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Objective—Carbon monoxide (CO) is a weak soluble guanylyl cyclase stimulator, leading to transient increases in cGMP and vasodilation. The aim of the present work was to measure the effect of CO-releasing molecules (CORMs) on the cGMP/nitric oxide (NO) pathway and to evaluate how selected CORMs affect NO-induced vasorelaxation.

Methods and Results—Incubation of smooth muscle cells with some but not all of the CORMs caused a minor increase in cGMP levels. Concentration-response curves were bell-shaped, with higher CORMs concentrations producing lower increases in cGMP levels. Although exposure of cells to CORM-2 enhanced cGMP formation, we observed that the compound inhibited NO-stimulated cGMP accumulation in cells and NO-stimulated soluble guanylyl cyclase activity that could be reversed by superoxide anion scavengers. Reactive oxygen species generation from CORMs was confirmed using luminol-induced chemiluminescence and electron spin resonance. Furthermore, we observed that NO is scavenged by CORM-2. When used alone CORM-2 relaxed vessels through a cGMP-mediated pathway but attenuated NO donor-stimulated vasorelaxation.

Conclusion—We conclude that the CORMs examined have context-dependent effects on vessel tone, as they can directly dilate blood vessels, but also block NO-induced vasorelaxation. (***Arterioscler Thromb Vasc Biol***. 2011; 31:2570-2576.)

Carbon monoxide (CO), which was previously considered only as a highly toxic and life-threatening pollutant, has been recognized as a signaling molecule having regulatory roles in many physiological and pathophysiological processes within the cardiovascular, immune, and nervous systems.[1,2] It is generated in mammalian tissues via the endogenous degradation of heme by a family of constitutive (heme oxygenase-2 and heme oxygenase-3) and inducible (heme oxygenase-1) heme oxygenase enzymes.[2] CO gas binds to heme-containing proteins and exerts antiinflammatory and antioxidant effects.[2,3] In the cardiovascular system, CO exhibits vasodilatory properties, prevents endothelial cell apoptosis, controls vascular smooth muscle cell proliferation, and inhibits platelet aggregation.[3–5]

It has been suggested that CO can be used as a therapeutic agent; testing the beneficial actions of CO has been facilitated by the discovery of molecules that release CO under appropriate conditions.[1,6] Initial CO-releasing molecules (CORMs) were transition metal carbonyls soluble only in organic

solvents and required the use of physical or strong chemical stimuli to release CO.[7,8] More recently, water-soluble CORMs have been reported that liberate CO in a controlled fashion in biological systems and have the potential to deliver it to tissues and organs.[9,10] Several uses have been proposed for CORMs, ranging from inflammatory diseases and vascular dysfunction to tissue ischemia, organ rejection, and sepsis.[1] In the cardiovascular system, CORMs recapitulate many of the effects of CO gas. CORMs induce vessel relaxation in isolated aortic tissue and prevent coronary vasoconstriction, as well as acute hypertension in vivo.[11]

Similar to nitric oxide (NO), CO can activate the hemoprotein soluble guanylyl cyclase (sGC), but only weakly; CO is reported to enhance sGC activity by 3- to 4-fold compared with the maximal 200-fold activation triggered by NO.[12] On activation, sGC converts GTP to the second messenger molecule cyclic guanosine monophosphate (cGMP), which has an impact on many cellular functions.[13] For example, sGC is important in lowering smooth muscle tone and

preventing platelet aggregation and leukocyte adhesion to the vessel wall.[13–15]

In spite of the observations that CORMs reduce vessel tone, the potential interactions between CORMs and vasodilators that activate sGC (ie, NO donors) have not been studied. To this end, we have synthesized several CORMs and determined their ability to alter cGMP levels in cells and to modulate NO-stimulated cGMP formation and vasodilation.

Methods

Synthesis of ALFs and Detection of CO Release
The metal carbonyl CORMs (ALFXXX) were synthesized according to published or modified procedures for the synthesis of related complexes (see supplemental material, available online at http://atvb.ahajournals.org). All compounds were characterized by Fourier transform infrared spectroscopy, 1H nuclear magnetic resonance, and C,H,N analysis. The compounds were prepared, purified, and kept under N_2 atmosphere in the dark. The solutions of CORMs were freshly prepared before the experiments by dissolving the compound in the appropriate solvent. The release of CO from the metal carbonyl complexes (ALFXXX) under normoxic conditions was assessed by a chromatographic method (see supplemental material).

Cell Culture
Rat aortic smooth muscle cells (RASMCs) were isolated from 12- to 14-week-old male Wistar rats, 5 rats per isolation, as previously described.[16] More than 95% of cells isolated stained positive for smooth muscle α-actin. Cells between passages 2 and 5 were used for all experiments. RASMCs were routinely cultured in Dulbecco's modified Eagle medium containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum and antibiotics.

Determination of cGMP Levels in RASMCs
RASMCs were plated in 24-well plates. Cells were washed twice with Hanks' balanced salt solution and incubated in Hanks' balanced salt solution in the presence of 3-isobutyl-1-methylxanthine (1 mmol/L) for 5 minutes. Cells were then treated with vehicle, tempol (1 mmol/L), sodium nitroprusside (SNP) (10 μM), diethylamine NONOate (DEA/NO) (1 μmol/L), a nonsaturated CO solution (100 μmol/L), or CORMs at various concentrations (1 to 300 μmol/L) for 15 minutes. Media were then aspirated, and 200 μL of 0.1 N HCl was added into each well to extract cGMP. After 30 minutes, HCl extracts were collected and centrifuged at 600g for 10 minutes to remove debris. The supernatants were directly analyzed for cGMP by enzyme immunoassay.

Determination of sGC Activity
Guanylyl cyclase activity was determined by formation of [α-^{32}P]cGMP from [α-^{32}P]GTP. In short, reactions were performed for 10 minutes at 30°C in a final volume of 100 μL, in a 50 mmol/L HEPES, pH 8.0, reaction buffer containing 500 μmol/L GTP, 1 mmol/L dithiothreitol, and 5 mmol/L MgCl$_2$. Basal and DEA/NO (1 μmol/L)–stimulated guanylyl cyclase activity was measured in the presence of CORMs (10 and 100 μmol/L). To inhibit reactive oxygen species (ROS) production superoxide dismutase (SOD) (100 IU/mL) was added in the mixture. Typically, 5 μL of purified sGC (10 ng/μL) was used in each assay reaction.

Assessment of ROS Production by CORMs
Chemiluminescence was used to measure ROS production by freshly prepared CORMs. L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-day]pyridazine-1,4-(2H,3H)dione sodium salt) is a very sensitive chemiluminescence probe used to detect reactive oxygen and nitrogen species formation. Various CORMs (1 to 300 μmol/L) were added to 40 μL of a PBS buffer containing 400 μmol/L L-012.

L-012-enhanced chemiluminescence was immediately measured with a chemiluminometer (Luminoskan, Labsystems, Melbourne, Victoria, Australia) and reported as relative light units. In some cases, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (tempol) (1 mmol/L) was added to the mixture.

NO Quantification in Solution in the Presence of CORM-2
The release of NO was measured using an amperometric NO-specific probe (ISO-NOP) connected to the single channel free radical analyzer (TBR 1025, World Precision Instruments). A 4-channel recording system (Laboratory-Trax-4/16, World Precision Instruments) was used to digitize the analog signal from the probe. The ISO-NOP sensor was calibrated by mixing NaNO$_2$ solution of desired concentration with H$_2$SO$_4$ (0.1 mol/L) and NaI (0.1 mol/L). The release of NO by DEA/NO in phosphate buffer, pH 7.4, was then monitored continuously. Cumulative concentration responses curves to DEA/NO were performed in the presence of vehicle or CORM-2 (10 and 100 μmol/L); the solution was constantly bubbled with N$_2$ to remove atmospheric oxygen.

Assessment of Hydroxyl Production by ALF186, ALF409, and ALF436
The formation of oxygen-based radicals and ROS in solution from the reaction between low-oxidation-state complexes and O$_2$ was tested in electron spin resonance experiments using 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) as a spin trap.[17] A stock solution of each CORM was prepared by dissolving the compound in 1 mL of deoxygenated distilled water (MeOH was used in the case of ALF436) under N$_2$. Another stock solution of BMPO was prepared with a concentration of 250 mmol/L. An aliquot was taken from the CORM solution and 100 μL from the BMPO solution to a closed vial under N$_2$, and deoxygenated distilled water was added to make 1 mL total volume. The final concentration of CORM was 200 μmol/L, whereas the final concentration of BMPO was 12.5 mmol/L for the ALF186 experiment and 25 mmol/L for the others, ALF409 and ALF436. A sample was taken from this solution, and a control spectrum was acquired before bubbling O$_2$. Oxygen was then bubbled in the solution for 3 to 4 minutes, and another sample was taken and analyzed. The experiment was performed in a quartz, planar, electron spin resonance cell, at room temperature, microwave power of 2 mW, modulation frequency 100 kHz, modulation amplitude 0.1 mT.

Aortic Ring Preparation
Male Wistar rats (250 to 300 g; Harlan, Bresso, Italy) of 8 to 10 weeks of age were euthanized, and the thoracic aorta was rapidly dissected and cleaned from fat and connective tissue. Rings were denuded of the endothelium and placed in organ baths filled with oxygenated (95% O$_2$ to 5% CO$_2$) Krebs solution at 37°C, mounted to isometric force transducers, and connected to a Graphtec recorder. Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 minutes, during which tension was adjusted. In each experiment aortic rings were first challenged with phenylephrine (PE) (1 μmol/L) until the responses were reproducible. Aortic rings were contracted with PE (1 μmol/L). Once the plateau was reached, a cumulative concentration-response curve to DEA/NO or S-nitroso-N-acetyl-L,L-penicillamine (SNAP) (1 nmol/L to 10 μmol/L) and CORM-2 (1 μmol/L to 200 μmol/L) in the presence or absence of 1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one (ODQ) (5 μmol/L) were performed. In another set of experiments, rings were pretreated with 200 μmol/L CORM-2 for 15 minutes at resting tension, and then PE was added to constrict the preparation. The increase in tension induced by PE after CORM-2 preincubation was not significantly different from control. DEA/NO or SNAP cumulative concentration-response curves were then performed in the presence or absence of SOD (100 IU/mL) or tempol (3 mmol/L).

Statistical Analysis

Data are expressed as means±SE. Statistical comparisons between groups were performed using 1- or 2-way ANOVA (data from cell culture experiments were analyzed with 1-way ANOVA, whereas data from vasorelaxation studies were analyzed by 2-way ANOVA) followed by a post hoc test or Student t test as appropriate. Differences were considered significant at $P<0.05$. GraphPad Prism software (version 4.02, GraphPad Software, San Diego, CA) was used for all the statistical analysis.

Results

Detection of CO Liberated From CORMs

CO release form CORMs in cell-culture medium (Roswell Park Memorial Institute medium/fetal bovine serum) is summarized in the supplemental Table. Some CORMs (ALF186, ALF409, ALF411, ALF432, and ALF436) liberate >0.9 molar equivalents of CO after 2 hours. Interestingly, when dissolved in PBS (pH 7.4), CO liberation was enhanced in all cases, with the exception of ALF411. This indicates that molecules present in the Roswell Park Memorial Institute medium/fetal bovine serum medium protect CORMs complexes from decomposition or quench the CO formed. ALF411 behaves differently because it dissolves in Roswell Park Memorial Institute medium/fetal bovine serum, but is completely insoluble in PBS. As expected, once in solution its stability decreases and CO release occurs. The role of O_2 as the trigger for the liberation of CO from these complexes is quite clear because under anaerobic conditions (N_2), CO release is blocked in all cases except that of ALF409, where a small amount of CO is still liberated. In contrast to other CORMs and in accordance to previous reports,[1] the well-known Ru(II) CORMs, CORM-2 $[Ru(CO)_3Cl_2]_2$ and CORM-3 $[Ru(CO)_3Cl(H_2NCH_2COO)]$ do not release any CO in the headspace. On the contrary, they slowly release CO_2 over 24 hours in solution.[18]

Effect of CORMs on cGMP Levels in Cultured Cells

Exposure to a nonsaturated CO solution (100 μmol/L) caused a small, ≈50%, increase in cGMP levels in rat aortic smooth muscle cells (Figure 1A). For comparison, under the same conditions, stimulation of cells with the NO donor at 10 μmol/L leads to a 30-fold increase in cGMP accumulation (Figure 1B). Most CORMs when used at 1 and 10 μmol/L failed to increase cGMP levels in smooth muscle cells (Supplemental Table I); among the compounds tested, ALF436 increased cGMP by 3-fold at the lowest concentration used. For the more widely studied CORM-2 and CORM-3, higher concentrations were also used. Cells treated with CORM-2 showed a bell-shaped increase in cGMP accumulation, whereas exposure to CORM-3 caused an inhibition at the highest concentration used (300 μmol/L) (Figure 1C and 1D). The ability of CORM-2 to stimulate cGMP levels was blocked by the sGC inhibitor ODQ or by incubation of cells with hemoglobin (Supplemental Figure IA and IB). Moreover, the inactive form of CORM-2 (lacking the CO groups) did not alter cGMP accumulation (Supplemental Figure IC). The above data taken together suggest that CORM-2 increased cGMP accumulation through CO-dependent activation of sGC.
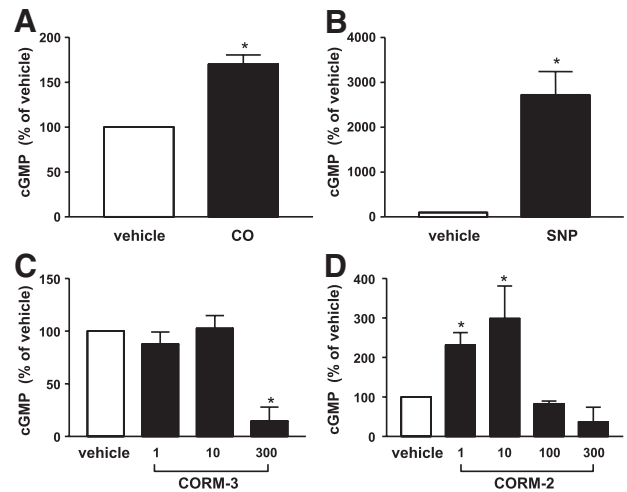

Figure 1. cGMP levels in rat aortic smooth muscle cells (RASMCs) treated with NO and CO donors. Confluent RASMCs were treated with vehicle, 100 μmol/L CO (A), 10 μmol/L sodium nitroprusside (SNP) (B), or the indicated concentrations of CO-releasing molecule (CORM)-3 (C) or CORM-2 (D) in the presence of 3-isobutyl-1-methylxanthine for 15 minutes. cGMP was extracted and measured as described in the Methods section. Data are expressed as percentage of the respective vehicle, which was set as 100%; n=4 to 12; *$P<0.05$ compared with vehicle.

Effect of CORMs on Recombinant sGC Activity

We next evaluated whether CORMs exert a direct effect on sGC activity by using recombinant rat sGC. In contrast to the increase in cGMP accumulation observed with cultured cells, no increase in sGC activity was observed after incubation with CORM-3 and CORM-2 at the concentrations used (Figure 2A). On the other hand, the NO-stimulated sGC activity was markedly reduced in a concentration-dependent manner by these CORMs (Figure 2B).

Generation of ROS and Scavenging of NO by CORMs

To study the mechanism through which CORMs reduce NO-stimulated cGMP formation, we initially measured the ability of CORMs to generate ROS. ROS production from CORM-2 was concentration dependent, plateauing at a 100 μmol/L concentration of this CORM, and could be abolished by addition of tempol, an O_2^- scavenger (Figure 3A and 3B). Two other molybdenum-based CORMs, ALF186 and ALF436, also increased ROS production (Supplemental
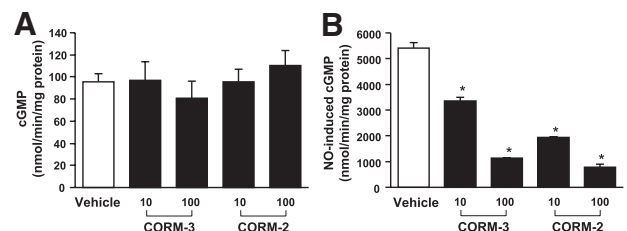
Figure 2. Effect of selected CORMs on purified soluble guanylyl cyclase (sGC) enzyme activity. The activity of recombinant sGC was measured following addition of 10 or 100 μmol/L of the indicated CO-releasing molecules (CORMs) in the absence (A) or presence (B) of diethylamine NONOate (1 μmol/L); n=3; *$P<0.05$ compared with vehicle.
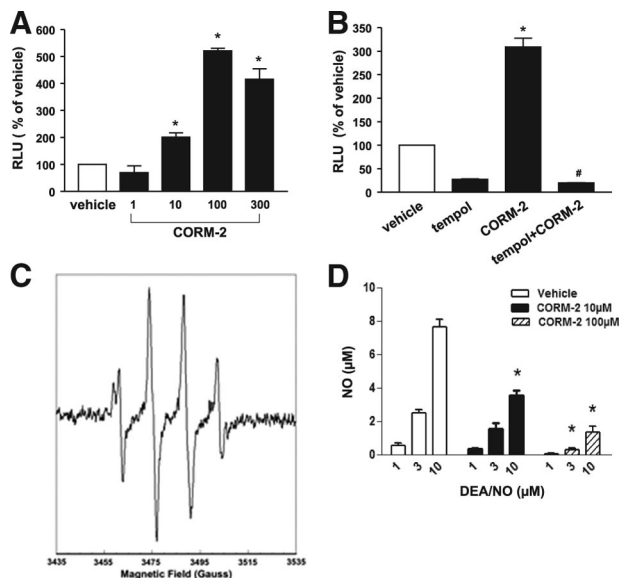
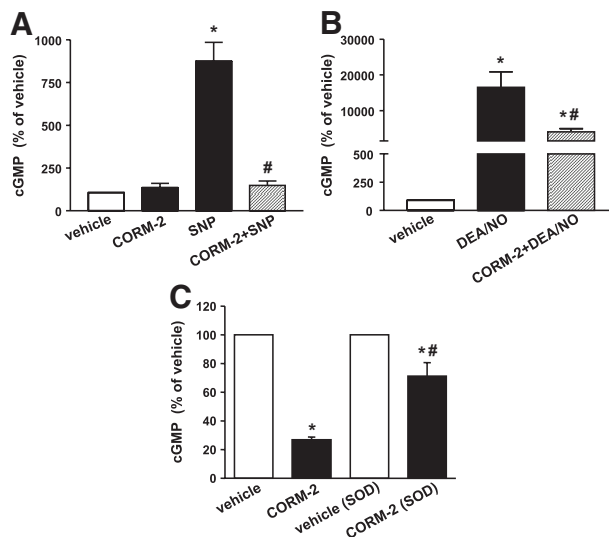Figure 3. CO-releasing molecules (CORMs) release reactive oxygen species. Superoxide anion release by the indicated concentration of CORM-2 was measured by chemiluminescence (A). The superoxide anion scavenger tempol (1 mmol/L) was used to quench O_2^- produced from CORM-2 (100 μmol/L) (B). Electron spin resonance spectrum obtained after bubbling O_2 in a water solution containing 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) and ALF186 (C). NO concentration in a solution containing 1 to 10 μmol/L diethylamine NONOate (DEA/NO) in the presence of different CORM-2 concentrations (10 to 100 μmol/L) (D); n=3; *$P<0.05$ compared with vehicle. $P>0.05$ compared with CORM-2. RLU indicates relative light units.

Figure 4. CO-releasing molecule (CORM)-2 reduces NO-induced cGMP formation. Rat aortic smooth muscle cells were treated with vehicle, sodium nitroprusside (SNP) alone (0.1 μmol/L, A), diethylamine NONOate (DEA/NO) alone (1 μmol/L, B) or a combination of the NO donor with CORM-2 (200 μmol/L) in the presence of 3-isobutyl-1-methylxanthine for 15 minutes. cGMP was extracted and measured as described in Methods section. The activity of the NO-stimulated purified soluble guanylyl cyclase (sGC) was measured after addition of CORM-2 (10 μmol/L) in the presence of the endogenous superoxide dismutase (SOD) (100 IU/mL) (C). Data are expressed as percentage of the respective vehicle, that was set as 100%; n=3 to 8; *$P<0.05$ compared with vehicle, #$P>0.05$ compared with CORM-2. #$P<0.05$ compared with SNP, DEA/NO, or CORM-2.

Figure IIA and IIB). The ALF186- and ALF436-induced ROS released was inhibited by tempol (Supplemental Figures IIIB and IVB). In a separate series of experiments, we observed that the electron spin resonance spectra obtained for the solutions of ALF186 (or CORM-3, ALF409, ALF436; data not shown) after bubbling O_2 revealed a very strong and clean signal assignable to the hydroxyl adduct of BMPO (Figure 3C). Thus, although CO from CORMs activates sGC in an NO-independent and, thus, superoxide-insensitive manner, the ROS generated by CORMs would be expected to inhibit NO-stimulated guanylyl cyclase activity.

To assess whether CORM-2 can react with NO, we measured NO using an NO electrode (Figure 3D). In these experiments, we observed that CORM-2 attenuated the amount of NO in solution released by DEA/NO in a concentration-dependent manner. When NO levels were measured in a 10 μmol/L DEA/NO solution, they were found to be decreased by 80% when a 10-fold excess of CORM-2 was present. Similar observations were made using SNP as a donor (data not shown). Using a different experimental setup, we confirmed that CORM-2 quenched NO; scavenging of NO by CORM-2 was favored when CORM-2 is present in excess ($>$10-fold) relative to NO (Supplemental Figure V).

Interaction of CORMs With NO Donors in Cells
To study whether CORM-2 reduces NO donor-stimulated cGMP accumulation in cells, RASMCs were exposed to SNP or DEA/NO, and cGMP was measured. Indeed, CORM-2 attenuated cGMP formation in response to both NO donors,

confirming and extending in a cellular milieu the in vitro biochemical findings that CORMs release ROS and attenuate cGMP formation. Interestingly, responses to SNP (Figure 4A) were affected more than those to DEA/NO (Figure 4B). In cells treated with tempol, the ability of CORM-2 to reduce DEA/NO-induced cGMP accumulation was attenuated (Supplemental Figure VI). The 2 molybdenum-based CORMs, ALF186 and ALF436, also reduced NO-stimulated cGMP accumulation in cultured cells (Supplemental Figure IIC and IID) in a tempol-reversible manner (Supplemental Figures IIIA and IVA).

Additional evidence for the role of ROS in CORMs-mediated inhibition of NO-induced cGMP formation was obtained in sGC activity assays. High concentrations of CO (700 μmol/L) decreased NO-stimulated sGC activity by $\approx$20% (data not shown); cGMP formation was even more reduced after exposure to CORM-2; when SOD was added to the sGC activity assay mixture to remove any O_2^- formed, the effect of CORM-2 was partially reversed (Figure 4C).

Effect of CORM-2 on Vascular Tone
CORM-2 administration caused a concentration-dependent relaxation that reached approximately 60% relaxation at the highest concentration used (Figure 5A). CORM-2-induced relaxation was abolished by ODQ, suggesting that the relaxation is mediated by sGC. Exposure to the NO donor DEA/NO led to a full relaxation that was observed at a much lower concentration than the one needed for maximal
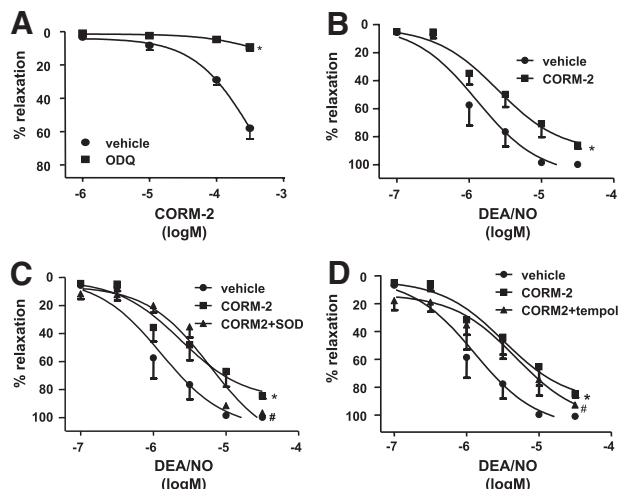
Figure 5. CO-releasing molecule (CORM)-2 causes vasodilation, but inhibits NO-stimulated relaxation. Rat aortic rings were pre-contracted with phenylephrine, and relaxation was triggered with increasing concentrations of CORM-2 (1 to 200 μmol/L) in the presence or absence of 5 μmol/L 1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one (ODQ) (A). Rings were pretreated with CORM-2 (200 μmol/L) or vehicle for 15 minutes and then contracted with phenylephrine; they were then exposed to increasing concentrations of the NO donor diethylamine NONOate (DEA/NO) (B). Phenylephrine-contracted rings were incubated with SOD (100 IU/mL, C) or tempol (3 mmol/L, D) for 15 minutes before being exposed to CORM-2 (200 μmol/L); DEA/NO was then added at the indicated concentration to elicit vasorelaxation. Data are mean$\pm$SEM; n=4; *$P<0.01$ compared with vehicle, #$P<0.05$ compared with CORM-2.

CORM-2 relaxation (Figure 5B). To assess whether CORM-2 could interfere with the vasodilation triggered by exogenously administered NO, relaxation curves to DEA/NO were repeated in the presence of a maximally dilating CORM-2 concentration. It should be noted that the contractile response to PE was not significantly affected by the preincubation with CORM-2. This might be due to the fact that the CORM-2-induced vasodilation is short-lived and is no longer observed after the preincubation, or due to the fact that the vasorelaxing action of CORM-2 is only displayed when this agent is applied to a tissue after, but not before, it develops an elevated tone. In line with what we observed in the experiments with purified sGC and cultured cells, NO-induced relaxation was attenuated by CORM-2. The inhibitory effect of CORM-2 in NO-induced vasodilation was reduced by SOD (Figure 5C) and tempol (Figure 5D). Similar results were obtained using a different NO donor SNAP. In a manner analogous to that observed with SNP responses in cultured cells, the vasorelaxing action of SNP was inhibited to a greater extent by CORM-2 (Supplemental Figure VII).

Discussion

The derivatives of the fragment fac-[Ru^{II}(CO)$_3$)], CORM-2, and CORM-3 are the most commonly reported examples of metal carbonyl complexes used as vehicles to deliver CO to cells and tissues.[1] The CO releasing capacity of these compounds has been measured by their ability to rapidly ($<$1 minute) transfer 1 CO to deoxy-Mb, forming carbonmonoxy-myoglobin. However, we and others[1,19] have observed that

these compounds do not release CO as a free gas. Thus, CO from CORM-2 and CORM-3 is assumed to be first transferred to a biological molecule forming an intermediate that delivers CO in living systems.[1] In the present study, we introduced a different group of CORMs based on zero-valent molybdenum carbonyls. In contrast to the previous Ru(II) complexes, these Mo(0) compounds are able to release CO as a free gas to their biological environment once they are activated by molecular oxygen.

All but one of the molybdenum-based CORMs, in spite of releasing measurable amounts of CO in cell culture media, failed to stimulate cGMP production in cultured vascular smooth muscle cells. The one that did (ALF436) increased cGMP only marginally, and this effect was lost when higher concentrations were used. From our in vitro studies, it became apparent that cGMP accumulation in cultured smooth muscle cells is not a good bioassay to test for biological activity of molybdenum-based CORMs. Among the ruthenium-based CORMs (CORM-2 and CORM-3), neither of which release CO to the headspace but rather transfer CO to heme-containing biological molecules, only CORM-2 increased cGMP accumulation. However, CORM-3 has been reported to promote vasodilation in an ODQ-inhibitable manner and to increase cGMP levels in vascular tissue.[9] The cGMP-elevating effect of CORM-3 was shown to be diminished by endothelium removal or endothelial nitric oxide synthase inhibition, suggesting that CORM-3 might be acting, at least in part, by enhancing endogenous NO release rather than through a direct effect on sGC.[9] Of note, cGMP-independent mechanisms of CORM-induced smooth muscle relaxation have also been shown to exist. These are mediated through big conductance calcium-activated potassium channels: the opening of these channels leads to membrane hyperpolarization, which in turn closes voltage-dependent calcium channels, reduces calcium levels, and relaxes smooth muscle.[20]

To examine whether CORMs directly activate sGC, we tested the 2 most studied CORMs to date (CORM-2 and CORM-3) for their ability to stimulate recombinant sGC. In line with previous observations, CORM-3 did not enhance sGC activity in vitro[9]; neither did CORM-2. CORM-3 has been shown to increase sGC activity in the presence of the sGC sensitizer YC-1.[9] The fact that CORM-2 increased cGMP formation in cells but not in the sGC activity assay could be explained by the existence of an endogenous YC-1-like sGC sensitizer[21] in smooth muscle cells. Alternatively, the formation of an intermediate/metabolite might be required for the transfer of CO from these CORMs to sGC and this only occurs in cells. Surprisingly, we found that both CORMs markedly and dose-dependently inhibited NO stimulated sGC activity. This inhibitory action of CORM-2 and CORM-3 on NO-stimulated sGC has not been reported before and could be explained by (1) a partial agonist effect of CO on sGC activation, (2) ROS generation by the CORMs that inactivates NO, or (3) quenching of NO by the transition metal complexes. Experiments were therefore designed to test the above-mentioned hypotheses.

CO binds to the sGC heme with considerably lower affinity and is a much weaker sGC stimulator compared with NO.[22,23]

tion of NO-stimulated sGC activity, suggesting that CO, can act as partial agonist if present in great excess. Because of the different affinity for sGC exhibited by NO and CO, the antagonistic action of CO would only be observed if pharmacological amounts of CO are delivered and would be of no importance under physiological conditions. Incubation of recombinant sGC with CORM-2 caused an even greater decrease in NO-stimulated sGC activity than that caused by the CO-saturated solution. The decrease in sGC activity was partially restored by SOD, suggesting that the inhibition of sGC activity by CORM-2 involves at least 2 mechanisms (partial agonism and ROS generation). To provide experimental evidence that CORM-2 generates ROS, we measured the production of ROS from CORM-2 using a chemiluminescent dye. In these experiments, we could demonstrate a concentration-dependent increase in chemiluminescence that was blocked by incubation with tempol, indicating that superoxide anions are generated from CORM-2. In addition, scavenging of superoxide anions limited the ability of CORM-2 to inhibit DEA/NO-stimulated cGMP formation in cells. ROS generation was also noted with the molybdenum complexes (ALF186 and ALF436). In addition, the inhibition of NO-induced cGMP formation by ALF186 and ALF436 could be rescued by tempol. It thus seems that Mo-based CORMs are not devoid of the drawbacks seen with the Ru-based compounds.

CORM-3, ALF186, ALF409, and ALF436 released hydroxyl radicals after exposure to O_2. For the Mo(0) CORMs, it is quite plausible that hydroxyl radicals originate from the metal catalyzed decomposition of O_2^- initially formed, but we have not investigated this issue further. It is worth noting that in spite of the known antioxidant properties of CO, CORM-3 has also been shown to increase O_2^- production and constrict renal arteries in a dose-dependent manner.[24] The effect of CORM-3 in renal arteries was, however, proposed to involve the activity of multiple oxidases including nitric oxide synthase, NADPH oxidase, xanthine oxidase, and complex IV of the mitochondrial electron chain. Thus, CORM-3 might generate ROS both directly and indirectly in biological systems. As sGC activity is known to depend on the existence of reduced thiol groups,[13,25] it is tempting to speculate that cGMP accumulation in cells is biphasic, showing reduction at higher CORM concentrations (see Figure 1) due to the generation of ROS from CORMs and oxidation of critical sGC cysteine residues. If ROS production from CORM is excessive, it would be possible to observe an inhibition of sGC activity that masks any activation of sGC by the CO delivered.

It has been previously reported that ruthenium complexes have a high affinity for NO and effectively scavenge NO in biological systems.[26,27] In fact, efforts to design drugs that scavenge NO and prevent some of its deleterious effects include the synthesis of structures based on Ru complexes.[28] To test whether CORM-2 quenches NO, we used 2 different approaches. Both in the NO electrode experiments and in the measurements of NO remaining in solution after incubation with CORM-2, we observed that CORM-2 scavenged NO and removed it from the solution in a time and concentration-dependent manner. Thus, when ruthenium-based CORMs are used as pharmacological tools, they would be expected to lower NO concentrations in tissues.

It has been previously reported that CORM-2 relaxes precontracted vessels.[11] In agreement with these observations, we observed a vasorelaxing effect of this CORM that was first evident at 100 μmol/L and reached an $\approx$60% decrease in vessel tone at 200 μmol/L. In addition, inhibition of sGC abolished the effects of CORM-2, indicating that sGC activation and cGMP elevation mediate the vasodilatory effect of CORM-2 in this preparation. However, in a manner analogous to that seen with recombinant sGC and cells, we also observed that CORM-2 attenuated NO donor–induced vasorelaxation. The magnitude of the inhibitory responses of CORM-2 toward NO-driven dilation varied with the donor used; responses to SNAP were affected more than those to DEA/NO. In agreement with what we observed with purified sGC and cultured cells we saw that scavenging O_2^- reduced the defect in relaxation caused by CORM-2. However, the effect of both SOD and tempol in preventing the effects of CORM-2 on NO-stimulated dilation was weaker compared with that observed with the purified enzyme or cells. Given that tissues have a higher antioxidant capacity, the inhibitory action of CORM-2 in aortic ring relaxations might be mainly due to scavenging of the NO released by CORM-2; the CORM-2:DEA/NO ratio was, for most of the concentration-response curves in the relaxation experiments, much higher than the CORM-2:NO donor ratio in the activity experiments; thus, even if ROS are removed by antioxidants, NO would still be scavenged in the organ bath experiments by CORM-2.

In summary, we have shown that CORMs do not activate purified sGC, but are capable of elevating cGMP levels in cells and causing sGC-mediated vasorelaxation. More importantly, we have shown that currently available CORMs based on transition metal carbonyl scaffolds inhibit NO-induced signaling and vasorelaxation through multiple mechanisms that include ROS generation and direct scavenging of NO. The final biological effect of any CORM would depend on the amount of CO released by the CORM, the levels of endogenously produced NO, the concentration of CORM present that might react with NO, the amount and species of ROS produced as a byproduct of CORM breakdown, and the levels of antioxidants enzymes and chemicals present in the tissues. Design of improved, "second-generation" CORMs devoid of unwanted properties (ROS generation, NO scavenging) is needed for this class of compounds to be used in establishing the therapeutic utility of CO in preclinical and clinical models.
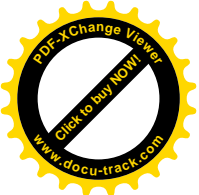
Disclosures

None.

References

1. Motterlini R, Otterbein LE. The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov*. 2010;9:728–743.
2. Wu L, Wang R. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev*. 2005;57:585–630.
3. Ryter SW, Alam J, Choi AMK. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev*. 2006;86:583–650.
4. Brouard S, Otterbein LE, Anrather J, Tobiasch E, Bach FH, Choi AM, and Soares MP. Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *J Exp Med*. 2000;192:1015–1026.
5. Durante W, Schafer A. Carbon monoxide and vascular cell function (review). *Int J Mol Med*. 1998;2:155–262.
6. Szabo S. Gaseotransmitters: new frontiers for translational science. *Sci Transl Med*. 2010;2:59ps54.
7. Motterlini R, Mann BE, Johnson TR, Clark JE, Foresti R, Green CJ. Bioactivity and pharmacological actions of carbon monoxide-releasing molecules. *Curr Pharm Des*. 2003;9:2525–2539.
8. Motterlini R, Foresti R, Green CJ. Studies on the development of carbon monoxide-releasing molecules: potential applications for the treatment of cardiovascular dysfunction. In: Wang R, ed. *Carbon Monoxide and Cardiovascular Functions*. Boca Raton, FL: CRC Press; 2002:249–271.
9. Foresti R, Hammad J, Clark JE, Johnson TR, Mann BE, Friebe A, Green CJ, Motterlini R. Vasoactive properties of CORM-3, a novel water-soluble carbon monoxide-releasing molecule. *Br J Pharmacol*. 2004;142:453–460.
10. Motterlini R, Sawle P, Hammad J, Bains S, Alberto R, Foresti R, Green CJ. CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. *FASEB J*. 2005;19:284–286.
11. Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ. Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res*. 2002;90:e17–e24.
12. Kharitonov VG, Sharma VS, Pilz RB, Magde D, Koesling D. Basis of guanylate cyclase activation by carbon monoxide. *Proc Natl Acad Sci U S A*. 1995;92:2568–2571.
13. Lucas KA, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, Waldman SA. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev*. 2000;52:375–414.
14. Ahluwalia A, Scotland RS, McLean PG, Mathur A, Perretti M, Moncada S, Hobbs AJ. Anti-inflammatory activity of soluble guanylate cyclase: cGMP-dependent down-regulation of pselectin expression and leukocyte recruitment. *Proc Natl Acad Sci U S A*. 2004;101:1386–1391.
15. Moncada S, Higgs E. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*. 1991;43:109–142.
16. Papapetropoulos A, Marczin N, Mora G, Milici A, Murad F, Catravas JD. Regulation of vascular smooth muscle soluble guanylate cyclase activity, mRNA, and protein levels by cAMP-elevating agents. *Hypertension*. 1995;26:696–704.
17. Zhao H, Joseph J, Zhang H, Karoui H, Kalyanaraman B. Synthesis and biochemical applications of a solid cyclic nitrone spin trap: a relatively superior trap for detecting superoxide anions and glutathiyl radicals. *Free Rad Biol Med*. 2001;31:599–606.
18. Santos-Silva T, Mukhopadhyay A, Seixas JD, Bernardes GJL, Romão CC, Romão MJ, CORM-3 Reactivity toward proteins: the crystal structure of a Ru(II) dicarbonyl-lysozyme complex. *J Am Chem Soc*. 2011;133:1192–1195.
19. Obirai JC, Hamadi S, Ithurbide A, Wartelle C, Nyokong T, Zagal J, Top SaB, F. UV-visible and electrochemical monitoring of carbon monoxide release by donor complexes to myoglobin solutions and to electrodes modified with films containing hemin. *Electroanalysis*. 2006;18:1689–1695.
20. Wang R, Wu L. The chemical modification of KCa channels by carbon monoxide in vascular smooth muscle cells. *J Biol Chem*. 1997;272:8222–8226.
21. Lamothe M, Chang FJ, Balashova N, Shirokov R, Beuve A. Functional characterization of nitric oxide and YC-1 activation of soluble guanylyl cyclase: structural implication for the YC-1 binding site? *Biochemistry*. 2004;43:3039–3048.
22. Stone J, Marletta M. Synergistic activation of soluble guanylate cyclase by YC-1 and carbon monoxide: implications for the role of cleavage of the iron-histidine bond during activation by nitric oxide. *Chem Biol*. 1998;5:255–261.
23. Hoenicka M, Becker EM, Apeler H, Sirichoke T, Schröder H, Gerzer R, Stasch JP. Purified soluble guanylyl cyclase expressed in a baculovirus/Sf system: stimulation by YC-1, nitric oxide, and carbon monoxide. *J Mol Med*. 1999;77:14–23.
24. Lamon BD, Zhang FF, Puri N, Brodsky SV, Goligorsky MS, Nasjletti A. Dual pathways of carbon monoxide-mediated vasoregulation: modulation by redox mechanisms. *Circ Res*. 2009;105:775–783.
25. Sayed N, Baskaran P, Ma X, van den Akker F, Beuve A. Desensitization of soluble guanylyl cyclase, the NO receptor, by S-nitrosylation. *Proc Natl Acad Sci U S A*. 2007;104:12312–12317.
26. Mosi R, Seguin B, Cameron B, Amankwa L, Darkes MC, Fricker SP. Mechanistic studies on AMD6221: a ruthenium-based nitric oxide scavenger. *Biochem Biophys Res Commun*. 2002;292:519–529.
27. Bencini A, Failli P, Valtancoli B, Bani D. Low molecular weight compounds with transition metals as free radical scavengers and novel therapeutic agents. *Cardiovasc Hematol Agents Med Chem*. 2010;8:128–146.
28. Chatterjee D, Mitra A, van Eldik R. Kinetics and mechanism of the reaction of [RuIII(edta)(H2O)]- with HOBr to form an intermediate RuV=O complex in aqueous solution. *Dalton Trans*. 2006:4691–4695.

SUPPLEMENT MATERIAL

Materials and Methods

Materials

DMEM, HBSS, and FBS were obtained from GIBCO-BRL (Paisley, UK). Cell culture flasks and plates were obtained from Greiner Labortechnik (Frickenhausen, Germany). Dulbecco's PBS, penicillin, and streptomycin were from Biochrom (Berlin, Germany). cGMP enzyme immunoassay kits were purchased from Assay Designs (Ann Arbor, MI). Tempol, L-NAME, ODQ, IBMX, S-nitroso-N-acetylpenicillamine (SNAP), 1,1-diethyl-2-hydroxy-2-nitroso-hydrazine (DEA/NO), superoxide dismutase (SOD), sodium nitroprusside (SNP), CORM-2 and all other chemical reagents were purchased from Sigma (St. Louis, MO). L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was purchased from Wako Pure Chemical Industries (Osaka, Japan). The spin trap NWT-BMPO, >99% was purchased from Northwest Life Sciences Specialties, LLC (Vancouver, WA).
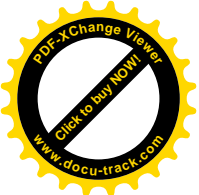
Synthesis of ALFs and detection of CO release

The metal carbonyl CO-RMs ALF186[1], ALF411[2], CORM-3[3] were synthesized according to published procedures: ALF436 was adapted from van Staveren et al[4]; ALF409 was prepared by a modification of the method reported for the analogue [Et$_4$N]$^+$ salt[5] and ALF432 was prepared in a similar way as described below.

Preparation of tricarbonyl(N-(*p*-carboxy-benzyl)bis(2-picolyl)amine)Mo(0) (ALF436)

The N-(*p*-carboxy-benzyl)bis(2-picolyl)amine ligand was prepared as follows:
To a clear THF solution of methyl-4-(bromomethyl)benzoate (1.13 g, 4.85 mmol; 229.08 g/mol) was added at once the yellow di-2(-picolyl)amine (0.903 ml; 4.85

mmol; 1.107 g/ml; 199.25 g/mol) at room temperature. The solution turns light yellow. Triethylamine (675 μL; 4.85 mmol; 0.727 g/ml; 101.19 g/mol) is added and the solution turns immediately deep yellow. The mixture was allowed to reach room temperature and subsequently filtered to remove a white precipitate. After removal of the solvent under reduced pressure, the orange oily residue was dissolved in diethyl ether (40 ml) and filtered to remove some more precipitate. Evaporation of the solvent gives a dark orange oil which was used in the next step.

¹H NMR (CDCl₃, 400 MHz, rt; δ in ppm): δ = 8.49 (d,2H), 7.98 (d,2H), 7.64 (dt,2H), 7.53 (d,2H), 7.48 (d,2H), 7.12 (tt,2H), 3.89 (s,3H), 3.81 (s,4H), 3.75 (s,2H). Note: There is a 5% of the di-2(-picolyl)amine used in slight excess;

The orange oil was dissolved in methanol (20 ml) and a solution of aqueous NaOH (0.8 g in 5 ml H₂O; 20 mmol; 40 g/mol) was added. The solution gets immediately darker and is stirred for two hours at room temperature. The pH was adjusted to 7 by dropwise addition of 2M HCl (50 ml HCl 37% in 250 ml water solution) followed by removal of the solvent under reduced pressure. The sticky yellow residue was ground with CHCl₃ (200 ml) followed by filtration to remove NaCl. Removal of the solvent under reduced pressure afforded an orange sticky oil to which acetonitrile (30 ml) was added, followed by vigorous stirring to dissolve all compound. Precipitation is seen after 15 minutes. The solution was placed in the refrigerator to effect further precipitation. The white precipitate was filtered and dried under vacuum. **Yield**: < 20%.
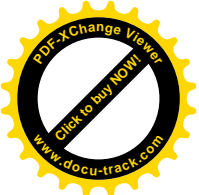
E.A. Calc. for N₃O₂C₂₀H₁₉: %C:72.05, %H:5.74, %N:12.60; Found: %C:72.57, %H:5.98, %N:13.04;

¹H NMR (CDCl₃, 400 MHz, rt, δ in ppm): δ = 8.61 (d,2H), 8.01 (d,2H), 7.70 (dt, 2H), 7.60 (d,2H), 7.44 (d,2H), 7.12 (t,2H), 3.88 (s,4H), 3.77 (s,2H).

Synthesis of the complex ALF436:

Mo(CO)₃(η⁶-C₇H₈) (0.800 g; 2.94 mmol; 272.1117 g/mol) was dissolved in 40 ml of MeOH and added to (C₅H₄CH₂)₂NCH₂C₆H₄COOH (0.980g; 2.94 mmol; 333.39 g/mol) in 25 ml of MeOH.

The red clear solution became turbid with an abundant insoluble precipitate after 5minutes. The reaction was carried on for 2h at room temperature. The precipitate

was allowed to rest and was filtered. It was washed with 25 ml of Et_2O and dried in vacuum giving a bright orange powder. **Yield:** 86%.

IR (KBr/cm^{-1}; C≡O): 1899(vs), 1759(s);

E.A. Calc. for $C_{23}H_{19}N_3MoO_5$: %C=53.81, %H=3.73, %N=8.19; Found: %C=54.01, %H=3.79, %N=7.79;

^{1}H NMR ((CD_3)$_2$CO, 400MHz, rt, δ in ppm): δ = 8.85 (d,2H); 8.14 (d,2H), 7.88 (d,2H), 7.61 (d,2H), 7.21 (d,2H), 7.11 (d,2H), 4.80 (s,2H), 4.73 (d,2H), 3.92 (d,2H).

Preparation of Na$_3$[Mo(CO)$_3$(citrate)] (ALF409)

The general procedure is presented for Na$_3$[Mo(CO)$_3$(citrate)]:

Sodium citrate dihydrate (0.844 g; 2.801 mmol; 294.1 g/mol) and (η^6-C_7H_8)Mo(CO)$_3$ (0.780 g; 2.866 mmol; 272.12 g/mol) were suspended in 100 ml of dry MeOH and stirred for 24 hours at room temperature. The light orange solution was filtered and concentrated under vacuum. This was transferred to another schlenk, partially filled with a large amount of Et_2O. A flocculate precipitate was obtained and filtered. It was washed with Et_2O and dried in vacuum to afford a light brown powder. **Yield:** 74%.

IR (KBr/cm^{-1}): 1984(w), 1901(s) (C≡O); 1754(br), 1599(br); 1410(m) (C=O);

E.A. Calc. for $MoC_9H_5O_{10}Na_3.2(CH_3OH)$: %C:26.31, %H:2.61; Found: %C:26.49, %H:2.21;

^{1}H NMR (D$_2$O, 400MHz, rt, δ in ppm): δ = 2.67 (s,1H); 2.63 (s,2H), 2.57 (s,1H), 2.53 (s,1H).

Preparation of Na$_5$[Mo(CO)$_3$(diethylenetriaminepentaacetate)] (ALF432)
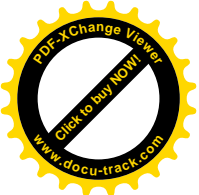
The compound was prepared as ALF409 above.

Yield: 80%;

IR (KBr/cm^{-1}):1897(s) (C≡O); 1753(br), 1600(br), 1401(m) (C=O);

E.A. Calc. for $MoC_{17}H_{18}O_{13}N_3Na_5.(CH_3OH)$: %C=30.23, %H=3.10, %N=5.87; Found: %C=29.83, %H=3.66, %N=5.66;

^{1}H NMR (D$_2$O, 400MHz, rt, δ in ppm): δ = 2.8-3.9 (broad band).

The solutions of CO-RMs were freshly prepared before the experiments by dissolving the compound in the appropriate solvent. The release of CO from the metal carbonyl complexes (ALFXXX) under normoxic conditions was assessed by a chromatographic method. Typically, 10-30mg of each compound were incubated with 3ml of RPMI/FBS containing 10% Fetal Bovine Serum, with magnetic stirring in a closed vessel at 37^0C, in the dark. The atmosphere of this vessel was CO_2 free, reconstituted air. Samples of the homogenized headspace were taken at 2hr and the amount of CO gas quantified by GC with TCD detection. Depending on the compound, the total amount of gas liberated in these experiments was in the order of 1-3 ml (Table I).

NO Detection

In the present study we assessed the effect of CORM-2 in the NO release by DEA/NO using an amperometric NO-specific probe (ISO-NOP) connected to the single channel free radical analyzer (TBR 1025, World Precision Instruments). A four channel recording system (Lab-Trax-4/16, World Precision Instruments) was used to digitize the analog signal from the probe. The ISO-NOP sensor was calibrated immediately before the experiment following the manufacturing specifications. Briefly, the calibration procedure involves two solutions (0.1M H2SO4 + 0.1 M KI, and 50μM NaNO2) and it is based on the following reaction

2NaNO2 + 2KI +2H2SO4 → 2NO +I2+ 2H2O +2K2SO4
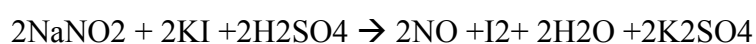
The ISO-NOP sensor was immersed in 5ml of the solution containing iodide and a positive potential of 860 mV (Ag/AgCl reference electrode) was applied. Increasing amount of $NaNO_2$ (12.5, 25, 50, 100, and 200 μl) were added to produce a known amount of NO. The concentration of NO generated can be calculated directly from the

stoichiometry since KI and H_2SO_4 are present in great excess and the reaction goes to completion. NO diffuses across the gas permeable (NO-selective) membrane and is oxidized at the working electrode surface producing a redox current which is measured. Once the calibration procedure ended the probe was immersed in 5ml of phosphate buffer at pH 7.4 and the release of NO by the NO donor DEA/NO was monitored continuously. Cumulative concentration response curves to DEA/NO were performed by adding increasing DEA/NO concentrations in presence or absence of CORM-2 (10 and 100 µmol/L) under constant nitrogen bubbling. A higher DEA/NO concentration was added once a plateau was reached (typically within 2-3min). Vehicle (DMSO) or CORM-2 were added immediately before the first addition of DEA/NO.

NO quantification in solution in the presence of CO-RM

NO in solution was determined by measuring nitrite (NO_2^-) concentration using the Griess reagent. As NO reacts with oxygen producing NO_2^- in equimolar concentrations, the reaction between NO and CORM-2 was performed in the absence of air, in deoxygenated vials using deoxygenated water. At each time point an aliquot of the reaction mixture was removed from the vials. A NO saturated solution (2 mM) was mixed with different concentrations of CORM-2 [solubilized in 10% dimethyl sulfoxide (DMSO)] in order to have the following ratios of CORM-2 to NO: 1:1 (0.5 mM CORM-2: 0.5 mM NO); 10:1 (5 mM CORM-2: 0.5 mM NO) and 20:1 (10 mM CORM-2: 0.5 mM NO). After 5-60min a sample of each reaction mixture was removed from the vial and allowed to stand in room air; some samples were incubated with nitrate reductase (0.0025U/ml nitrate reductase) for 30 min at 30ºC to convert nitrate to nitrite. After the addition of the Griess reagent, the mixtures were left at

room temperature for 10 minutes and then the absorbance of the solutions was measured at 550 nm in a microplate reader. Standard curves were constructed using $NaNO_2$.

Assessment of hydroxyl production by ALF186, ALF409 and ALF436

The formation of oxygen based radicals and ROS species in solution from the reaction between low oxidation state complexes and O_2, was tested in ESR experiments using BMPO as a spin trap [6]. A stock solution of each CO-RM was prepared by dissolving the compound in 1 mL of deoxygenated distilled water (MeOH was used in the case of ALF436) under N_2. Another stock solution of BMPO was prepared with a concentration of 250 mM. An aliquot was taken from the CO-RM solution and 100 μL from the BMPO solution to a closed vial under N_2 and deoxygenated distilled water added to perform 1 ml total volume. The final concentrations of CO-RM were 200μM whereas the final concentration of BMPO was 12.5 mM for the ALF186 experiment and 25mM for the other ALF409 and ALF436. A sample was taken from this solution and a control spectrum was acquired before bubbling O_2. Oxygen was then bubbled in the solution for 3-4 min and another sample was taken and analyzed. The experiment was performed in a quartz, planar, ESR cell, at room temperature, microwave power of 2 mW, modulation frequency 100 kHz, modulation amplitude 0,1 mT.

Aortic ring preparation

Male Wistar rats (250–300 g; Harlan, Bresso, Italy) of 8–10 wk of age were killed, and the thoracic aorta was rapidly dissected and cleaned from fat and connective tissue. Rings of 2–3mm length were cut, denuded of the endothelium and placed in

organ baths (2–5 ml) filled with oxygenated (95% O2-5% CO2) Krebs solution at 37°C and mounted to isometric force transducers (type 7006, Ugo Basile, Comerio, Italy) and connected to a Graphtec recorder (WR 3310). The composition of the Krebs solution was as follows (mol/l): NaCl 0.118, KCl 0.0047, $MgCl_2$ 0.0012, KH_2PO_4 0.0012, $CaCl_2$ 0.0025, $NaHCO_3$ 0.025, and glucose 0.010. Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 min during which tension was adjusted, when necessary, to a 0.5 g, and bathing solution was periodically changed. In a preliminary study, a resting tension of 0.5 g was found to develop the optimal tension to stimulation with contracting agents. In each experiment aortic rings were first challenged with PE (1µM) until the responses were reproducible. Aortic rings were contracted with PE (1µM). Once the plateau was reached, a cumulative concentration-response curve to DEA/NO or SNAP with or without CORM-2 was performed; in some experiments rings were exposed to increasing concentrations of CORM-2 (1µM-1mM) in the presence of ODQ (5µM). In another set of experiments, rings were incubated with 200µM CORM-2 and SNAP cumulative concentration-response curve was performed in the presence or absence of SOD (300IU/ml) or tempol (3mM).

Determination of soluble guanylyl cyclase activity

GC activity was determined by formation of [α-^{32}P]cGMP from [α-^{32}P]GTP as previously described using semi-purified rat sGC[7]. In short, reactions were performed for 10 min at 30°C in a final volume of 100 µl, in a 50 mM HEPES, pH 8.0, reaction buffer containing 500 µM GTP, 1 mM DTT, and 5 mM $MgCl_2$. Basal and DEA-NO(1µM)-stimulated guanylyl cyclase activity was measured in the presence of CO-

RMs (10 and 100µM). To inhibit ROS production SOD (100IU/ml) was added in the mixture. Typically, 5 µl of purified sGC (10 ng/µl) was used in each assay reaction.

REFERENCES

1. Beck W, Petri W, Meder J. Metal-Complexes with Biologically Significant Ligands. XVIII. Tricarbonylhistidinato Complexes of Molybdenum and Tungsten. *J. Organomet. Chem.* 1980; 191: 73-77

2. Abel EW, Bennett MA, Burton R, Wilkinson G, Transition-Metal Complexes of 7-Membered Ring Systems. Part I. The Cycloheptatriene-Metal Complexes and Related Compounds *J. Chem. Soc.* 1958; 4559-4563

3. Clark JE, Naughton P, Shurey S, Green CJ, Johnson TR, Mann BE, Foresti R, Motterlini R. Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule *Circ. Res.* 2003: 93: e2-8

4. van Staveren DR, Bothe E, Weyhermuller T, Metzler-Nolte N. Spectroscopic properties, electrochemistry, and reactivity of Mo-0, Mo-I, and Mo-II complexes with the Mo(bpa)(CO)(3) unit bpa = bis(2-picolyl)amine and their application for the labelling of peptides. *Eur. J. Inorg. Chem.* 2002: 1518-1529

5. Takuma M, Ohki Y, Tatsumi K. Molybdenum carbonyl complexes with citrate and its relevant carboxylates. *Organometallics* 2005, 24: 1344-1347.

6. Zhao H, Joseph J, Zhang H, Karoui H, Kalyanaraman B. Synthesis and biochemical applications of a solid cyclic nitrone spin trap: A relatively superior trap for detecting superoxide anions and glutathiyl radicals. *Free Rad Biol Med*. 2001; 31:599-606

7. Lamothe M, Chang FJ, Balashova N, Shirokov R, Beuve A. Functional characterization of nitric oxide and YC-1 activation of soluble guanylyl

cyclase: structural implication for the YC-1 binding site? *Biochemistry* 2004; 43: 3039-3048

Figure Legends

Figure I. **CORM-2 stimulates cGMP by releasing CO and activating sGC in cells**. Confluent RASMCs were treated with vehicle, CORM-2 10μM in the presence or absence of hemoglobin (1μM; A), or ODQ (10μM, 30min pre-treatment; B) in the presence of IBMX for 15min. In (C), cells were treated with the inactive CORM-2 (RuCl$_3$, 20μM) in the presence or absence of DEA/NO (1μM). cGMP was extracted and measured as described in Methods section. Data are expressed as percentage of respective vehicle which was set as 100%; n=4-8; *P<0.05 from vehicle, #P<0.05 from CORM-2 or DEA/NO.

Figure II. Molybdenum-based CO-releasing molecules release ROS and reduce NO-induced cGMP accumulation. ROS release by the indicated concentration of CO-RMs was measured by chemiluminescence (A and B). RASMC were treated with vehicle, CO-RM alone (100μM), DEA/NO alone (1μM, B) or a combination of the NO-donor with CO-RM in the presence of IBMX for 15min (C and D). cGMP was extracted and measured as described in Methods section. Data are expressed as percentage of respective vehicle which was set as 100%; n=8; *P<0.05 from vehicle and [#]P<0.05 from DEA/NO.

Figure III. Tempol inhibits ALF186-induced ROS production and ALF186-triggered inhibition of cGMP accumulation. cGMP accumulation was measured in RASMCs in the presence of IBMX. Cells were treated with DEA/NO (1μM) or a combination of DEA/NO with ALF186 (100μM) in the presence of tempol (30min

pretreatment; 1mM; A); n=8; *P<0.05 from vehicle and #P<0.05 from DEA/NO. ROS release by ALF186 (100µM) was measured by chemiluminescence in the absence or presence of tempol (1mM; B); n=4; *P<0.05 from vehicle; #P<0.05 from ALF186.

Figure IV. Tempol inhibits ALF436-induced ROS production and ALF436-triggered inhibition of cGMP accumulation. cGMP accumulation was measured in RASMCs in the presence of IBMX. Cells were treated with DEA/NO (1µM) or a combination of DEA/NO with ALF436 (100µM) in the presence of tempol (30min pretreatment, 1mM; A); n=8; *P<0.05 from vehicle and #P<0.05 from DEA/NO. ROS release by ALF436 (100µM) was measured by chemiluminescence in the absence or presence of tempol (1mM; B); n=4; *P<0.05 from vehicle; #P<0.05 from ALF436.

Figure V: *CORM-2 scavenges NO*. NO was mixed with CORM-2 in deoxygenated vials and after the indicated time and aliquot was removed. The amount of NO in solution was quantified by measuring nitrite formation following air exposure. NO_2^- concentration was determined using the Griess reagent in the absence (A) or presence of nitrate reductase (B). The relative concentrations of CORM-2: NO used in the experiment were: 1:1; 10:1 or 20:1. The remaining NO is solution (after the reaction with CORM-2) upon exposure to air is mainly converted to NO_2^- since treatment with nitrate reductase did not alter the levels of NO_2^- detected.

Figure VI. Tempol reduces the effect of CO-RMs on cGMP accumuation. cGMP accumulation was measured in RASMCs in the presence of IBMX. Cells were treated with DEA/NO (1µM), CORM-2 (100µM) or a combination of both in the absence (A)

or presence (B) of tempol (30min pretreatment, 1mM); n=8; *P<0.05 from vehicle, #P<0.05 from DEA/NO; p=0.05 for DEA/NO vs DEA/NO+CORM-2 in the presence of tempol.

Figure VII. **CORM-2 inhibits SNAP-stimulated relaxation**. Rings were pre-treated with CORM-2 (200μM) or vehicle for 15min and then contracted with phenylephrine; they were then exposed to increasing concentrations of the NO donor SNAP (S-nitroso-N-acetyl-penicillamine) (A). Pheylephrine-contracted rings were incubated with SOD (100IU/ml) or tempol (3mM) for 15min prior to being exposed to CORM-2 (200μM); SNAP was then added at the indicated concentration to elicit vasorelaxation. Data are mean ± SEM; n=4-6 *P<0.001 from vehicle, ^{o}P<0.001 from CORM-2.

Suppl I

Suppl II

Suppl III

Suppl IV

A

B

Suppl VI
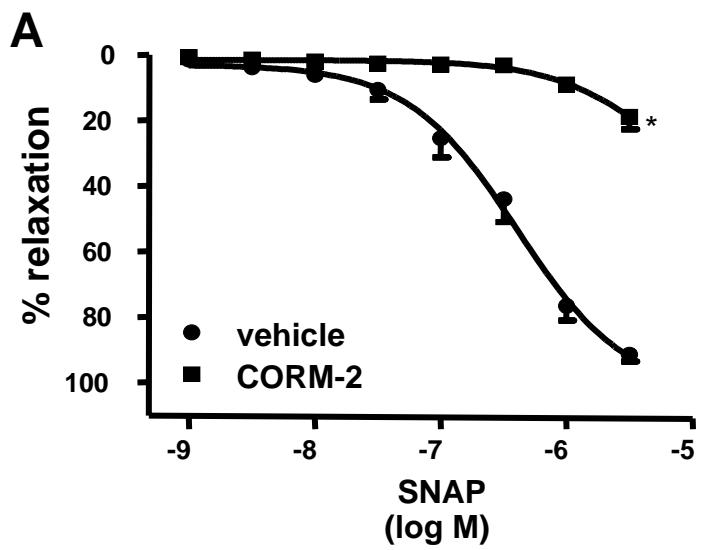
A

cGMP (% of vehicle)
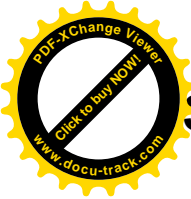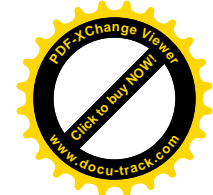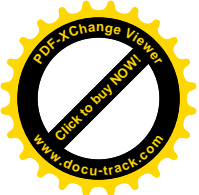
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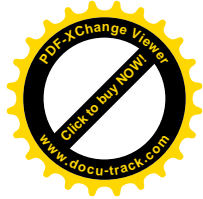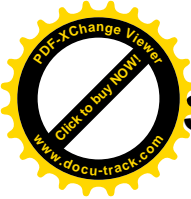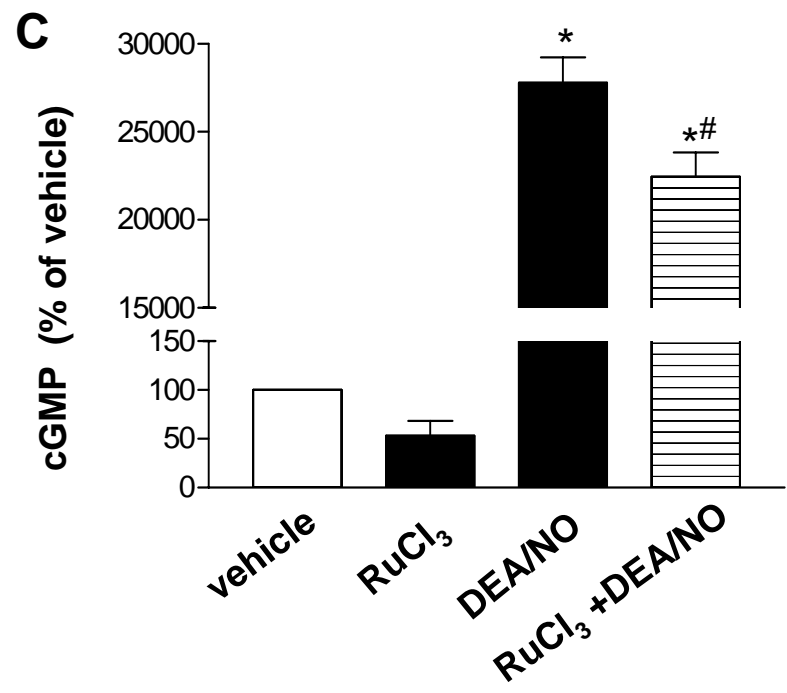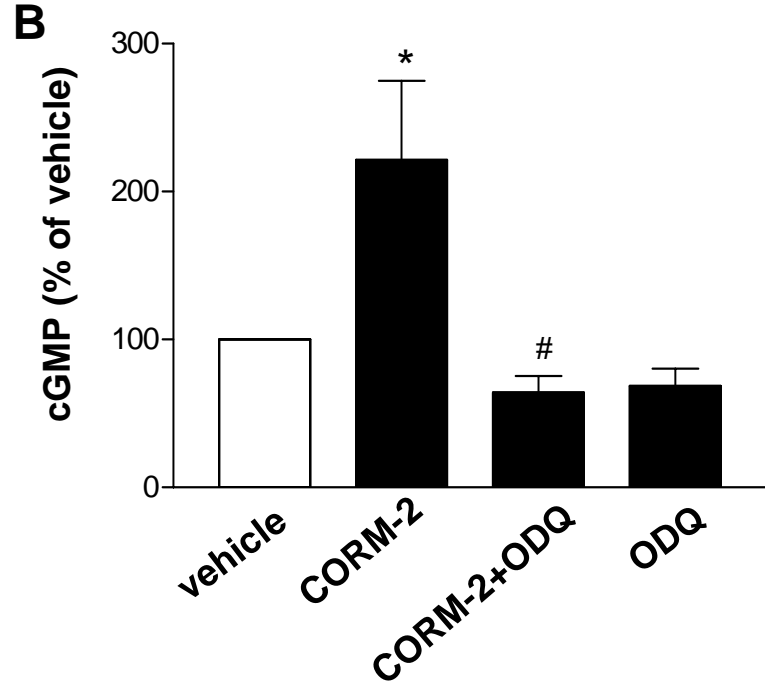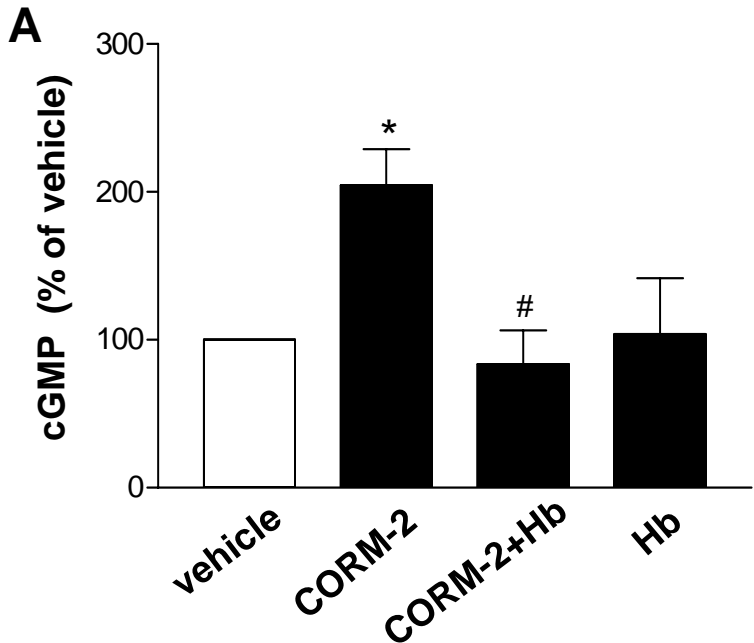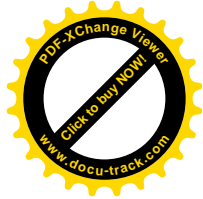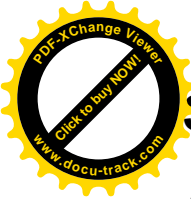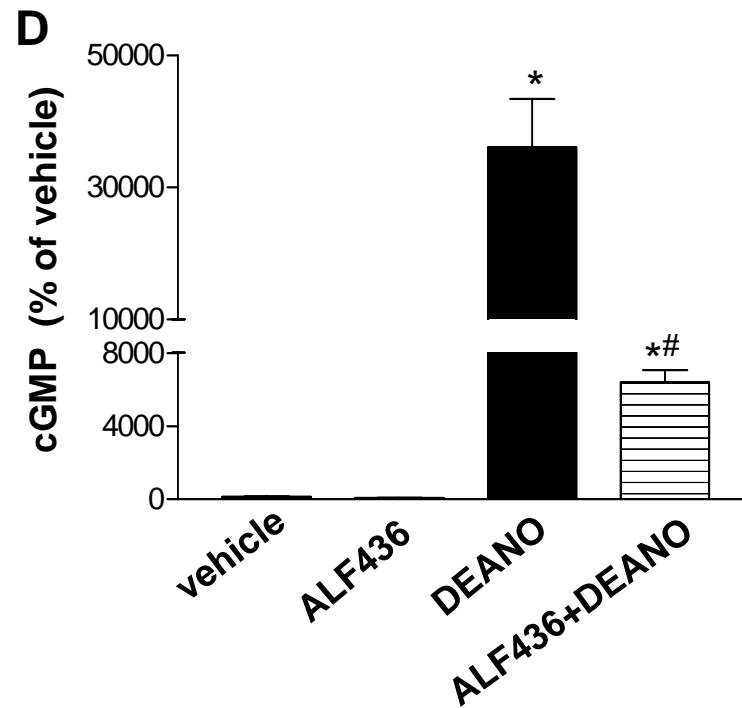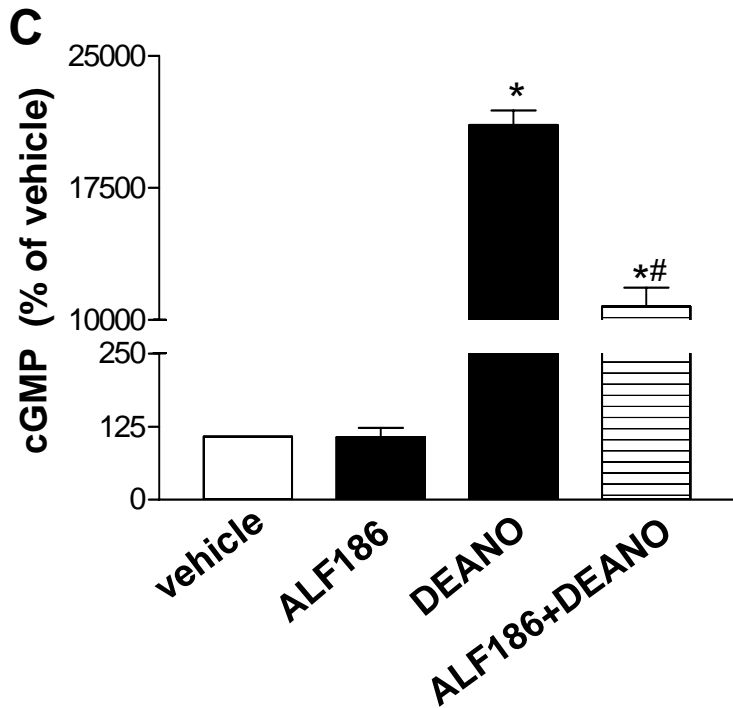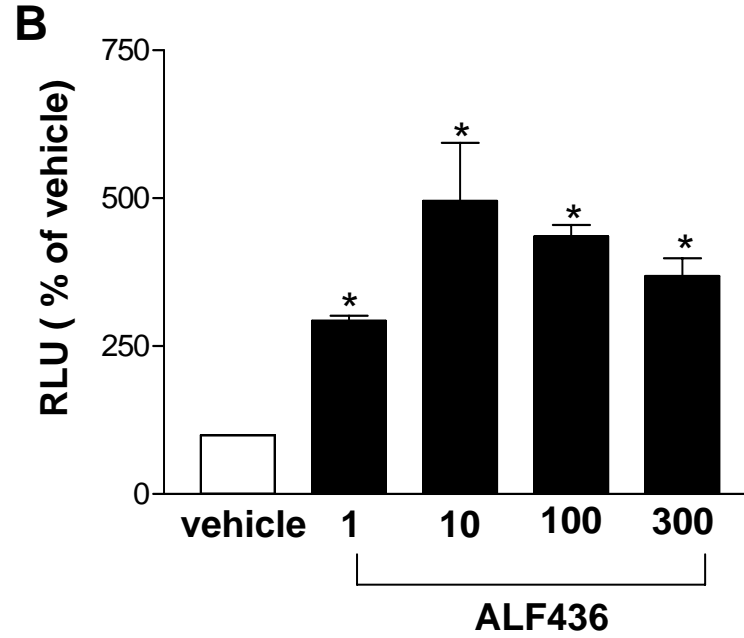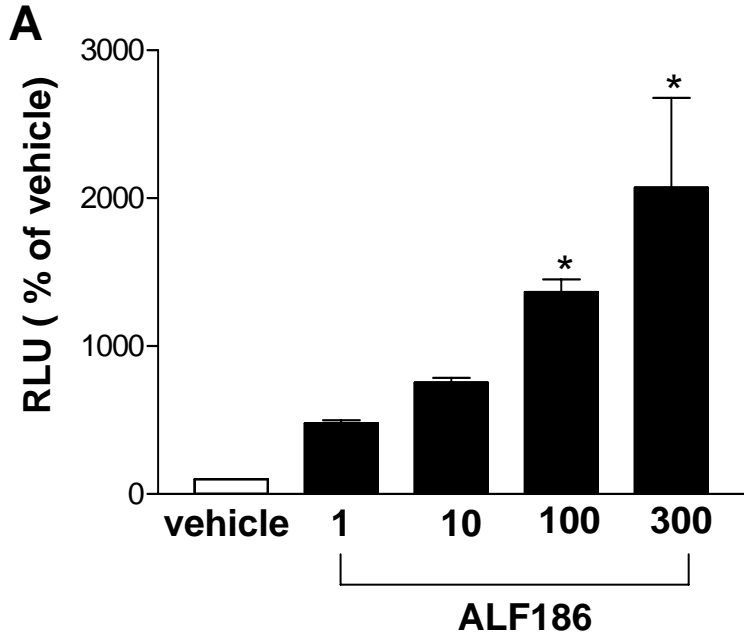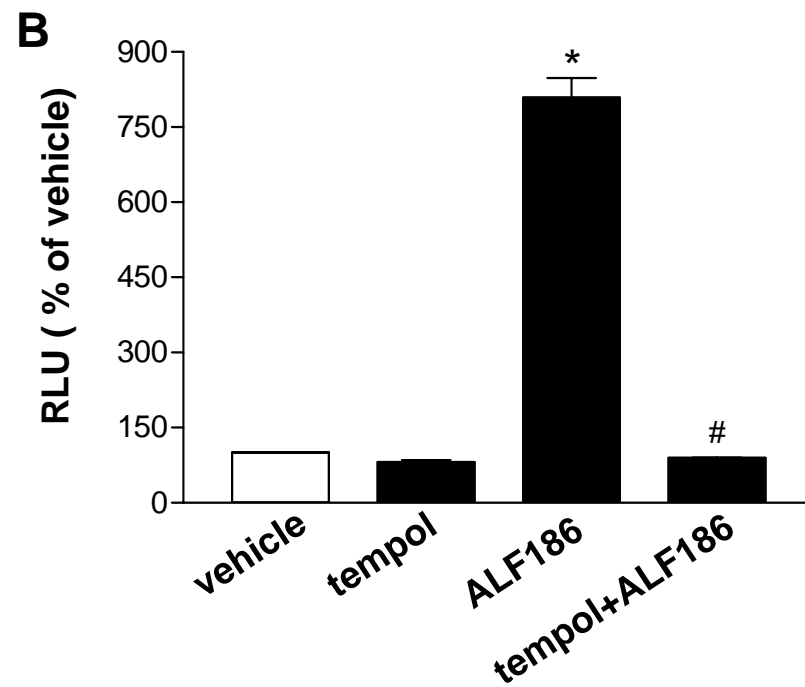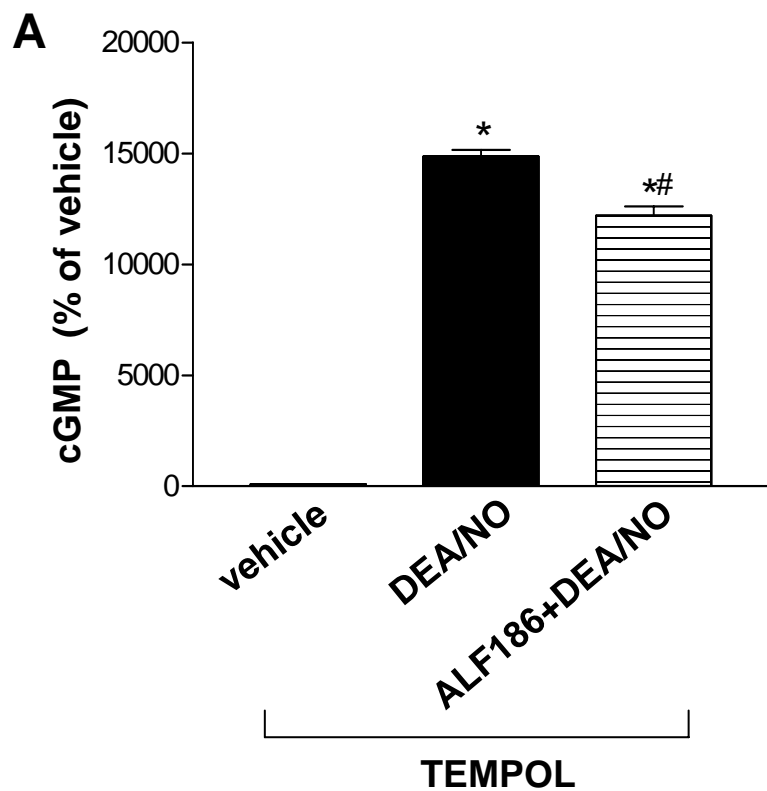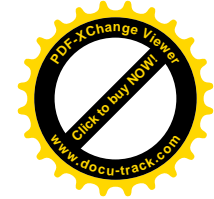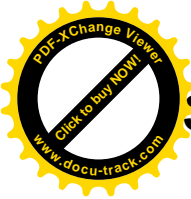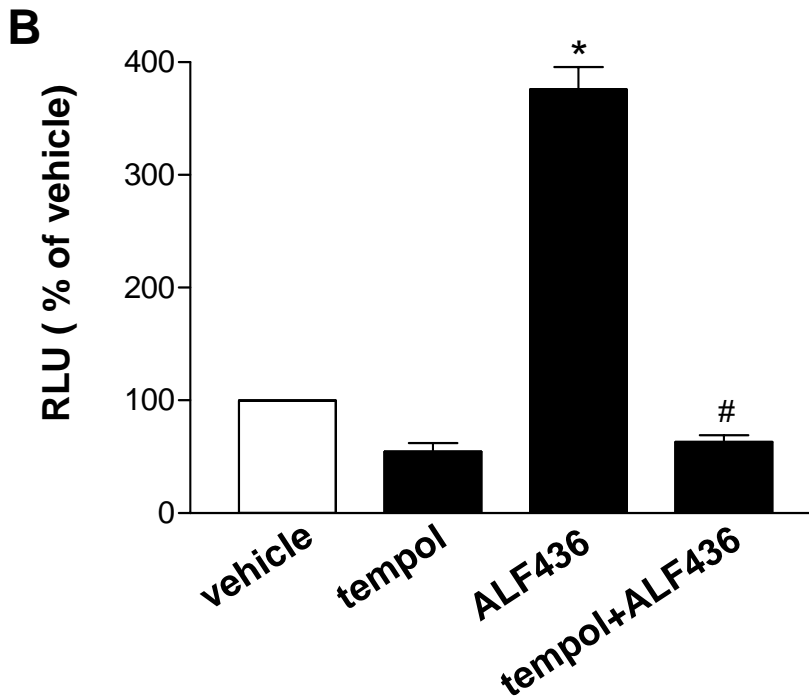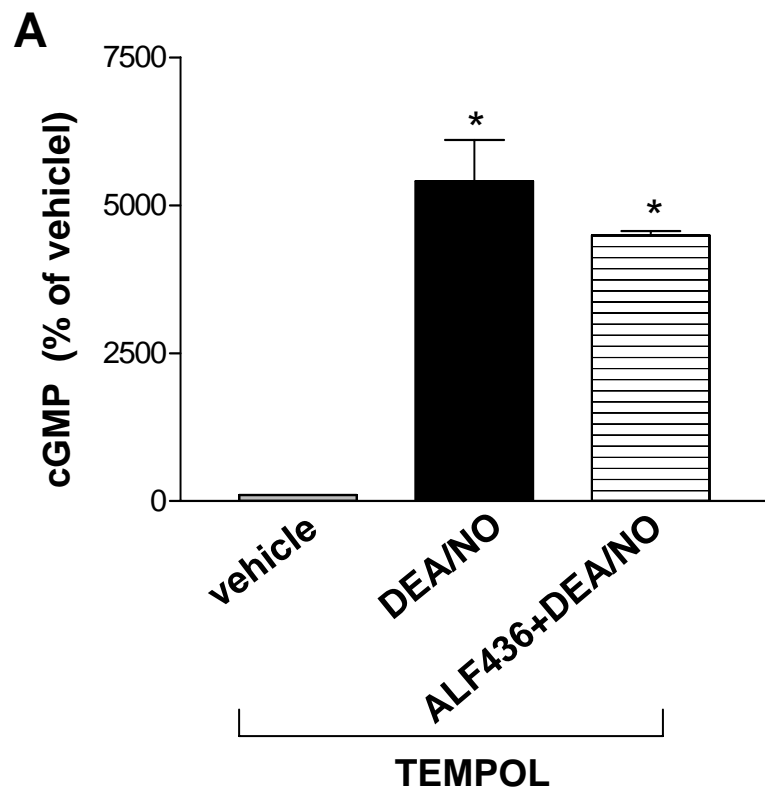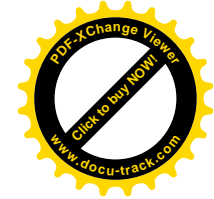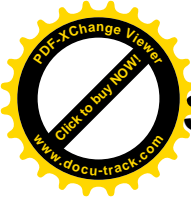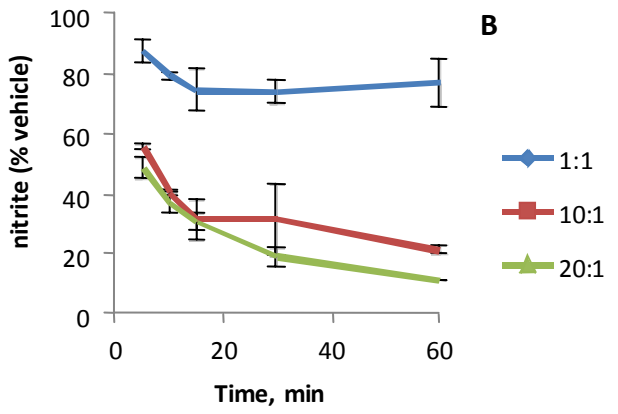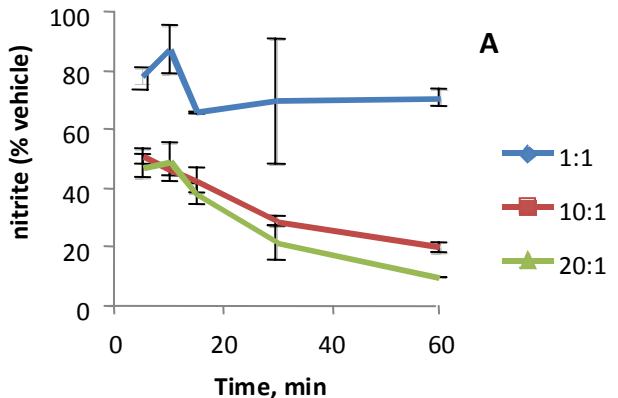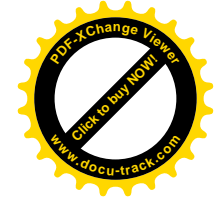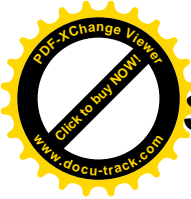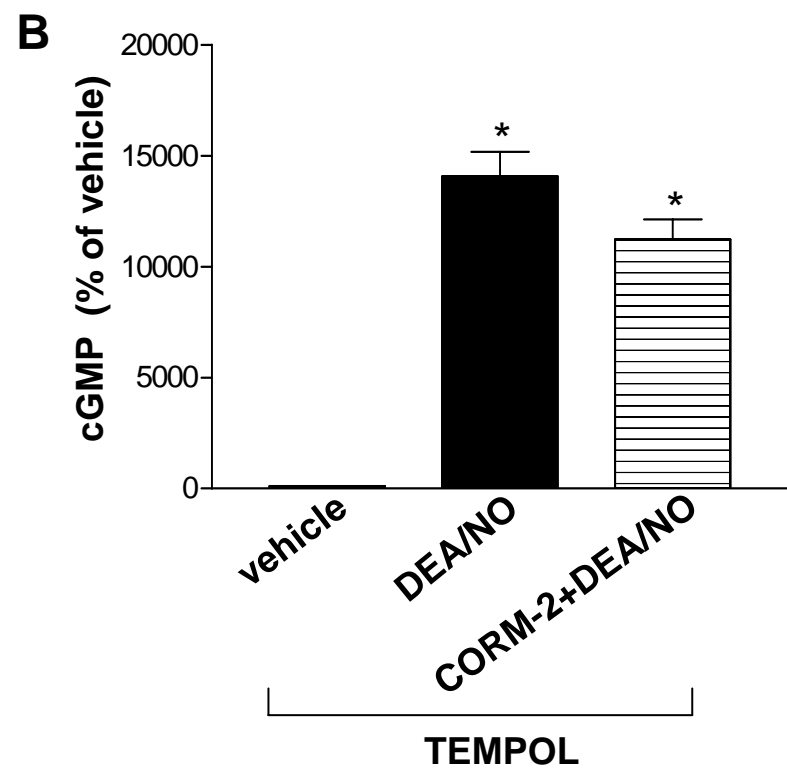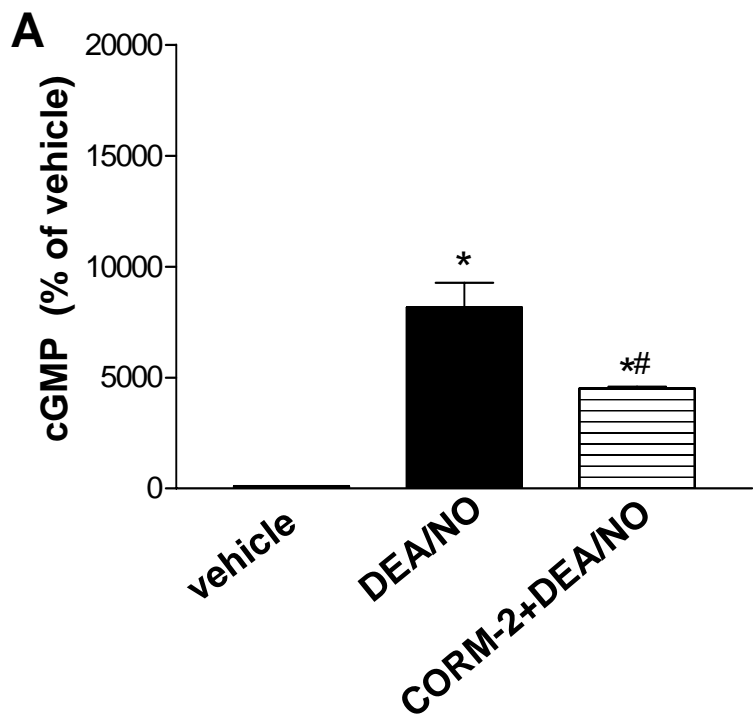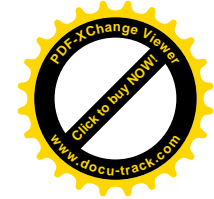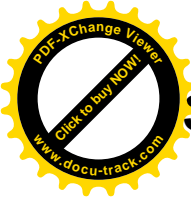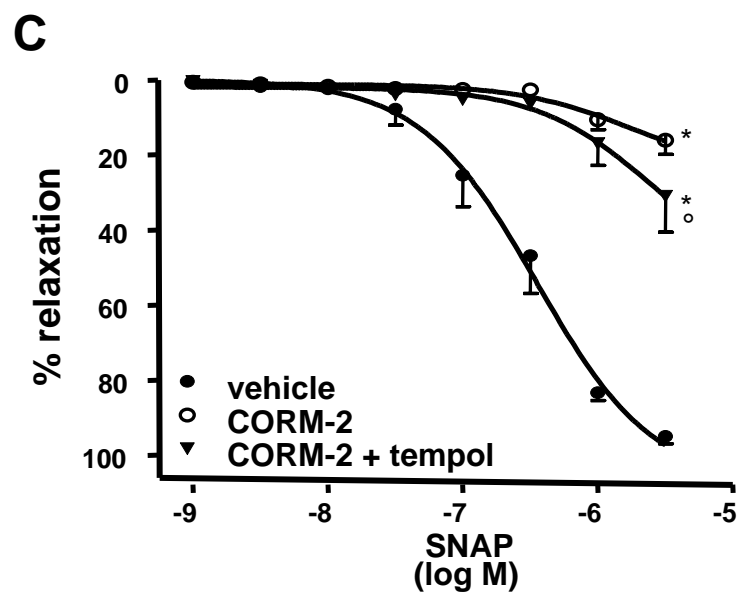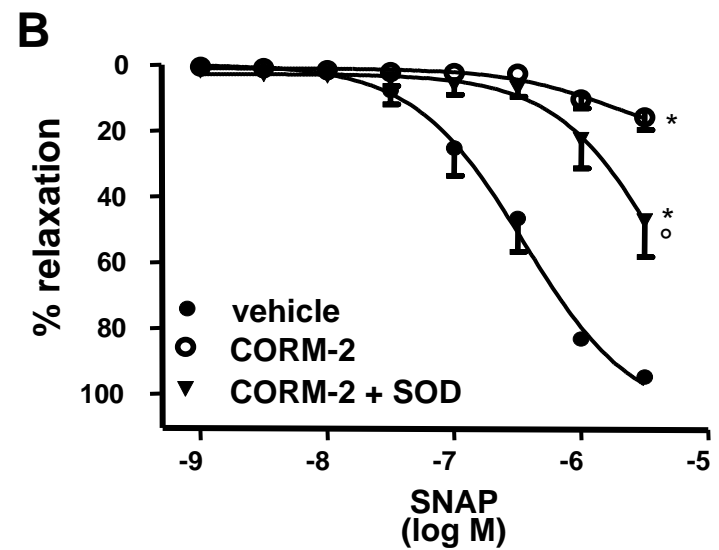
cGMP (% of vehicle)

Table I

Compound	Structure	Equiv. CO released in RPMI/PBS in air	Equiv. CO released in PBS in air	Equiv. CO released in PBS in N_2	Time/ hours	cGMP production % of vehicle 1μM	cGMP production % of vehicle 10μM
ALF 186		1.9	2.7	0	2	145.77 ± 30.7	127.78 ± 19.04
ALF409		0.9	1.3	0.1	2	93.76 ± 10.23	89.45 ± 14.39
ALF411		1.3	0.0	0.0	2	67.56 ± 11.98	84.81 ± 11.99
ALF432		1.7	3.0	0.0	2	100.19 ± 2.34	86.60 ± 5.86
ALF436		0.9	1.5	0.0	2	335.28* ± 108.57	152.42 ± 20.29
CORM-2		0.0	0.0	--	2	231.32 ± 31.91	298.51* ± 82.82
CORM-3		0.0	0.0	--	2	87.75 ± 8.14	102.53 ± 12.3

Table I. **Spontaneous Release of CO and cGMP production by CO-RMs.**

Molar equivalents of CO released by the indicated CO-RMs in RPMI/FBS or PBS. Experiments were performed at room temperature under normoxic and anoxic conditions. CO released in the headspace of a closed vial was determined by a gas chromatographic method that uses a thermal conductivity detector (GC-TCD). Confluent RASMC were treated with vehicle or a CO-releasing molecule (1 and 10μM) in the presence of IBMX for 15min. cGMP was extracted and measured as described. Data are expressed as percentage of respective vehicle which was set as 100%; n=8-12; *P<0.05 from vehicle.