p21-Dependent Protective Effects of a Carbon Monoxide–Releasing Molecule-3 in Pulmonary Hypertension

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**Objective**—Carbon monoxide–releasing molecules (CORMs) represent a pharmacological alternative to CO gas inhalation. Here, we questioned whether CORM-3, a well-characterized water-soluble CORM, could prevent and reverse pulmonary hypertension (PH) in chronically hypoxic mice and in smooth muscle promoter 22 serotonin transporter mice overexpressing the serotonin transporter in smooth muscle cells (SMCs).

**Approach and Results**—Treatment with CORM-3 (50 mg/kg per day once daily) for 3 weeks prevented PH, right ventricular hypertrophy, and distal pulmonary artery muscularization in mice exposed to chronic hypoxia and partially reversed PH in smooth muscle promoter 22 serotonin transporter mice by reducing Ki67 dividing pulmonary artery SMCs (PA-SMCs). In these models, CORM-3 markedly increased lung p21 mRNA and protein levels and p21-stained PA-SMCs. These effects contrasted with the transient pulmonary vasodilatation and rise in lung cGMP levels induced by a single injection of CORM-3 in mice exposed to acute hypoxia. Studies in cultured rat PA-SMCs revealed that the inhibitory effects of CORM-3 on cell growth were independent of cGMP formation but associated with increased p21 mRNA and protein levels. Protection against PH by CORM-3 required increased lung expression of p21, as indicated by the inability of CORM-3 to prevent chronic hypoxia-induced PH in p21-deficient mice and to alter the growth of PA-SMCs derived from p21-deficient mice. CORM-3–induced p21 overexpression was linked to p53 activation as assessed by the inability of CORM-3 to prevent PH and induce p21 expression in p53-deficient mice and in PA-SMCs derived from p53-deficient mice.

**Conclusions**—CORM-3 inhibits pulmonary vascular remodeling via p21, which may represent a useful approach for treating PH. (Arterioscler Thromb Vasc Biol. 2014;34:304-312.)

**Key Words:** carbon monoxide • pulmonary circulation

Pulmonary hypertension (PH) either occurs as a complication of various pathological conditions or develops as a primary disease for which no underlying cause can be found.1,2 Drugs recommended for treating PH improve the symptoms but have a limited impact on the prognosis.1,2 Thus, there is a need to define new therapeutic strategies for arresting or reversing the underlying structural remodeling of pulmonary vessels, which results mainly from proliferation of pulmonary artery smooth muscle cells (PA-SMCs).

Previous studies using transgenic animal models demonstrated that heme oxygenase-1 (HO-1) and its enzymatic product, carbon monoxide (CO), are protective against the development of hypoxia-induced PH in rats.3,4 Most recently, studies using mice briefly exposed to CO gas inhalation showed that CO could reverse established PH in several animal models.5 This protection was attributed mainly to the relaxation of vascular tone and inhibition of PA-SMC proliferation by CO. The antiproliferative effect of CO on SMCs was investigated previously in various models of systemic or pulmonary vascular remodeling and shown to involve several pathways.6–8 These include activation of guanylate cyclase and subsequent generation of cGMP, induction of endothelial NO synthase (eNOS), activation of p38 mitogen-activated protein kinases (MAPKs), and expression of the cell cycle inhibitor p21. Of note, the protective effects of CO were associated with upregulation of p21 gene expression in a model of carotid stenosis after balloon injury, which led to the hypothesis that increased expression of p21 could explain the long-term inhibitory effects on vascular remodeling by short exposure to CO gas inhalation.5 In accordance with these results, we recently demonstrated that p21 could represent a major target for new therapies targeting PA-SMC proliferation during progression of PH.6

Delivery of CO gas to remodeled vessels may therefore represent a therapeutic approach for treating PH. However,
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CORM</td>
<td>carbon monoxide–releasing molecule</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<tr>
<td>HO-1</td>
<td>heme oxygenase-1</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>ODQ</td>
<td>1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one</td>
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<tr>
<td>PA-SMC</td>
<td>pulmonary artery smooth muscle cell</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PH</td>
<td>pulmonary hypertension</td>
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<tr>
<td>PPAR-γ</td>
<td>peroxisome proliferator–activated receptor-γ</td>
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<tr>
<td>RVSP</td>
<td>right ventricular systolic pressure</td>
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<tr>
<td>SM22-5-HTT+</td>
<td>smooth muscle promoter 22 serotonin transporter</td>
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<td>WT</td>
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Despite the convincing data mentioned above, a critical point in relation to its clinical applications is whether CO gas can be administered at doses and times that do not compromise the oxygen-carrying capacity of blood, knowing that CO has a much higher affinity for hemoglobin compared with oxygen.

In this context, transition metal carbonyls capable of liberating CO in biological systems, known as CO-releasing molecules (CORMs), may represent an ideal alternative to CO gas because they have been designed to deliver CO in a controlled and physiological fashion in vivo.7–9 Different classes of CORMs have been reported to be pharmacologically active and mimic many of the effects mediated by CO gas, among them tricarbonylchloro(glycinato)ruthenium(II) (CORM-3) is the best characterized one.10 It is a stable compound and fully soluble in aqueous solutions, rapidly liberates CO in the target tissue (half-life <1 minute), and has been shown to restore vascular function in different pathological conditions including vasoconstriction, systemic hypertension, and vascular thrombosis.10–13 One important note is that systemic administration of therapeutic doses of CORM-3 in animal models of disease does not result in increased carboxyhemoglobin (HbCO) levels in blood.13,14

In the present study, we questioned whether CORM-3 could prevent or reverse established PH in mice. To this end, we first investigated the effects of chronic administration of CORM-3 in mice after hypoxia-induced PH and in transgenic mice overexpressing the serotonin transporter in SMCs (SM22-5-HTT+ mice), which develop spontaneous PH in normoxia.15 Furthermore, we examined the mechanisms underlying the effects of CORM-3 using a combination of cultured PA-SMCs studies and by investigating the specific role of p21 in p53 knockout mice exposed to chronic hypoxia.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Effects of CORM-3 on PH in Mice Exposed to Chronic Hypoxia and in SM22-5-HTT+ Mice

Daily oral treatment of chronically hypoxic mice with 50 mg/kg per day of CORM-3 attenuated the development of PH as indicated by a marked decrease in right ventricular systolic pressure (RVSP), RV hypertrophy, and distal pulmonary artery muscularization (Figure 1A). This protective effect of CORM-3 was associated with a simultaneous decrease in PA-SMC proliferation and apoptosis, as assessed by the percentage of Ki67- and Caspase-3–stained cell, respectively, as well as a marked increase in p21-stained cell count (Figure 1A and 1B). Studies in SM22-5-HTT+ mice were performed to investigate whether CORM-3 reversed established PH and altered p21 activity under normoxia. SM22-5-HTT+ mice, which spontaneously develop PH, were treated for 3 weeks with 50 mg/kg per day CORM-3. CORM-3 partially reversed PH in SM22-5-HTT+ mice, decreasing RVSP, RV hypertrophy, pulmonary vessel muscularization, Ki67- and Caspase-3–stained cell count, and substantially increased p21-stained cell counts (Figure 1C and 1D). It is important to note that during the chronic treatment with CORM-3, HbCO levels remained within physiological values (0.83±0.05% versus 0.76±0.05% in controls; NS).

Short-Term Effects of CORM-3 Administration on Pulmonary Arterial Pressure in Wild-Type and SM22-5-HTT+ Mice

Mice were intubated and subjected to hypoxia (9%) for 5 minutes to record changes in RVSP caused by hypoxic vasoconstriction. As shown in Figure 2A, exposure to acute hypoxia in control mice treated with vehicle resulted in a significant increase in RVSP (from 24.9±0.2 to 30.6±0.5 mm Hg; P<0.05). The rise in RVSP was reduced partially in mice pretreated with CORM-3 1 hour before the hypoxic challenge, whereas a total suppression in the pressure increase was observed in mice pretreated with CORM-3 3 hours prior hypoxia (Figure 2A).

A partial decrease in RVSP was also observed 6 hours after CORM-3 pretreatment, suggesting that the effect of CORM-3 did not last much longer than 6 hours. This inhibitory effect of CORM-3 on the pressure response to hypoxia was associated with a simultaneous rise in lung cGMP levels. Interestingly, administration of CORM-3 did not alter basal RVSP in SM22-5-HTT+ mice in which PH was shown to result mainly from pulmonary vascular remodeling rather than to vasoconstriction16 (Figure 2B). In WT mice treated with 50 mg/kg dose of CORM-3 or vehicle, systemic arterial pressure remained unchanged (113±8 versus 117±5.3 mm Hg; NS; respectively).

In addition, on the basis of echocardiographic assessment, we found that 3 hours after treatment with CORM-3, heart rate (609±15 versus 597±10 bpm; P=NS), pulmonary velocity time integral (2.42±0.07 versus 2.43±0.04 cm; P=NS), and left ventricular ejection fraction (85±3 versus 87±1%; P=NS) were similar to the control group.

Effects of CORM-3 Treatment on p38-MAP Kinase, eNOS, and p21 in the Lung of Chronically Hypoxic and SM22-5-HTT+ Mice

Compared with animals treated under normoxic conditions, administration of CORM-3 in chronically hypoxic mice markedly increased lung p21 mRNA and protein levels. In addition, eNOS expression increased sharply, whereas p38 protein remained unchanged (Figure 3A). Despite the lack of change in p53 protein levels observed, treatment with CORM-3 was
associated with an increase in p53 target genes as assessed by increased B-cell translocation gene 2, B-cell lymphoma-2–associated X protein, and p53 upregulated modulator of apoptosis mRNA levels in the lung of chronically hypoxic mice (Figure 3A). Similar to the observation made in chronically hypoxic mice, CORM-3 treatment increased p21 mRNA and p21 protein levels as well as eNOS mRNA in the lung of SM22-5-HTT+ mice with no significant changes in p53 and p38 protein levels (Figure 3B). Among the genes regulated downstream of p53, only p21 and B-cell translocation gene 2 expression were increased by CORM-3 in SM22-5-HTT+ mice (Figure 3B).

Figure 1. A, Right ventricular systolic pressure (RVSP), right ventricular hypertrophy index (RV/left ventricular [LV]+septum [S]), and pulmonary vessel muscularization in control animals (normoxia) or mice exposed to hypoxia for 15 days. Carbon monoxide–releasing molecule (CORM)-3 (50 mg/kg) was administered daily by oral gavage. The percentages of Ki67-positive dividing cells, p21-stained cells, and Caspase-3–positive cells were assessed in the lung. B, Representative micrographs of pulmonary vessels stained for Ki67, Caspase-3, or p21. Data are mean±SEM (n=6–10). *P<0.05, **P<0.01, and ***P<0.001 vs vehicle-treated normoxic mice. †P<0.05 and †††P<0.001 vs vehicle-treated hypoxic mice. §P<0.5 and §§§P<0.001 vs vehicle-treated normoxic mice. C, RVSP, RV/LV+S, and muscularization of pulmonary vessels, percentages of Ki67, p21, and Caspase-3–stained cells in wild-type (WT) or smooth muscle promoter 22 serotonin transporter (SM22-5-HTT+) mice after 21 days of treatment with vehicle or of CORM-3 (50 mg/kg). D, Representative micrographs of pulmonary vessels stained for Ki67, Caspase-3, or p21. Data are mean±SEM of 6 to 10 animals. *P<0.05, **P<0.01, and ***P<0.001 vs vehicle-treated mice. †††P<0.001 vs vehicle-treated WT mice. §P<0.05, §§P<0.01, and §§§P<0.001 vs vehicle-treated WT mice.
levels induced by CORM-3 was markedly abrogated in the presence of ODQ. In contrast, the marked rise in p21 mRNA expression after CORM-3 treatment was unaltered in the presence of ODQ (Figure 4C). To question whether the increase in p21 expression induced by CORM-3 was preceded by a rise in p53, we simultaneously measured p53 and p21 gene expression at various times after CORM-3 treatment. As shown in Figure 4D, p21 mRNA increased sharply at 2 hours after CORM-3 treatment, whereas p53 protein levels significantly increased at 4 hours with a peak at 12 hours (Figure 4D).

Effects of CORM-3 on PH in p21-Deficient Mice and on Proliferation in Cultured p21-Deficient PA-SMC
To determine whether p21, a cell cycle inhibitor, was required to prevent PH in chronically hypoxic mice, we investigated the effects of CORM-3 treatment in p21-deficient (p21−/−) mice. As shown in Figure 5A and 5B, treatment with CORM-3 failed to protect p21−/− mice against PH induced by hypoxia. The tendency for a lower RVSP in CORM-3–treated mice was interpreted as a consequence of the pulmonary vasodilatory effects of CORM-3. We then examined the ability of CORM-3 to inhibit proliferation of PA-SMC derived from WT and p21−/− mice. A shown in Figure 5C, pretreatment of PA-SMCs with CORM-3 for 24 hours inhibited PDGF-induced proliferation of PA-SMC derived from WT mice but not in PA-SMCs from p21−/− mice. Inactive CORM-3 did not have any effect on PDGF-induced proliferation.

Effects of CORM-3 on PH in p53−/− Mice and on p53 Activation in Cultured PA-SMC
To determine whether the effects mediated by CORM-3 require p53 activation, we conducted experiments using p53−/− mice and their littermate controls exposed to chronic hypoxia. In contrast to its protective effect in control nonmutant mice, CORM-3 did not prevent hypoxic-induced PH in p53−/− mice (Figure 6A). Administration of CORM-3 in chronically hypoxic mice markedly increased p21 mRNA levels, which were completely abrogated in p53−/− mice (Figure 6B). Similarly, pretreatment of PA-SMCs with CORM-3 for 24 hours resulted in inhibition of PDGF-induced proliferation in cells derived from WT mice but was ineffective in PA-SMCs extracted from p53−/− mice (Figure 6C). In PA-SMC from WT mice treated with CORM-3, we observed a pronounced increase in p21 mRNA, which peaked at 4 hours; this rise in p21 by CORM-3 was completely abolished in PA-SMCs from p53−/− mice (Figure 6D). In addition, incubation of cells transfected with the luciferase p53 reporter in the presence of CORM-3 led to a marked increase in luciferase activity compared with vehicle-treated control cells (Figure 6E). Because peroxisome proliferator–activated receptor-γ (PPAR-γ) has been linked to p21 expression, we also examined the effects of CORM-3 on this transcription factor. We found that in PA-SMC transfected with the luciferase PPAR-γ reporter, CORM-3 failed to increase luciferase activity (data not shown).

Discussion
During the past years, efforts have been undertaken to define the therapeutic potential of CO against vascular diseases. This
has been achieved either by subjecting animals for brief periods of time to CO gas inhalation or by increasing the production of endogenous CO by pharmacological upregulation of the HO-1 gene.3 Interestingly, a remarkable protective effect of CO gas has been demonstrated against vascular lesions produced in systemic or pulmonary vessels.17–20 More specifically, in different rodent models of PH, a daily brief exposure to CO gas has been reported to reverse established PH by decreasing right ventricular hypertrophy, restoring right ventricular and pulmonary arterial pressures, and preserving pulmonary vascular architecture.5 The effect of CO gas required functional eNOS/NOS3 and was associated with a simultaneous increase in apoptosis and decreased proliferation of vascular SMCs.17

In the present study, we adopted an alternative pharmacological approach to CO gas using CORM-3, a water-soluble compound that liberates controlled quantities of CO in vitro and in vivo.8,10,12 Our aim was to assess the therapeutic properties of CORM-3 against the development of PH and delineate its mechanism of action using animal models and lung-derived cells, respectively. Two distinct experimental mouse models of PH were studied: mice exposed to hypoxia and 5-HTT+ mice, which spontaneously develop PH. We found that daily treatment with a single administration of CORM-3 effectively prevented or reversed PH, with marked reduction in muscularization of pulmonary arteries and decreased number of proliferating PA-SMCs. Notably, these effects were observed without any change in blood HbCO levels, indicating that CORM-3 can effectively limit PH without significantly compromising the oxygen-carrying capacity of Hb.

Contrasting with the effects of chronic treatment, a single administration of CORM-3 in mice exposed to acute hypoxia resulted in pulmonary vasodilatation which did not last >6 hours. When a single dose of CORM-3 was given to SM22-5-HTT+ mice, no changes in pulmonary arterial pressure were recorded. These observations suggest that the protective effects of CORM-3 are not related to its vasodilatory properties. Administration of CORM-3 was associated with a transient increase in lung cGMP levels, which returned to basal levels within 12 hours after oral administration. Given the effectiveness of drugs, such as phosphodiesterase type 5 inhibitors or soluble guanylate cyclase activators, to counteract PH progression through cGMP-mediated effects, these results indicate that CORM-3 possesses similar interesting therapeutic properties. However, a more sustained increase in cGMP levels in the lung of mice affected by PH has been reported in the case of phosphodiesterase type 5 inhibitors or soluble guanylate cyclase activators.1 To investigate further the role of cGMP in mediating the protective effects of CORM-3, we performed in vitro studies on cultured PA-SMC. The fact that CORM-3 still prevented PA-SMC proliferation in the presence of the soluble guanylate cyclase inhibitor ODQ clearly indicates that the growth inhibitory effects of CORM-3 on...
PA-SMC do not require activation of guanylate cyclase and was independent of intracellular cGMP accumulation.

Different mechanisms underlying the antiproliferative effects of CO have been proposed in addition to or in association with intracellular cGMP generation. These include activation of p38 MAPKs, increased expression of NO synthase and NO production, and the involvement of the cell cycle inhibitor p21.17 In our study, we found that p38 MAPK increased in cells treated with CORM-3. However, the increase in p38 MAPK was dependent clearly on intracellular cGMP accumulation as it was almost completely inhibited in the presence of ODQ in cultured PA-SMC. This result is in line with previous studies showing that CO, like NO, activates p38 MAPK via cGMP.17 Because eNOS expression dramatically increased after treatment with CORM-3 in hypoxia-induced PH, it is likely that this effect contributed to the beneficial effects of CO liberated from this compound in vivo, as emphasized by previous studies on CO gas.17 Moreover, L-NAME was unable to reverse CORM-3–mediated inhibition of proliferation induced by PDGF. This is in line also with published evidence that PA-SMCs may not express eNOS.21 However, CORM-3 directly inhibited proliferation of cultured PA-SMC even in the presence of ODQ. Thus, the NO/cGMP pathway, which probably contributed to the beneficial effect of CORM-3 administration to mice, could not explain its direct antiproliferative effects on PA-SMC. What we found most interesting from our study was

Figure 4. A, Rat pulmonary artery smooth muscle cells (PA-SMCs) were exposed to carbon monoxide–releasing molecule (CORM)-3 (50 μmol/L) in the absence or presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ) or Nω-nitro-l-arginine methyl ester hydrochloride L-NAME before stimulation of cell proliferation with 20 ng platelet-derived growth factor (PDGF). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay measured in optical density (O.D.; left y axis). Percentage of apoptotic cells (right y axis). Inactive CORM-3 (Ru(DMSO)4Cl2), which does not release CO, was used as a negative control. Each bar represents the mean±SEM of ≥5 independent experiments (means±SEM; n=30 at each time point). †††P<0.001 for comparison with 0% fetal calf serum (control); ***P<0.001 compared with PDGF. B, Effects of CORM-3 and ODQ on cGMP levels in PA-SMCs. Values are mean±SEM. ***P<0.001 compared with vehicle-treated cells. C, Time-dependent changes in total p21 mRNA and total p38 protein expression in PA-SMC treated with CORM-3 in the presence or absence of ODQ. Data are mean±SEM of 5 values. *P<0.05 vs ±ODQ. D, Changes in total p21 protein, p21 mRNA, total p53 protein, and p53 mRNA expression over time after treatment of PA-SMC with CORM-3. Data are mean±SEM of 6 values. †P<0.01, ††P<0.001 compared with vehicle.
a marked increase in p21 mRNA and p21 protein levels in lungs from chronically hypoxic and SM22-5-HTT+ mice as well as cultured PA-SMC treated with CORM-3. Moreover, chronic treatment with CORM-3 in vivo was associated with an increased number of p21-stained PA-SMC. These results are therefore consistent with the previous observation of an increase in p21 induced by CO gas. In contrast to these previous studies, we found that CO-induced p21 upregulation in PA-SMC was independent of cGMP formation and was maintained in cells treated with ODQ. To further investigate the role of p21 in mediating the protective effects of CORM-3 on PH, we performed studies in p21−/− mice. We found that the protective action of CORM-3 against PH required p21, as indicated by the inability of CORM-3 to prevent chronic hypoxia-induced PH in p21−/− mice and to inhibit growth of p21−/− mice derived PA-SMC. These results therefore indicate a prominent role for p21 in mediating the effects of CORM-3 in our experimental models of PH, independently of cGMP.

An interesting observation originating from our study is that the increase in p21 induced by CORM-3 was far greater in hypoxic and SM22-5-HTT+ mice than in control mice without PH. Moreover, treatment with CORM-3 inhibited proliferation of PA-SMC without inducing cell apoptosis or senescence. These findings indicate that the protective effects of CORM-3 on PH are most likely a result of its antiproliferative action mediated by p21. In support of this are also our data showing that that CORM-3 was extremely effective in targeting dividing PA-SMC in mice with PH, but no major effects of this drug were observed in control mice. Because of its property as a cell cycle inhibitor, p21 expression can be considered as the main effector of CORM-3 treatment.

To explore the signaling pathways linking CO to p21, we questioned whether the transcription factors PPAR-γ or p53 were involved. Activation of PPAR-γ in PH by CO gas has been reported recently, and this might contribute to the protective actions attributed to CO. In our cell studies, however, we found that this was not the case because in SMCs transfected with the luciferase PPAR-γ reporter CORM-3 failed to increase luciferase activity. In contrast, treatment with CORM-3 resulted in a major increase in luciferase activity in cells transfected with the luciferase p53 reporter. These observations led us to investigate the possibility of a primary effect of CO on p53 followed by a transcriptional expression of p21. This possibility was further corroborated by the fact that in the lung of chronically hypoxic mice as well as in cultured PA-SMCs, we found that CORM-3 induced the expression of several genes that are under the control of p53, including p21, B-cell lymphoma-2–associated X protein, Bcl2, and B-cell translocation gene 2. In cultured PA-SMCs treated with CORM-3, the increase in p21 mRNA was observed to precede the increase in total p53 protein levels. Because only total p53 protein levels were measured, it is possible that some transcriptional activity occurred before any detectable change in total p53 protein levels. Using p53−/− mice exposed to chronic hypoxia, we found that the protective effects of CORM-3 were suppressed compared with WT mice. In addition, the antiproliferative effect of CORM-3 was abrogated in cultured PA-SMC derived from p53−/− mice, as it was in cells derived from p21−/− mice. Moreover, the rise in p21

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**Figure 5.**

A, Right ventricular systolic pressure (RVSP), right ventricular hypertrophy index ([RV]/[left ventricular ([LV])+septum ([S])], pulmonary vessel muscularization, and percentages of Ki67-positive dividing cells in wild-type (WT) and p21 knockout (KO) mice analyzed on 15 days after exposure to hypoxia or normoxia. Carbon monoxide–releasing molecule (CORM)-3 (50 mg/kg) was administered daily by oral gavage. B, Representative micrographs of pulmonary vessels stained for Ki67. Data are means±SEM of 6 to 10 animals. *P<0.05, **P<0.01, and ***P<0.001 compared with vehicle-treated mice. †††P<0.001 compared with vehicle-treated normoxic WT mice. §§§P<0.001 vs vehicle-treated WT normoxic mice. C, Effects of CORM-3 (50 μmol/L) and inactive CORM-3 (Ru(DMSO)$_2$Cl$_2$) on proliferation of pulmonary artery smooth muscle cells from WT and p21 KO mice 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay measured in optical density (O.D.). Each bar represents the mean±SEM of ≥5 independent experiments (mean±SEM; n=30). †††P<0.001 compared with platelet-derived growth factor (PDGF). ***P<0.001 compared to control mice (vehicle).
mRNA following CORM-3 treatment in PA-SMC from WT mice was not detected in p53−/− cells, nor was any increase in p21 mRNA levels in the lungs from chronically hypoxic p53−/− mice treated with CORM-3.

The present findings showing that a CO-releasing agent can prevent or reverse PH by activating the p53/ P21 pathway is novel and of major significance. In a recent report, we have shown that nutlin-3a, which prevents the ubiquitin ligase murine double minute 2, a negative p53 regulator, exerts therapeutic effects in PH by inducing PA-SMC senescence.6 Similarities can be drawn from these 2 independent studies as in both cases the interplay between p53 and p21 pathways seems to play a determinant role in the setting of PH. A major difference, however, is that cells treated with nutlin-3a stopped proliferating because they became senescent, whereas in the case of CORM-3 inhibition of cell proliferation is not associated with cells entering into a senescent or an apoptotic program. The precise mechanism that drives arrested cells into senescence is not fully understood but depending on the extent or duration of p21 and p53 expression, cells may become quiescent or senescent.23 Whether induction of PA-SMC quiescence or senescence has differential consequences in the resolution of PH remains to be defined. Moreover, the precise mechanisms underlying the activation of p53 by CO-releasing agents need to be further investigated. Because both CO gas and CORMs have been reported extensively to possess antiproliferative, anti-apoptotic, antioxidative, and anti-inflammatory properties,8 it is unlikely that activation of p53 occurred in response to DNA damage produced in the cells by CO. Moreover, several
studies including ours now converge to confirm that differential approaches using either CO gas or CORMs are associated with an increase in tissue p21 expression.\textsuperscript{17,24} Thus, modulation of p21 expression and activity by CO gas or CORMs may represent an interesting therapeutic target in the treatment of PH although further studies are required to better delineate the functional effects of p21 activation in the context of vascular remodeling in the lung.

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Disclosures
None.

References

Significance
This study reports for the first time that a therapeutic approach using a compound that releases CO in vivo prevents and reverses experimental-induced pulmonary hypertension. We also identified p21 activation as a critical pathway responsible for the CO-mediated protective effects against pulmonary hypertension.
p21-Dependent Protective Effects of a Carbon Monoxide–Releasing Molecule-3 in Pulmonary Hypertension
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MATERIALS AND METHODS

Animals and reagents
Adult male mice (C57Bl/6j) were used in conformity with institutional guidelines that complied with national and international laws and policies. All experiments on animals were approved by the Institutional Animal Care and Use Committee of the French National Institute of Health and Medical Research (INSERM)-Unit 955, Créteil, France. Transgenic male mice over-expressing 5-HTT in smooth muscle cells under the control of the SM22 promoter (SM22-5-HTT+) were produced and bred on a C57Bl/6j background as previously described1. SM22-5-HTT+ mice are fertile and have a normal life span and normal growth1. These mice were used for the experiments at 18 weeks of age. Male mice with deletion of the p53 (p53/-/-) or p21 (p21/-/-) gene were obtained from Jackson laboratory (Bar Harbor, ME). Ru(CO)3Cl(glycinate) (CORM-3) and Ru(DMSO)4Cl2 (inactive CORM-3), a molecule in which the carbonyl groups have been replaced with DMSO and used as a negative control, were synthesized as previously described2, 3. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma Aldrich (France).

Hemodynamic response of normoxic mice to acute hypoxia
Mice 8–10 weeks of age, weighing approximately 25 g were anesthetized with an intraperitoneal injection of ketamine (6 mg/100 g) and xylazine (1 mg/100 g). The trachea was cannulated and the lungs were ventilated with room air at a tidal volume of 0.2 mL and a rate of 90 breaths per minute. A 26-gauge needle was then introduced percutaneously into the right ventricle via the subxiphoid approach. Right ventricular systolic pressure (RVSP) was measured using a Gould P10 EZ pressure transducer connected to pressure modules and a Gould TA 550 recorder. Right ventricular systolic pressure and heart rate were recorded initially while the animals were ventilated with room air and then after 5 min of ventilation with the hypoxic gas mixture (8% O2, 92% N2). Under these conditions, only animals with a heart rate between 300 and 500 beats per minute (bpm) were analyzed. If the heart rate fell below 300 bpm, animals were excluded from the study.

Echocardiography
Closed-chest transthoracic echocardiography was performed in non-sedated mice using a 13-MHz linear-array transducer with a digital ultrasound system (Vivid 7, GE Medical Systems). Conventional measurements [end-systolic and end-diastolic LV diameters, LV shortening fraction and LV ejection fraction] were obtained from M-mode tracings at the level of the papillary muscles. In order to assess stroke volume and estimate therefore cardiac output, velocity-time integral of pulmonary flow (VTI, cm) was measured at the level of the pulmonary valve tips by pulsed wave Doppler as previously described\(^4\). All measures were averaged over three cardiac cycles and the reader was blinded to the treatment of the mice.

**Exposure to chronic hypoxia**

Male mice (10-15 weeks of age) with a mean weight of 25 g were exposed to chronic hypoxia (9% O\(_2\)) in a ventilated chamber (Biospherix, New York, NY). The hypoxic environment was established by flushing the chamber with a mixture of room air and nitrogen then recirculating the gas mixture. The chamber was opened every other day for 1 h to clean the cages, administer the drugs and replenish it with food and water. Normoxic mice were kept in the similar conditions with the same light-dark cycle.

**Assessment of pulmonary hypertension**

Mice were anesthetized with an intraperitoneal injection of ketamine (60 mg/Kg) and xylazine (10 mg/Kg). A 26-gauge needle connected to a pressure transducer was inserted into the right ventricle and RVSP and heart rate were recorded immediately\(^5\). If the heart rate fell below 300 bpm, animals were excluded from the study. After the assessment of hemodynamic parameters, all mice were killed by cardiac puncture and exsanguination. The heart was dissected out and weighed for calculation of the right ventricular hypertrophy index (ratio of right ventricular free wall weight over the sum of the septum plus left ventricular free wall weights: RV/LV+S, expressed in percentage). The right lung was quickly removed and divided into two parts that were immediately snap-frozen in liquid nitrogen and then stored at -80°C for measurement of tissue 5-HT contents. The left lung was fixed in the distended state with formalin buffer for assessments of pulmonary vessel muscularization. This was assessed by counting both the partial and complete muscularized pulmonary vessels and expressed this value as percentage of the total number of vessels in a given lung.
section. PA-SMC proliferation was measured using Ki67 immunostaining, as described previously\(^6,7\).

**Determination of smooth muscle cells proliferation and apoptosis**

Pulmonary artery smooth muscle cells (PA-SMCs) from rat pulmonary arteries were cultured and characterized as previously described\(^8\), similar technique was use to sample PA-SMCs from mice. Before treatment, PA-SMCs were placed for 48 hours in Dulbecco modified Eagle medium containing 0.1% serum. Cells were exposed to CORM-3, inactive CORM-3 or vehicle in serum-free medium for 30 minutes and then treated with PDGF-BB (20 ng/mL). To assess a possible contribution of soluble guanylyl cyclase (sGC) activation by CO on proliferation, the inhibitor of sGC, ODQ was added to cells 45 min before exposure to CORM-3. In addition, to study the role of eNOS, an inhibitor of eNOS (L-NAME) was added at the time of exposure to CORM-3. After 48 h incubation at 37°C, 0.2 mg/mL tetrazolium salt (MTT from Sigma) was added to each well). The culture medium was then removed and formazan crystals were solubilized by adding 100 µL dimethyl sulfoxide. The reduction of tetrazolium salt to formazan within the cells was quantified spectrophotometrically at 520 nm and taken as an indicator of the number of cells. Annexin V flow cytometry was performed with a commercially available annexin V–FITC assay (Sigma-Aldrich). Cells were trypsinized and resuspended in media at 1 x10\(^6\) cells per 1 mL and then incubated with annexin V-FITC–conjugated antibody and stained with propidium iodide according to the manufacturer’s instructions. Annexin V and propidium iodide staining were detected by fluorescence-activated cell sorting (Becton Dickinson, Franklin Lakes, NJ) as an index of apoptosis.

**Cyclic GMP immunoassay and cell viability**

Levels of cGMP in lung tissues and cells were quantified using cGMP EIA kit (Cayman Chemical, USA). SMCs were incubated with CORM-3 in the presence or absence of ODQ and cell lysates were analyzed for cGMP content. Cells were seeded at 5 × 10\(^3\) cells/well and cultured overnight as previously described\(^8\). Cells were serum starved for an additional 48 h (0% serum) and were then treated as indicated. Cells were counted daily using a Neubauer hemocytometer and viability was assessed using Trypan blue.
Biological measurements in mouse lungs and cultured rat pulmonary-artery smooth muscle cells (PA-SMCs)

Western blotting was used to detect and quantify p53 and p21 protein expression in lung tissue and cells. Levels of p21 and p53 mRNAs in lung tissue and cells were determined using RT-qPCR. Total mRNA was extracted from PA-SMC using RNeasy Mini Kit (Qiagen, ZA Courtaboeuf, France). First-strand cDNA was synthesized in reversed transcribed samples as follows: 1 μg total RNA isolated from cells or lung tissues, 200 U/μL SuperScrip II reverse transcriptase, 100 ng Random primers, and 10 mM mixed dNTP (Invitrogen, Life Technologies, Cergy-Pontoise, France). Quantitative PCR was performed in a 7900HT Real8 Time PCR system (Applied Biosystems, ZA Courtaboeuf, France) using SYBR green Mix from Invitrogen.

Real-time reverse transcription quantitative PCR (RT-qPCR)
The levels of p53 downstream genes and eNOS were determined by real-time reverse transcription (RT) qPCR using SYBR Green (Invitrogen). To normalize for cDNA input load, mouse 18s was used as internal standard. After addition of cDNA to SYBR Green Master Mix PCR (300 nM of each specific primer), PCR was performed in a total volume of 25 μL using a 7900HT real-time PCR cycler (Applied Biosystems). All cDNA samples were tested in duplicate and analyzed using ABI Prism Sequence Detection Software v.1.7 (PE Applied Biosystems). Samples were compared using the relative Ct method. The Ct value, which is inversely proportional to the initial template copy number, is the calculated number of cycles 9 with a fluorescence signal significantly above background levels. Fold induction or repression was measured relative to controls and calculated after adjusting for 18s using $2^{-[\Delta\Delta Ct]}$, where $\Delta Ct = Ct$ tested gene $-$ Ct 18s and $\Delta\Delta Ct = \Delta Ct$ control $-$ $\Delta Ct$ treatment.

Western Blot Analysis
Total protein from lung tissue (60 μg) or cell sample (50 μg) was subjected to 10% SDS-PAGE, and the separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Molsheim, France). After incubation in blocking solution (TBS/5% milk), the membranes were incubated sequentially with the following antibodies: anti–p21 (Cell Signaling Technology, Boston, Mass) and anti–p53 (BD Transduction Laboratories, Franklin Lakes, NJ); and anti β-actin (Sigma,
Saint-Quentin-Fallavier, France). Densitometric quantification was normalized for the β-actin level in each sample (Gene Tools, Ozyme, Montigny le Bretonneux, France).

**Immunohistochemistry**

Paraffin-embedded sections were deparaffinized using xylene and a graded series of ethanol dilutions then incubated in citrate buffer (0.01 M, pH 6) at 90°C for 20 min. Tissues were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Endogenous peroxidase activity was blocked with 3% H$_2$O$_2$ and 10% methanol in PBS for 10 min. Slides were incubated for 60 min in 1% bovine serum albumin and 5% goat serum in PBS then incubated overnight with anti-caspase-3, anti-p21 mouse antibody (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-Ki67 rabbit antibody (1:300, Abcam). The slides were incubated with biotinylated goat anti-rabbit antibody (1:200, Vector Labs, Burlingame, CA) and the signal was then developed with an immunoperoxidase reagent (ABC-HRP, Vector Labs) and DAB (Sigma-Aldrich) as the substrate.

**Experimental protocol for in vivo experiments**

To assess the vasodilatory effects of CORM-3 in mice subjected to acute hypoxia, mice were initially treated with a single oral administration of CORM-3 (50 mg/kg/day) and then exposed to acute hypoxia for various periods of time. In mice subjected to hypoxia-induced pulmonary hypertension, animals received an oral daily dose of 50 mg/kg of CORM-3 during the hypoxia. In SM22-5-HTT+ mice, CORM-3 was administered at the same dose for 21 days. The last dose of CORM-3 was given 3 hours prior to the measurements. The dose of CORM-3 we used (50 mg/kg) is, from our historical data, the one that gives a maximal effect without causing visible toxicity. Indeed, we have tested this compounds administered at higher doses (100 and 150 mg/kg) daily for 1 week and found a significant increase in mortality.

**Transient tranfection of pulmonary-artery smooth muscle cells**

Cells were seeded in 24-well plates and transiently transfected the following day with a p53 reporter construct (p53 Cignal Reporter Assay, Qiagen, Courtaboeuf, France), as well as with positive and negative controls, using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. After 18 h of transfection, the medium was changed and replaced by complete growth medium and cells were treated with
CORM-3 (25 and 50 μM), or ethanol alone. A dual-luciferase assay (Promega, Charbonnières les Bains, France) was performed 24 h after treatment. Cells were lysed with lysis buffer, and luciferase activity was quantified using a Tristar luminometer (Berthold, Thoiry, France). Values are expressed as arbitrary units using a construct constitutively expressing Renilla luciferase for internal normalization. Firefly/Renilla activity ratios were generated from experimental and control transfections. Experiments were done in triplicate. A similar technique was used to transfect cells with a PPAR-γ reporter gene.

**Statistical analysis**
Data are expressed as mean±SEM. Parametric tests were used after verification that the variables in each group were normally distributed. One-way analysis of variance (ANOVA) was performed to analyze the treatment effects of CORM-3. *P* values <0.05 were considered significant for the initial ANOVA, and a Bonferroni correction test was then used for multiple comparisons (*P*<0.017).

**REFERENCES**


