inhibits PDGF-induced ASMC Proliferation—To investigate how CO influences cell proliferation, we analyzed the effect of CORM-2 on PDGF-induced ASMC proliferation (2). We found that PDGF induced a 4-fold increase in ASMC proliferation. Treatment of cells with CORM-2 (10 μM) 30 min before stimulation with PDGF reduced cell proliferation by about 50%. In contrast, the negative control (Ru(Me2SO)4Cl2, 10 μM), which does not contain CO groups, did not modify the proliferative response (Fig. 1), confirming that CO liberated from CORM-2 is directly involved in the observed effect. This confirms that CORM-2 has effects similar to CO administrated as a gas in ASMCs (6) and is a useful tool for studying the mechanisms of action of CO. Indeed we observed that myoglobin (10 μM) effectively reversed the antiproliferative effect of CORM-2 (Fig. 1).
To assess whether CO inhibits PDGF-induced ASMC proliferation by affecting NAD(P)/H oxidase-dependent signaling pathway, we tested the effect of CORM-2 on NAD(P)/H oxidase function by evaluating the extracellular superoxide anion production in whole cells and NAD(P)/H oxidase activity in ASMC membranes. The results obtained by measuring the reduction of cytochrome c in whole ASMCs revealed that PDGF induced a 5.7-fold increase in superoxide production (Fig. 3, Panel A). CORM-2 inhibited cytochrome c reduction in a concentration-dependent manner with a maximum effect reached at 10 μM. Ru(Me₂SO)₄Cl₂ had no effect on ROS production. DPI and apocynin also significantly inhibited superoxide production, thus supporting the role of NAD(P)/H oxidase (Fig. 3, Panel A). This was further confirmed by analysis of NAD(P)/H oxidase activity in ASMC membranes prepared from cells incubated for 24 h in the presence or absence of PDGF (Fig. 3, Panel C). The results of these experiments showed a significant reduction in NAD(P)/H oxidase activity from isolated ASMC membranes after incubation with 10 μM CORM-2 (Fig. 3, Panel C).

To investigate the molecular target of CO within the NAD(P)/H oxidase, we analyzed in a whole cell lysate how CORM-2 affected the reduced minus oxidized spectrum of cytochrome b₅₅₃ the heme-containing membrane-bound component of the NAD(P)/H oxidase. CORM-2 induced changes in the cytochrome b₅₅₃ spectrum (Fig. 3, Panel B). Specifically the reduced minus oxidized spectrum of control ASMCs shows absorption at two main wavelengths, 426 and 558 nm, as described previously (25). In CORM-2-treated cells, we found the disappearance of the 558 nm peak and a decrease in absorbance at 426 nm. The negative control (Ru(Me₂SO)₄Cl₂) did not modify NAD(P)/H oxidase activity and did not change the cytochrome b₅₅₃ spectrum emphasizing the involvement of CO in the observed chemical modification. Because multiple cytochromes coexist in the cell, especially in the mitochondria, we purified ASMC membranes and performed a similar analysis of cytochrome b₅₅₃ spectra. The reduced spectra were similar to that of entire cells, and CORM-2 induced a similar change in the spectra, further supporting the effect of CO on NAD(P)/H oxidase (Fig. 3, Panel D).

Finally to further confirm the inhibition of NAD(P)/H oxidase by CO, we conducted experiments on fresh human polymorpho-nuclear cells as they contain very high levels of NAD(P)/H oxidase (17). In the absence of CORM-2, phorbol myristate acetate-stimulated superoxide production was 44.6 ± 2.2 nmol/2 million neutrophils/10 min. As in ASMCs, CORM-2 (25 and 50 μM, 30-min incubation) significantly inhibited superoxide production and changed cytochrome b₅₅₃ spectra in these cells (Fig. 4).

Inhibition of the Respiratory Chain by CORM-2 Contributes to the Attenuation of Cell Proliferation—Mitochondria-derived ROS are involved in the control of cell proliferation (13–16). To address this issue in our system, we first analyzed the muscular effects of rotenone, an agent that increases mitochondrial ROS production by blocking the respiratory chain at complex I (14, 15). As expected, rotenone increased PDGF-stimulated ROS production as revealed by quantification of DCFH-DA oxidation (Fig. 5, Panel C) and inhibited PDGF-induced cyclin D1 expression (Fig. 6) and cell proliferation (Fig. 1). It must be noted that the reduced cell proliferation observed with rotenone was not related to rotenone-induced apoptosis (36) because the percentage of ASMCs positively stained with propidium iodide was low and not statistically different in both untreated and rotenone-treated cells (0.85 ± 0.4 versus 2.09 ± 1.59%, n = 6, respectively).

We then investigated whether CORM-2 stimulates the intracellular production of ROS by the mitochondria. CORM-2 enhanced intracellular ROS production in a concentration-dependent manner as demonstrated by the increase in DCFH-DA fluorescence (Fig. 5, Panel A). The mitochondrial origin of these intracellular oxidants is supported by the similar concentration-dependent effect of CORM-2 on oxidation of MitoTracker CM-H₂XRos, a fluorescent probe specific for mitochondria-produced ROS (28) (Fig. 5, Panel B). Furthermore no effect of CORM-2 on DCFH-DA fluorescence was observed when cells were pre-treated with rotenone (Fig. 5, Panel C), suggesting that CORM-2 acts on the respiratory chain downstream of site I. We are confident that rotenone did not induce a maximal oxidation of DCFH-DA, which could theoretically mask the additive effect with CORM-2, because tumor necrosis factor-α induced a higher fluorescent signal (Fig. 5, Panel C). Therefore, we can conclude that CORM-2 causes antiproliferative effects via either inhibition of the NAD(P)/H oxidase and/or the mitochondrial respiratory chain.

CORM-2 Inhibits Cyclin D1 Expression and ERK1/2 Mitogen-activated Protein Kinase Phosphorylation—As cyclin D1 expression is a key element in ASMC proliferation (37), we tested whether CORM-2, similar to CO gas, modulates PDGF-induced cyclin D1 expression. CORM-2, but not the negative control Ru(Me₂SO)₄Cl₂ (data not shown), inhibited cyclin D1 expression in a dose-dependent manner (Fig. 6, Panels A and B). Because we hypothesized that CO causes antiproliferative effects via inhibition of the NAD(P)/H oxidase and/or the mitochondrial respiratory chain, we used the oxidase inhibitors DPI and apocynin and the mitochondrial respiratory chain inhibitor rotenone to assess whether the oxidase or the respiratory chain was involved in the changes of cyclin D1 systems. We found that both oxidase inhibitors (DPI and apocynin) blocked PDGF-induced cyclin D1 expression (Fig. 6). A similar reduction in cyclin D1 expression was observed with rotenone (Fig. 6, Panels A and B), which increased intracellular ROS secondary to respiratory chain inhibition (14, 15).

Cyclin D1 expression is known to be regulated by ERK1/2 (37, 38). Therefore, we investigated whether CORM-2 could modulate PDGF-induced ERK1/2 phosphorylation as demonstrated with 1% CO gas (6). We found that CORM-2 inhibited ERK1/2 phosphorylation in a concentration-dependent manner (Fig. 7, Panels A and B). Interestingly DPI (10 μM) did not modify ERK phosphorylation, whereas rotenone significantly
FIG. 3. Effect of CORM-2 on NAD(P)H oxidase activity and cytochrome \( b_{558} \) spectra in ASMCs. Panel A, effect of CORM-2 on PDGF-induced superoxide anion production in ASMCs assessed by cytochrome c reduction assay. CORM-2, Ru(Me$_2$SO)$_4$Cl$_2$, DPI, and apocynin were added to the medium 30 min before PDGF. Each bar represents the mean ± S.E. for four to six experiments. *, significantly different from 1% fetal calf serum (FCS), \( p < 0.05 \); #, significantly different from PDGF alone, \( p < 0.05 \). Panels B and D, spectrum analysis of flavocytochrome \( b_{558} \) in whole ASMCs and ASMC cell membranes, respectively, treated with 10 \( \mu M \) CORM-2 or Ru(Me$_2$SO)$_4$Cl$_2$. The difference between reduced and oxidized spectra was analyzed as described under “Materials and Methods.” Panel C, effect of CORM-2 on PDGF-induced superoxide anion production in ASMC membranes assessed by lucigenin-enhanced chemiluminescence. Membranes were prepared from cells incubated 24 h in the presence and in the absence of PDGF. CORM-2, Ru(Me$_2$SO)$_4$Cl$_2$, and DPI were added to the medium 30 min before measurement. Each bar represents the mean ± S.E. for four to six experiments. *, significantly different from 1% fetal calf serum (FCS), \( p < 0.05 \); #, significantly different from PDGF alone, \( p < 0.05 \). DMSO, Me$_2$SO; Abs, absorbance.
inhibited this process (Fig. 8, Panels A and B). Similar findings were obtained with apocynin (data not shown). The effects of rotenone and CORM-2 were reversed by preincubation of ASMCs with N-acetylcysteine (NAC) (Fig. 8, Panels A and B). Interestingly NAC also reversed the effect of both molecules on cell proliferation, although the effect on down-regulation of ASMC proliferation elicited by CORM-2 was only partial (Fig. 1).

Altogether these results provide evidence that CORM-2 inhibits two important signaling pathways involved in ASMC proliferation: a mitochondrial ROS-ERK1/2-cyclin D1 pathway and an NAD(P)H oxidase-cyclin D1 pathway. The involvement of the former pathway is further supported by the results showing that NAC reversed the effects of CORM-2 on ERK1/2 phosphorylation and cell proliferation.

**DISCUSSION**

The main results of this study are that CORM-2, a carbon monoxide-releasing molecule 1) inhibits NAD(P)H oxidase cytochrome b$_{558}$ activity, 2) increases oxidant production by the mitochondria, and 3) inhibits ASMC proliferation and phosphorylation of the ERK1/2 mitogen-activated protein kinase and expression of cyclin D1, two critical pathways involved in muscle proliferation (35). Because both DPI and apocynin (inhibitors of NAD(P)H oxidase) and rotenone (a molecule that increases mitochondrial ROS production by blocking the respiratory chain) mimicked the effect of CORM-2 on cyclin D1 expression and ASMC proliferation, we propose that the anti-proliferative effect of CORM-2 is probably related to inhibition of cytochromes on both NAD(P)H oxidase and the respiratory chain. The partial inhibition of CORM-2-mediated effects on proliferation by the antioxidant NAC supports a role for the increase in mitochondrial oxidants. Interestingly the participation of the ERK1/2 pathway on the CORM-2-mediated antiproliferative effect was probably related only to inhibition of the mitochondrial respiratory chain because a decreased ERK1/2 phosphorylation was observed with rotenone but not with NADP(H) oxidase inhibitors and was completely reversed by NAC in the case of both rotenone and CORM-2. To the best of our knowledge these data provide for the first time evidence that both NAD(P)H oxidase and the respiratory chain are targets for CO in ASMCs.

The role of CO in modulating smooth muscle cell proliferation has been previously investigated. It was first studied in vascular smooth muscle (4) and subsequently in airway smooth muscle (6), revealing that in the presence of CO the cycle progression is arrested at the G0/G1 phase. Although some of the mechanisms involved in CO signaling have been described, such as the involvement of ERK1/2 mitogen-activated protein kinase family (6) and the guanylyl cyclase-cGMP pathway (5), the true target for CO and the chemical modification required for controlling cell proliferation remain to be fully characterized. The current knowledge on the biochemistry of CO and especially its high affinity for heme moieties strongly suggest that heme-containing enzymes are likely targets for CO-dependent cellular activities (39). For example, the activity of nitric-oxide synthase, which contains heme, is inhibited by CO (40), and this effect seems responsible for the paradoxical increase in vascular reactivity of arterioles recently described by Johnson and Johnson (41). In addition, the well known toxicity of CO is strictly based on the high affinity of heme groups for CO gas, which prevents binding of oxygen and redox reactions required for controlling cell proliferation. Heme-binding enzymes are likely targets for CO-dep

![Image](http://www.jbc.org/)

**FIG. 4. Effects of CORM-2 on NAD(P)H oxidase activity and cytochrome b$_{558}$ spectra in human polymorphonuclear cells.** Panel A, effect of CORM-2 on phorbol myristate acetate-induced superoxide anion production in polymorphonuclear cells. CORM-2 and Ru(Me$_2$SO)$_3$Cl$_2$ were added to the medium 30 min before phorbol myristate acetate. Control (100%) superoxide production was 44.6 ± 2.0 nmoles/million neutrophils/10 min. Each bar represents the mean ± S.E. for four to six experiments. *, significantly different from Ru(Me$_2$SO)$_3$Cl$_2$, p < 0.05. Panel B, spectrum analysis of flavocytochrome b$_{558}$ in polymorphonuclear cells treated with 50 μM CORM-2 or Ru(Me$_2$SO)$_3$Cl$_2$. The difference between reduced and oxidized spectra was analyzed as described under “Materials and Methods.” DMSO, Me$_2$SO; Abs, absorbance.
Ru(Me$_2$SO)$_4$Cl$_2$ were applied directly to the membranes, we can ensure that CO directly interacts with NAD(P)H oxidase, excluding an effect mediated by intracellular events, as it should occur in experiments using whole cells. Furthermore the ability of CO to bind and inhibit cytochrome b$_{558}$ was confirmed in human neutrophils, which express high levels of this protein complex.

Our study further demonstrates that CO increased the production of intracellular ROS originating from mitochondria because CORM-2 increased the fluorescence of MitoTracker CM-H$_2$XRos, a probe specific for mitochondria-produced ROS (43, 44). This was probably secondary to inhibition of cytochromes in the respiratory chain as demonstrated previously by Poderoso et al. (45). Furthermore we observed an increase in intracellular oxidants in ASMCs using rotenone, an agent that increases mitochondrial ROS production by blocking the respi-

**FIG. 5.** Effect of CORM-2 on intracellular ROS production in ASMCs. Panel A, effect of CORM-2 on PDGF-induced intracellular ROS production in ASMCs assessed by measurement of DCFH-DA oxidation as described under “Materials and Methods.” Control, Ru(Me$_2$SO)$_4$Cl$_2$. As described under “Materials and Methods” Ru(Me$_2$SO)$_4$Cl$_2$ and CORM-2 were added to the medium 30 min before PDGF. Each point represents the mean ± S.E. for five to seven experiments. *, significantly different from 1% fetal calf serum (FCS), p < 0.05; #, significantly different from PDGF alone, p < 0.05. Panel B, effect of CORM-2 on PDGF-induced mitochondrial ROS production in ASMCs assessed by measurement of CM-H$_2$XRos oxidation as described under “Materials and Methods.” Each bar represents the mean ± S.E. for six to eight experiments. *, significantly different from 1% fetal calf serum (FCS), p < 0.05; #, significantly different from PDGF alone, p < 0.05. Panel C, effect of CORM-2 and/or the mitochondrial complex I inhibitor rotenone on PDGF-induced intracellular ROS production in ASMCs assessed by measurement of DCFH-DA oxidation as described under “Materials and Methods.” The effects of tumor necrosis factor-α (TNF-α) were also quantified. Each point represents the mean ± S.E. for four to seven experiments. *, significantly different from 1% fetal calf serum (FCS), p < 0.05; #, significantly different from PDGF alone, p < 0.05; §, significantly different from CORM-2 + rotenone. DMSO, Me$_2$SO.
FIG. 6. Effect of CORM-2, DPI, apocynin, and rotenone on cyclin D1 expression in ASMCs. Panel A, typical Western blot analysis of cyclin D1 expression after a 24-h stimulation with PDGF-AB in the presence of CORM-2, DPI, or rotenone added to the media 30 min before PDGF. Panel B, densitometric analysis of the cyclin D1 bands. Each bar represents the mean ± S.E. of the optical density of the bands compared with the expression of β-actin for four experiments. *, significantly different from 1% fetal calf serum (FCS), p < 0.05; #, significantly different from PDGF alone, p < 0.05.

Panel C, typical Western blot analysis of cyclin D1 expression after a 24-h stimulation with PDGF-AB in the presence of apocynin added to the media 30 min before PDGF. Panel D, densitometric analysis of the cyclin D1 bands. Each bar represents the mean ± S.E. of the optical density of the bands compared with the expression of β-actin for four experiments *.}, significantly different from 1% fetal calf serum (FCS), p < 0.05; #, significantly different from PDGF alone, p < 0.05.
Interestingly there was no additive effect by CORM-2 and rotenone on intracellular ROS concentration, suggesting that CORM-2 inhibits the respiratory chain downstream of complex I, probably at the cytochrome c oxidase level (19, 46), as observed previously with nitric oxide (45). However, we cannot rule out an effect of CO on other components of the respiratory chain resulting in an increased mitochondrial ROS generation. It must be noted that in PDGF-stimulated cells we found a discrepancy between the decrease in extracellular ROS production and the increase in intracellular ROS concentration elicited by CORM-2. Because smooth muscle NAD(P)H oxidase produces superoxide anion toward both the extracellular and the intracellular sides of the cell, this discrepancy is at first glance counter intuitive because CO inhibited this protein complex. However, it could be explained by a predominance of the increase in mitochondrial ROS production. CO inhibits the respiratory chain prior to cytochrome c oxidase, which is downstream to complex I (14, 15).
ROS generation over NAD(P)/H oxidase inhibition, thus resulting in a net increase in intracellular ROS concentration. At present, we do not know whether the oxidase produces equal amounts of superoxide anion toward both the exterior and interior of the cell, but a less important contribution of intracellular production could explain this dissociation.

Both inhibition of NAD(P)/H oxidase and the respiratory chain could explain the decrease in ASMC proliferation elicited by CORM-2. Indeed inhibition of the oxidase by either DPI or apocynin and inhibition of the respiratory chain by rotenone blocked PDGF-induced cyclin D1 expression and the ensuing proliferation; the effect of rotenone was secondary to an increase in intracellular ROS because it was blocked by the antioxidant NAC. However, only rotenone attenuated ERK1/2 phosphorylation, which is known to regulate cyclin D1 expression (37, 38), suggesting that only the increase in mitochondrial ROS is involved in modulation of this mitogen-activated protein kinase. Because similar reversing effects by NAC on ERK1/2 phosphorylation and muscle proliferation were found in the case of CORM-2, we postulate that inhibition of the respiratory chain, resulting in an increased mitochondrial ROS production, was clearly involved in the antiproliferative effect of CORM-2 in ASMCs. This result is in agreement with previous data showing that mitochondria-derived ROS can modulate ERK phosphorylation in different cell types (14, 16, 47, 48). For example, Alonso and co-workers (48) have shown that increasing mitochondrial ROS production in rat brain in culture significantly decreased ERK1/2 phosphorylation, whereas decreasing ROS concentration with catalase had the opposite effect. However, because NAC reversed the effects of CORM-2 on cell proliferation only partially, we cannot exclude inhibition of an NAD(P)/H oxidase-cyclin D1 pathway in the decreased proliferation elicited by CO.

In a recent study published by our laboratory on the role of HO-1 in modulation of redox signaling in ASMCs, we found that bilirubin inhibited PDGF-induced ROS production, ERK phosphorylation, and cell proliferation, whereas scavenging endogenous CO with myoglobin or inhibiting the cGMP-guanylyl cyclase pathway did not modify the effect of HO-1 induction on ASMC proliferation (2). We concluded that HO-1-induced CO was not involved in this model. The discrepancy between those results and the ones presented here is difficult to explain. In the present study, we are confident about the specificity of CORM-2 as a CO-releasing molecule because its effect was reversed by myoglobin, and the negative control molecule did not significantly modify any of the parameters analyzed. One could argue that variations of the amount and/or of the cellular targets of endogenously produced versus exogenously administered CO could explain these differences. In this context, it is interesting to note that HO-1 induction reduced intracellular ROS production (2), whereas the opposite effect was observed in the present study with CO. It is possible that if HO-1 activity would have been more strongly induced, CO could have played a more important role. In fact, most of the studies investigating the cytoprotective role of CO have been performed in pathological conditions (such as hypoxia, hyperoxia, ischemia-reperfusion, etc.; for a review, see Ref. 42). Perhaps CO becomes predominant in situations where HO-1 is induced, whereas bilirubin is more strongly involved in the control of proliferation in normal conditions.

In conclusion, this study is the first to report that NAD(P)/H oxidase and the mitochondria respiratory chain are targets for the antiproliferative effects of CO in human ASMCs. Given the wide distribution of these heme proteins, we can imagine that they could act, separately or in concert, as “CO sensors,” transducing signals via specific redox pathways upon CO fixation on the heme component. This could constitute a general mechanism explaining the wide protective effects of CO, as those observed in sepsis, transplantation, or ischemia-reperfusion.

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