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Carbon Monoxide Releasing Molecule-2 Inhibits Pancreatic Stellate Cell Proliferation by Activating p38 Mitogen-Activated Protein Kinase/Heme Oxygenase-1 Signaling

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ABSTRACT

Proliferation of pancreatic stellate cells (PSCs) plays a cardinal role during fibrosis development. Therefore, the suppression of PSC growth represents a therapeutic option for the treatment of pancreatic fibrosis. It has been shown that up-regulation of the enzyme heme oxygenase-1 (HO-1) could exert antiproliferative effects on PSCs, but no information is available on the possible role of carbon monoxide (CO), a catalytic byproduct of the HO metabolism, in this process. In the present study, we have examined the effect of CO releasing molecule-2 (CORM-2) liberated CO on PSC proliferation and have elucidated the mechanisms involved. Using primary rat PSCs, we found that CORM-2 inhibited PSC proliferation at nontoxic concentrations by arresting cells at the G_0/G_1 phase of the cell cycle. This effect was associated with activation of p38 mitogen-activated protein kinase (MAPK) signaling, induction of HO-1 protein, and up-regulation of the cell cycle inhibitor $p21^{Waf1/Cip1}$. The p38 MAPK inhibitor 4-(4-flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB203580) abolished the inhibitory effect of CORM-2 on PSC proliferation and prevented both CORM-2-induced HO-1 and $p21^{Waf1/Cip1}$ up-regulation. Treatment with tin protoporphyrin IX, an HO inhibitor, or transfection of HO-1 small interfering RNA abolished the inductive effect of CORM-2 on $p21^{Waf1/Cip1}$ and reversed the suppressive effect of CORM-2 on PSC growth. The ability of CORM-2 to induce cell cycle arrest was abrogated in $p21^{Waf1/Cip1}$-silenced cells. Taken together, our results suggest that CORM-2 inhibits PSC proliferation by activation of the p38/HO-1 pathway. These findings may indicate a therapeutic potential of CO carriers in the treatment of pancreatic fibrosis.

Chronic pancreatitis is a progressive fibroinflammatory disease that, in its end stages, results in the loss of organ architecture and functional insufficiency of the gland (Witt et al., 2007). Therapeutic options are rare, and treatment modalities focus on improvement of the patient's quality of life. With the identification, isolation, and characterization of the pancreatic stellate cell (PSC) (Apte et al., 1998; Bachem et al., 1998), a considerable amount of knowledge has been gained about the pathogenesis of this disease, particularly related to the mechanisms responsible for the development of pancreatic fibrosis, a dominant feature of chronic pancreatitis (Apte and Wilson, 2004). In the normal pancreas, PSCs are quiescent and show a similar morphology to hepatic stellate cells, the principal effector cells in liver fibrosis (Friedman, 1993; Bachem et al., 1998). When activated by toxic stimuli such as ethanol or by cytokines, PSCs undergo a transition into highly proliferative myofibroblast-like cells (Haber et al., 1999). Activated PSCs express the cytoskeletal protein α-smooth muscle actin and regulate both synthesis and degradation of the extracellular matrix components that comprise fibrous tissue (Apte and Wilson, 2004). Taking this into account, compounds that inhibit PSC proliferation or induce apoptosis in activated PSCs may have the potential to become a new approach for the treatment of pancreatic fibrosis (Omary et al., 2007).

Carbon monoxide (CO) has shown promising potential as a therapeutic agent in animal models of lung fibrosis (Zhou et

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ABBREVIATIONS: PSC, pancreatic stellate cell; ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; Cdk, cyclin-dependent kinase; CORM, carbon monoxide-releasing molecule; CORM-2, tricarbonyldichlororuthenium(II) dimer; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HO-1, heme oxygenase-1; IMDM, Iscove's modified Dulbecco's medium; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; SB203580, 4-(4-flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole; siRNA, small interfering RNA; SnPP, tin protoporphyrin IX; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; MKK, mitogen-activated protein kinase kinase; MAPKAPK, mitogen-activated protein kinase-activated protein kinase; SB202474, 4-ethyl-2-(*p*-methoxyphenyl)-5-(4′-pyridyl)-1H-imidazole.

al., 2005; Zheng et al., 2009), despite its limitations as a toxic gas and environmental hazard. Endogenous CO arises principally as the byproduct of heme catabolism by action of heme oxygenase (HO) enzymes (Tenhunen et al., 1968). In recent years, the physiological importance of CO as a gaseous mediator of many biological and cellular processes has been realized (Maines, 1997; Taille et al., 2005). The effect of CO on cell proliferation is variable and seems to be cell type-specific. For example, it has been reported that exposure of cells to CO gas suppresses proliferation in airway smooth muscle cells (Song et al., 2002), vascular smooth muscle cells (Otterbein et al., 2003), and T lymphocytes (Song et al., 2004), whereas an increase in proliferation was observed in endothelial cells (Wegiel et al., 2008).

CO-releasing molecules (CORMs) are metal carbonyl compounds capable of delivering defined amounts of CO into cellular systems, thereby reproducing the biological effects of CO derived from HO activity (Motterlini et al., 2002; Sawle et al., 2006). CORMs have shown antiproliferative effects in airway smooth muscle cells (Taille et al., 2005). Previous observations from our laboratory suggest an antifibrotic action of HO-1 in PSCs (Schwer et al., 2008), and several lines of evidence indicate that CO mediates many of the biological actions of HO-1 (Ryter et al., 2006).

We therefore tested the hypothesis that CO, liberated from CORM-2 [tricarbonyldichlororuthenium(II) dimer] may suppress PSC proliferation. The aims of this study were as follows: 1) to investigate whether CORM-2 affects PSC proliferation; 2) to characterize the effects of CORM-2 on cell cycle progression of PSCs; and 3) to explore the role of the MAPK pathways, HO-1 and the cell cycle inhibitor $p21^{Waf1/Cip1}$, under these experimental conditions.

Materials and Methods

Reagents. Collagenase P and 5-bromo-2′-deoxyuridine (BrdU) cell proliferation kits were purchased from Roche Diagnostics (Mannheim, Germany). Iohexol (Nycodenz) was obtained from Nycomed (Oslo, Norway), and Hanks' buffered salt solution was from Invitrogen (Karlsruhe, Germany). Iscove's modified Dulbecco's medium (IMDM) and supplements for cell culture were obtained from Invitrogen. The polyclonal rabbit antibodies anti-phospho-p38, anti-total-p38, anti-phospho-ERK1/2, anti-total-ERK1/2, anti-phospho-JNK, anti-total-JNK, anti-phospho-MKK3/MKK6, anti-total-MKK3/MKK6 and anti-phospho-MAPKAPK-2 were obtained from Cell Signaling Technology (Danvers, MA), and polyclonal rabbit anti HO-1 antibody was from Assay Designs (Ann Arbor, MI). Monoclonal mouse anti-$p21^{Waf1/Cip1}$ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and monoclonal mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from Nventa Biopharmaceuticals (San Diego, CA). Horseradish peroxidase-conjugated antibodies and the ECL kit were obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). All other reagents were purchased from Sigma Chemie (Deisenhofen, Germany) unless indicated otherwise.

Isolation and Culture of PSCs. All animal procedures were performed in accordance with the Institute of Laboratory Animal Resources (1996). Rat PSCs were prepared from the pancreas of male Wistar rats (Charles River, Sulzfeld, Germany) weighing 250 to 300 g according to the procedure described by Shinji et al. (2002). In brief, the pancreas was digested with 0.03% collagenase P in Hanks' buffered salt solution. The resultant suspension of cells was centrifuged in a 13.2% iohexol gradient at 1400*g* for 20 min. Stellate cells separated into a fuzzy band just above the interface of the iohexol solution and the aqueous buffer. This band was harvested, and the cells were washed and resuspended in IMDM containing 10% fetal calf serum (FCS), 4 mM glutamine, and antibiotics (penicillin 100 U/ml and streptomycin 100 mg/ml). Cell purity was always more than 90% as assessed by vitamin A autofluorescence. After reaching confluence, cells were harvested and replated at equal seeding densities. All experiments were performed using culture-activated cells (passages 2–4). PSCs were incubated in serum-free medium for 24 h before the addition of experimental reagents.

Treatment of Cells. The carbon monoxide-releasing molecule tricarbonyldichlororuthenium(II) dimer (CORM-2; Sigma Chemie), the p38 MAPK inhibitor SB203580 (Calbiochem, Bad Soden, Germany), and the HO inhibitor tin protoporphyrin IX (SnPP; Frontier Scientific Europe, Carnforth, UK) were dissolved in dimethyl sulfoxide (DMSO) and then diluted in culture medium [0.1% (v/v)]. Control cells were treated with the same vehicle. Potential cytotoxic effects of the administered solutions were assessed by lactate dehydrogenase (LDH) release assays of the collected culture media.

Lactate Dehydrogenase Release Assay. Serum-starved PSCs were treated with CORM-2 at the indicated concentrations for 24 h. LDH in conditioned media was determined as medium LDH. LDH in cell lysates was analyzed as cellular LDH. LDH in IMDM with 10% FCS was considered as contamination arising from FCS and was subtracted from medium and cellular LDH. LDH activities were determined by an LDH assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Fluorogenic Caspase Activity Assay. Total protein cell extracts of 5×10^6 cells per sample (10 μl) were mixed with 90 μl of assay buffer (100 mM HEPES, pH 7.5, 2 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride). The respective fluorogenic substrate for caspase-3 and -7, 7-amino-4-methylcoumarin (1 μl, 60 μM; Alexis Corporation, Gruenberg, Germany) was added and the fluorescence was measured at 30°C for 30 min in a Microplate SpectraMax Gemini XS reader (Molecular Devices, Sunnyvale, CA) at 380/460 nm.

Determination of Cell Growth. Cultured PSCs were passaged twice and replated at equal seeding densities into 24-well culture plates. After a 24-h serum deprivation, triplicate wells of cells were then exposed to CORM-2 (12.5–100 μM) for 30 min, followed by a 24-h stimulation with 10% FCS. Cells incubated in medium without FCS served as controls. Cells were washed twice in phosphate-buffered saline, harvested by trypsination using 0.5% trypsin/0.2% EDTA, resuspended in 200 μl of culture medium, and counted by a Casy TT cell counter according to the manufacturer's instructions (Schärfe System, Reutlingen, Germany). Cell count analyses were performed in quadruplicate with $n = 3$ separate cell preparations, respectively.

Determination of Cell Proliferation. Serum-starved PSCs (~80% density) were left untreated or were treated with 10% FCS in the presence of CORM-2 at the indicated concentrations. Cell proliferation was evaluated with a BrdU-based enzyme-linked immunosorbent assay (Roche Diagnostics) according to the manufacturer's instructions. After 30-min incubation with the indicated substances, cells were labeled with BrdU for 20 h at 37°C. Cells were fixed and incubated with a peroxidase-conjugated anti-BrdU antibody for 90 min at room temperature. After adding the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine, BrdU incorporation was determined by measuring the optical densities at 370 nm minus the background at 492 nm. BrdU assays were performed in quadruplicate with at least $n = 3$ separate cell preparations, respectively.

Cell Cycle Analysis by Flow Cytometry. Culture-activated PSCs were starved from serum for 24 h followed by a 24- or 48-h stimulation with 10% FCS in the presence or absence of CORM-2 (100 μM). Cells were washed twice in phosphate-buffered saline and harvested by trypsin digestion (0.5% trypsin-0.2% EDTA). Preparation of nuclei and propidium iodide staining was performed using a commercial kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's recommendations and analyzed on a FACSCalibur

(BD Biosciences) using both CELLQuest (BD Biosciences) and FlowJo softwares (TreeStar, Ashland, OR).

Western Blot Analysis. Total cell lysates were prepared by the addition of 100 μl of radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). The protein content of the cell lysates was determined using a commercial protein assay kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of proteins (40 μg) were separated on a 10 or 13% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), and the membranes were blocked with 5% skim milk in Tween 20/phosphate-buffered saline and incubated with the indicated protein-specific antibodies overnight at 4°C. After incubation with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig antibody, proteins were visualized using the Enhanced Chemiluminescence Kit (GE Healthcare). For normalization, blots were reprobed with antibodies to detect total amounts of GAPDH, p38 MAPK, ERK1/2, or JNK. Blots were analyzed by laser-scanning densitometry (Personal Densitometer; GE Healthcare). The results shown are representative of experiments with at least $n = 3$ different cell preparations.

siRNA Transfection. PSCs (1×10^5/ml) were transfected with 100 nM concentration of a validated scrambled control siRNA (All-Stars nonsilencing siRNA; QIAGEN GmbH, Hilden, Germany) or siRNA specifically targeting genes of interest, HO-1 [*hmox1_4* (HO-1 siRNA A); *hmox1_3* (HO-1 siRNA B); both obtained from QIAGEN GmbH] and $p21^{Waf1/Cip1}$ [Cdkn1a_2 (p21 siRNA A); Cdkn1a_3 (p21 siRNA B); QIAGEN GmbH] using HiPerFect transfection reagent (QIAGEN GmbH) according to the manufacturer's recommendations. Twelve hours after transfection, the medium was changed and PSCs were incubated in serum-free IMDM for 24 h before treatment.

Statistical Analysis. Results are expressed as means ± S.E.M. for the indicated number of separate cell preparations per experimental protocol. Data were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls post hoc test. Differences between groups were considered to be significant at $p < 0.05$. Analyses were performed using the SigmaStat statistical software package (Systat Software GmbH, Erkrath, Germany).

Results

CORM-2 Suppresses PSC Proliferation. To evaluate the effect of CORM-2 on PSC growth, cells were exposed to CORM-2 (12.5–100 μM) before stimulation with 10% FCS. Cell growth was determined by cell counts (Fig. 1A) and native microscopy (Fig. 1C). As shown in Fig. 1A, 10% FCS induced a profound increase in cell counts by 57% compared with cells incubated with 0.1% FCS. The administration of CORM-2 in 10% FCS-stimulated cells caused a significant and dose-dependent decrease in cell numbers (129 ± 7.5% at 50 μM and 123 ± 3.7% at 100 μM), respectively. To further investigate the mechanism underlying the inhibitory effect of CORM-2 on PSC growth, cell proliferation in response to serum stimulation was assessed using BrdU incorporation assays. DNA synthesis in cells exposed to 0.1% FCS was defined as 100%. As shown in Fig. 1B, 10% FCS significantly stimulated PSC proliferation (154 ± 10.2%). Treatment of serum-stimulated cells with increasing amounts of CORM-2 (12.5–100 μM) reduced BrdU incorporation in a dose-dependent manner. At higher concentrations, CORM-2 significantly and completely inhibited DNA synthesis (101 ± 4.8% at 50 μM and 89 ± 4.6% at 100 μM).

Cytotoxicity Profile of CORM-2 in PSCs. We then analyzed whether the reduced cell growth after CORM-2 treatment was a consequence of necrosis or apoptosis. CORM-2 toxicity to culture-activated PSCs was investigated by examining LDH release. Tween 20 was added to the cell culture media to a final concentration of 1% for the determination of maximal LDH release (100%; Fig. 2A). Compared with control (DMSO treatment only), CORM-2 did not cause a relevant increase in LDH release when used at concentrations between 12.5 and 100 μM. At higher concentrations, administration of CORM-2 results in a 10.4 ± 2.4 (200 μM), 13.7 ± 3.6 (500 μM), and 32.1 ± 4.2% (1000 μM) increase in LDH

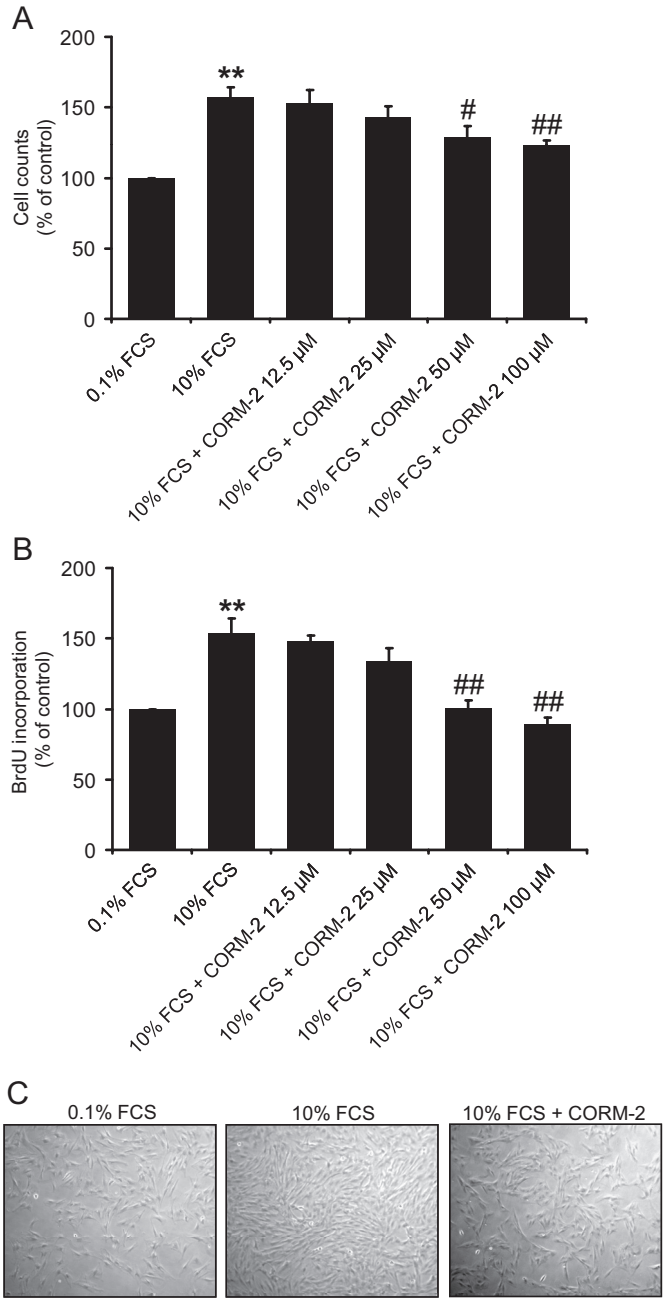

Fig. 1. Effect of CORM-2 on PSC proliferation. Serum-starved PSCs were incubated with CORM-2 at the indicated concentrations for 30 min followed by a 24-h stimulation with 10% FCS. A, cell numbers were determined by a computer-equipped cell counter. B, DNA synthesis was estimated by measuring the incorporation of BrdU into cellular DNA. Data are presented as mean ± S.E.M. for $n = 3$ separate cell preparations. **, $p < 0.01$ versus 0.1% FCS; #, $p < 0.05$ versus 10% FCS; ##, $p < 0.01$ versus 10% FCS. C, native microscopy. Pictures were taken from random fields of vision (original magnification, 10× objective).

release (Fig. 2A). On the basis of these observations, it was concluded that CORM-2 up to 100 μM was not toxic to cultured PSCs. We subsequently investigated whether CORM-2 induces apoptosis in PSCs by measuring the activity of caspase-3, a crucial enzyme in the induction of apoptosis, using a fluorogenic assay. Compared with control (DMSO), treatment of PSCs with the proapoptotic compound staurosporine (2 μM) led to a significant increase in caspase-3-like activity (Fig. 2B). In contrast, CORM-2 (12.5–1000 μM) had no effect.

CORM-2 Arrests PSCs at the G_0/G_1 Phase of the Cell Cycle. After synchronization by serum starvation for 24 h, PSCs were stimulated with 10% FCS in the presence or absence of CORM-2. Cells were stained with propidium iodide, and cell cycle distribution was analyzed at 24 or 48 h after stimulation by flow cytometry. Compared with 10% FCS alone, CORM-2 decreased the percentage of cells in S and G_2/M phases by 41% (53.4 ± 4.3 versus 31.3 ± 2.1%) at 24 h and 55% (59.2 ± 6.2 versus 26.8 ± 3.7%) at 48 h (Fig. 3, A and B).

CORM-2 Up-Regulates $p21^{Waf1/Cip1}$ and HO-1 Protein Expression in PSCs. To explore a possible mechanism that may account for the inhibitory effects of CORM-2 on cell cycle progression, the expression of $p21^{Waf1/Cip1}$, a potent inhibitor of G_1 cyclin-dependent kinases (Harper et al., 1993), was examined. As shown in Fig. 4A, CORM-2 significantly induced $p21^{Waf1/Cip1}$ expression compared with cells incubated with 10% FCS alone. Furthermore, we could show that treatment of PSCs with 10% FCS led to a slight increase in HO-1 protein expression, whereas CORM-2 profoundly up-regulated HO-1 (Fig. 4B).

CORM-2 Modulates MAPK Activation in PSCs. To elucidate the involvement of the MAPK pathways in the inhibitory effect of CORM-2 on PSC proliferation, serum-starved PSCs were preincubated with CORM-2 for 30 min followed by a 4-h stimulation with 10% FCS. As demonstrated by Western blot analysis, 10% FCS caused a profound increase in phosphorylation of ERK1/2 (Fig. 5B) and JNK (Fig. 5C), whereas the level of phosphorylated p38 (Fig. 5A) was only slightly enhanced in the presence of 10% FCS. The FCS-induced activation of ERK1/2 (Fig. 5B) or JNK (Fig. 5C) was not affected by CORM-2 treatment. In sharp contrast, CORM-2 led to a significant increase in p38 MAPK phosphor-

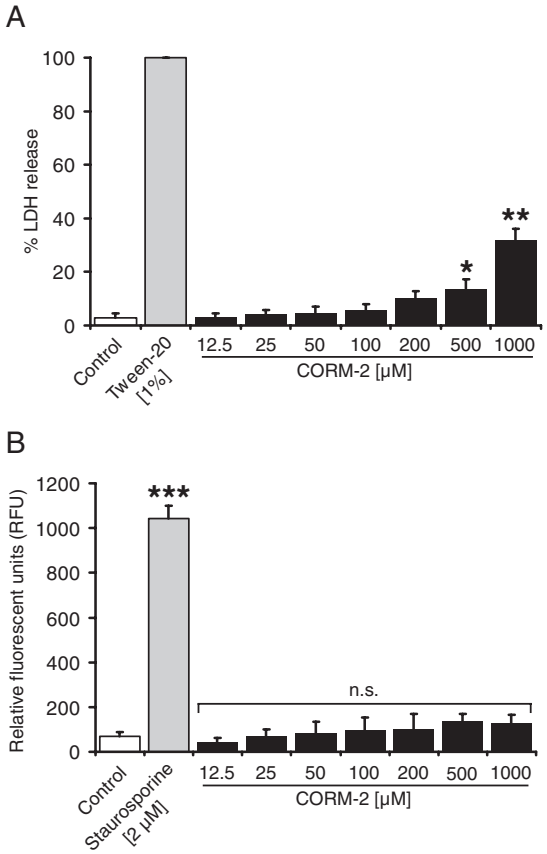

Fig. 2. Effects of CORM-2 on LDH release and caspase-3-like activity in PSCs. A, percentage of LDH released from PSCs after a 24-h incubation with increasing amounts of CORM-2 (12.5–1000 μM). Data are expressed as a percentage of the total LDH released after treatment of cells with Tween 20 (1%). B, caspase-3-like activity was assessed using a fluorogenic caspase activity assay. The results were given in relative fluorescent units. Data are presented as mean ± S.E.M. for $n = 3$ separate cell preparations. *, $p < 0.05$ versus control (DMSO only); **, $p < 0.01$ versus control; ***, $p < 0.001$ versus control; n.s., not significant versus control.

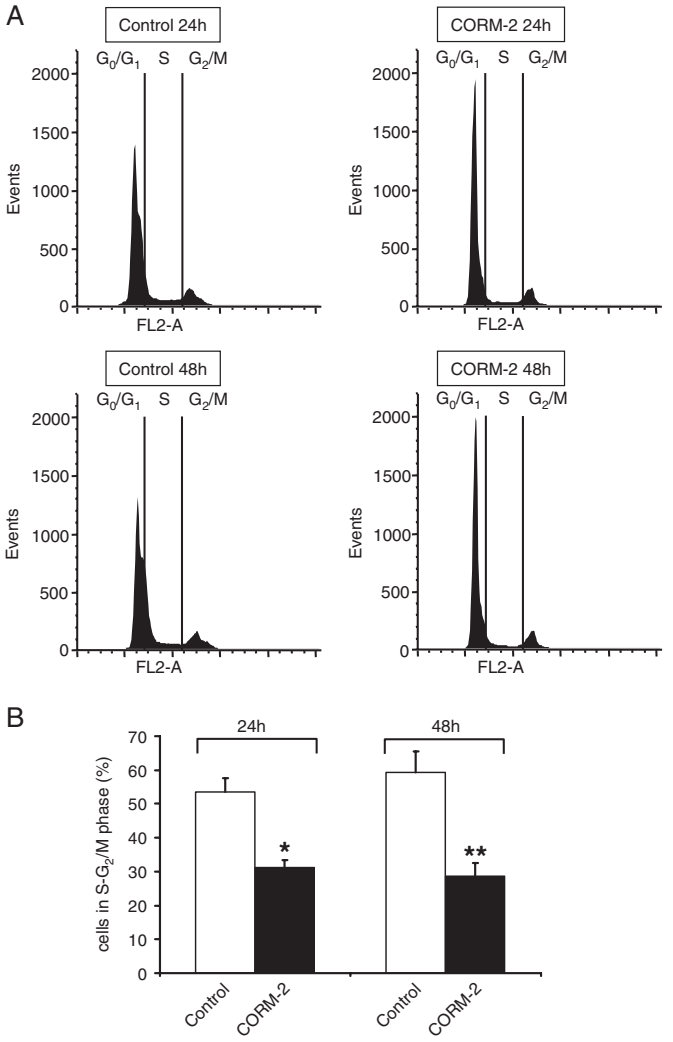

Fig. 3. Effect of CORM-2 on cell cycle distribution. A, serum-starved PSCs were stimulated with 10% FCS for 24 or 48 h in the presence or absence of CORM-2 (100 μM). Cells were harvested, and cell cycle distribution was analyzed by flow cytometry. B, quantitative analysis of cells at S–G_2/M phase. Percentage of cells in S–G_2/M phase obtained by flow cytometry. Data are presented as the mean ± S.E.M. for $n = 3$ separate cell preparations. *, $p < 0.05$ versus 10% FCS; **, $p < 0.01$ versus 10% FCS.

ylation (Fig. 5A). Next, we examined the effect of CORM-2 on MKK3 and MKK6, both of which have been shown to phosphorylate and activate p38 MAPK but not ERK1/2 or JNK (Derijard et al., 1995; Raingeaud et al., 1996). As shown in Fig. 5D, 10% FCS alone had no effect on the level of phosphorylated MKK3/MKK6, whereas CORM-2 significantly induced the phosphorylation of MKK3/6.

The Role of p38 MAPK Signaling in CORM-2-Mediated Effects. To assess the role of the p38 MAPK pathway on CORM-2-mediated effects, PSCs were preincubated with SB203580, a selective inhibitor of the p38α and $-\beta$ isoforms (Cuenda et al., 1995), or with the inactive analog SB202474 before treatment with CORM-2. First we explored the effect of CORM-2, SB203580, and SB202474 treatment on the phosphorylation of MAPKAPK-2, a downstream target of p38 (Young et al., 1997). As shown in Fig. 6A, compared with the untreated control, 10% FCS alone had no effect on the level of phosphorylated MAPKAPK-2, whereas CORM-2 significantly induced phosphorylation of MAPKAPK-2. SB203580, but not SB202474, completely abrogated the CORM-2 induced increase in phosphorylated MAPKAPK-2. To investigate whether p38 MAPK activation confers to the inhibitory effect of CORM-2 on PSC proliferation, BrdU incorporation assays were performed. As shown in Fig. 6B, SB203580 treatment blocked the ability of CORM-2 to inhibit PSC proliferation, whereas SB202474 had no effect. Finally we assessed the role of p38 signaling in CORM-2-induced $p21^{Waf1/Cip1}$ and HO-1 up-regulation. We found that coadministration of SB202474 did not prevent the induction of $p21^{Waf1/Cip1}$ (Fig. 6C) or HO-1 (Fig. 6D). By contrast, SB203580 significantly attenuated the increase in both $p21^{Waf1/Cip1}$ and HO-1 protein after CORM-2 treatment.

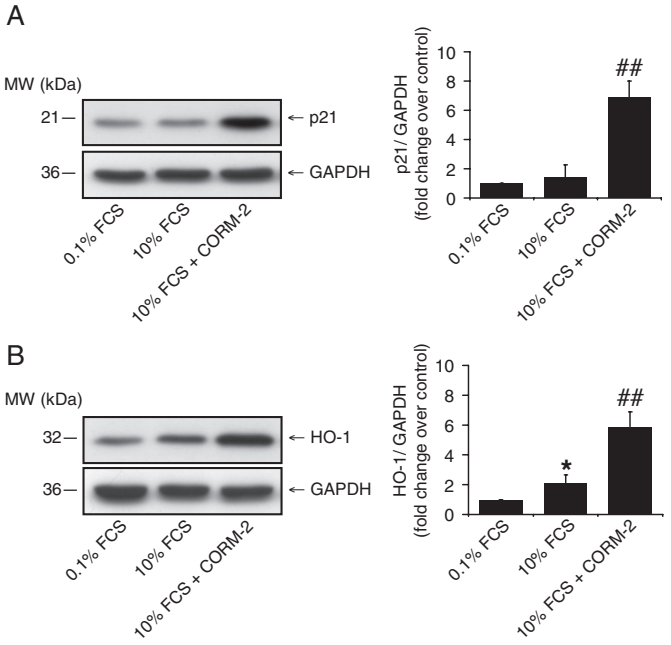

Fig. 4. Effect of CORM-2 on $p21^{Waf1/Cip1}$ and HO-1 expression in PSCs. Serum-starved PSCs were incubated with CORM-2 (100 μM) for 30 min followed by a stimulation with 10% FCS for 24 h. $p21^{Waf1/Cip1}$ (A) and HO-1 (B) protein expression were determined by Western blot analysis. To show equal loading, the membranes were stripped and reprobed with an anti-GAPDH protein-specific antibody. The histograms represent the ratios between $p21^{Waf1/Cip1}$ or HO-1 optical densities and that of GAPDH. Data are presented as mean ± S.E.M. ($n = 3$). *, $p < 0.05$ versus 0.1% FCS; ##, $p < 0.01$ versus 10% FCS.

HO-1 Mediates the Inhibitory Effects of CORM-2 on PSC Proliferation. To prove a causal relationship between CORM-2-induced HO-1 expression and the inhibitory effect of CORM-2 on PSC growth, cells were pretreated with the HO inhibitor SnPP for 45 min before administration of CORM-2. As shown in Fig. 7, SnPP treatment alone had no effect on FCS-induced increase in PSC proliferation. In large contrast, the inhibitory effect of CORM-2 on PSC proliferation was significantly attenuated in the presence of SnPP. To further explore whether CORM-2 suppresses PSC proliferation specifically via HO-1, we performed knockdown experiments by transfection of PSCs with two different HO-1 siRNAs. As indicated in Fig. 8A, transfection of nonsilencing siRNA had no effect on CORM-2-induced HO-1 up-regulation in FCS-stimulated PSCs. In the absence of CORM-2, the level of HO-1 protein was comparably low in control-transfected and in anti-HO-1 siRNA-transfected cells. By contrast, transfection with both anti-HO-1 siRNAs tested significantly attenuated CORM-2-induced up-regulation of HO-1 and reduced its inhibitory effect on PSC proliferation as demonstrated by BrdU proliferation assays (Fig. 8B).

Effect of HO-1 Knockdown on CORM-2-Induced $p21^{Waf1/Cip1}$ Expression. To clarify the role of HO-1 in CORM-2-induced $p21^{Waf1/Cip1}$ up-regulation, we examined the effect of HO-1 knockdown on $p21^{Waf1/Cip1}$ protein expression. We found that in control-transfected PSCs, CORM-2 strongly and significantly increased the level of $p21^{Waf1/Cip1}$, whereas in cells transfected with different anti-HO-1 siRNAs, the ability of CORM-2 to up-regulate $p21^{Waf1/Cip1}$ was significantly attenuated (Fig. 9).

Effect of $p21^{Waf1/Cip1}$ Silencing on CORM-2-Induced Cell Cycle Arrest. To determine whether a causal relationship exists between the ability of CORM-2 to up-regulate $p21^{Waf1/Cip1}$ and to induce G_0/G_1 cell cycle arrest, PSCs were transfected with different siRNAs to knock down $p21^{Waf1/Cip1}$ gene expression before exposure to CORM-2. Cell cycle analyses revealed that compared with cells transfected with control siRNA and treated with 10% FCS alone (Fig. 10A), transfection of p21 siRNA A (Fig. 10C) or p21 siRNA B (Fig. 10E) did not significantly alter the percentage of cell in S and G_2/M phases [50.7 ± 3.5 versus 51.9 ± 4.1 (p21 siRNA A) or 51.2 ± 3.9% (p21 siRNA B)]. In line with previous data from our study, administration of CORM-2 decreased the percentage of nonsilenced cells in S and G_2/M phases by 28% (Fig. 10B; 50.7 ± 3.5 versus 36.5 ± 2.9%). When cells were transfected with p21 siRNA A (Fig. 10D) or p21 siRNA B (Fig. 10F) before CORM-2 treatment, the ability of CORM-2 to induce G_0/G_1 cell cycle arrest was almost completely abrogated [Fig. 10G; 36.5 ± 2.9 versus 46.4 ± 3.6 (p21 siRNA A) or 45.7 ± 3.4% (p21 siRNA B)].

Discussion

The present study demonstrates for the first time, that CORM-2 inhibits PSC proliferation. This effect involves the activation of p38 MAPK signaling, up-regulation of HO-1 protein, and $p21^{Waf1/Cip1}$-dependent cell cycle arrest at the G_0/G_1 phase.

Carbon monoxide is increasingly recognized as a relevant mediator of numerous cellular functions (Ryter et al., 2006). It is generated endogenously during heme metabolism in a

reaction catalyzed by HO enzymes. CO has been shown to exert antiproliferative effects in smooth muscle cells (Song et al., 2002; Otterbein et al., 2003) and T lymphocytes (Song et al., 2004). In an experimental model of lung fibrosis, Zhou et al. (2005) have demonstrated previously that suppression of fibroblast proliferation contributes to the antifibrotic effects of CO. To our knowledge, no such data are currently available concerning CO-associated effects in the exocrine pancreas.

We have reported recently that HO-1 inhibits the proliferation of PSCs, a cell type responsible for pancreatic fibrosis development (Schwer et al., 2008). In the present study, we investigated the effect of the HO-1 reaction product CO on PSC growth. Transition carbonyls acting as CO carriers could represent a way of supplying CO to tissues such as the pancreas in a more controllable fashion than achieved with CO gas, thereby reducing the risk of systemic toxicity (Motterlini et al., 2002; Sawle et al., 2006). We therefore used the carbon monoxide-releasing compound CORM-2 to assess the effects of CO on PSC proliferation.

Using this approach, we could demonstrate by BrdU assays and cell count analyses that CORM-2 inhibits PSC proliferation. This effect was dose-dependent and significantly detectable starting at 50 μM. In addition, an increase in caspase-3-like activity could not be observed, suggesting that apoptosis is not involved in the inhibitory effect of CORM-2 on PSC growth. It should be noted, however, that the concentrations of CORM-2 used in the present study are higher than those reported to exert antiproliferative effects in airway smooth muscle cells (Taille et al., 2005). We assume that this discrepancy might reflect differences in the experimental design, including starving conditions or seeding densities of the two different cell types.

In an effort to understand possible mechanisms responsible for the inhibition of PSC growth by CORM-2, we examined a possible effect on cell cycle progression. In mammalian

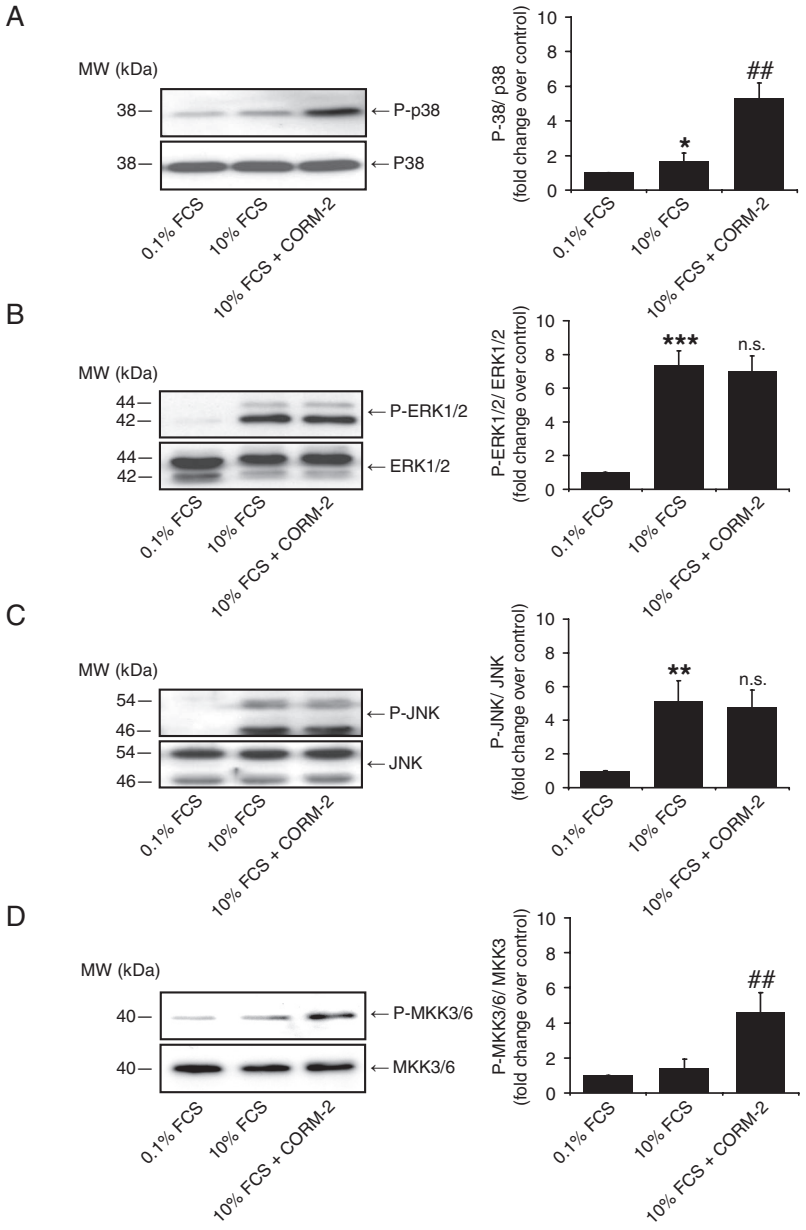

Fig. 5. Effect of CORM-2 on MAPK activation and MKK3/MKK6 phosphorylation in PSCs. Serum-starved PSCs were pretreated with CORM-2 (100 μM) for 30 min followed by a 4-h stimulation with 10% FCS. Phospho-p38 (P-p38; A), phospho-ERK1/2 (P-ERK1/2; B), phospho-JNK (P-JNK; C), and phospho-MKK3/MKK6 (P-MKK3/6; D) MAPK levels were determined by Western blot analysis using protein-specific antibodies. The membranes were stripped and reprobed with anti-total-p38, anti-total-ERK1/2, anti-total-JNK, and anti-total-MKK3/MKK6 antibodies. The histograms represent the ratios between phosphorylated MAPKs optical densities and that of their nonphosphorylated isoforms. Data are presented as mean ± S.E.M. (n = 3). *, $p < 0.05$ versus 0.1% FCS; **, $p < 0.01$ versus 0.1% FCS; ***, $p < 0.001$ versus 0.1% FCS; ##, $p < 0.01$ versus 10% FCS; n.s., not significant versus 10% FCS.

cells, proliferation is controlled primarily in the G_1 phase of the cell cycle through the action of two cyclin-dependent kinases (Cdks), Cdk4 and Cdk2 (Koff et al., 1992). These kinases are activated by association with cyclin E or cyclin D. The activation of Cdk2 also requires dephosphorylation of Thr14 and Thr15 by Cdc25A and an activating phosphorylation event at Thr160 by Cdk-activating kinase (Gu et al., 1992). Another mode of regulating Cdk2 kinase is through the association with $p21^{Waf1/Cip1}$. $p21^{Waf1/Cip1}$ acts by binding to the cyclin E-Cdk2 complex and inhibiting its kinase activity (Gu et al., 1993). It is interesting that Hitomi et al. (1998) have shown that $p21^{Waf1/Cip1}$ might function even

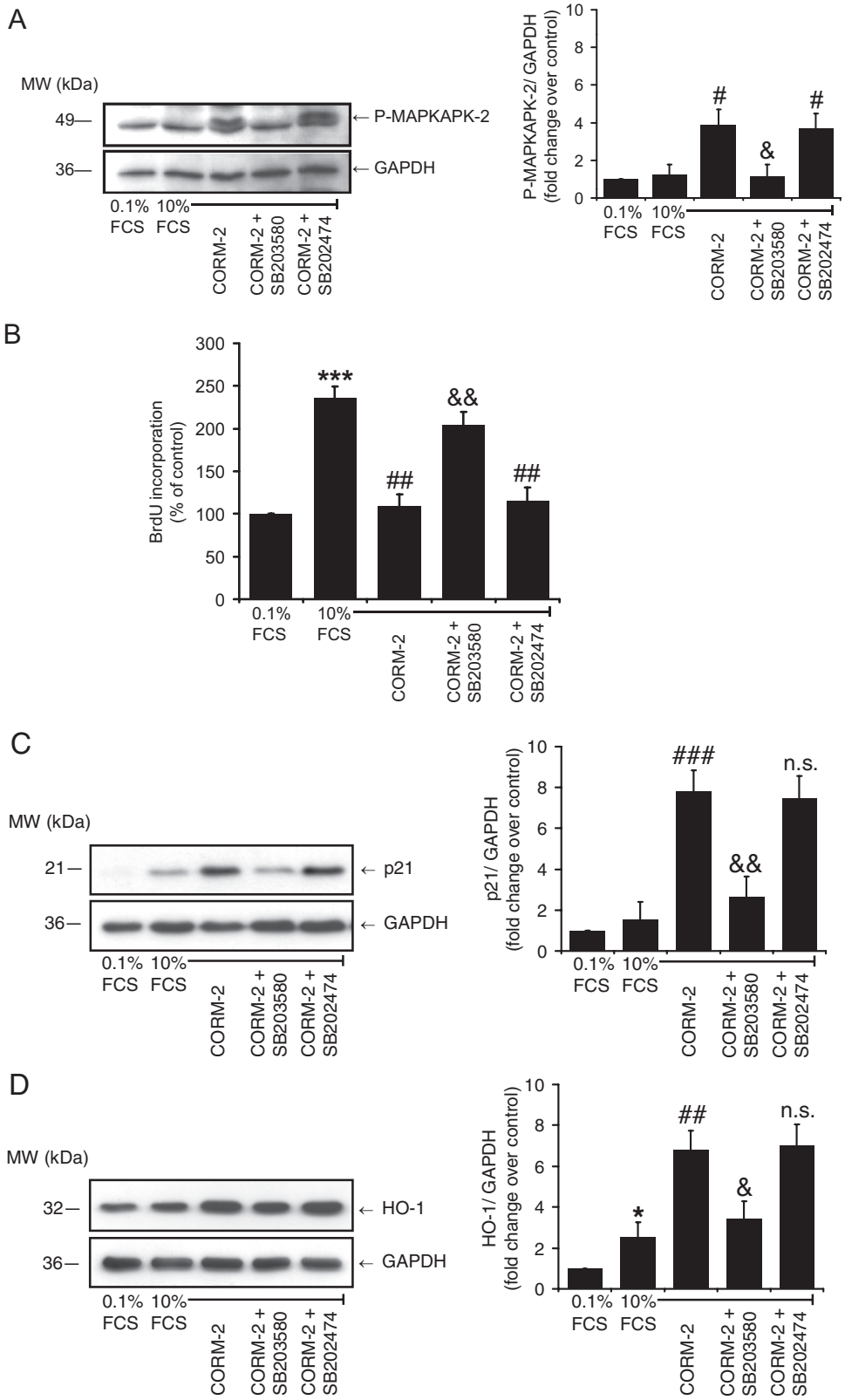

Fig. 6. Role of p38 MAPK inhibition on CORM-2 mediated effects on PSC proliferation, $p21^{Waf1/Cip1}$, and HO-1 expression. Serum-starved PSCs were pretreated with SB203580 (10 μM) or SB202474 (10 μM) for 30 min before exposure to CORM-2 (100 μM). A, representative Western blot analysis of phospho-MAPKAPK-2 (P-MAPKAPK-2) expression after an 8-h stimulation with 10% FCS. The graph shows the ratios between phospho-MAPKAPK-2 (P-MAPKAPK-2) optical densities and that of GAPDH (n = 3). B, cell proliferation as assessed by BrdU assays after a 24-h incubation with 10% FCS. The results are expressed as a percentage of the untreated control (n = 4). C, Western blot analysis of $p21^{Waf1/Cip1}$ and HO-1 protein expression (D). Whole-cell proteins were extracted from PSCs after a 24-h stimulation with 10% FCS. To demonstrate equal loading, the membranes were stripped and reprobed with an antibody against GAPDH. The histograms represent the ratios between $p21^{Waf1/Cip1}$ or HO-1 optical densities and that of GAPDH. The data are presented as mean ± S.E.M. (n = 3). *, $p < 0.05$ versus 0.1% FCS; ***, $p < 0.001$ versus 0.1% FCS; #, $p < 0.05$ versus 10% FCS; ##, $p < 0.01$ versus 10% FCS; ###, $p < 0.001$ versus 10% FCS; &, $p < 0.05$ versus 10% FCS + CORM-2; &&, $p < 0.01$ versus 10% FCS + CORM-2; n.s., not significant versus 10% FCS + CORM-2.

more efficiently by binding to the cyclin-Cdk complex before its activation in late G_1 and preventing the phosphorylation event that is essential to its kinase activity. The results from the current study indicate that CORM-2 arrests PSCs at the G_0/G_1 phase of the cell cycle and this effect depends on up-regulation of $p21^{Waf1/Cip1}$ protein. These findings are consistent with previous studies implying $p21^{Waf1/Cip1}$ as an important regulator of PSC growth arrest (Manapov et al., 2005). Because we observed that CORM-2 prevented serum-induced phosphorylation of retinoblastoma tumor suppressor protein (data not shown), a downstream target of Cdk2, the inhibitory effect of $p21^{Waf1/Cip1}$ on cell cycle progression could reflect a direct inhibition of cyclin E-Cdk2 kinase activity. However, it must be taken into consideration that the accessibility and cellular use of cyclin E-Cdk2 could also be targeted by CORM-2 treatment.

Many biological effects attributed to CO have been linked to its ability to modulate MAPK signaling pathways. MAPKs are activated by a wide range of extracellular stimuli and have been shown to play a pivotal role in the regulation of PSC growth (Jaster et al., 2002; Masamune et al., 2003). Our laboratory has reported previously that HO-1 inhibits PSC proliferation by repression of the ERK1/2 pathway (Schwer et al., 2008). Effects of CO gas or CORMs on MAPK activation are variable and depend on the cell type. In airway smooth muscle cells and T cells, CO exerts its antiproliferative effects via inhibition of ERK1/2 MAPK phosphorylation, whereas in vascular smooth muscle cells, CO acts through activation of the p38 MAPK pathway (Wegiel et al., 2008). In the present study, we found a marked activation of p38 MAPK after CORM-2 treatment, whereas an effect on ERK1/2 or JNK signaling could not be detected. The activation of p38 MAPK was associated with an activation of the upstream kinases MKK3/MKK6 and accompanied by the phosphorylation of a p38 target, MAPKAPK-2. SB203580, a pharmacological inhibitor of p38 MAPK, but not SB202474, an inactive analog, blocked the inhibitory effect of CORM-2

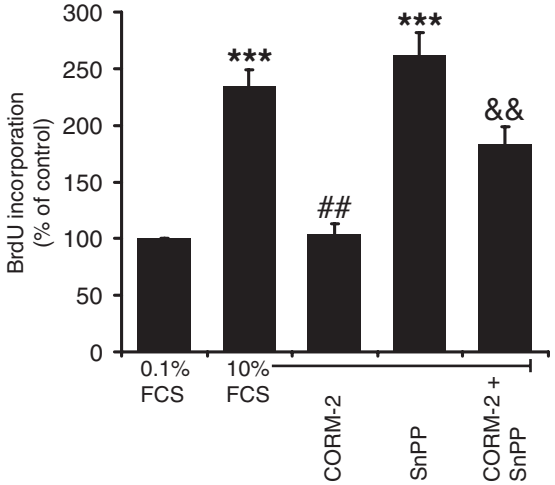

Fig. 7. Effect of CORM-2 and/or SnPP on FCS-induced PSC proliferation. Serum-starved PSCs were preincubated with SnPP (20 μM) for 45 min before exposure to CORM-2 (100 μM). Cells were stimulated to proliferate with 10% FCS, and DNA synthesis was estimated by measuring the incorporation of BrdU into cellular DNA. Results are expressed as a percentage of control values observed in PSCs not incubated with 10% FCS. Data are presented as mean ± S.E.M. for $n = 3$ separate cell preparations. ***, $p < 0.001$ versus 0.1% FCS; ##, $p < 0.01$ versus 10% FCS; &&, $p < 0.01$ versus 10% FCS + CORM-2.

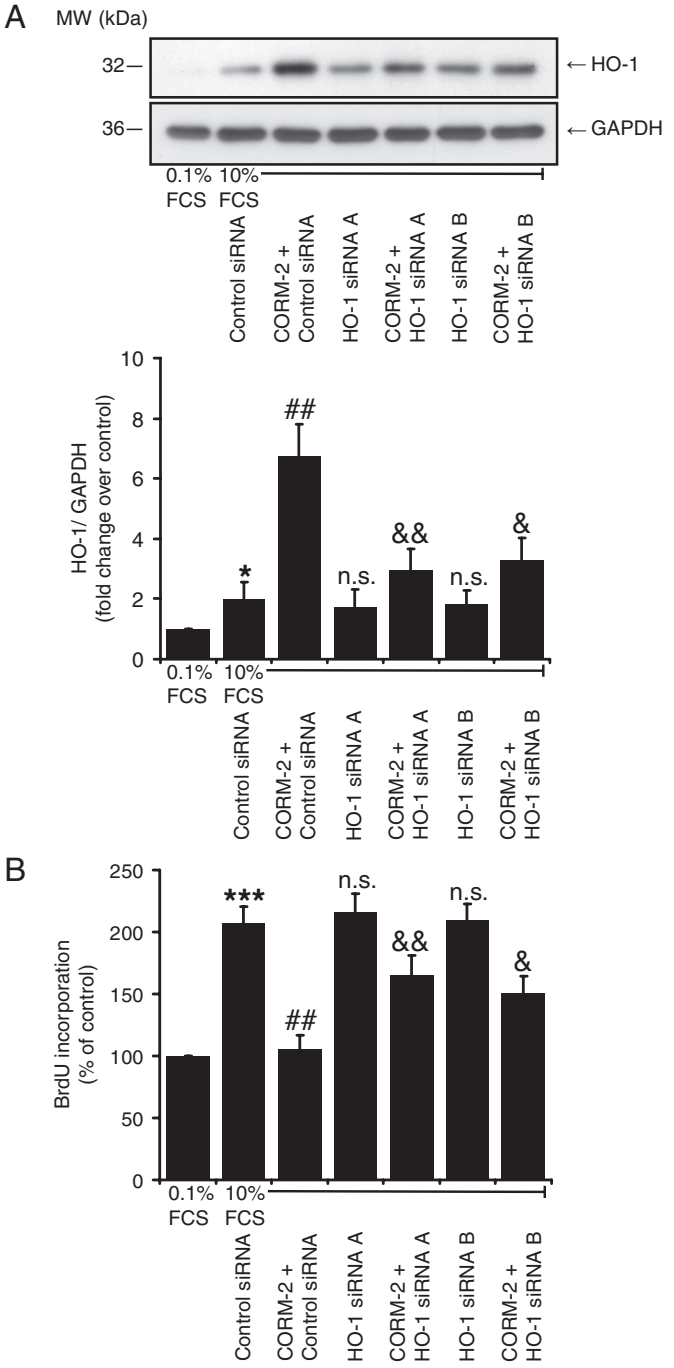

Fig. 8. Role of HO-1 in the inhibitory effect of CORM-2 on PSC proliferation. PSCs were transfected with siRNAs directed against hmox1-mRNA (HO-1 siRNA A, HO-1 siRNA B) or nonsilencing siRNA not targeting any gene product (Control siRNA). After transfection, cells were incubated in serum-free medium for 24 h. CORM-2 (100 μM) was added to the cells for 30 min followed by a 24-h stimulation with 10% FCS. A, HO-1 protein expression was assessed by Western blot analysis. The membrane was stripped and reprobed with an anti-GAPDH protein-specific antibody. The histogram represents the ratios between HO-1 optical densities and that of GAPDH. B, DNA synthesis was estimated by measuring the incorporation of BrdU into cellular DNA. Data are presented as mean ± S.E.M. ($n = 3$). *, $p < 0.05$ versus 0.1% FCS; ***, $p < 0.001$ versus 0.1% FCS; ##, $p < 0.01$ versus 10% FCS + control siRNA; &, $p < 0.05$ versus 10% FCS + CORM-2 + control siRNA; &&, $p < 0.01$ versus 10% FCS + CORM-2 + control siRNA; n.s., not significant versus 10% FCS + control siRNA.

on PSC proliferation. These findings suggest that specific activation of the p38 MAPK cascade contributes to the CORM-2-induced cell growth arrest. In vascular smooth muscle cells, p38 MAPK signaling is involved in $p21^{Waf1/Cip1}$ induction (Lee et al., 2006). Because up-regulation of $p21^{Waf1/Cip1}$ was observed as the result of CORM-2 treatment, this prompted us to examine the role of p38 MAPK in the regulation of $p21^{Waf1/Cip1}$ expression in PSCs. SB203580 prevented CORM-2 induced $p21^{Waf1/Cip1}$ expression, indicating that CORM-2 up-regulates $p21^{Waf1/Cip1}$ via activation of p38 MAPK.

The critical role of p38 MAPK signaling in the regulation of HO-1 is well documented (Alam et al., 2000; Kietzmann et al., 2003). De Backer et al. (2009) have shown that CORMs could exert their effects via p38-dependent HO-1 induction in a mouse model of postoperative ileus. However, inconsistent data exist about the ability of CORM-2 to up-regulate HO-1 (Sawle et al., 2005; Taille et al., 2005; Sun et al., 2008). In this respect, it was important to investigate whether CORM-2 induces HO-1 in PSCs. In the present study, we could show a significant increase in HO-1 protein after CORM-2 treatment. These findings suggest that CORM-2-liberated CO might be responsible for HO-1 induction. Pharmacological p38 MAPK inhibition attenuated CORM-2-induced HO-1 up-regulation, indicating that HO-1 acts downstream of p38 in our experimental setting. To investigate whether HO-1 induction contributes to the protective effects of CORM-2, experiments with the phar-

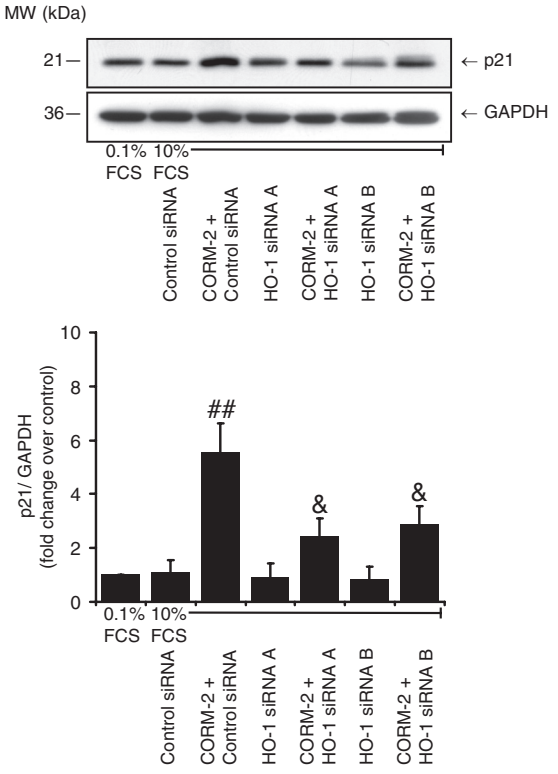

Fig. 9. Role of HO-1 on CORM-2-induced $p21^{Waf1/Cip1}$ expression. PSCs were transfected with siRNAs directed against hmox1-mRNA (HO-1 siRNA A, HO-1 siRNA B) or nonsilencing siRNA (Control siRNA) before the addition of CORM-2 (100 μM) for 24 h. The level of $p21^{Waf1/Cip1}$ protein was examined by Western blot analysis. The graph represents the optical density ratios of $p21^{Waf1/Cip1}$ and GAPDH. Data are presented as mean ± S.E.M. ($n = 3$). ##, $p < 0.01$ versus 10% FCS + control siRNA; &, $p < 0.05$ versus 10% FCS + CORM-2 + control siRNA.

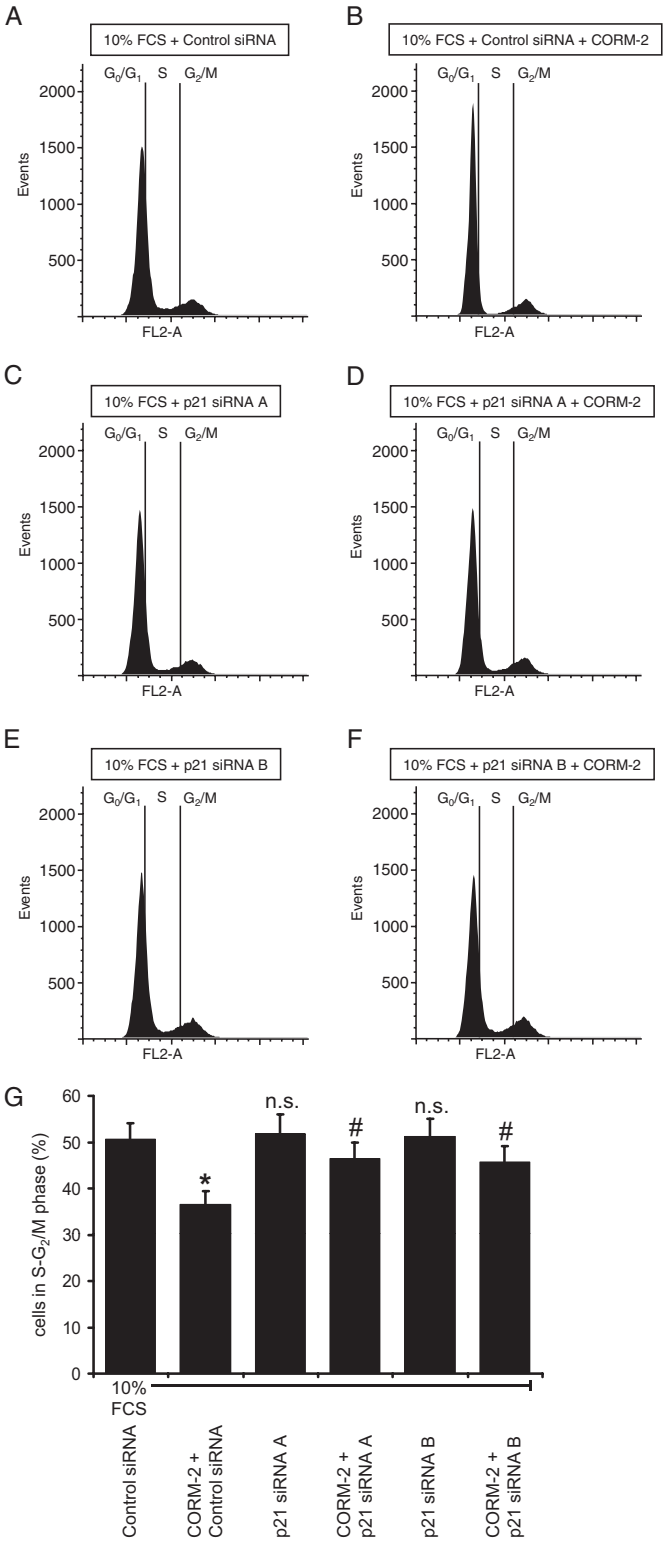

Fig. 10. Role of $p21^{Waf1/Cip1}$ in CORM-2-induced G_0/G_1 cell cycle arrest. A–F, PSCs were transfected with siRNAs directed against $p21^{Waf1/Cip1}$-mRNA (p21 siRNA A, p21 siRNA B) or nonsilencing siRNA (Control siRNA) followed by a 24-h starvation from serum. Cells were stimulated with 10% FCS in the presence or absence of CORM-2 (100 μM) and cell cycle distribution at 24 h was analyzed by flow cytometry. G, quantitative analysis of cells at S–G_2/M phase. The percentage of cells in S–G_2/M phase was obtained by flow cytometry. Data are presented as mean ± S.E.M. for $n = 3$ separate cell preparations. *, $p < 0.05$ versus 10% FCS + control siRNA; n.s., not significant versus 10% FCS + control siRNA; #, $p < 0.05$ versus 10% FCS + CORM-2 + control siRNA.

macological HO inhibitor SnPP and HO-1 siRNA were performed. In the presence of SnPP or by transfecting PSCs with HO-1 siRNA, CORM-2 failed to suppress PSC proliferation and up-regulate $p21^{Cip1/WAF1}$ protein. These data provide evidence for a causal involvement of HO-1 in the observed CORM-2-mediated inhibitory effects on PSC growth. It is noteworthy that HO-1 overexpression has been shown to up-regulate $p21^{Waf1/Cip1}$ in kidney proximal tubule cells (Inguaggiato et al., 2001). This observation would be in line with the data obtained in PSCs presented in this study.

On the basis of our current findings, we conclude that CO released by CORM-2 exerts potent antiproliferative effects in PSCs. Furthermore, CORM-2 inhibits PSC growth by a mechanism involving activation of p38 MAPK signaling and induction of HO-1 protein expression. The inhibitory effect of CORM-2 on PSC proliferation seems to be linked to its ability to up-regulate $p21^{Waf1/Cip1}$. These findings suggest a beneficial role of CORMs in the treatment of pancreatic fibrosis.

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