

Caveolin-1 expression by means of p38 β mitogen-activated protein kinase mediates the antiproliferative effect of carbon monoxide

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During vascular injury, the proliferation and migration of smooth muscle cells leads to characteristic neointima formation, which can be exacerbated by genetic depletion of caveolin-1 or heme oxygenase 1 (HO-1), and inhibited by carbon monoxide (CO), a by-product of heme oxygenase 1 activity. CO inhibited smooth muscle cell proliferation by activating p38 mitogen-activated protein kinase (MAPK) and p21^{Waf1/Cip1}. Exposure to CO increased caveolin-1 expression in neointimal lesions of injured aorta and *in vitro* by activating guanylyl cyclase and p38 MAPK. p38 $\beta^{-/-}$ fibroblasts did not induce caveolin-1 in response to CO, and exhibited a diminished basal caveolin-1 expression, which was restored by p38 β gene transfer. p38 β MAPK down-regulated extracellular signal-regulated protein kinase 1/2 (ERK-1/2), which can repress caveolin-1 transcription. Genetic depletion of caveolin-1 abolished the antiproliferative effect of CO. Thus, we demonstrate that CO, by activating p38 β MAPK, up-regulates caveolin-1, which acts as a tumor suppressor protein that mediates the growth inhibitory properties of this gas.

Activation of the p38 mitogen-activated protein kinase (MAPK) by genetic overexpression or cellular stimulation with noxious environmental agents can result in permanent cell cycle arrest and premature cell senescence (1–3). Many cell differentiation programs also involve the activation of p38 MAPK, including the neuronal differentiation of PC12 cells (4), the erythropoietin-induced differentiation of erythroid precursors (5), as well as C2C12 myogenesis (6), and 3T3-L1 adipogenesis (7).

Caveolin-1, which serves as the principle structural component of plasma membrane caveolae, potentially regulates many downstream signaling processes that originate in the membrane (8). Caveolin-1-null mice develop hypercellularity in the lungs, mammary gland, and heart associated with hyperactivation of the extracellular regulated kinase-1/2 (ERK1/2) MAPK (9–11). Interestingly, elevated levels of caveolin-1 appear in senescent cells or in aged animals (12, 13), and in fully differentiated cells, such as endothelial cells, epithelial cells, fibroblasts, and adipocytes (13). On the other hand, caveolin-1 seems down-regulated in human tumors, in cell lines derived from human tumors, and in oncogene-transformed cell lines (i.e., H-Ras and v-Abl) (14, 15). Conversely, up-regulation of caveolin-1 reverts the transformed phenotype of oncogene-transformed cell lines (15). Consistent with these observations, caveolin-1 negatively regulates smooth muscle cell (SMC) proliferation in neointimal lesions of injured aorta (16). These studies suggest that caveolin-1 may possess tumor suppressor activity. In support of this hypothesis, overexpression of caveolin-1 in mouse embryonic fibroblasts arrests these cells in the G₀/G₁ phase of the cell cycle through a pathway dependent on p53 and the p21 cyclin-dependent kinase inhibitor (p21^{Waf1/Cip1}) (17).

We have previously demonstrated that carbon monoxide (CO) can suppress arteriosclerotic lesions associated with graft rejection and with vascular injury, by inhibiting smooth muscle

proliferation (18). Despite the well known toxicity of this gas, CO potentially acts as a signaling molecule at trace levels in cellular and biological systems (19). CO arises physiologically in most cell types during the oxidative catabolism of heme by the heme oxygenase (HO, E.C. 1:14:99:3) enzymes (19). Expression of the inducible isozyme, heme oxygenase-1 (HO-1) represents a protective response to injury associated with proapoptotic stimuli or inflammation (20–22). Expression of HO-1-inhibited cellular proliferation in pulmonary epithelial cells (23) and in vascular SMC *in vivo* and *in vitro*, associated with G₁/S growth arrest and up-regulation of p21^{Waf1/Cip1} (24). CO, when applied exogenously confers potent antiproliferative effects in airway and vascular SMC, which depend on cGMP and p38 MAPK (18, 25, 26).

In this study, we have further investigated the signaling pathways that mediate the antiproliferative effects of CO. We show that the activation of the p38 β MAPK signaling pathway by CO led to an enhanced expression of caveolin-1 in fibroblasts and SMC, which in turn mediated the increased expression of p21^{Waf1/Cip1}, and the down-regulation of cyclin A, leading to growth arrest. The inhibition of intimal hyperplasia by CO treatment in a vascular injury model involved the enhanced expression of caveolin-1 in vascular smooth muscle. We demonstrate that CO failed to inhibit cellular proliferation in the absence of caveolin-1 expression, strongly supporting a critical role for caveolin-1 in the antiproliferative activity of this gas.

Materials and Methods

Materials. Platelet-derived growth factor (PDGF, rat recombinant BB-form) and all other reagent chemicals were from Sigma.

Cell Culture and Treatments. Primary rat pulmonary artery SMC were cultured as described (18) and used for experiments as subconfluent monolayers at passages 7–12. SMC were grown in DMEM high glucose media containing 10% FBS and antibiotics. Fibroblasts were cultured from the lungs of p38 $\beta^{-/-}$, c-Jun-NH₂-terminal kinase $-/-$ (*Jnk^{-/-}*), or caveolin-1-null (*cav^{-/-}*) mice as described (27). Cells were grown in humidified incubators containing an atmosphere of 5% CO₂ and 95% air at 37°C. Chemical inhibitors (Calbiochem), including SB 203580 (10

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Abbreviations: CO, carbon monoxide; ERK1/2, extracellular signal-regulated protein kinase 1/2; HO-1, heme oxygenase-1; p21^{Waf1/Cip1}, p21 cyclin-dependent kinase inhibitor; PDGF, platelet-derived growth factor; MAPK, mitogen-activated protein kinase; SMC, smooth muscle cells; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; 8-Br-cGMP, 8-bromo-cyclic guanylyl monophosphate; siRNA, small interfering RNA; SnPP, tin proto-phorphyrin-IX.

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μM), 1H-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, 20 μM), tin protoporphyrin-IX (SnPP, 20 $\mu\text{mol/liter}$) (Frontier Scientific, Logan, UT), and 8-bromo-cyclic guanylyl monophosphate (8-Br-cGMP), a cell-permeable cGMP analog, were applied to the culture media at the indicated final concentrations.

Carbon Monoxide Exposures. Cell cultures or animals were exposed to CO in modular exposure chambers as described (18). The final CO levels in the chambers (250 ppm) were monitored by using a CO analyzer (Interscan Corporation, Chatsworth, CA).

Balloon Injury Model and Animal Exposure. Inbred LEW rats, 200–250 grams (Harlan–Sprague–Dawley), were maintained in laminar flow cages in a pathogen-free facility at the University of Pittsburgh. Rats were acclimated for 1 week with rodent chow and water ad libitum. All procedures were performed in accordance with the Council on Animal Care at the University of Pittsburgh and the National Research Council's Guide for the Humane Care and Use of Laboratory animals. For induction of intimal hyperplasia, the left carotid artery was exposed and subjected to balloon catheter injury as described (18). The animals were exposed to compressed air or CO (250 ppm) for a 2-h interval before surgery, which was maintained for an additional 24 h postsurgery. Animals were killed at 21 days after balloon injury. Histological analysis was done in a blind manner.

Transfections. SMC were infected with adenoviral constructs containing HO-1 or LacZ cDNA for 48 h before the experiment as described (28). A Flag-tagged p38 α dominant-negative mutant (TGY \rightarrow AGF) construct ligated in pcDNA3.1 (29) was transfected into wild-type fibroblasts with Lipofectamine 2000 (Invitrogen). A p38 β cDNA in expression vector pcDNA3.1 was also transfected into p38 β ^{-/-} fibroblasts. Transiently transfected cells were incubated for an additional 48 h and harvested for the determination of caveolin-1 expression.

Small interfering RNA (siRNA) were designed against the coding sequence of caveolin-1 cDNA by using software by Dharmacon Research (Lafayette, CO). Sequences corresponding to the siRNAs were as follows: rat caveolin-1 coding region, 257–275 (CCAGAAGGGACACACAGTT, GenBank accession no. BC078744). Transfection of siRNA in rat SMC was carried out by using Lipofectamine 2000 (Invitrogen).

Western Blot Analysis and Immunoprecipitation. The following antibodies were used for immunoblotting: monoclonal anti-caveolin-1, (BD Transduction Laboratories, Lexington, KY), anti- α smooth muscle actin (α -SMA) (Sigma), polyclonal anti-caveolin-1, anti-p21, anti-cyclin A, (Santa Cruz, Santa Cruz, CA), anti-Flag, anti-p38 α MAPK, anti-phospho ERK1/2, anti-phospho p38 MAPK, as well as antibodies against corresponding inactive dephospho-forms (Cell Signaling, Beverly, MA). Western immunoblot analysis was performed as described (30).

Immunocytochemistry. Carotid arteries were harvested at 21 days after injury. Vessels were fixed, embedded and serially sectioned (5 μm) *in toto*. Sections were stained by a modified elastic tissue-Masson Trichrome or hematoxylin and eosin. Immunocytochemistry was performed as described (18). Paraffin embedded tissues were hydrated, retrieved, and immunostained with polyclonal anti-caveolin-1 (Santa Cruz) or anti- α smooth muscle actin (α -SMA, Sigma) antibodies. Bound primary antibodies were visualized with diaminobenzidine staining by using ABC kits (Vector Laboratories).

Cell Counts and [³H]Thymidine Incorporation. For [³H]thymidine incorporation studies, SMC were serum-starved overnight and then stimulated with 10% serum containing 5 $\mu\text{Ci/ml}$ [³H]thymidine (1 Ci = 37 GBq) (New England Nuclear). [³H]Thymidine

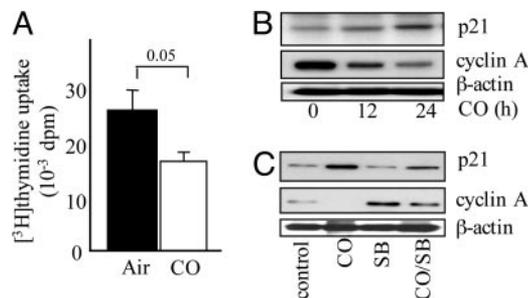


Fig. 1. Inhibition of cell growth by CO *in vitro*. (A) SMC were serum-starved overnight, and then stimulated by addition of serum (10% FBS) to the culture to initiate cell growth. Cellular proliferation was assessed by measuring [³H]thymidine incorporation [counts per min (cpm) $n = 3$] after serum stimulation in the absence or presence of CO (250 ppm for 24 h). (B) The relative expression levels of cell cycle-related proteins p21^{Waf1/Cip1}, and cyclin A, were determined in SMC after CO exposure for the indicated times by Western blot analysis. (C) SMC were pretreated with CO (1 h) followed by addition of SB 203580 (10 μM) (1 h), a specific p38 α/β MAPK inhibitor, followed by CO exposure in the absence or presence of SB for an additional 24 h. Expression of p21 and cyclin A was determined by Western blot analysis.

incorporation was measured by scintillation counting and presented as the mean counts per min per well. The proliferation assay was conducted by direct cell counting (18).

Statistical Analysis. All values were expressed as the mean \pm SD. from at least three independent experiments. Differences in measured variables between experimental and control group were assessed by using the Student's *t* test (STATVIEW II statistical package, Abacus Concepts, Berkeley, CA). Statistically significant difference was accepted at $P < 0.05$.

Results

Essential Role of Caveolin-1 in the AntiProliferative Properties of CO.

Exposure to CO (250 ppm) significantly inhibited the serum-stimulated proliferation of SMC (Fig. 1A). The antiproliferative effect of CO in SMC was accompanied by the increased expression of the cyclin-dependent kinase inhibitor p21^{Waf1/Cip1}, and by the decreased expression of cyclin A (Fig. 1B). Treatment of SMC with SB203580, a chemical inhibitor of p38 α/β MAPK, abrogated the stimulatory effect of CO on p21^{Waf1/Cip1} expression (Fig. 1C). Furthermore, SB203580 prevented the inhibitory effect of CO on cyclin A expression (Fig. 1C). Because PDGF acts as a potent mitogen for cells of mesenchymal origin (31, 32), we treated SMC with PDGF (10 ng/ml) in the absence or presence of CO (250 ppm). PDGF induced SMC proliferation, which was inhibited in the presence of CO (Fig. 2A). Under serum-starved conditions in the absence of mitogenic stimuli, CO had no antiproliferative effect (Fig. 2A). PDGF treatment reduced caveolin-1 expression (Fig. 2B), as predicted from previous studies (33). In contrast, CO treatment reversed the inhibitory effect of PDGF on caveolin-1 expression (Fig. 2A). PDGF treatment down-regulated p21^{Waf1/Cip1} expression in SMC and up-regulated cyclin A expression. In contrast, CO treatment up-regulated the expression of p21^{Waf1/Cip1}, and down-regulated cyclin A expression, even in the presence of PDGF (Fig. 2B).

Next, we examined the relationship between CO and caveolin-1 expression in an *in vivo* model of vascular SMC proliferation. The interruption of blood flow caused by ligation of the common carotid artery induces the migration and proliferation of SMC from the media to the intima, with the concomitant infiltration and activation of circulating leukocytes, leading to neointima formation (16). PDGF plays a key role in promoting the migration and proliferation of SMC during this process (32, 33). We examined caveolin-1 expression in neointimal lesions

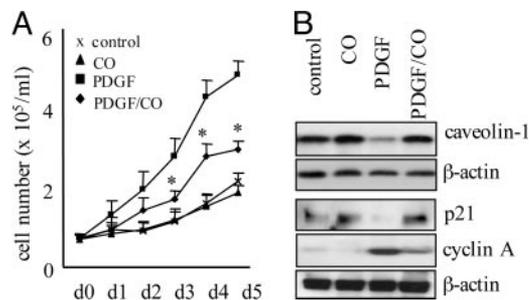


Fig. 2. Reciprocal effects of CO and growth factor on the expression of caveolin-1 and cell cycle-related proteins. (A and B) Serum-starved SMC were treated with PDGF (10 ng/ml) for 24 h in the absence or presence of CO (250 ppm), and cellular proliferation was assessed by direct cell counting. (B) Total cell lysates were probed with caveolin-1 antibody, or antibodies to p21 and cyclin A. β -actin served as the loading control. The results represent three independent experiments.

induced by balloon injury. The lesions were characterized by intimal hyperplasia, and loss of medial SMC relative to sham injury (Fig. 3A, B, E–G, and K and Fig. 7, which is published as supporting information on the PNAS web site). Caveolin-1 expression in the neointima was reduced after balloon-injury (Fig. 3F), as reported elsewhere (33). Exposure to CO (250 ppm) 2 h before, and during the first 24 h postsurgery, inhibited neointima formation in this model (Fig. 3C, D, H–J, and K), and increased caveolin-1 expression in the intima as well as the media (Fig. 3I and J) relative to air-treated controls (Fig. 3F and G).

To explore a possible role of caveolin-1 in the regulation of cell growth by CO, we transfected SMC with siRNA corresponding to caveolin-1, and measured thymidine uptake in the absence and presence of CO. Transfection of siRNA corresponding to caveolin-1 efficiently reduced the basal expression of caveolin-1 as expected, and also diminished caveolin-1 expression in the presence of CO. CO exposure alone increased the expression of caveolin-1 (Fig. 4A). Genetic silencing of caveolin-1 abrogated the antiproliferative effect of CO in SMC (Fig. 4A).

Treatment of SMC with caveolin-1 siRNA abrogated the stimulatory effect of CO on p21^{Waf1/Cip1} expression and prevented the inhibitory effect of CO on cyclin A expression (Fig. 4B).

To examine the potential for endogenous CO produced by HO-1 activity in the regulation of cellular proliferation, SMC were transfected with an adenoviral vector containing HO-1

cDNA (AdHO-1). AdHO-1 transfection inhibited SMC proliferation and increased caveolin-1 expression, both effects that were reversed by tin protoporphyrin (SnPP), a competitive inhibitor of HO activity. In the presence of SnPP, the antiproliferative effect was completely restored by exogenous CO (Fig. 4C).

The Stimulation of Caveolin-1 Expression by CO Depends on cGMP and p38 MAPK. We examined whether exposure to CO could up-regulate caveolin-1 expression through cGMP and p38 MAPK pathways in mesenchymal cells. The increased expression of caveolin-1 in SMC stimulated by CO was abolished by SB203580, which also inhibited basal caveolin-1 expression (Fig. 5A), implying a role of p38 α/β MAPK in caveolin-1 regulation. Treatment with PDGF caused a transient increase in the activation (phosphorylation) of p38 MAPK at 10 min posttreatment that rapidly declined thereafter. CO increased and sustained p38 MAPK activation in SMC treated with PDGF within the first 60 min (Fig. 5B). A cell-permeable analog of cGMP, 8-Br-cGMP, activated p38 MAPK within 15 min of treatment, and up-regulated caveolin-1 expression in a dose-dependent manner (Fig. 5C). Finally, the expression of caveolin-1 observed in the presence of CO was abrogated by treatment with ODQ, an inhibitor of guanylate cyclase (Fig. 5D).

p38 β MAPK Inhibits Cellular Proliferation Through the Up-Regulation of p21^{Waf1/Cip1} and Caveolin-1, and the Down-Regulation of Cyclin A Expression. Because the effect of CO on p21^{Waf1/Cip1} expression could be inhibited by SB 203580, which targets p38 α/β MAPK (Fig. 1C), we further examined the subtype specificity of this effect in cells derived from p38 β -null mice. We isolated p38 β -deleted fibroblasts (p38 β ^{-/-}), which are readily harvestable from the lungs of the knockout mice. The use of genetically altered cells provides a powerful tool for the elucidation of signaling pathways, because it removes the questions of selectivity often associated with the use of chemical inhibitors.

To elucidate the function of p38 β MAPK in the control of cellular proliferation, we examined the basal expression of cell cycle related proteins in wild-type and p38 β ^{-/-} fibroblasts. As expected, p38 β ^{-/-} fibroblasts displayed hypercellularity under normal culture conditions. Furthermore, the antiproliferative effect of CO was lost in p38 β ^{-/-} fibroblasts (Fig. 6A). The deletion of p38 β MAPK inhibited the expression of tumor suppressor proteins (p21^{Waf1/Cip1}), and promoted the expression of cyclin A, enabling the null fibroblasts to grow rapidly (Fig. 8A, which is published as supporting information on the PNAS web site). p38 β ^{-/-} fibroblasts displayed

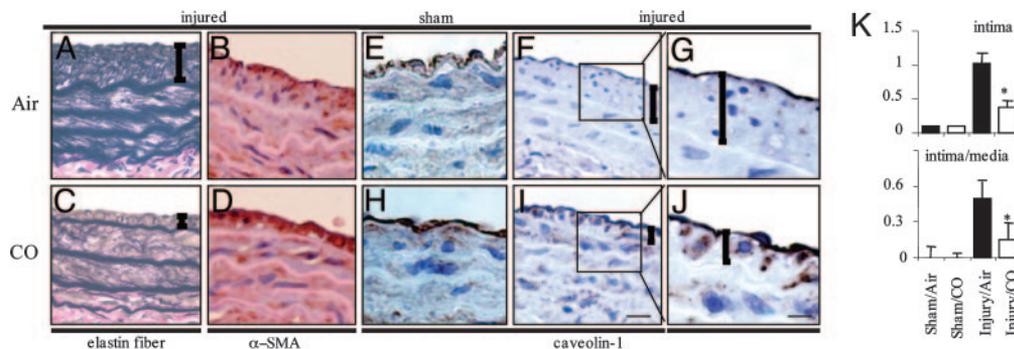


Fig. 3. CO increases caveolin-1 expression *in vivo*. LEW rats were subjected to balloon injury of the carotid artery, as described in *Materials and Methods*. The rats were exposed to either room air (A, B, and E–G) or CO (C, D, and H–J) before and during balloon or sham injury. Carotid arteries were analyzed by immunohistochemical staining 21 days after balloon angioplasty. Tissue samples were stained with a modified elastic tissue-Masson's trichrome stain for elastic fiber (A and C). Sections were immunostained with anti- α -SMA for the detection of SMC (B and D), or anti-caveolin-1 (E–G and H–J), which was detected in both SMC and endothelial cells (G and J). [Scale bar: 25 μ m (E, F, H, and I) or 10 μ m (G and J).] Samples shown are representative of three to six animals analyzed per group. Tissue sections corresponding to sham or balloon injury in the absence or presence of CO were quantified for intimal thickness, as well as the ratio of intima to media (K). Results represent the mean \pm SD for three independent determinations (arbitrary units). *, $P < 0.05$.

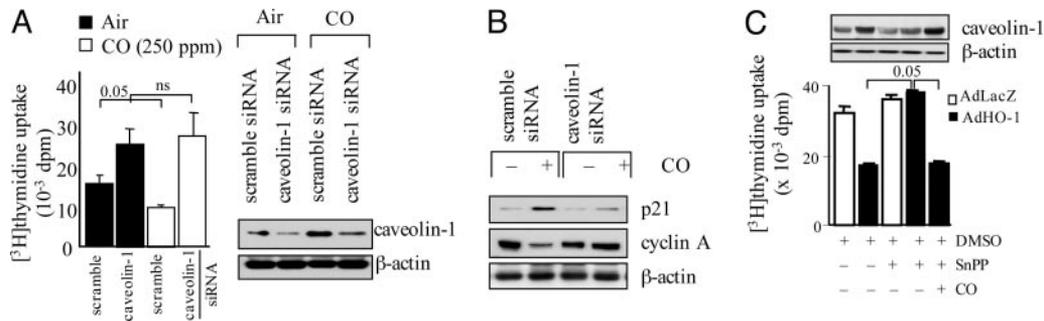


Fig. 4. Down-regulation of caveolin-1 abolishes the antiproliferative effect of CO. (A) [³H]Thymidine incorporation assay was conducted in SMC transfected with siRNA for caveolin-1 or control siRNA (Dharmacon) and treated with CO (250 ppm) or room air. Cell lysates were assayed for caveolin-1 expression in the absence or presence of CO. (B) SMC were transfected with siRNA for caveolin-1 or control siRNA (48 h), followed by an additional 24 h in the absence or presence of CO. Cell lysates were analyzed for the expression of p21, and cyclin A by Western immunoblotting. β -actin served as loading control. Results represent at three independent experiments. (C) SMC were transfected with adenoviral constructs containing HO-1 or LacZ cDNA for 48 h, followed by incubation in the absence or presence of CO (250 ppm) or SnPP for an additional 24 h before determination of cell proliferation by [³H]thymidine incorporation. Proliferation data represent three independent experiments using triplicate wells. *, $P < 0.05$; ns, not significant (A and C).

a reduced basal expression of caveolin-1, relative to wild-type cells during exponential growth (Fig. 8B). Silencing of p38 α by transfection of fibroblasts with a dominant negative mutant exerted little effect on caveolin-1 expression (Fig. 8C). CO failed to stimulate caveolin-1 expression in p38 $\beta^{-/-}$ fibroblasts (Fig. 6B), again implying an essential role for p38 β MAPK in the regulation of caveolin-1 by CO. Fibroblasts deleted of c-Jun-NH₂-terminal kinase-1 (*jdk-1^{-/-}*) displayed similar caveolin-1 expression relative to wild-type fibroblasts (Fig. 8D).

To determine the downstream regulatory targets of p38 β MAPK, wild-type and p38 $\beta^{-/-}$ fibroblast cultures were stimulated with PDGF (10 ng/ml). PDGF stimulated ERK1/2 phosphorylation within 5–30 min of treatment, to a greater extent in the p38 $\beta^{-/-}$ fibroblasts relative to wild-type fibroblasts. The activation of ERK1/2 in the presence of PDGF was amplified by CO in the p38 $\beta^{-/-}$ fibroblasts but not in the wild-type fibroblasts (Figs. 6C and 8E).

Reconstitution of p38 β expression in p38 $\beta^{-/-}$ fibroblasts restored basal caveolin-1 expression (Fig. 6D), suggesting an exclusive contribution of the β -isoform of p38 MAPK in mediating caveolin-1 expression. Reconstitution of p38 β in p38 $\beta^{-/-}$ fibroblasts also restored the ability of CO treatment to induce caveolin-1 expression in these cells (Fig. 6D). Finally, the antiproliferative effect of CO was lost in fibroblasts derived from caveolin-1 knockout mice (*cav^{-/-}*) (Fig. 6E).

Discussion

We have examined the mechanisms underlying the antiproliferative activity of CO in vascular smooth muscle cells and fibroblasts. We have identified caveolin-1, whose expression is modulated by CO, as a cardinal mediator of this process. Previously, we found that inducible HO-1 protein and activity, the principle source of endogenous CO, localize to caveolae fractions of endothelial cells. Caveolin-1 residing in the caveolae negatively regulates HO-1 activity by directly interacting with the HO-1 protein (30). Previously, we have also shown that inhibition of p38 MAPK activation abrogated the growth inhibitory effect of CO in vascular smooth muscle (18, 26). Likewise, the expression of HO-1 also induced growth arrest by means of activation of cGMP- and p38 MAPK-dependent pathways (24). Our current data strongly support that activation of p38 MAPK is crucial in the regulation of caveolin-1 expression. Collectively, our studies point to the complex participation of caveolin-1 in the biological effects of CO, with implications in both the negative regulation of endogenous CO production in endothelial cells, and in the antiproliferative activity of CO in smooth muscle.

Our current study shows that SMC exposed to CO (250 ppm) exhibit a growth-inhibited phenotype dependent on p38 MAPK and downstream modulation of cell-cycle check point proteins such as p21^{Waf1/Cip1} and cyclin A (Fig. 1 A–C). In contrast, p38 $\beta^{-/-}$ fibroblasts display a growth-accelerated phenotype, with reciprocal

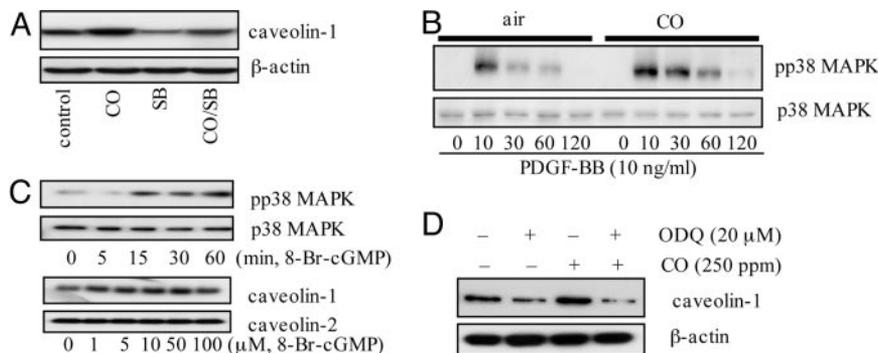


Fig. 5. The stimulation of caveolin-1 expression by CO requires cGMP and p38 MAPK-dependent pathways. (A) SMC were pretreated with CO (1 h) followed by addition of SB 203580 (10 μ M) (1 h), a specific p38 α/β MAPK inhibitor, followed by continuous CO exposure in the absence or presence of SB for an additional 24 h. The effect of CO on caveolin-1 expression was determined by Western blot analysis. (B) Serum-starved SMC were treated with PDGF (10 ng/ml) for the indicated times in the absence or presence of CO. Total cell lysates were immunoblotted with anti-phospho p38 MAPK antibody. Total p38 MAPK served as a loading control. (C) SMC were treated with a cGMP analog, 8-Br-cGMP (20 μ M) for the indicated times, or with the indicated concentrations for 24 h. Total cell lysates were immunoblotted with anti-phospho p38 MAPK antibody, or anti-caveolin-1. Total p38 MAPK or caveolin-2 served as loading controls. (D) The effect of CO on caveolin-1 expression was determined in SMC in the absence or presence of ODQ, a specific guanylate cyclase inhibitor.

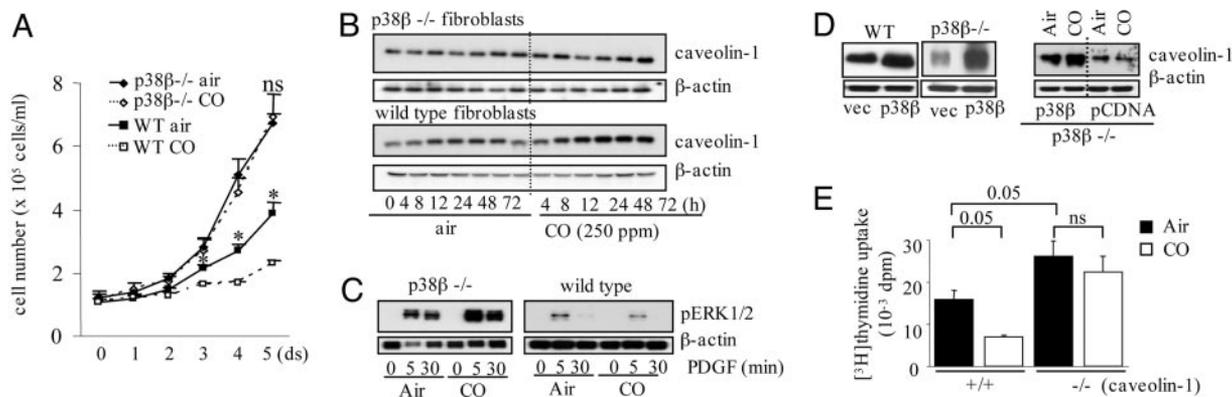


Fig. 6. p38 β MAPK inhibits cellular proliferation, by increasing p21^{Waf1/Cip1} and decreased cyclin A expression. (A–E) Fibroblasts were isolated from the lungs of wild-type or p38 β -null mice (A–D) or from caveolin-1-null mice (E). (A) Growth curves of wild-type and p38 β - fibroblasts in the absence of CO (250 ppm) were determined by direct cell counting (initial cell number: 0.5×10^5 cells per ml). Data represent the mean \pm SD of four to six experiments. (B) Wild-type or p38 β - fibroblasts were treated with air or CO (250 ppm) for the indicated times (0–72 h) and assessed for caveolin-1 expression by Western analysis with anti-caveolin-1 antibody. β -actin served as loading control. Results represent at least three independent experiments. (C) Wild-type or p38 β - fibroblasts were treated with PDGF in the absence or presence of CO and assayed for ERK1/2 phosphorylation at the indicated times by Western analysis. (D) Wild-type and p38 β - fibroblasts were transfected with a p38 β MAPK expression vector or control vector for 48 h (Left) or incubated for a further 24 h in the absence or presence of CO (250 ppm) (Right). Cell lysates were immunoblotted with anti-caveolin-1 antibody. β -actin served as loading control. (E) Fibroblasts derived from caveolin-1-null mice (*cav*^{-/-}) or wild-type mice were serum-starved overnight, and then stimulated by addition of serum (10% FBS) to the culture to initiate cell growth. Cellular proliferation was assessed by measuring [³H]thymidine incorporation after serum stimulation in the absence or presence of CO (250 ppm for 24 h).

regulation of p21^{Waf1/Cip1} and cyclin A (Figs. 6A and 8A). These experiments underscore the critical role for p38 MAPK in growth control. Because many effects of CO, including the inhibition of inflammation, apoptosis, and proliferation, depend on the activation of p38 MAPK, we sought the existence of a downstream effector of p38 MAPK with tumor-suppressing activity. Caveolin-1 met these criteria *in vivo* and *in vitro* (Figs. 2–4).

Many of the known physiological effects of CO depend on the activation of guanylate cyclase, which produces cGMP for signaling processes (19). The current results support a role for guanylate cyclase/cGMP in the up-regulation of caveolin-1 by CO, because an inhibitor of guanylate cyclase (ODQ) blocked the effect (Fig. 5D). Furthermore, a cell-permeable analog of cGMP, 8-Br-cGMP, mimicked the effects of CO on p38 MAPK activation and caveolin-1 expression (Fig. 5C).

Many biological programs activated during premature cellular senescence, or by genotoxic stimuli and oxidative stress, also use p38 MAPK-dependent pathways (1–3, 17). By using gene-deleted mice of the type-3 phosphatase Wip1, Bulavin *et al.* (34) have demonstrated that the up-regulation of p38 MAPK inhibits mammary tumorigenesis by activating p53 and the p16(Ink4a)-p19(Arf) pathway, strongly supporting a critical role of p38 MAPK in cell growth arrest. However, the specific roles of individual isoforms of p38 MAPKs (α , β , γ , and δ) remain incompletely understood. The initiation of a G₂/M cell cycle checkpoint after UV radiation exposure required two isoforms (α and β) of p38 MAPK (3). CO treatment in the presence of PDGF activated both α and β isoforms of p38 MAPK, as determined by immunoprecipitation experiments (data not shown). Here, we demonstrate that the β -isoform of p38 MAPK specifically regulates caveolin-1 expression in fibroblasts in response to CO treatment (Fig. 6B). The antiproliferative effects of CO in T lymphocytes, in direct contrast to observations in smooth muscle and fibroblasts, excluded a role for p38 MAPK activation, (35, 36), suggesting that cell type-specific variations

may occur in the distinct pathways that mediate this effect. Given the variable cellular distribution of p38 MAPK isoforms (37), it is plausible that cell type-specific variations may also occur in the pathways mediating the activation of caveolin-1 by CO.

Cyclin-dependent kinase inhibitors such as p21^{Waf1/Cip1}, p16^{Ink4}, and p27^{Kip1} participate in the regulation of cell cycle progression (38). The expression of p21^{Waf1/Cip1} increases in arteries after vascular injury. In SMC, expression of p21^{Waf1/Cip1} resulted in G₁ arrest and inhibition of cell growth (24). We have observed that exposure of SMC to CO up-regulated p21^{Waf1/Cip1} protein expression (Fig. 1B), strongly suggesting a role for p21^{Waf1/Cip1} in the antiproliferative effect of CO. We have also shown that the genetic deletion of p38 β or chemical inhibition of p38 α/β MAPK, dramatically reduces the expression of p21^{Waf1/Cip1} and of caveolin-1 in smooth muscle (Figs. 1C and 5A), or fibroblasts (Figs. 6D and 8A). Galbiati *et al.* (17) have shown that overexpression of caveolin-1 regulates p21^{Waf1/Cip1} expression, leading to G₀/G₁ cell cycle arrest. We propose that the antiproliferative effect of CO, which involves the induction of p21^{Waf1/Cip1}, seems to be governed by the expression of caveolin-1. We have shown that down-regulation of caveolin-1, in addition to blocking the antiproliferative effect of CO, also abolished the modulatory effects of CO on p21^{Waf1/Cip1} and cyclin A expression in SMC (Fig. 4B).

Collectively, our data demonstrated that CO up-regulated caveolin-1 expression under the specific control of p38 β MAPK pathway, which in turn down-regulated the growth of target cells. An understanding of the effects of CO on cell growth and its underlying mechanisms may provide useful tools for the study or treatment of various cell-proliferative disorders, including cancer.

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Corrections

PHARMACOLOGY. For the article “5-Methyltetrahydrofolate and tetrahydrobiopterin can modulate electrotonically mediated endothelium-dependent vascular relaxation,” by Tudor M. Griffith, Andrew T. Chaytor, Linda M. Bakker, and David H. Edwards, which appeared in issue 19, May 10, 2005, of *Proc. Natl. Acad. Sci. USA* (102, 7008–7013; first published May 2,

2005; 10.1073/pnas.0408919102), the authors note that previously published data had been included in error. Specifically, in Fig. 3A of the PNAS article, representative traces of the endothelial cell patch clamp data were identical to those in figure 2A of ref. 4. The corrected figure and its legend appear below.

4. Chaytor, A. T., Bakker, L. M., Edwards, D. H. & Griffith, T. M. (2005) *Br. J. Pharmacol.* 144, 108–114.

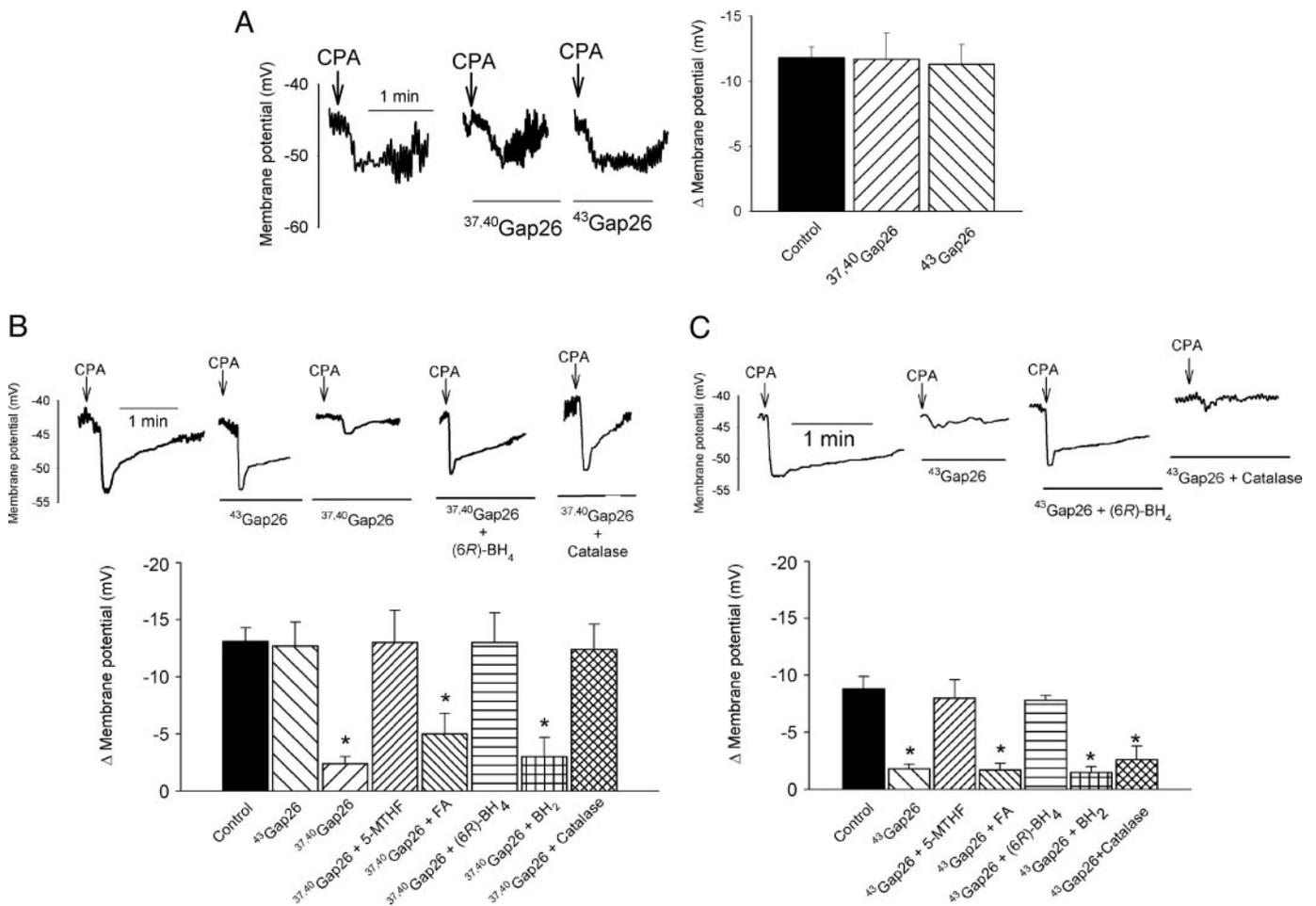


Fig. 3. Electrophysiological studies with connexin-mimetic peptides. (A) Whole-cell patch-clamp recordings and histogram confirming that 600 μM $^{37,40}\text{Gap}26$ and 100 μM $^{43}\text{Gap}26$ did not depress endothelial hyperpolarizations evoked by 30 μM CPA. (B and C) Original recordings and histograms comparing the effects of the peptides and 2,000 units/ml catalase on subintimal (B) and subadventitial (C) hyperpolarizations evoked by CPA. $^{37,40}\text{Gap}26$ attenuated the transmission of endothelial hyperpolarization to subintimal smooth muscle, whereas $^{43}\text{Gap}26$ selectively impaired transmission of subintimal hyperpolarization across the vessel wall. The effects of both peptides were prevented by 100 μM 5-MTHF and (6R)-BH₄ but not by 100 μM FA or BH₂. Catalase prevented the effects of $^{37,40}\text{Gap}26$ on subintimal hyperpolarization, but not those of $^{43}\text{Gap}26$ on subadventitial hyperpolarization. *, $P < 0.05$, compared with control.

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CELL BIOLOGY. For the article “Caveolin-1 expression by means of p38 β mitogen-activated protein kinase mediates the antiproliferative effect of carbon monoxide,” by Hong Pyo Kim, Xue Wang, Atsunori Nakao, Sung Il Kim, Noriko Murase, Mary E. Choi, Stefan W. Ryter, and Augustine M. K. Choi, which appeared in issue 32, August 9, 2005, of *Proc. Natl. Acad. Sci. USA* (**102**, 11319–11324; first published July 28, 2005; 10.1073/pnas.0501345102), the authors note that the following statements should be added to the acknowledgments: “We thank M. Drab (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany) for providing the caveolin-1 knockout mice. We thank K. Kuida (Vertex Pharmaceuticals, Boston) and Richard Flavell (Yale University School of Medicine, New Haven, CT) for the gifts of p38 $^{-/-}$ and jnk-1 $^{-/-}$ mice, respectively.”

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