The International Journal of Biochemistry & Cell Biology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Carbon monoxide releasing molecule-2 CORM-2 represses global protein synthesis by inhibition of eukaryotic elongation factor eEF2

Q1 Christian Ingo Schwer[a,*], Patrick Stoll[a], Sabine Rospert[b], Edith Fitzke[b], Nils Schallner[a], Hartmut Bürkle[a], Rene Schmidt[a], Matjaz Humar[a]

[a] Department of Anesthesiology and Critical Care Medicine, University Medical Center Freiburg, Hugstetterstrasse 55, D-79106 Freiburg, Germany
[b] Institute of Biochemistry and Molecular Biology and Centre for Biological Signalling Studies (BIOSS), University Freiburg, Stefan-Meier-Straße 17, D-79104 Freiburg, Germany

ARTICLE INFO

Article history:
Received 15 February 2012
Received in revised form 16 August 2012
Accepted 25 September 2012
Available online xxx

Keywords:
Carbon monoxide
Pancreatic stellate cells
Pancreatic fibrosis
Protein synthesis
Eukaryotic elongation factor 2

ABSTRACT

Carbon monoxide (CO) is an endogenous gaseous transmitter that exerts antiproliferative effects in many cell types, but effects of CO on the translational machinery are not described. We examined the effects of the carbon monoxide releasing molecule-2 (CORM-2) on critical steps in translational signaling and global protein synthesis in pancreatic stellate cells (PSCs), the most prominent collagen-producing cells in the pancreas, whose activation is associated with pancreatic fibrosis. PSCs were isolated from rat pancreatic tissue and incubated with CORM-2. CORM-2 prevented the decrease in the phosphorylation of eukaryotic elongation factor 2 (eEF2) caused by serum. By contrast, the activation dependent phosphorylation of initiation factor 4E-binding protein 1 (4E-BP1) was inhibited by CORM-2 treatment. The phosphorylation of eukaryotic initiation factor 2α (eIF2α) and eukaryotic initiation factor 4E (eIF4E) were not affected by CORM-2 treatment. In consequence, CORM-2 mediated eEF2 phosphorylation and inactivation of 4E-BP1 suppressed global protein synthesis. These observations were associated with inhibition of phosphatidylinositol 3-kinase-Akt-mammalian target of rapamycin (PI3K-Akt-mTOR) signaling and increased intracellular calcium and cAMP levels. The CORM-2 mediated inhibition of protein synthesis resulted in downregulation of cyclin D1 and cyclin E expression, a subsequent decline in the phosphorylation of the retinoblastoma tumor suppressor protein (Rb) and cell growth arrest at the G_0/G_1 phase checkpoint of the cell cycle. Our results suggest the therapeutic application of CO releasing molecules such as CORM-2 for the treatment of fibrosis, inflammation, cancer, or other pathologic states associated with excessive protein synthesis or hyperproliferation. However, prolonged exogenous application of CO might also have negative effects on cellular protein homeostasis.

1. Introduction

Carbon monoxide (CO) is a freely diffusible gas that acts as a physiological mediator of many biological and cellular processes, including angiogenesis and hormone secretion (Lundquist et al., 2003; Otterbein et al., 2003; Ryter et al., 2006). Endogenous CO is released during the catabolism of heme by heme oxygenase (HO) enzymes (Tenhunen et al., 1968). In the past decade, the therapeutic potential of CO has been increasingly recognized. CO exerts potent beneficial effects in animal models of inflammatory or oxidative tissue injury, organ transplantation and fibrosis (Motterlini and Otterbein, 2010). 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 bactericidal (Desmard et al., 2009), antiinflammatory (Bani-Hani et al., 2006; Sawle et al., 2005), antiapoptotic (Li et al., 2006; Schallner et al., 2011), and antiproliferative effects (Schwer et al., 2010; Taille et al., 2005) in many cell types.

Pancreatic fibrosis is a common histopathological feature in chronic pancreatitis and pancreatic cancer. It is now generally accepted that pancreatic stellate cells (PSCs) play a crucial role in the development of fibrosis (Apte et al., 1998; Bachem et al., 1998; Omary et al., 2007). In response to profibrogenic stimuli, PSCs undergo transdifferentiation from quiescent phenotypes into highly proliferative myofibroblast-like cells, which synthesize and secrete increased amounts of the extracellular matrix proteins that comprise fibrous tissue (Apte et al., 2004; Apte and Wilson, 2004). Therefore, compounds that inhibit PSC proliferation or suppress protein synthesis in activated PSCs may have the potential to become a new approach for the treatment of pancreatic fibrosis (Bulow et al., 2007; Omary et al., 2007).

The acute phase of mRNA translation in mammalian cells in response to mitogens is regulated through changes in the

* Corresponding author. Tel.: +49 761 270 23060; fax: +49 761 270 23960.
E-mail address: christian.schwer@uniklinik-freiburg.de (C.I. Schwer).

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http://dx.doi.org/10.1016/j.biocel.2012.09.020

phosphorylation states of eukaryotic initiation factors (eIFs) and eukaryotic elongation factors (eEFs) (Diggle et al., 1998; Kimball, 1999; Proud et al., 2001). eEF2 is a monomeric 100 kDa protein that mediates the translocation step in peptide-chain elongation by promoting transfer of peptidyl-tRNA from the A site to the P site on the ribosome (Proud, 1994, 2006; Proud and Denton, 1997; Ryazanov et al., 1988). Phosphorylation of eEF2 at threonine 56 (Thr56) by eEF2 kinase interferes with its ability to bind the ribosome, and thus inhibits peptide-chain elongation (Browne and Proud, 2002, 2004). eEF2 kinase activity is dependent on calcium (Ca^{2+}) ions, calmodulin, and has been reported to be regulated by cyclic adenosine monophosphate (cAMP) (Diggle et al., 1998; Hovland et al., 1999), mitogen-activated protein kinase (MAPK) (Wang et al., 2001) and mammalian target of rapamycin (mTOR) (Browne and Proud, 2004; Proud, 2004) signaling pathways.

The effect of CORMs or CO on components of the translational machinery or global protein synthesis has not been elucidated yet. In this study we characterized the effect of CORM-2 on components of the translational machinery, we explored whether CORM-2 blocks global protein synthesis in PSCs, and revealed regulatory mechanisms and consequences.

2. Materials and methods

2.1. Isolation and culture of PSCs

Male Wistar rats (Charles River, Sulzfeld, Germany), weighing between 250 and 300 g, were used for all experiments. The experimental protocol was approved by the Local Animal Care and Use Committee, and all animals were housed in accordance with the guidelines from the American Association of Laboratory Animal Care.

Primary rat PSCs were isolated according to the procedure described by Shinji et al. (2002). Briefly, the dissected pancreas was minced with scissors and digested with 0.03% collagenase P (Roche Diagnostics, Mannheim, Germany) in HBSS (Hanks' buffered salt solution) (Invitrogen, Karlsruhe, Germany). The resultant suspension of cells was centrifuged in a 13.2% iohexol (Nycodenz) (Nycomed, Oslo, Norway) gradient for 20 min at 1400 × *g*. 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 cultured in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS), 4 mM glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml) (all from Invitrogen). Cell purity was determined by vitamin A autofluorescence and was always higher than 90%. After reaching confluency, cells were harvested and seeded in a density of 1×10^5 cells/ml.

2.2. Treatment of cells

Experiments were performed using PSCs in passages 2–4. PSCs were incubated in serum-free medium for 24 h before addition of CORM-2 (100 μM; Sigma, Deisenhofen, Germany), ruthenium(III) chloride ($RuCl_3$; 100 μM; Sigma), the mTOR inhibitor rapamycin (100 nM; Sigma), the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (10 μM; Calbiochem, Bad Soden, Germany), or the mitogen/extracellular signal-regulated kinase (MEK) inhibitor PD98059 (20 μM; Calbiochem). All reagents were dissolved in dimethyl sulfoxide (DMSO). Control cells were treated with similar amounts of the solvent.

2.3. Western blot analysis

Total cell lysates were prepared in 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 by the BCA protein assay kit (Thermo Fisher Scientific; Rockford, IL, USA) and equal amounts of proteins per sample were loaded on SDS-polyacrylamide gels, separated by electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk in Tween-20/phosphate buffered saline and incubated with antibodies raised against phospho-eEF2 (Thr56), total-eEF2, phospho-eIF2α (Ser51), total-eIF2α, phospho-eIF4E (Ser209), total-eIF4E, phospho-4E-BP1 (Ser65), total-4E-BP1, phospho-Akt (Ser473), total-Akt, phospho-p70 S6K (Thr389), total-p70 S6K, phospho-S6 Ribosomal Protein (Ser235/236), total-S6 Ribosomal Protein, phospho-ERK1/2 (Thr202/Tyr204), total-ERK1/2, phospho-p90 RSK (Thr359/Ser363), total-p90 RSK, phospho-AMPKα (Thr172), total-AMPKα, phospho-Rb (Ser608, Ser795, and Ser807/811) and total-Rb (all Cell Signaling Technology, Danvers, MA, USA) or cyclin D1 and cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Specific protein bands were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgGs (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and the Enhanced Chemiluminescence Kit (GE Healthcare).

2.4. Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was measured in cells using the Fura 2-AM assay (Calbiochem). Briefly, cultured cells were starved from serum overnight and incubated with 1 μM Fura2-AM for 30 min at 37 °C followed by three washes with calcium-free HBSS to remove any extracellular dye. The fluorescence value was measured at 510 nm and an excitation wavelength of 340 nm by a TECAN infinite M200 spectrofluorophotometer (Tecan, Crailsheim, Germany). To detect the effect of CORM-2 on resting $[Ca^{2+}]_i$, serum-starved PSCs were treated with vehicle (0.1% DMSO) or 100 μM CORM-2 for the indicated time periods before Fura 2-AM loading.

2.5. Determination of intracellular cAMP concentration

The effect of CORM-2 on intracellular cAMP concentration was determined with the Cyclic AMP XP® Assay Kit (Cell Signaling). PSCs were plated in 96-well plates (1×10^4 cells/well) and incubated overnight in IMDM containing 10% FCS. Cells were rinsed with 200 μl warm PBS before test compounds in serum-free medium were added. Cell lysis and calculation of absolute amounts of cAMP was performed according to the manufacturer's instructions.

2.6. Metabolic labeling

PSCs were seeded at a density of 2×10^5 cells/ml in 24-well tissue culture plates in 10% FCS/DMEM and grown to 80% confluence. Then cells were serum-starved for 24 h and treated with 100 μM CORM-2 or 2.5 μg/ml cycloheximide (CHX) for 8 h. Subsequently, cell culture supernatants were replaced by 500 μl/well of serum-free DMEM deficient in cold methionine (MP Biomedicals, Solon, OH, USA) for 1 h. Cultures were then pulse-labeled for 1 h with 50 μCi of [^{35}S]-methionine (PerkinElmer, Rodgau, Germany), washed with ice-cold PBS and lysed by addition of 200 μl 1× SDS sample buffer [250 mM Tris (pH 6.8), 10% SDS, 500 mM dithiothreitol, 50% glycerol, and 0.5% bromphenol blue]. Proteins were denatured by boiling for 5 min, and [S^{35}]-methionine incorporation into proteins was analyzed by 10% SDS-PAGE and X-ray film autoradiography. To demonstrate equal loading, gels were stained for 2 h in 0.06% Coomassie G250 and 10% acetic acid after fixing the separated proteins in 25% isopropanol and 10% acetic acid for 60 min. Subsequently, gels were destained for in 10% acetic acid for

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6 h and dried on a 3 MM Whatman paper for 2 h at 80 °C before exposure to X-ray films (GE Healthcare).

2.7. Ribosome profiles

Ribosome profiles were performed as described previously (Jaiswal et al., 2011). Briefly, quiescent PSCs were pretreated with 100 μM CORM-2 for 30 min followed by a 8-h stimulation with 10% FCS. 20 min before trypsinization, 100 μg/ml CHX was added. Cells were collected and resuspended in 2× the volume of the cell pellet in lysis buffer containing 50 mM HEPES-KOH, pH 7.4, 1 mM Mg acetate, 1 mM PMSF, 1× PIC. Mechanical cell disruption was performed by vortexing cells in the presence of 1× the volume of the cell pellet of glas beads six times for 20 s at 4 °C, followed by a 40 s interval on ice. The supernatant was collected after a clarifying spin at 2500 × *g* for 10 min. Supernatant corresponding to 10 units of optical density at 260 nm (OD_{260}) were loaded on a linear 15–55% sucrose gradient. After centrifugation at 200,000 × *g* for 2.5 h at 4 °C, gradients were fractionated from top to bottom with a density gradient fractionator monitoring A_{254} (Teledyne ISCO, Lincoln, NE, USA).

2.8. Detection of cyclin D1 and cyclin E

PSCs were seeded at a density of 2×10^5 cells/ml in 24-well tissue culture wells and were starved from serum for 24 h. Subsequently, cells were incubated in 500 μl/well of serum-free DMEM deficient in cold methionine for 1 h prior to the addition of 100 μM CORM-2 or vehicle (0.1% DMSO). After 30 min, cells were stimulated with 10% FCS for 30 min before they were pulse-labeled with 50 μCi/well of [^{35}S]-methionine (PerkinElmer) for 6 h. Then, PSCs were washed with ice-cold PBS and lysed in 200 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, 2 mM AEBSF, 1.6 μM Aprotinin, 80 μM Bestatin, 28 μM E-64, 40 μM Leupeptin, 30 μM Pepstatin, 4 mM PMSF, 2 mM sodium orthovanadate). After centrifugation at 14,000 × *g*, supernatants were incubated with 1 μg of anti-cyclin D1 or anti-cyclin E antibodies and 40 μl Protein A/G-plus agarose slurry (Santa Cruz) at 4 °C on a wheel over night. Protein beads were washed three times with 500 μl radioimmunoprecipitation assay buffer, boiled in 40 μl of 2× SDS sample buffer, and resolved on 13% SDS-PAGE gels. Separated proteins were fixed in 25% isopropanol, 10% acetic acid for 60 min and dried on a 3MM Whatman paper for 2 h at 80 °C before exposure to X-ray films.

2.9. Real-time polymerase chain reaction

RNA was isolated using the RNeasy Mini Kit (Qiagen, Erkrath, Germany). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using cDNA reverse transcription kit (Applied Biosystems, Inc, Foster City, Calif) according to the manufacturer's protocol. The resulting cDNA was used in semiquantitative real-time polymerase chain reaction analysis. Reactions were performed in duplicate for each sample on an ABI Prism 7000 (Applied Biosystems). All primer/probes and mixtures were purchased from Applied Biosystems; Taq Man probe rat cyclin D1 (*Ccnd1*; assay ID: Rn00432360_m1), and Taq Man probe rat cyclin E (*Ccne1*; assay ID: Rn01457762_m1).

Parameters for quantitative PCR were as follows: 10 min at 95 °C, followed by 40 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C. As endogenous control, *GAPDH* gene expression was measured for each probe using a VIC/MGBYlabeled probe (rat: 4352338E-0703008). The obtained data from GAPDH were used to standardize the sample variation in the amount of input cDNA by the ΔΔCT method.

2.10. Design of small interfering RNA and transfection of PSCs

PSCs (1×10^5/ml) were transfected with 100 nM *hmox1_4* small interfering RNA (siRNA) directed against hmox1-mRNA: sense (5′ > 3′) r(AAA UGG CAU UAU CUA AUA A)dTdT, antisense r(UUA UUA GAU AAU GCC AUU U)dAdT, or AllStars nonsilencing siRNA (both obtained from 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.

2.11. Cell cycle analysis by flow cytometry

Serum-starved PSCs (80% density) were left untreated or were stimulated with 10% FCS for 12 h in the presence of vehicle (0.1% DMSO) or 100 μM of CORM-2. Cells were washed twice in PBS and harvested by trypsin digestion (0.5% trypsin–0.2% EDTA). Preparation of nuclei and propidium iodide staining was performed using the CycleTEST™ PLUS DNA Reagent Kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's recommendations and samples were analyzed on a FACS Calibur (BD Biosciences) using both CELLQuest™ (BD Biosciences) and FlowJo™ softwares (FlowJo, Ashland, OR, USA).

2.12. Statistical analysis

Results are expressed as means ± SEM for the indicated number of separate cell preparations per experimental protocol. Unless indicated otherwise, data were analyzed using the one-way ANOVA followed by the Student–Newman–Keuls post hoc test. Data from $[Ca^{2+}]_i$ or cAMP assays were analyzed by two-way ANOVA, followed by the Bonferroni's post hoc test. Differences between groups were considered to be significant at $p < 0.05$. Statistical analyzes were carried out using the Prism software package (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. CORM-2 induces posttranslational modification of regulators of the translational machinery in PSCs

Translation in mammalian cells is regulated by phosphorylation of various translation initiation and elongation factors (Proud and Denton, 1997; Rhoads et al., 1999; Wang et al., 2001). To evaluate whether CO affects components of the translational machinery in PSCs, cells were stimulated with 10% FCS in presence of the CO-releasing molecule CORM-2. Serum-starved PSCs displayed strong phosphorylation of eukaryotic translation elongation factor eEF2 on Thr56 and eukaryotic translation initiation factor eIF2α on Ser51, whereas only low amounts of phosphorylated eukaryotic initiation factor eIF4E (Ser209) or initiation factor 4E binding protein 1 (Ser65) could be observed (Fig. 1). Treatment of PSCs with 10% FCS resulted in a decrease in the level of both phosphorylated eEF2 and eIF2α and an increase in the level of phosphorylated eIF4E and 4E-BP1. Compared to cells incubated with 10% FCS alone, the addition of CORM-2 had no effect on phosphorylation of eIF2α or eIF4E. However, CORM-2 prevented the decrease of phosphorylated eEF2 caused by serum and markedly reduced the level of phosphorylated 4E-BP1. These effects occur at the posttranslational level, since total amounts of eEF2 or 4E-BP1 protein were not affected by CORM-2 treatment.

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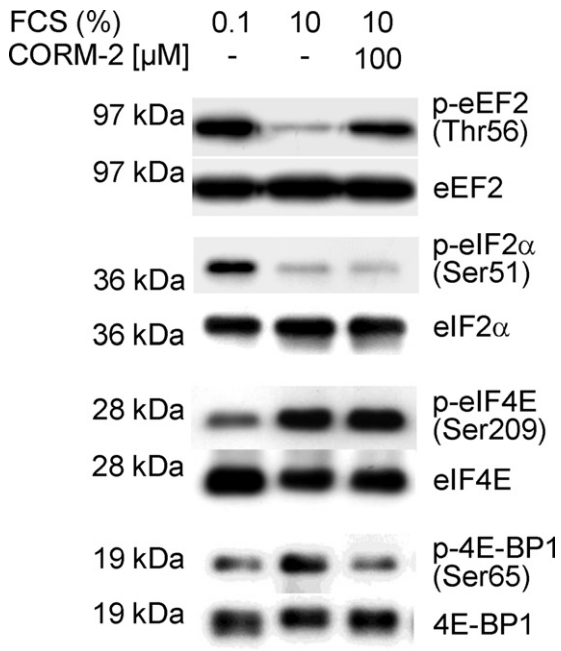

Fig. 1. CORM-2 modulates phosphorylation of translational regulators in PSCs. Serum-starved PSCs were incubated with CORM-2 (100 μM) or vehicle (DMSO) for 30 min followed by a 8-h stimulation with 10% FCS. Phosphorylation of translational regulators eEF2, eIF2α, eIF4E and 4E-BP1 was analyzed by immunoblotting. To demonstrate equal loading, membranes were stripped and reprobed with anti-total eEF2, anti-total eIF2α, anti-total eIF4E, and anti-total 4E-BP1 antibodies. All blots shown are representative for the results of experiments with $n = 3$ separate cell preparations.

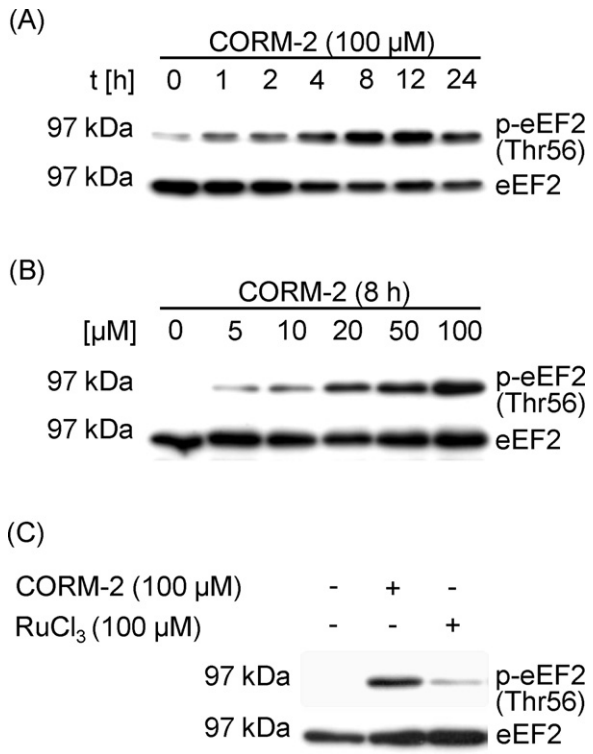

Fig. 2. CORM-2 induces phosphorylation of p-eEF2 in a time- and dose-dependent manner. PSCs were exposed to vehicle (DMSO) or the indicated concentration of CORM-2 for various time periods (A) and 8 h (B). (C) Serum-starved PSCs were incubated with CORM-2 (100 μM) or the negative control $RuCl_3$ (100 μM) for 30 min followed by a 8-h stimulation with 10% FCS. p-eEF2 and eEF2 in cell lysates were analyzed by immunoblotting. The data shown are representative for the results of experiments with $n = 3$ separate cell preparations.

3.2. Time- and dose-dependent effect of CORM-2 on eEF2 phosphorylation

The influence of CORM-2 on the posttranslational modification of eEF2 was analyzed by a time and dose kinetic. PSCs were incubated with CORM-2 (100 μM) for increasing time periods (Fig. 2A). Consistent with the data shown in Fig. 1, CORM-2 prevented FCS-induced eEF2 dephosphorylation but did not affect total eEF2 levels. This effect was time-dependent and already apparent after 1 h of treatment. It peaked at 8 h and remained elevated for at least 24 h. Exposure of PSCs for 8 h to various concentrations of CORM-2 (0–100 μM) resulted in a concentration-dependent increase in phospho-eEF2 (Fig. 2B). The negative control $RuCl_3$, a CORM-2 analog unable to release CO, at 100 μM did not affect eEF2 phosphorylation (Fig. 2C). The concentration of CORM-2 that caused the strongest increase in eEF2-phosphorylation (100 μM) was chosen for further experiments. Toxicity studies revealed that CORM-2 at 100 μM did not cause any cellular damage whereas concentrations greater than 200 μM led to significant cell injury as measured by an LDH-release cytotoxicity assay (Schwer et al., 2010).

3.3. CORM-2 inhibits PI3K-Akt-mTOR but not ERK1/2 signaling in PSCs

eEF2 kinase is the only known kinase to phosphorylate eEF2 at Thr56 (Browne and Proud, 2002). eEF2 kinase itself is regulated by numerous mechanisms in the eukaryotic cell (Kaul et al., 2011). The phosphorylation of eEF2 kinase by p70 S6K and p90 RSK results in the inactivation of eEF2 kinase, which facilitates the dephosphorylation of eEF2, and thus promotes translation (Frodin and Gammeltoft, 1999; Hovland et al., 1999; Wang et al., 2001). While p70 S6K is under the control of mTOR, p90 RSK is activated via the ERK1/2 signaling pathway in response to many growth factors, hormones and neurotransmitters (Frodin and Gammeltoft, 1999).

To assess the role of the PI3K-Akt-mTOR and ERK1/2 signaling pathways in CORM-2 mediated effects, the PI3K inhibitor LY294002, the mTOR inhibitor rapamycin, and the ERK1/2 inhibitor PD98059 were added to PSCs before they were incubated with CORM-2 and 10% FCS. As shown in Fig. 3, CORM-2 (A, B and C, column 3), LY294002 (A, column 4) and rapamycin (B, column 4) prevented the decrease of phosphorylated eEF2 caused by serum. By contrast, the levels of phosphorylated Akt, p70 S6K and 4E-BP1 were reduced in both CORM-2 and LY294002 treated cells (Fig. 3A). Rapamycin only slightly decreased the phosphorylation of Akt, but did cause the complete dephosphorylation of 4E-BP1, p70 S6k and S6, another target of mTORC1 (Fig. 3B, column 4). Finally, CORM-2 attenuated FCS-induced phosphorylation of S6 (Fig. 3B, column 3). These data suggest that CORM-2 acts as an inhibitor of the PI3K-Akt-mTOR signaling pathway in PSCs. As expected, the FCS-induced phosphorylation of ERK1/2 was prevented in presence of PD98059 (Fig. 3C, column 4). The level of phosphorylated eEF2 was increased in CORM-2 but not PD98059 treated cells. By contrast, CORM-2 treatment had no significant effect on the phosphorylation of ERK1/2 or p90 RSK (Fig. 3C, column 3), demonstrating that modification of eEF2 by CORM-2 is independent of the ERK1/2 signaling pathway.

3.4. Increased phosphorylation of eEF2 correlates with profound inhibition of protein synthesis in PSCs

Posttranslational phosphorylation of eEF2 and 4E-BP1 has been associated with the repression of global protein synthesis (Browne and Proud, 2002). To investigate whether CORM-2 affects translation in PSCs, we measured the incorporation of [S^{35}]-methionine into newly synthesized proteins (Fig. 4). Preliminary experiments

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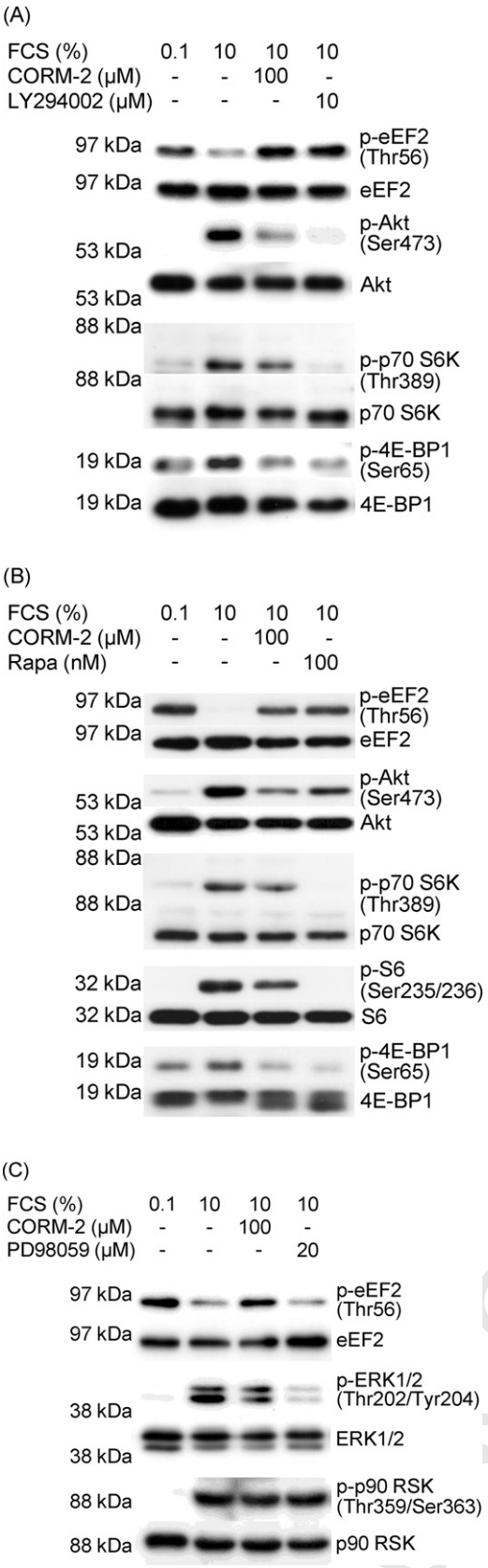

Fig. 3. CORM-2 inhibits PI3K-Akt-mTOR, but not ERK1/2-dependent pathways in PSCs. Serum-starved PSCs were pretreated with CORM-2 (100 μM), LY294002 (10 μM; A), rapamycin (100 nM; B) or PD98059 (10 μM; C) for 30 min. (A) Representative Western blot analyses of phospho-eEF2 (p-eEF2), phospho-Akt (p-Akt), phospho-p70 S6K (p-p70 S6K), and phospho-4E-BP1 (p-4E-BP1) expression after an 8-h stimulation with 10% FCS. (B) Representative Western blot analyses of phospho-eEF2 (p-eEF2), phospho-Akt (p-Akt), phospho-p70 S6K (p-p70 S6K), phospho-S6 Ribosomal Protein (p-S6), and phospho-4E-BP1 (p-4E-BP1) expression after an 8-h stimulation with 10% FCS. (C) Representative Western blot analyses of phospho-eEF2 (p-eEF2), phospho-ERK1/2 (p-ERK1/2), and phospho-p90 RSK (p-p90 RSK) expression after an 8-h stimulation with 10% FCS. To demonstrate equal loading, blots were reprobed with antibodes to detect total amounts of eEF2, Akt, p70 S6K, S6 Ribosomal Protein, 4E-BP1, ERK1/2 and p90 RSK.

demonstrated a linear increase in [S^{35}]-methionine incorporation into proteins in a time-dependent manner, when PSCs were metabolically labeled for 10 min to 6 h (data not shown). Administration of CORM-2 resulted in a significant reduction in protein synthesis by 51% (48.9 ± 8.8%), after labeling PSCs for 1 h compared to vehicle treated controls (Fig. 4A). Translational inhibition by CORM-2 was more pronounced than using the translational repressor cycloheximide that caused a 36% (64.1 ± 11.9%) decrease in global translational activity (Fig. 4B). Loading of equal amounts of total protein in each lane was verified by Coomassie Blue G-250 staining of gels before autoradiography (data not shown). To determine the rate limiting step in the inhibition of global protein synthesis by CORM-2, the association of ribosomes to mRNA was assessed by polysome fractionation. The absence of polysomes would argue for inhibition of translational initiation by CORM-2. However, we observed that pretreatment of PSCs with CORM-2 did not prevent the subsequent formation of polysomes after adding 10% FCS (Fig. 4C, fractions 8–20), although peaks in fractions 8–14 seem to be less distinguished in CORM-2 treated cells.

3.5. CORM-2 increases intracellular calcium [Ca^{2+}]$_i$ and cAMP levels in PSCs

Phosphorylation of eEF2 at Thr56 by eEF2 kinase is stimulated by calcium/calmodulin (Nairn et al., 1985), increased cAMP levels (Diggle et al., 1998) and elevated AMP/ATP ratios (Thomson et al., 2008). To determine whether CORM-2 impairs intracellular calcium homeostasis, [Ca^{2+}]$_i$ levels were measured by Fura 2-AM labeling in PSCs. Our results indicate that CORM-2 significantly increases cytoplasmatic calcium in a time-dependent manner (Fig. 5A). Peak emission levels of calcium-bound Fura-2 were determined at 30 min, but were declining to basal levels within 1 h. The observed short-termed and early [Ca^{2+}]$_i$ burst induced by CORM-2 might be due to the fact that the CO-release from CORM-2 once added to physiological solutions is nearly instantaneous (Motterlini et al., 2002).

Next we examined the effect of CORM-2 on intracellular cAMP levels by a competition enzyme-linked cAMP immunoassay (Fig. 5B). Cells treated with cholera toxin, a stimulator of adenylate cyclase, served as control and demonstrated a progressive increase in intracellular cAMP-mobilization. CORM-2 also induced a significant and time-dependent increase in intracellular cAMP, reaching maximum levels at 15 min of treatment. In this phase CORM-2 mediated effects were comparable to the biological activity of cholera toxin. Afterwards cAMP levels declined in CORM-2 treated PSCs, an observation that might again be attributed to the short termed release of CO by CORM-2 (Motterlini et al., 2002).

CO causes impaired oxygen delivery and utilization at the cellular level. CO inhibits cytochrome c oxidase, a rate-limiting enzyme within the respiratory electron transport chain of mitochondria, by competing with an oxygen-binding site (Alonso et al., 2003; Zuckerbraun et al., 2007). This affects the AMP/ATP ratio and consequently AMP-activated protein kinase (AMPK), a cellular energy sensor, is induced. AMPK blocks protein synthesis by inhibition of eEF2K-dependent eEF2 phosphorylation (Carling et al., 2011; Zhang et al., 2007). Therefore we speculated that CO liberated from CORM-2 might inhibit the eEF2 kinase/eEF2 pathway via activation of AMPK. The induction of Thr172 auto-phosphorylation within the

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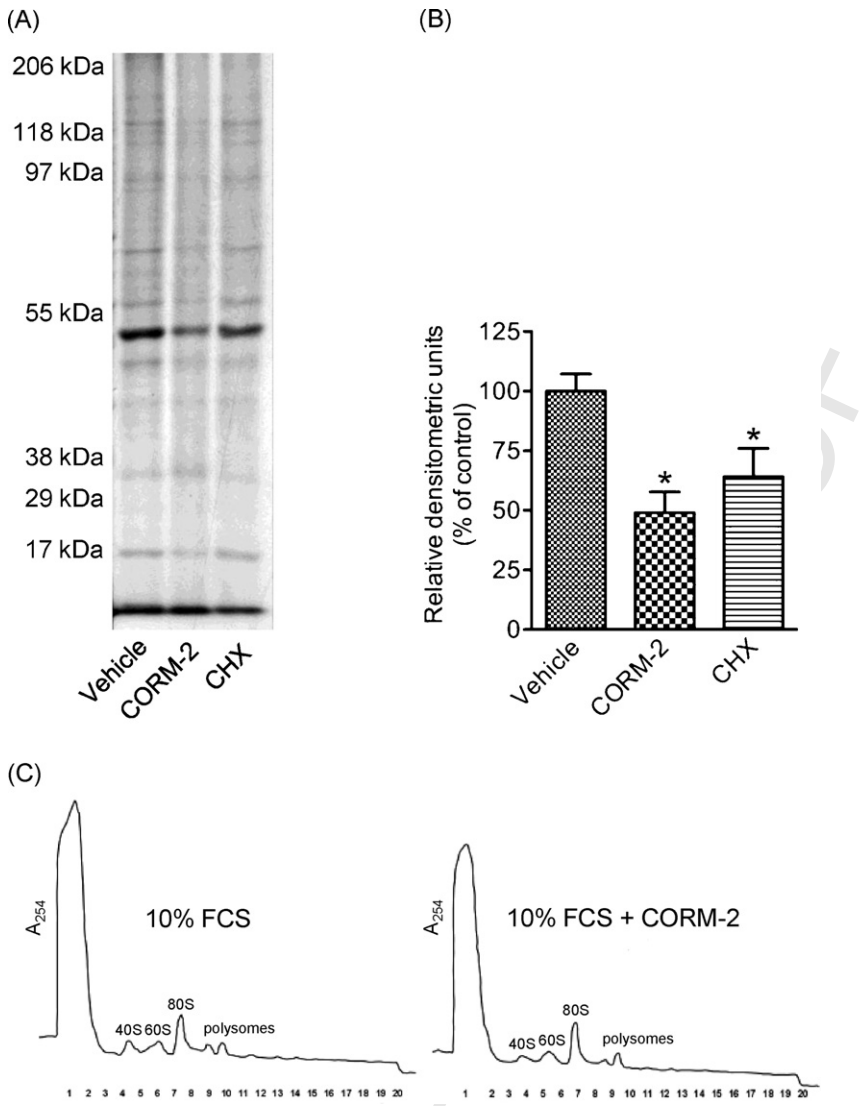

Fig. 4. CORM-2 represses protein synthesis in PSCs. Serum-starved PSCs were preincubated with vehicle (DMSO), CORM-2 (100 μM), or cycloheximide (CHX; 2.5 μg/ml) for 30 min followed by a 8-h stimulation with 10% FCS. To measure protein synthesis, cells were pulse-labeled with 5 μCi/well of [^{35}S]-methionine for 1 h and cell lysates were subjected to SDS-PAGE and X-ray autoradiography (A). [^{35}S]-methionine incorporation was statistically analyzed (B) and presented as relative densitometric units with the mean ± SEM ($n = 3$). *$p < 0.05$ versus control group (Vehicle). In (C), sucrose gradient velocity sedimentation analysis of polyribosomes from the cytoplasm of PSCs were performed. The UV-absorption profiles of the gradients using PSCs, pretreated with 10% FCS for 8 h (left profile) or pretreated with 100 μM CORM-2 30 min before addition of 10% FCS (right profile) are shown. The peaks corresponding to 40S, 60S, and 80S ribosomes as well as the position of the polysomes are indicated.

activation loop of AMPK was used as a marker for AMPK activation. However, our results demonstrate that CORM-2 had no effect on the level of phosphorylated AMPKα in PSCs (Fig. 5C, compare bands 1 and 3 or bands 2 and 4). In contrast to CORM-2, the AMPK activator phenformin potently induced phosphorylation of AMPKα at Thr172, demonstrating the inducibility of AMPK in PSCs (Fig. 5C).

3.6. HO-1 is not involved in the effect of CORM-2 on eEF2 phosphorylation

To elucidate a possible involvement of HO-1 in CORM-2 mediated effects, we examined the effects of HO inhibition on eEF2 phosphorylation. In line with data from our previous report (Schwer et al., 2010), treatment of PSCs with CORM-2 led to a profound increase in HO-1 protein expression (Fig. 6). Transfection of PSCs with HO-1 siRNA blocked HO-1 protein expression, whereas transfection of nonsilencing siRNA had no effect on CORM-2-induced HO-1 up-regulation in FCS-stimulated PSCs (Fig. 6). In non-silenced cells, CORM-2 led to an increase in eEF2 phosphorylation but did not affect total eEF2 levels. The ability of CORM-2 to prevent the decrease of phosphorylated eEF2 caused by serum was not altered in PSCs transfected with HO-1 siRNA. Similarly, the pharmacological HO-1 inhibitor tin protoporphyrin IX (SnPP IX) did not prevent CORM-2 dependent elevation of phosphorylated eEF2. These data suggest that HO-1 induction is not involved in the inhibitory effect of CORM-2 on global protein synthesis.

3.7. CORM-2 induces cell cycle arrest by decreased translation of cyclin D1, cyclin E and inhibition of Rb phosphorylation

The repression of global protein translation by CORM-2 (Fig. 7A) might have serious implications on substantial cellular functions or the progression of diseases associated with increased protein synthesis. PSCs are intimately involved in the pathogenesis of pancreatic diseases and excessive PSC proliferation might lead to fibrosis (Omary et al., 2007). We therefore investigated whether CORM-2 influences the proliferation of PSCs. Proliferation of cells is regulated by a sequential translational activation

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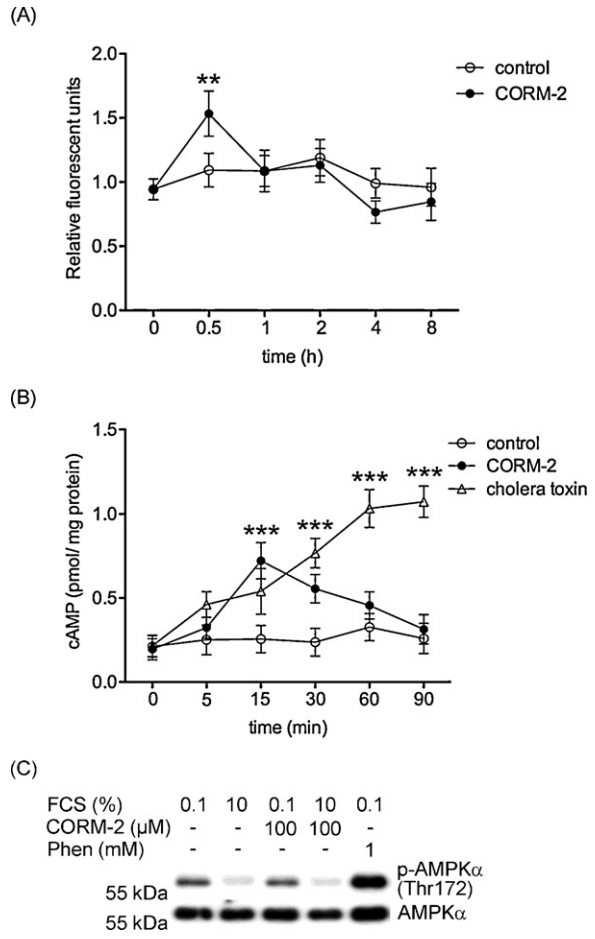

Fig. 5. CORM-2 increases intracellular calcium and cAMP levels but has no effect on AMPKα phosphorylation. Serum-starved PSCs were exposed to vehicle (DMSO) or CORM-2 (100 μM) for the times indicated. (A) $[Ca^{2+}]_i$ levels were assessed by Fura-2 labeling. Data represent mean values of dublicate determinations from three separate experiments. $^{**}p < 0.01$ versus control. (B) cAMP concentrations were determined by a competition enzyme-linked immunoassay. Data represent mean values of three independent experiments done in duplicate. $^{***}p < 0.001$ versus control. (C) PSCs were exposed to vehicle (DMSO), CORM-2 (100 μM), or phenformin (Phen; 1 mM) in the presence or absence of 10% FCS for 1 h. AMPKα phosphorylation was analyzed by immunoblotting. The membrane was stripped and reprobed with an antibody to detect total amounts of AMPKα.

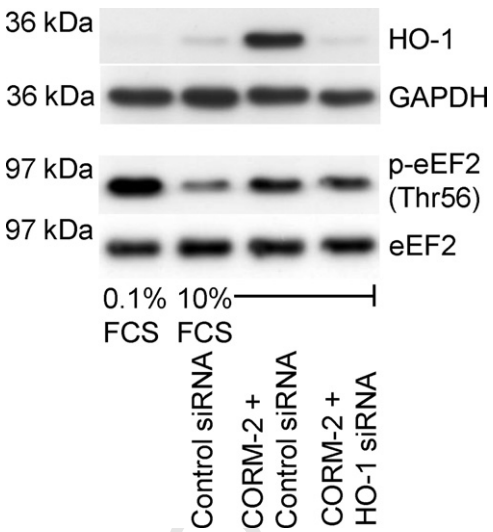

Fig. 6. Role of HO-1 in the effect of CORM-2 on eEF2 phosphorylation. PSCs were transfected with siRNAs directed against hmox1-mRNA (HO-1 siRNA) or nonsilencing siRNA (Control siRNA). After transfection, cells were incubated in serum-free medium for 24 h. CORM-2 (100 μM) was added to the cells for 30 min followed by a 8-h stimulation with 10% FCS. HO-1 and phospho-eEF2 (p-eEF2) protein expression was examined by Western blot analysis. To demonstrate equal loading, membranes were stripped and reprobed with anti-GAPDH or anti-total eEF2 antibodies.

of cyclins that release cell cycle checkpoints. To analyze whether CORM-2 represses the synthesis of cyclins, synchronized PSCs were metabolically labeled with [S^{35}]-methionine for 6 h to allow the successive translational activation of G_1-phase cyclins. Subsequently, cyclin D1 and cyclin E proteins were immunoprecipitated and resolved by SDS-PAGE and quantified by autoradiography (Fig. 7A). Our results demonstrate that addition of 10% FCS for 6 h led to a strong increase in the level of newly synthesized, radiolabeled cyclin D1 and cyclin E. This effect was almost completely abrogated by CORM-2. The loading of equal amounts of total protein in each lane was verified by Coomassie Blue G-250 staining of gels before autoradiography (data not shown).

Inhibition of cyclin D1 and cyclin E synthesis may occur at the translational or transcriptional level. To test whether CORM-2 inhibits the transcription of cyclin D1 or cyclin E, real-time PCR experiments were performed. As demonstrated in Fig. 7B, exposure of PSCs to 10% FCS for 6 h led to a significant increase in both cyclin D1 and cyclin E mRNA levels. However, incubation of PSCs with CORM-2 did not significantly decrease cyclin D1 and cyclin E mRNA levels in FCS-stimulated PSCs (Fig. 7B). This demonstrates that reduction of cyclin D1 and cyclin E synthesis is primary due to translational repression.

To further investigate the molecular mechanism resulting from CORM-2 induced repression of cyclin D1 and cyclin E synthesis, the phosphorylation of Rb was analyzed by Western blotting. Hyperphosphorylation of Rb by cyclin-CDK (cyclin-dependent kinase) complexes leads to the release of E2F transcription factors and S-phase entry (Lundberg and Weinberg, 1998). As shown in Fig. 7C, CORM-2 inhibited FCS-induced phosphorylation at serine 608 and serine 807/811 sites that have been described to be specifically phosphorylated by cyclin D-CDK4/6 and cyclin E-CDK2 complexes (Lundberg and Weinberg, 1998). In contrast, phosphorylation of Rb at serine 795 was not significantly affected by CORM-2 treatment. Phosphorylation of Rb at serine 795 involves ERK1/2 activation (Garnovskaya et al., 2004) that is not influenced by CORM-2 (Fig. 3C, panel 3).

Inhibition of cyclin synthesis and E2F release prevents proliferation of cells (Wu et al., 2001). To examine the antiproliferative effects of CORM-2, PSCs were stained with propidium iodide and cell cycle distribution was analyzed by flow cytometry (Fig. 8A and B). Proliferation was induced by 10% FCS in the presence or absence of CORM-2 using serum-starved, G_0/G_1 synchronized cells. Compared with untreated controls, 10% FCS led to a strong and significant increase in the percentage of cells in S and G_2/M phases (21.8 ± 3.6 versus 44.2 ± 5.2%; Fig. 8B). There was no significant difference in percentage of cells in S and G_2/M phases between 0.1 FCS and 10% FCS + CORM-2 treated PSCs (21.8 ± 3.6 versus 28.5 ± 3.8%; Fig. 8B). Compared with 10% FCS alone, CORM-2 significantly decreased the percentage of cells in S and G_2/M phases by 36% (44.2 ± 5.2 versus 28.5 ± 3.8%; Fig. 8B).

4. Discussion

Our study demonstrates that the CO-releasing molecule CORM-2 blocks global protein synthesis in PSCs. Translational inhibition is a result of eEF2 phosphorylation due to inhibition of PI3K-Akt-mTOR signaling, increased $[Ca^{2+}]_i$, and elevated cAMP levels. Translational repression leads to inhibition of cyclin D1 and cyclin E

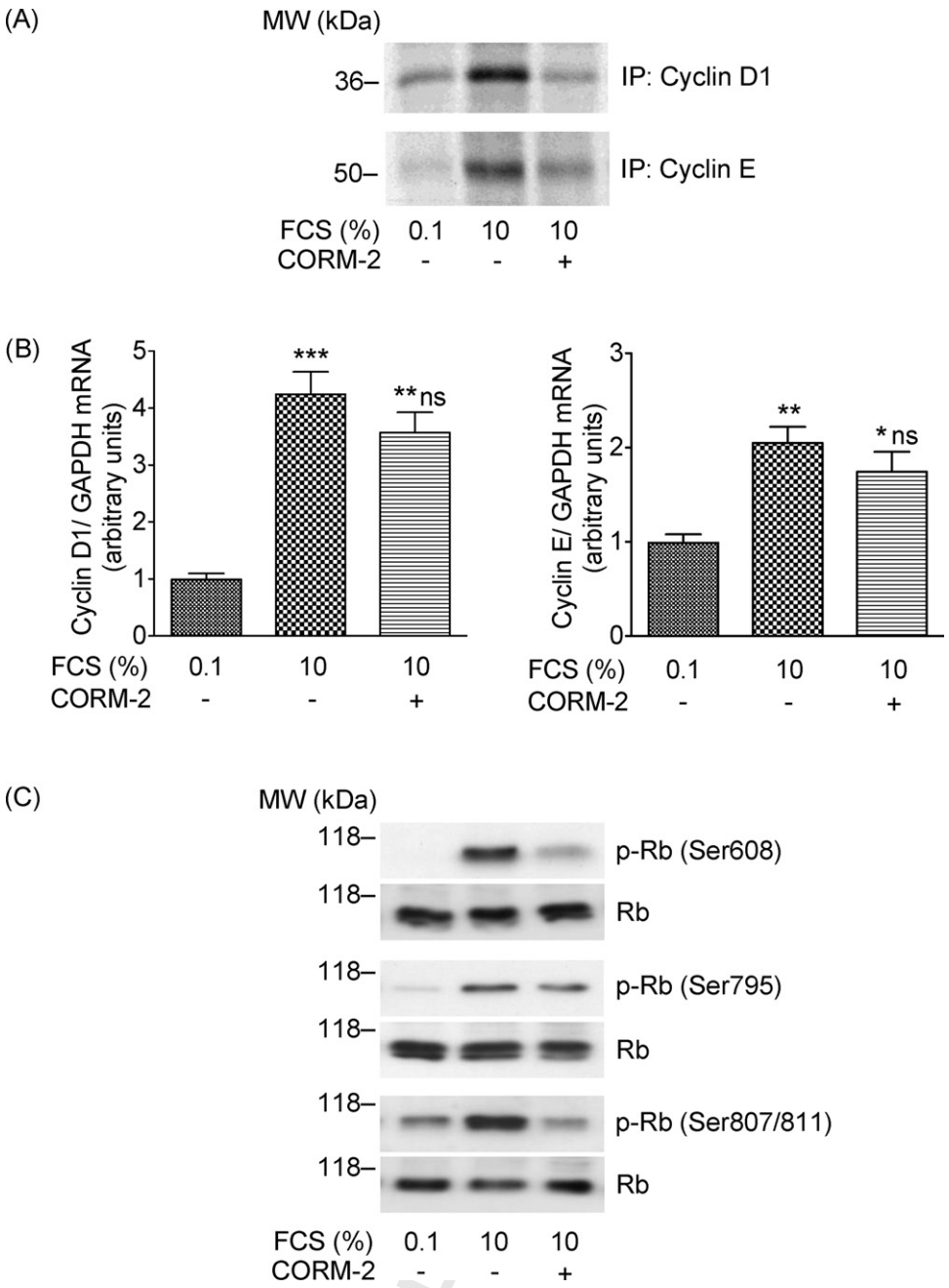

Fig. 7. CORM-2 inhibits the translation of cyclin D1 and cyclin E and represses the phosphorylation of Rb in PSCs. (A) Serum-starved PSCs were stimulated with 10% FCS in presence of vehicle (DMSO) or CORM-2 (100 μM) and 50 μCi/well of [^{35}S]-methionine for 6 h. Cell extracts were subjected to immunoprecipitation using antibodies against cyclin D1 and cyclin E. Immunoprecipitates were separated by SDS-PAGE, and protein levels of cyclin D1 and cyclin E were visualized by autoradiography. (B) Serum-starved PSCs were stimulated with 10% FCS in presence of vehicle (DMSO) or CORM-2 (100 μM) for 6 h. Cytoplasmatic RNA was used to perform real-time PCR experiments using primers for cyclin D1, cyclin E or GAPDH. Expression of cyclin D1 or E mRNA was normalized to GAPDH mRNA levels and presented as arbitrary units (mean ± SEM; $n = 3$). $^{*}p < 0.05$ versus 0.1% FCS; $^{**}p < 0.01$ versus 0.1% FCS; $^{***}p < 0.001$ versus 0.1% FCS; *ns*, not significant versus 10% FCS. (C) Rb phosphorylation (p-Rb) at Ser608, Ser795, and Ser807/811 in PSCs exposed to 10% FCS, vehicle, or CORM-2 (100 μM) for 24 h. The membranes were stripped and reprobed with an antibody to detect total amounts of Rb. The immunoblots shown are representative of three independent experiments.

synthesis, cyclin/Cdk-dependent repression of Rb phosphorylation and S-phase entry.

CORMs are metal carbonyl compounds capable of delivering defined amounts of CO into cellular systems, thereby mimicking the biological effects of intrinsic CO (Motterlini et al., 2002; Sawle et al., 2006). CORMs have shown antiinflammatory (Sawle et al., 2005), antiproliferative (Schwer et al., 2010; Taille et al., 2005), and bactericidal properties (Desmard et al., 2009, 2012; Tavares et al., 2011). However, effects of CORM-2 on the translational machinery were previously undisclosed. Our present study demonstrates that CORM-2 released CO acts as a global translational repressor by inducing phosphorylation of eEF2. Repression of global protein synthesis provides a possible mechanism through which CORM-2 mediates antiinflammatory and antiproliferative effects.

Protein synthesis in eukaryotes is regulated at the transcriptional and the translational level. The elongation stage of mRNA requires eEF2, which promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome (Kaul et al., 2011; Rennie, 2005). A major finding of the present study is that CORM-2 prevents the decrease in

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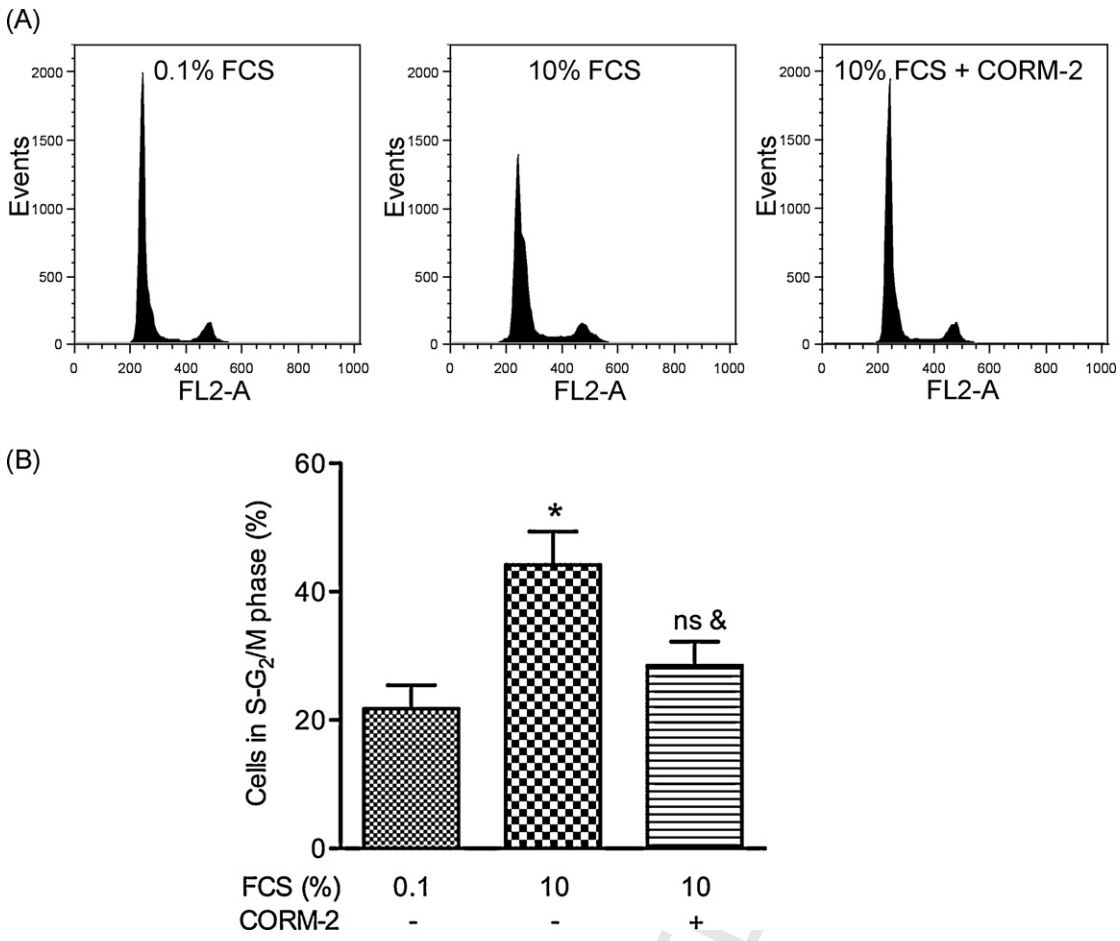

Fig. 8. CORM-2 arrests PSCs at the G_0/G_1 phase of the cell cycle. (A) Serum-starved PSCs were stimulated with 10% FCS in the presence or absence of CORM-2 (100 μM) and cell cycle distribution at 24 h was analyzed by flow cytometry. (B) Quantitative analysis of cells in S-G_2M phase obtained by flow cytometry. Data are shown as mean ± SEM ($n = 3$). $^{*}p < 0.05$ versus 0.1% FCS; $^{\&}p < 0.05$ versus 10% FCS.

phosphorylated eEF2 caused by serum, which is consistent with an inhibition of protein translation (Ryazanov et al., 1988). The effect of CORM-2 on eEF2 protein expression is time-dependent and significantly detectable already at 5 μM. We recently showed that CORM-2 in the range of 5–100 μM produced no reduction in PSC cell viability (Schwer et al., 2010). Our observations can directly be attributed to CO since $RuCl_3$ does not induce phosphorylation of eEF2. $RuCl_3$ is the chemical backbone of CORM-2 that is substituted with CO and therefore is unable to release carbon monoxide. Interestingly, recent reports describe that CORM-2, in contrast to $RuCl_3$, modulates redox signaling in smooth muscle cells (Taille et al., 2005). Thus translational impairment might be associated with the formation of reactive oxygen species (ROS). Taille et al. (2005) described that CO-released from CORM-2 inhibits membrane associated cytochromes, cyclin D1 synthesis, and proliferation but promotes the formation of ROS. Consistently we also observed diminished cyclin D1 synthesis and proliferation and it cannot be excluded that radicals contribute to translational inhibition. Experiments, exposing PSCs to 250 ppm CO confirmed, that translational impairment is directly attributed to CO (Schwer et al., unpublished observation). Binding of CO to cytochromes is most probably responsible for ROS generation (Taille et al., 2005) but it might simultaneously diminish mitochondrial ATP synthesis and thus activate AMPK, a kinase that has been shown to regulate cellular ROS/redox balance (Wang et al., 2012). Additionally, ROS formation by CO was associated with inhibition of ERK1/2 phosphorylation (Taille et al., 2005). However, neither activation of AMPK nor inhibition of ERK1/2 phosphorylation was evident in our experiments arguing for different and independent pathways regulating the translational response.

Other crucial proteins in the control of mRNA translation are initiation factors such as eIF4E and eIF2α (Gingras et al., 2001; Kimball, 1999; Kimball and Jefferson, 2010). In the present study, we found that CORM-2 has no impact on eIF4E or eIF2α modifications. These results are indicative for a specific effect of CORM-2 on translational regulators.

eEF2 phosphorylation is under control of eEF2k, whose activity is regulated by binding of calcium and calmodulin (Browne and Proud, 2002; Redpath et al., 1996; Ryazanov et al., 1997). Additionally, eEF2k can be activated by the cAMP-dependent protein kinase in response to elevated cAMP levels (Diggle et al., 1998; Hovland et al., 1999). In the present study we show that CORM-2 increases both $[Ca^{2+}]_i$ and cAMP levels, which might contribute to a CORM-2-mediated stimulation of eEF2k activity and thus explains the increase in phosphorylation of eEF2. Furthermore we demonstrate that CORM-2 inhibits serum-induced phosphorylation of Akt, p70 S6K and S6 Ribosomal Protein. p70 S6K lies on a mitogen-activated signaling pathway downstream of PI3K and mTOR (Pullen and Thomas, 1997), that has been shown to inactivate eEF2k and thus facilitates the dephosphorylation of eEF2 (Wang et al., 2001) and to phosphorylate the S6 protein of the 40S ribosomal subunit, leading to initiation of protein synthesis (Dufner and Thomas, 1999). The significance of the PI3K-Akt-mTOR pathway for CORM-2 mediated repression of global protein synthesis becomes evident by our observation, that CORM-2 additionally inhibits hyperphosphorylation of the translation repressor protein 4E-BP1, that is also

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regulated by this pathway and inhibits cap-dependent translation by binding to the eIF4E translation initiation factor (Brunn et al., 1997; Pause et al., 1994).

Phosphorylation of eEF2 and hypophosphorylation of 4E-BP1 both indicate suppression of global protein synthesis by CORM-2. Indeed we demonstrate a strong decrease in global protein synthesis following CORM-2 treatment. CORM-2 acts at the level of elongation rather than initiation, since compared to cells treated with 10% FCS alone, no substantial difference in the analytical polysome profile could be detected. For this reason effects of CORM-2 on regulators of translational elongation were further investigated. Interestingly, the translational repressor cycloheximide induces only a moderate inhibition of global protein synthesis in cultured PSCs. On plausible explanation might be the low concentration of cycloheximide used in the present study, however an increase in the concentration of cycloheximide was associated with pronounced cytotoxic effects on PSCs (unpublished observation).

Previous reports have shown that amino acid deprivation induces phosphorylation of eIF2α, leading to an inhibition of translational initiation (Everson et al., 1989). Since cells were pulse-labeled in methionine-deficient medium, an increase in eIF2α phosphorylation would provide a plausible mechanism underlying the inhibitory effect of CORM-2 on global protein synthesis. By incubating PSCs in medium lacking methionine we found that short-term exposure resulted in only a slight increase in phosphorylation of eIF2α (data not shown). Furthermore, control cells were treated under the same conditions and polysome profiles revealed, that translation initiation is not impaired by CORM-2 treatment.

CO acts as a mediator of numerous cellular functions and its potential as a therapeutic agent is increasingly recognized (Motterlini and Otterbein, 2010; Ryter et al., 2006). Our results indicate that the CO-releasing molecule CORM-2 affects cellular protein homeostasis and thereby might influence the execution of basic cellular functions. Cell cycle progression is mediated by the sequential translational activation of cyclins that associate with cyclin-dependent kinases (Cdks) and thus release cell cycle checkpoints (Koff et al., 1992). We demonstrate that CORM-2 mediated repression of global protein synthesis prevents the translation of cyclin D1 and cyclin E, the subsequent phosphorylation of the downstream target Rb by cyclin-Cdk complexes, and thus G_1/S phase progression. The significance of translation on CORM-2 mediated repression of the phospho-Rb dependent G_1/S phase progression is supported by our observation that hypophosphorylation of Rb was only demonstrated at cyclin dependent sites (serine 608, serine 807/811) but not the ERK1/2 dependent site (serine 795). In contrast to cyclins, ERK1/2 is not activated by protein synthesis but by posttranslational modification (Meloche and Pouyssegur, 2007). It has been reported that 4E-BP1 regulates cell proliferation by selectively inhibiting the translation of mRNAs that encode proliferation-promoting proteins and proteins involved in cell cycle progression (Dowling et al., 2010). Given that 4E-BP1 phosphorylation was altered by CORM-2 in the present study, 4E-BP1 might be involved in the inhibitory effect of CORM-2 on cyclin D1 and cyclin E expression. However, in a more recent report, the mTOR inhibitor Torin, which fully inhibits mTORC1, failed to signigicantly suppress translation of cyclin D1 mRNA (Thoreen et al., 2012). Furthermore, 24–48 h of mTOR inhibition are required to significantly exclude the cyclin D1 mRNA from polysomes (Dowling et al., 2010). In the present study, translational inhibition was apparent already at 6 h of treatment, indicating that 4E-BP1 is most probably not involved in the inhbitory effect of CORM-2 in the early phase of cyclin D1 and cyclin E synthesis.

Previously we have shown that up-regulation of HO-1 inhibits PSC proliferation (Schwer et al., 2008) and CORM-2 induced cell cycle arrest is associated with activation of p38 MAPK signaling, induction of HO-1 protein, and up-regulation of $p21^{Waf1/Cip1}$ (Schwer et al., 2010). The cell cycle inhibitor $p21^{Waf1/Cip1}$ acts by binding to the cyclin E-Cdk2 complex and inhibiting its kinase activity (Gu et al., 1993). To explore whether HO-1 induction contributes to CORM-2 mediated effects on protein synthesis, experiments with HO-1 siRNA were performed. We found that CORM-2 profoundly up-regulates HO-1 in PSCs, confirming data from our previous report. However, the ability of CORM-2 to prevent the decrease of phosphorylated eEF2 caused by serum was not prevented by transfection of HO-1 siRNA, suggesting that HO-1 is not involved in CORM-2 induced repression of cyclin D1 or cyclin E synthesis. Therefore it must be taken into consideration that both HO-1 dependent and independent mechanisms may account for the inhibitory effect of CORM-2 on cell cycle progression in PSCs: up-regulation of $p21^{Waf1/Cip1}$ protein and a reduced accessibility of cyclin-Cdk complexes.

The influence of CORM-2 liberated carbon monoxide on cellular protein synthesis most likely has significant implications on the therapeutic potential of CORMs and released CO respectively. A marked reduction in global protein synthesis describes a potential mechanism for the known antiproliferative properties of CORMs. This may be beneficial in disorders associated with excessive cellular proliferation and remodeling, such as cancer, hypertrophy, or fibrosis. However therapeutic application is not limited to cell cycle related effects. CORMs may gain increasing significance in the treatment of pancreatic fibrosis as they might be able to decrease the production of extracellular matrix components. Moreover, CORMs have been shown antiinflammatory properties in various cell types (Megias et al., 2007; Sawle et al., 2005) and organ systems (Cepinskas et al., 2008; Katada et al., 2010). It is tempting to speculate that inhibition of global protein synthesis may contribute to these CORM mediated effects.

Nevertheless, therapeutic inhibition of global protein synthesis has to be considered carefully. Although it has been described that prevention of synthesis of potentially cytotoxic mediators such as inducible nitric oxide synthase or cyclooxygenase-2 is protective (Surh et al., 2001) and inhibition of translational initiation or elongation factors may lead to a targeted expression of specific cytoprotective proteins by eEF2/eIF independent mechanisms (Lopez-Lastra et al., 2005), a prolonged inhibition of general protein synthesis has been associated with cellular damage. For instance, lack of recovery from protein synthesis inhibition has been shown to correlate with neuronal death following brain ischemia and reperfusion (Degracia et al., 2002; Thilmann et al., 1986) and inhibition of protein synthesis may result in apoptosis (Lennon et al., 1990; Martin, 1993). Compared to carbon monoxide as a gas, CORMs are easier to handle and offer the advantage of tissue- and organ-specific delivery (Motterlini et al., 2002). Compounds with fast CO release are more suitable for treatment, because application can be better controlled and lesser side effects are expected.

In conclusion, this study provides first evidence that CO acts as a global translational repressor by inducing phosphorylation of eEF2. This could constitute a general mechanism explaining the observed antiinflammatory and antiproliferative effects of CO. Our findings suggest a beneficial role of CORMs in the treatment of fibrosis, inflammation or other pathologic states being associated with excessive protein synthesis. However, application of exogenous CO might also have undesirable effects on cellular protein homeostasis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2012.09.020.

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Please cite this article in press as: Schwer CI, et al. Carbon monoxide releasing molecule-2 CORM-2 represses global protein synthesis by inhibition of eukaryotic elongation factor eEF2. Int J Biochem Cell Biol (2012), http://dx.doi.org/10.1016/j.biocel.2012.09.020

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