Carbon monoxide inhibits sprouting angiogenesis and vascular endothelial growth factor receptor-2 phosphorylation

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Summary

Carbon monoxide (CO) is a gaseous autacoid responsible for regulating inflammation, neural transmission and vascular tone. However, the role of CO in angiogenesis is unknown. The aim of this study was to investigate the effect of CO on angiogenesis and vascular endothelial growth factor (VEGF) receptor-2 phosphorylation. *In vitro* angiogenesis studies were performed using human umbilical vein endothelial cells (HUVECs) on growth factor-reduced Matrigel treated with a CO-releasing molecule (CORM-2) or exposed to CO gas (250 ppm). Here, we report the surprising finding that exposure to CO inhibits vascular endothelial growth factor (VEGF)-induced endothelial cell actin reorganisation, cell proliferation, migration and capillary-like tube formation. Similarly, CO suppressed VEGF-stimulated VEGFR-2 phosphorylation at key tyrosine residues and basic fibroblast growth factor (FGF-2) and VEGF mediated Akt phosphorylation in endothelial cells. Consistent with these data, mice exposed to CO gas (250 ppm, 1 hour/day for 14 days) exhibited a marked decrease in FGF-2-induced Matrigel plug angiogenesis ($p < 0.05$). These data establish a new biological function for CO in angiogenesis and point to a potential therapeutic use for CO as an anti-angiogenic agent.

Keywords

Carbon monoxide, vascular endothelial growth factor, angiogenesis, endothelial cells, vascular endothelial growth factor receptor-2

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Introduction

Angiogenesis is a complex process involving endothelial cell migration, proliferation, invasion, and tube formation and is obligatory for growth of solid cancers. Nitric oxide (NO) regulates blood vessel tone and angiogenesis in wound healing, inflammation, ischaemic cardiovascular disease and cancer (1). In the endothelium, endothelial NO synthase (eNOS) is activated by the Akt-dependent phosphorylation at Serine 1177 to generate NO (2), which is a prerequisite for vascular endothelial growth factor (VEGF)-induced angiogenesis (3, 4). Like NO, carbon monoxide (CO) also acts as a vasodilator and modulates endothelial cell permeability, making it potential candidate as a modulator of angiogenesis.

Carbon monoxide (CO) produced by the enzyme heme oxygenase-1 (Hmox1 / HO-1) shares a number of biological functions analogous to eNOS generated NO (5). It acts as a smooth muscle relaxant (6, 7) as well as an inhibitor of platelet aggregation (8). Atherosclerotic lesion formation and vascular remodelling is exacerbated in the absence of Hmox1 (9). Neovascularisation is associated with advanced atheromas in apolipoprotein E-deficient mice and endothelial cell inhibitors reduce plaque neovascularisation and growth (10). Indeed, pro-angiogenic growth factors and VEGF can induce lesion progression (11). There is increasing awareness that mammalian cells continuously generate CO by the Hmox pathway during heme catabolism (12). Similarly, there is mounting evidence that the stress response inducible Hmox1 and/or its catalytic byproduct CO confer cytoprotection against tissue and cellular injury (5, 13). Furthermore, pharmacologic inhibition of Hmox1 pathway induces leukocytic infiltration to enhance VEGF-induced angiogenesis, but blockade of leukocyte migration inhibits VEGF-induced angiogenesis by Hmox1 antagonists (14). Several *in vitro* studies reported that Hmox1 acts as a positive regulator of endothelial cell function (15–18) and Hmox1 can prevent graft rejection based on its ability to generate CO (19), which modulates inflammation, apoptosis and proliferation (20); however, the contribution of the gaseous product of Hmox1, CO as a regulator of angiogenesis remains unknown.

Recent studies in experimental tumours suggest that CO may exhibit anti-angiogenic activity (21–23). The discovery of transi-

tion metal carbonyls that act as CO-releasing molecules (CORM) has provided new impetus for the investigation of CO as cellular messenger as well as a potential therapeutic agent (24). In fact, low doses of CO were demonstrated to be non-toxic for healthy tissues, while still exerting beneficiary biological effects (24). Based on these data, a CO inhalation system for human use was developed and has been used in the first clinical trials (24).

The aim of this study was to investigate the effects of exposure to CO or CORM-2 (25) on angiogenesis. Here, we identify CO as an endogenous anti-angiogenic gaseous molecule that suppresses VEGF-mediated angiogenesis by inhibiting phosphorylation of VEGF receptor-2 (VEGFR-2) and Akt (PKB). This suggests that this diatomic signaling molecule could be exploited as a potential novel therapeutic agent for inhibiting pathological angiogenesis.

Methods

Reagents

Recombinant human VEGF-A, VEGF-E and FGF-2 were purchased from RELIATech (Brauschweig, Germany). We obtained mouse anti-rat CD31 antibody from BD Biosciences (Oxford, UK). Rabbit anti-phospho-Rb (Ser795), anti-phospho-Rb (Ser807/811), anti-phospho-Akt (Ser473) and anti-phospho-VEGFR-2 (Tyr1175) was obtained from New England Biolabs (Hertfordshire, UK). Rabbit anti-phospho-VEGFR-2 (Tyr1214) was purchased from Merck Chemicals (Nottingham, UK). Basement membrane matrix, growth factor-reduced MatrigelTM was from BD Biosciences (Oxford, UK). All other cell culture reagents and chemicals, including mouse anti-PY99 (sc-7020) and rabbit anti–VEGFR-2 (C-1158) antibodies, tricarbonyldichlororuthenium (II) dimer ([Ru(CO)3Cl2]2), more commonly known as CORM-2, were obtained from Sigma Aldrich (Poole, UK).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated, cultured in endothelial cell basal medium (EBM-2; Lonza, UK) supplemented with 2% fetal bovine serum (FBS) and used between passages 2–3 as previously described (26).

CORM-2 preparation

CORM-2 was prepared immediately prior to use in 10% DMSO solution as reported previously (27, 28). Inactivated CORM-2 (iCORM-2) was prepared by leaving CORM-2 at room tempera-

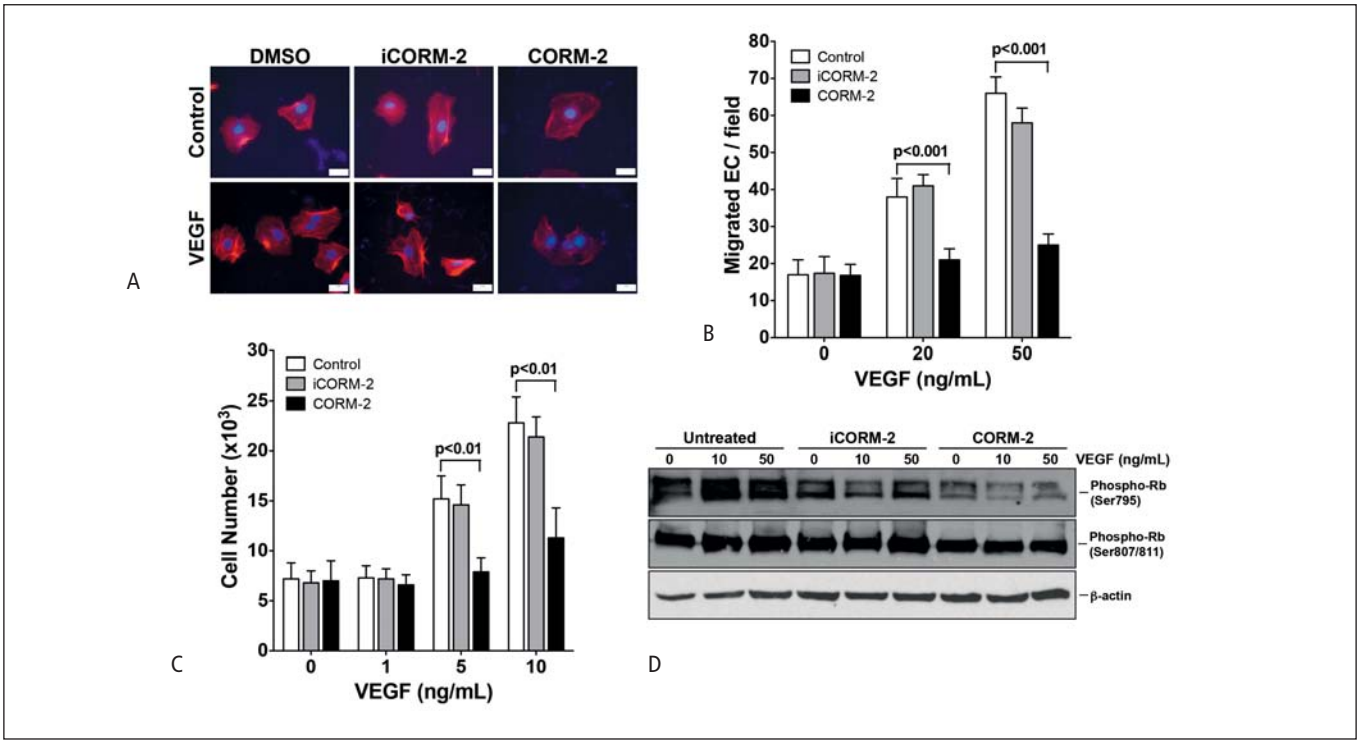

Figure 1: CO inhibits growth factor-induced endothelial cell migration and proliferation. A) Actin stress fibre formation in HUVECs following 15 min stimulation with VEGF (20 ng/ml) and in the presence of CORM-2 (50 µM), or iCORM-2 (50 µM). B) Migration of HUVECs pretreated with CORM-2 (50 µM) or iCORM-2 (50 µM) for 30 min and stimulated with VEGF (10 and 50 ng/ml) in a modified Boyden chamber. Results represent the mean (± SD) of three experiments (n=6). C) HUVECs were treated with increasing concentrations of VEGF (1, 5 and 10 ng/ml) in the presence of CORM-2 (50 µM) and the cell number determined after 48 h. D) Representative Western blot showing a decrease in VEGF (10 and 50 ng/ml) induced Rb phosphorylation (Serine 795 and 807/811) after 6 h in HUVECs pretreated with CORM-2 (50 µM). -actin was used as a loading control. Data are expressed as mean (± SD) or representative of five or more separate experiments performed in duplicate.

ture for two days, and flushing with nitrogen to remove residual CO. Before initiating the experiments, the release of CO from freshly diluted CORM-2 was confirmed by gas chromatography as described previously (23). Efficiency of CORM-2 inactivation was analysed in an analogous way. A fifty-micromolar concentration of CORM-2 was used based on our previous experience demonstrating 4–5 fold increase in CO concentration within the exposed cells (23).

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed using standard procedures as reported previously (28).

Cell migration assay

Cell migration was evaluated using a modified Boyden chamber assay as described (29). Gelatin-coated 5-µm PVP-free membranes were used to separate chambers, with an incubation time of 2 hours (h) after stimulation with the agonists. After staining of the membranes, with haematoxylin and eosin, cell migration was quantified by counting 8–10 fields of view at ×10 magnification.

Tube formation assay

The formation of capillary-like structures was examined on growth factor-reduced Matrigel in 24-well plates as described previously (4). Tube formation was quantified by measuring the total tube length in five random ×200 power fields per well using a Nikon phase-contrast inverted microscope with Image ProPlus image analysis software (Media Cybernetics, Silver Spring, MD, USA). Mean total tube length was calculated from three independent experiments performed in duplicate.

Endothelial cell sprouting assay

The sprouting assay was performed according to a modification of the method used by Nehls et al. (30). Briefly, HUVECs were grown to confluency on gelatin-coated Cytodex 3 beads (Amersham-Pharmacia, Piscataway, NJ, USA) for 5–7 days. Beads were em-

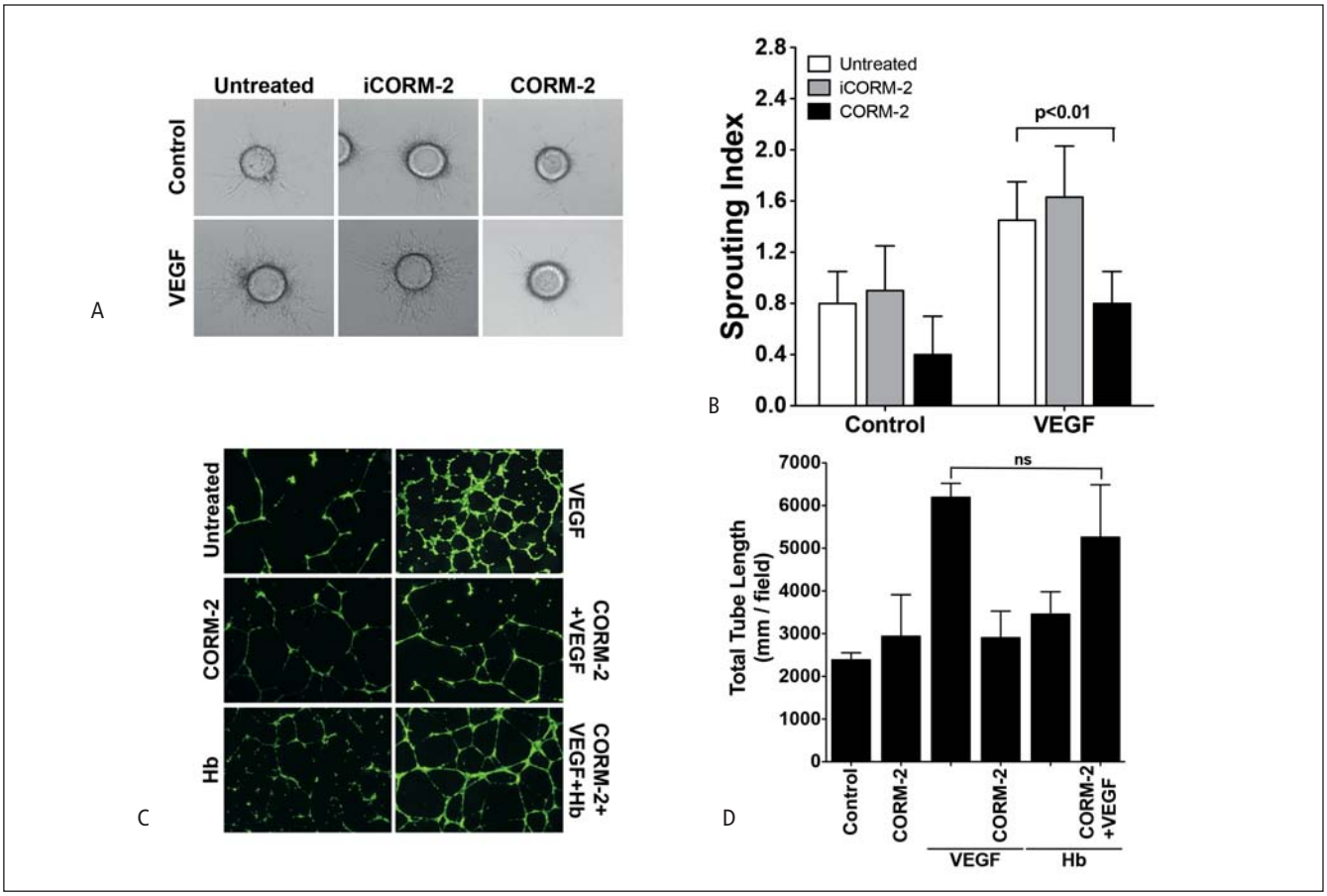

Figure 2: CO inhibits growth factor-induced angiogenesis. A) Representative photomicrographs showing the effect of CORM-2 (50 µM) on VEGF (50 ng/ml) stimulated sprouting of endothelial cells growing on dextran beads embedded in a fibrin gels for four days. B) Quantification of endothelial sprouting performed as described in *Methods*. C) Representative photomicrographs (C) and quantification (D) of VEGF-induced endothelial cell capillary-like network formation on growth factor-reduced Matrigel after 6 h showing the inhibition following CORM-2 (50 µM) pretreatment and abrogation of this effect with the CO scavenger haemoglobin (Hb; 100 µM). Data are expressed as mean (± SD) or representative of five or more separate experiments performed in duplicate.

bedded in a fibrin gel matrix. After 12 days of treatment, fibrin gels were fixed in 1% paraformaldehyde containing phosphate-buffered saline (PBS) and the sprouting visualised using a Nikon phase-contrast inverted microscope. The number of endothelial cell sprouts exceeding the diameter of the bead was determined and 40 beads counted per condition.

Complex IV activity assay

Cytochrome C oxidase activity was measured in total homogenates using a Mitochondrial Complex IV (Human) Activity Assay Kit (Merck Millipore, Feltham, UK). Briefly, HUVECs were grown to confluence on six-well plates and treated with CORM-2 or iCORM-2. Following a treatment period of 15 minutes (min) cells were lysed and 10 µl of lysate assayed at OD 550 nm. Sodium azide (5 mM) was used a positive control.

In vivo angiogenesis assay

The murine Matrigel plug assay was performed using a standard procedure as previously reported and plugs quantified using Image Pro Plus software (26). Briefly, 400 µl of growth factor-reduced Matrigel was injected subcutaneously into the abdomens of C57BL/6 mice. Following implantation of Matrigel plugs, animals were exposed to CO (250 ppm, 1 h/day) or air and sacrificed 14 days post-injection. The Matrigel plugs with adjacent subcutaneous tissues were carefully recovered by *en bloc* resection, fixed in 4% paraformaldehyde for histological analysis. The dose of CO used in *in vivo* study was selected according to safety criteria, as we had previously shown that exposure of animals to double this dose of CO (500 ppm, 1 h/day) resulted in CO-haemoglobin levels of only 9% (23). The local animal research committee had approved the protocols for all aspects of the animal studies in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the United States National Institutes of Health.

Statistical analyses

All *in vitro* data are expressed as the mean (± SD). Statistical comparisons were performed using one-way ANOVA followed by the Student–Newman–Keuls test as appropriate. All *in vivo* data are presented as mean ± SD, or median and 25–75% range when data were not normally distributed. The statistical significance of differences between variables was evaluated by the Mann-Whitney Rank Sum test. Statistical significance was set at a value of $p < 0.05$.

Results

CO inhibits VEGF-induced endothelial cell migration and proliferation

VEGF is a known endothelial-specific mitogen and actin reorganisation is an important component in VEGF-induced migration (31). To assess the effect of CORM-2 on VEGF-mediated actin reorganisation, cell migration and proliferation, endothelial cells were treated with VEGF in the presence of CORM-2 or iCORM-2, which served as a control. Exposure of endothelial cells to VEGF (20 ng/ml) for 15 min resulted in increased actin stress fiber formation characterized by distinct spindle-like processes, which was completely inhibited by 30 min preincubation with 50 µM CORM-2 (►Figure 1A). VEGF-dependent endothelial cell migration was also abrogated by CORM-2 (►Figure 1B) and CORM-2 treatment also significantly inhibited VEGF-stimulated endothelial cell proliferation (►Figure 1C).

During early and late G1 phase of the cell cycle, cyclin-dependent kinases promote phosphorylation of the tumour suppressor retinoblastoma protein (Rb), which plays a key role in the control of the G1/S phase transition in the cell cycle (32). To determine whether CORM-2 inhibits VEGF-induced Rb phosphorylation, endothelial cells were incubated with CORM-2 or iCORM-2 for 30 min prior to stimulation with VEGF for 6 h. Western blot analysis showed that CORM-2 markedly suppressed VEGF-induced phosphorylation of Rb at Serine 795 and 807/811 (►Figure 1D).

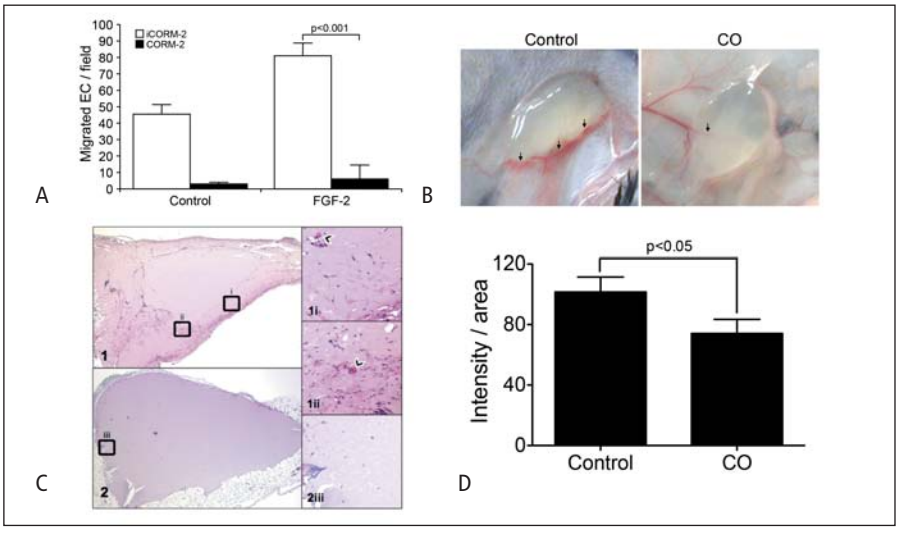

Figure 3: CO Inhibits *in vivo* angiogenesis. A) Photomicrographs of FGF-2 (20 ng/ml) induced vascular sprouts within Matrigel plugs 14 days after subcutaneous implantation in mice treated with either CO (250 ppm) or air. B) Detailed high magnification of Matrigel plugs showing a front of infiltrating vascular cells organised into blood-filled micro-capillaries (arrowhead in 1i and 1ii). C) Quantification of Matrigel plugs performed as described in *Methods*. Data are expressed as mean (± SD) or representative of five or more separate experiments performed in duplicate.

CO inhibits VEGF-induced angiogenesis

To determine whether CO could inhibit endothelial cell sprouting, primary HUVECs growing on micro-carrier beads, embedded in fibrin gel, were exposed to CORM-2 prior to VEGF treatment. VEGF increased sprout formation and this was inhibited significantly by CORM-2, but not by iCORM-2 (►Figure 2A-B). Similarly, VEGF induced capillary-like network formation on growth factor-reduced Matrigel was significantly blocked by CORM-2. Sequestration of CO using haemoglobin (Hb) at 100 µM restored VEGF-induced capillary networks (►Figure 2C-D). In addition, CORM-2 treatment also resulted in a significant reduction in FGF-2 mediated capillary network formation (data not shown). Together these data demonstrate that biologically relevant concentrations of CO inhibit angiogenesis.

CO inhibits FGF-2 induced angiogenesis

Fibroblast growth factors (FGF) also play a key role in neovascularisation. More recent discoveries have also highlighted their role in maintenance of blood vessels (33). To assess the effect of CORM-2 on FGF-mediated cell migration HUVECs were treated with FGF-2 in the presence of CORM-2. CORM-2 completely inhibited FGF-2 mediated endothelial cell migration (►Figure 3A). Moreover, Matrigel plugs supplemented with FGF-2 to enhance angiogenesis showed a marked decrease in newly formed vessels and vessel-like structures when animals were exposed to CO (250 ppm) for 1 h daily for 14 days as compared to mice exposed to air (►Figure 3B and C). Quantification of plug intensity showed a marked decrease in CO-treated mice compared to the control group (►Figure 3D).

CO inhibits VEGFR-2 receptor activation

VEGF receptor activation leads to phosphorylation of specific tyrosine residues and the association of different adaptor molecules to initiate signal transduction cascades (34). Treatment of HUVECs with CORM-2 prior to VEGF stimulation for 15 min suppressed VEGFR-2 phosphorylation at tyrosines 1175 and 1214 (►Figure 4A and B). Protein tyrosine phosphatases (PTP) scavenge phosphotyrosines in order to regulate a wide range of signalling pathways that underlie a broad spectrum of fundamental physiological processes. We investigated whether the CO-mediated decrease in VEGFR-2 phosphorylation was due to increased PTP activity. Endothelial cells were pre-treated with the pan-tyrosine phosphatase inhibitor, sodium orthovanadate. VEGF-induced VEGFR-2 tyrosine phosphorylation was potentiated by sodium orthovanadate. However, CORM-2 was still able to

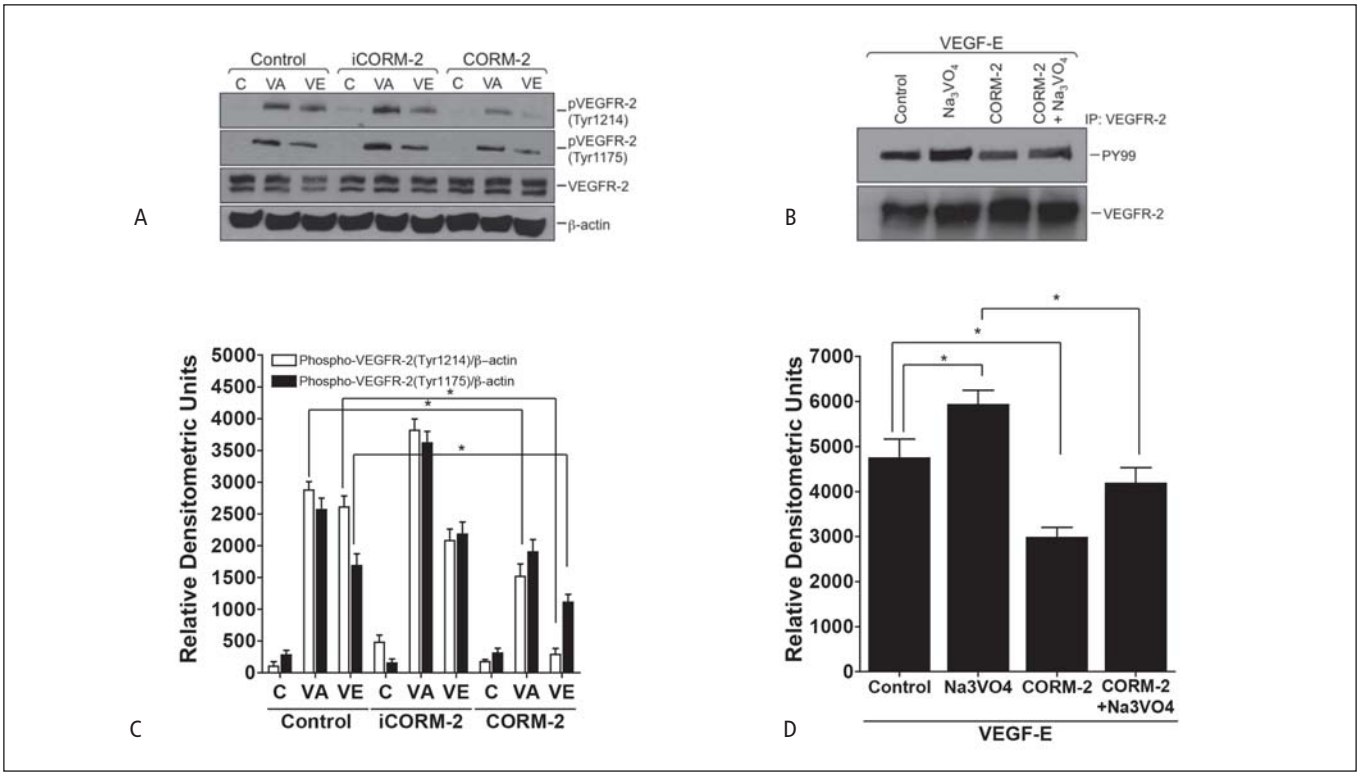

Figure 4: CO inhibits VEGF receptor activation. Western blot (A) and ensitometric quantification (B) showing the phosphorylation two key VEGFR-2 tyrosine residues (Tyr1214 and Tyr1175) in HUVEC pretreated with CORM-2 (50 µM) or iCORM-2 (50 µM) and stimulated with VEGF-A (V), VEGF-E (VE) (20 ng/mL) for 15 min and untreated controls (C). Western blot (C) and densitometric quantification (D) showing the effect of CORM-2 (50 µM) pretreatment on VEGFR-2 phosphorylation (phos-VEGFR-2) in HUVEC following VEGF stimulation for 10 min. Cell lysates were immunoprecipitated (IP) with rabbit anti-VEGFR-2 and blotted with an anti-phosphotyrosine antibody (PY99). Data are expressed as mean (± SD) or representative of five or more separate experiments. * $p < 0.05$.

suppress VEGF-induced VEGFR-2 phosphorylation to the same degree as the control stimulation in the presence of sodium orthovanadate suggesting that CO-mediated inhibition of VEGFR-2 phosphorylation is not due to increased tyrosine phosphatase activity (►Figure 4C and D).

CO inhibits growth factor-induced Akt phosphorylation

Both FGF-2 and VEGF induce Akt phosporylation to promote angiogenesis (35). Exposure of HUVECs to CORM-2 led to a marked inhibition in VEGF (V) or FGF-2 (F)-mediated Akt phosphorylation when compared to control (C) (►Figure 5A and B). The role of CO on cytochrome *c* oxidase activity was measured in whole cell HUVECs lysates. Exposure of cells to CORM-2 (50 μM) for 15 min had no significant effect on cytochrome c oxidase activity when compared to control (iCORM-2) (►Figure 5C). Sodium azide (5 mM) served as a positive control.

Discussion

For decades, CO has been regarded a potentially lethal gas; however, recent data point to its substantial beneficiary biological effects (36) with promising therapeutic potential. In our study, we investigated the effects of CO on angiogenesis using either CORM-2 or CO gas in several *in vitro* and *in vivo* experimental models. The concentration of CO used in our *in vitro* experiments (50 μM CORM-2) was shown previously to efficiently increase intracellular levels of CO (23), the dose of CO used in our *in vivo* study (250 ppm of CO for 1 h a day) was safe (23) and far below lethal dose for mice, which is equivalent to inhalation of 2400 ppm CO for 4 h (37).

Inhibition of growth factor mediated angiogenesis is a promising strategy for a number of vascular-based disorders (38). VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis *via* VEGFR-2 activation (39, 40). Anti-VEGF tumour therapy acts by reducing VEGF-dependent angiogenesis; however, this can lead to hypoxia, which initiates angiogenesis *via* other angiogenic factors (41). Our current study shows that CO inhibits both VEGF- and FGF-2-induced angiogenesis suggesting that CO may be a likely anti-angiogenic therapeutic candidate for targeting aberrant angiogenesis.

Specifically, exposure of endothelial cells to CORM-2 attenuated VEGF-induced phosphorylation of three crucial VEGFR-2 tyrosine residues, 951, 1175 and 1214. Earlier studies demonstrated that mutation of VEGFR-2 tyrosine 951 results in the inhibition of capillary morphogenesis (4) and crucial for F-actin filament reorganisation and endothelial cell migration (42). Furthermore, mutation of tyrosine 1175 results in embryonic lethality due to severe defects in endothelial cells (43). In contrast, the phosphorylation of tyrosine 1175 allows the binding of phospholipase Cγ1 and activation of protein kinase C and MAPK pathways (44), signals that are essential for VEGF-mediated proliferation. Collectively, these separate studies strongly suggest a direct effect of CO to limit VEGF-induced angiogenesis by suppressing

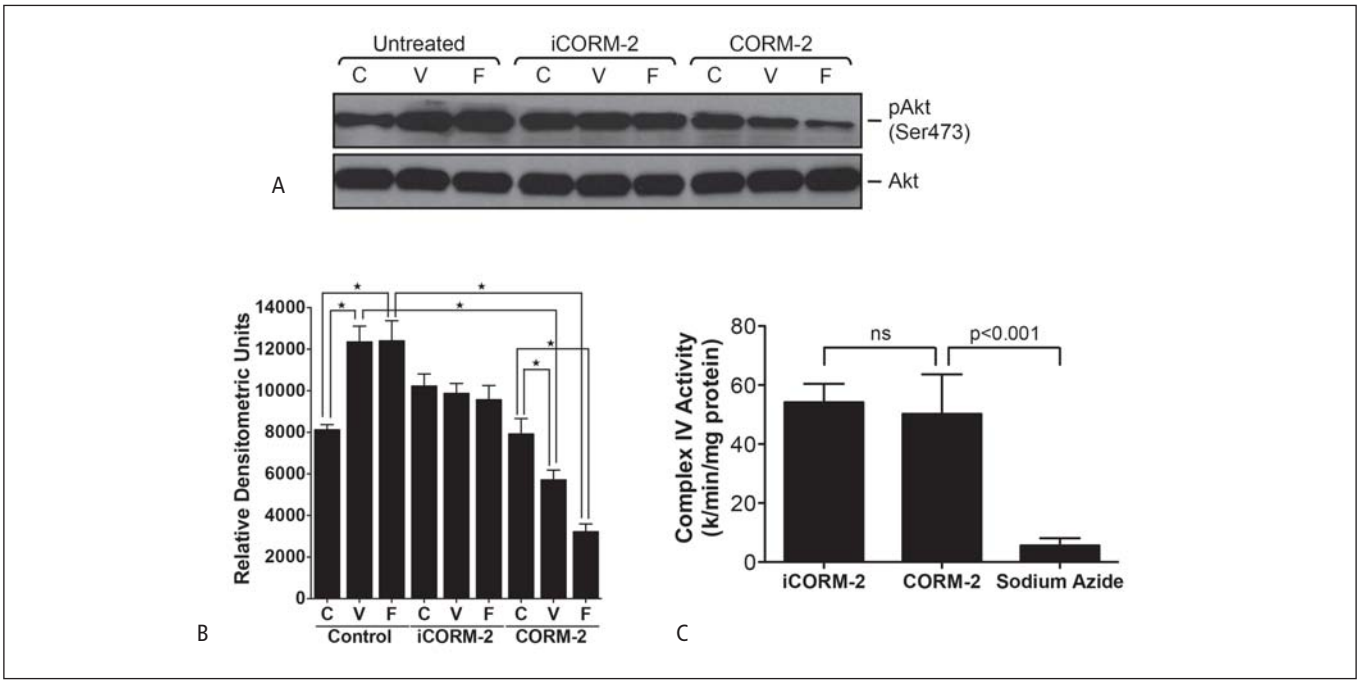

Figure 5: CO inhibits Akt phosphorylation. Western blot analysis (A) and densitometric quantification (B) of Akt phosphorylation in cells pretreated with CORM-2 (50 μM) or iCORM-2 and stimulated with vehicle (C), VEGF-A (V) or FGF-2 (F) for 15 min. C) Cytochrome C oxidase expressed as k/min/mg protein in cells treated with CORM-2 (50 μM), iCORM-2 or sodium azide (5 mM) for 15 min. Data are expressed as mean (± SD) or representative of five or more separate experiments.

VEGFR-2 receptor phosphorylation and thus limiting its activity (►Figure 6). Indeed, CORM-2 also inhibited VEGF- and FGF-2-mediated Akt phosphorylation, which concurs with an earlier study showing that oncoretroviral constitutive over-expression of Hmox1 in endothelial cells reduced endothelial cell proliferation and phosphorylation of eNOS and Akt (45). These findings are consistent with our recent observation demonstrating CORM-2-induced suppression of Akt phosphorylation in pancreatic cancer cells (23) pointing to a general effect of CO on this signalling cascade.

This study, despite being novel and demonstrating a new role for CO in inhibiting angiogenesis, will stimulate debate based on earlier reports that attributed a pro-angiogenic function to the Hmox1/CO pathway (18, 46). These studies, were conducted using the immortalised human dermal microvascular endothelial cell line (HMEC-1s) (47), or large vessel endothelial cells isolated from human heart (16) and aorta (17), as well as vascular smooth muscle cells (46). Endothelial functions and phenotypes differ depending on the vascular bed of origin (48). The differences between the angiogenic potential of HUVECs compared to HMECs was previously scrutinised (49). This group showed that the resting or stimulated HUVECs do not produce detectable amounts of VEGF. In contrast, resting HMEC-1s secrete significant amounts (~24.9 ng/ml) of VEGF, which can further increase in response to

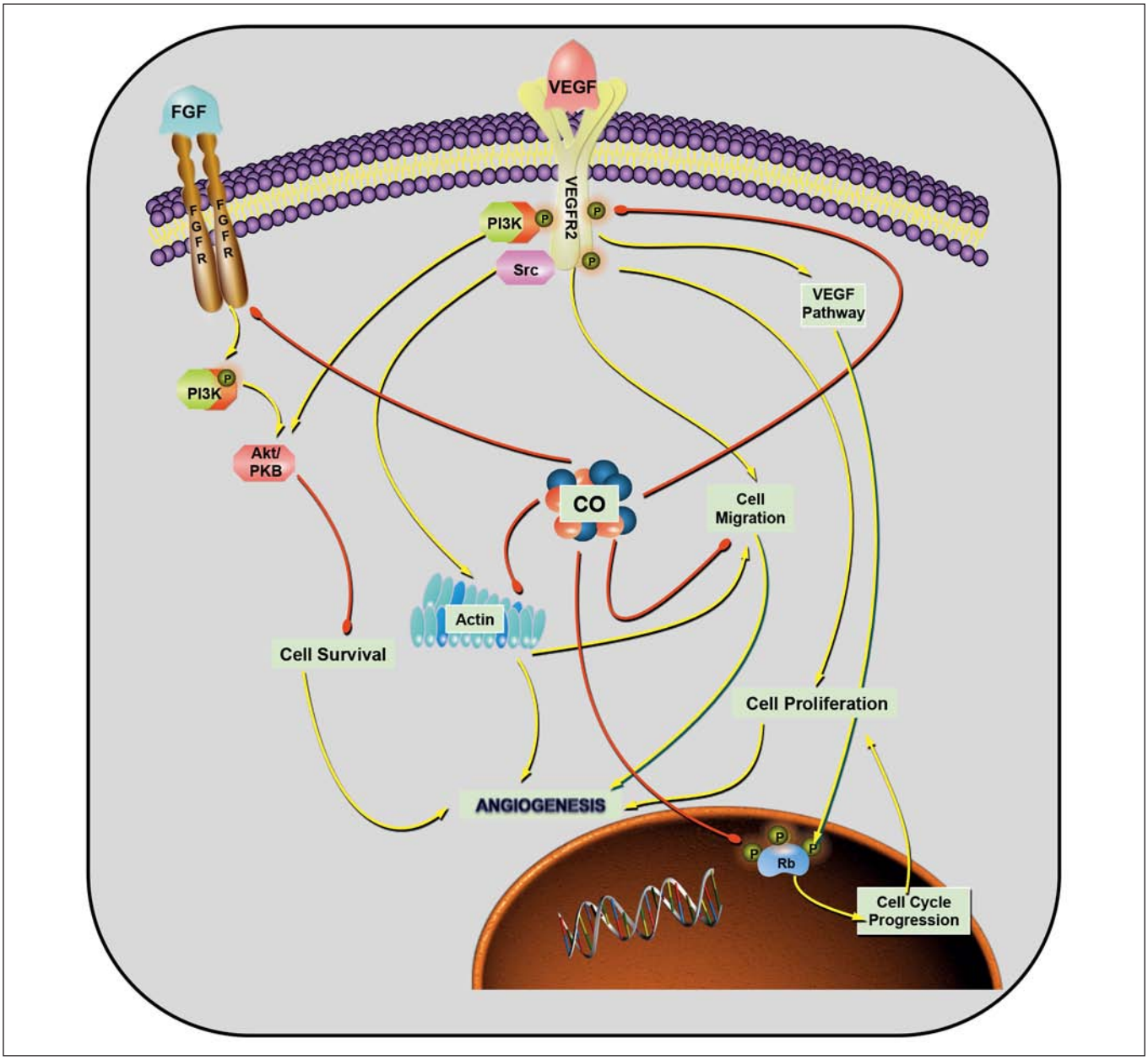

Figure 6: Schematic representation of the anti-angiogenic activity of CO. CO treatment impairs angiogenesis by inhibiting VEGFR-2 phosphorylation as well as downstream signalling events.

specific stimulus (49). Furthermore, HMECs and HUVECs differ in their sensitivity to exogenous growth factors (49). While under basal conditions, the proliferation of HUVECs is very low; this is increased with VEGF or FGF-2 whereas, HMECs proliferate spontaneously and do not require their proliferation is not enhanced by growth factors. Similarly, the spontaneous outgrowth of endothelial sprouts is negligible in unstimulated HUVECs, but more pronounced in HMECs (49).

Our findings provide mechanistic explanation for the reduction in tumour growth recently reported for prostate (21) and pancreatic (26) cancer. One of these studies focused on the ability of CO to inhibit tumour growth in mice and the proliferation of pancreatic cancer cell lines in culture (23), while Weigel et al. showed CO treatment sensitised prostate cancer cells to growth arrest and apoptosis *in vivo* (21). The current studies demonstrate that CO exposure significantly inhibited both *in vitro* and *in vivo* angiogenesis and the removal of CO using CO-scavenger restored the ability of endothelial cells to form tubular networks (21, 23). Similarly, CO suppressed VEGF-stimulated VEGFR-2 phosphorylation at key tyrosine residues (1175 and 1214) of VEGFR-2, and Akt phosphorylation in endothelial cells. These data demonstrate that this diatomic signalling species is a potent anti-angiogenic molecule and CO-releasing compounds may provide a simple and effective way to inhibit aberrant angiogenesis mediated by growth factors.

Acknowledgements

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Conflicts of interest

None declared.

What is known about this topic?

- Carbon monoxide (CO) is one of three products endogenously generated from heme degradation by the rate-limiting enzyme heme oxygenase (Hmox) and plays a role in regulating inflammation, neural transmission and vascular tone.
- Recent studies demonstrate that CO inhibits pancreatic and prostate tumour growth.

What does this paper add?

- The role of CO in angiogenesis is unknown. We sought to investigate the effect of CO on growth factor mediated angiogenesis and demonstrate that CO inhibits *in vivo* and *in vitro* angiogenesis.
- CO inhibits actin filament formation in endothelial cells.
- CO inhibits vascular endothelial growth factor (VEGF) receptor-2 and Akt phosphorylation in endothelial cells.

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