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Abstract

Carbon monoxide (CO) is emerging as an important and versatile mediator of physiological processes to the extent that treatment of animals with exogenous CO gas has beneficial effects in a range of vascular- and inflammatory-related disease models. The recent discovery that certain transition metal carbonyls function as CO-releasing molecules (CO-RMs) in biological systems highlighted the potential of exploiting this and similar classes of compounds as a stratagem to deliver CO for therapeutic purposes. Here we describe the biochemical features and pharmacological actions of a newly identified water-soluble CO releaser (CORM-A1) that, unlike the first prototypic molecule recently described (CORM-3), does not contain a transition metal and liberates CO at a much slower rate under physiological conditions. Using a myoglobin assay and an amperometric CO electrode, we demonstrated that the release of CO from CORM-A1 is both pH- and temperature-dependent with a half-life of approximately 21 min at 37 degrees C and pH 7.4. In isolated aortic rings, CORM-A1 promoted a gradual but profound concentration-dependent vasorelaxation over time, which was highly amplified by YC-1 (1 microM) and attenuated by ODQ, a stimulator and inhibitor of guanylate cyclase, respectively. Similarly, administration of CORM-A1 (30 micromol/kg i.v.) in vivo produced a mild decrease in mean arterial pressure, which was markedly potentiated by pretreatment with YC-1 (1.2 micromol/kg i.v.). Interestingly, an inactive form of CORM-A1 that is incapable of releasing CO failed to promote both vasorelaxation and hypotension, thus directly implicating CO as the mediator of the observed pharmacological effects. Our results reveal that the bioactivities exerted by CORM-A1 reflect its intrinsic biochemical behavior of a slow CO releaser, which may be advantageous in the treatment of chronic conditions that require CO to be delivered in a carefully controlled manner.

CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule

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ABSTRACT

Carbon monoxide (CO) is emerging as an important and versatile mediator of physiological processes to the extent that treatment of animals with exogenous CO gas has beneficial effects in a range of vascular- and inflammatory-related disease models. The recent discovery that certain transition metal carbonyls function as CO-releasing molecules (CO-RMs) in biological systems highlighted the potential of exploiting this and similar classes of compounds as a stratagem to deliver CO for therapeutic purposes. Here we describe the biochemical features and pharmacological actions of a newly identified water-soluble CO releaser (CORM-A1) that, unlike the first prototypic molecule recently described (CORM-3), does not contain a transition metal and liberates CO at a much slower rate under physiological conditions. Using a myoglobin assay and an amperometric CO electrode, we demonstrated that the release of CO from CORM-A1 is both pH- and temperature-dependent with a half-life of ~21 min at 37°C and pH 7.4. In isolated aortic rings, CORM-A1 promoted a gradual but profound concentration-dependent vasorelaxation over time, which was highly amplified by YC-1 (1 μM) and attenuated by ODQ, a stimulator and inhibitor of guanylate cyclase, respectively. Similarly, administration of CORM-A1 (30 μmol/kg i.v.) in vivo produced a mild decrease in mean arterial pressure, which was markedly potentiated by pretreatment with YC-1 (1.2 μmol/kg i.v.). Interestingly, an inactive form of CORM-A1 that is incapable of releasing CO failed to promote both vasorelaxation and hypotension, thus directly implicating CO as the mediator of the observed pharmacological effects. Our results reveal that the bioactivities exerted by CORM-A1 reflect its intrinsic biochemical behavior of a slow CO releaser, which may be advantageous in the treatment of chronic conditions that require CO to be delivered in a carefully controlled manner.

Key words: sodium boranocarbonate • CO electrode • vascular tone • blood pressure • heme oxygenase-1

Despite being notorious for its insidious and toxic effects, carbon monoxide (CO) is now recognized as a ubiquitous cellular mediator capable of controlling fundamental physiological and signaling processes in mammalian tissues (1). The paradigm of a potentially noxious molecule playing a crucial biological role at low concentrations should not be totally unexpected as CO is continuously generated in the cell through the degradation of heme by constitutive (HO-2) and inducible (HO-1) heme oxygenase proteins (2) and the local production of CO can be markedly increased upon activation of HO-1 (3, 4). This sensitive inducible enzyme, which beside CO generates the antioxidants biliverdin/bilirubin, is now regarded as an obligatory protective system necessary to restore the redox imbalance inflicted on cells and tissues by various forms of oxidative and nitrosative stress (5–10). Notably, the pleiotropic properties attributed to HO-1 within the cardiovascular, nervous, and immune systems dovetail with the multiplicity of activities that so far has also been ascribed to CO. Endogenous CO exerts vasodilatory effects through stimulation of soluble guanylate cyclase (4, 11, 12) and appears to alleviate acute hypertension in vivo as well as vascular resistance in the hepatic circulation (3, 13). Moreover, HO-1-derived CO controls the proliferation of vascular smooth muscle cells (14, 15), prolongs the survival of transplanted organs (16), and improves vascular reactivity of the mesenteric circulation in hepatic cirrhosis (17). The biological effects mediated by endogenous CO can be simulated when this gas is applied exogenously; this strategic approach revealed a variety of beneficial actions of CO, including potent anti-inflammatory and anti-apoptotic effects (18–20), suppression of atherosclerotic lesions following aortic transplantation (21), prevention of reperfusion-induced ventricular fibrillation in the myocardium (22), and protection against ischemic lung injury (23). The encouraging outcome of these studies led scientists to the tantalizing concept that CO could be used for therapeutic purposes in the treatment of vascular dysfunction and inflammatory disease states.

The discovery of compounds capable of carrying and delivering CO to tissues and organs would undoubtedly accelerate and facilitate the development of novel pharmaceutical agents suitable for therapeutic applications (24–26). Ideally, this stratagem could offer significant advantages over inhalation of CO gas as it may circumvent the problems related to the systemic effects imposed by CO on oxygen transport and delivery (27). Indeed, the therapeutic use of gas mixtures poses obvious limitations, not least the difficulty of storing and delivering CO in a controlled-directed fashion. To avoid these practical obstacles, we have focused on developing molecules having the inherent ability to liberate CO under appropriate conditions and function as CO-releasing molecules (CO-RMs) in biological systems. The first class of such compounds we evaluated are a group of transition metal carbonyls (28). Specifically, two carbonyl complexes initially identified as possessing CO-releasing properties were manganese decacarbonyl ($[Mn_2(CO)_{10}]$) and tricarbonyldichlororuthenium(II) dimer ($[Ru(CO)_3Cl_2]_2$) (termed CORM-1 and CORM-2, respectively [24]). Although these two compounds are soluble only in organic solvents and CORM-1 requires irradiation to induce CO loss, they both proved to be pharmacologically active by exerting effects that are typical of CO gas, including vessel relaxation, attenuation of coronary vasoconstriction, and suppression of acute hypertension (29).

Subsequently, we made further progress by synthesizing the first prototype of a water-soluble CO-RM; this was attained primarily to overcome the solubility constraints and the fact that the majority of carbonyl-based compounds described in the literature requires physical (e.g., irradiating light) or chemical (e.g., ligand substitution) stimuli to promote CO dissociation (28). Tricarbonylchloro(glycinato)ruthenium(II) (CORM-3), which can be obtained by coordinating

the amino acid glycine onto the metal center, is fully soluble in water and rapidly liberates CO in vitro, ex vivo, and in vivo biological models (24). Interestingly, based on the evidence that CO gas is beneficial against oxidative-stress-mediated damage (20) and prevents organ rejection following transplantation (16), we and our collaborators reported that CORM-3 protects myocardial tissues against ischemia-reperfusion injury both ex vivo (30) and in vivo (31) and prolongs the survival of cardiac allografts in mice (30). More recently, we have provided evidence for important vasodilatory properties of CORM-3 through mechanisms that involve guanylate cyclase and potassium channel activation (32). Thus, initial studies confirmed that transition metal carbonyls are versatile complexes that can be chemically modified to meet the criteria of biologically active CO carriers.

In our persistent search for agents that could be safely used as CO-RMs in biological systems, we have identified sodium boranocarbonate ($Na_2[H_3BCO_2]$, here termed CORM-A1) as an extremely promising water-soluble compound that spontaneously liberates CO in aqueous solutions. CORM-A1 differs from the original CO-RMs as it does not contain a transition metal and releases CO with a slower rate compared with CORM-3. Here we report on the biochemical reactivity and pharmacological effects of CORM-A1 and discuss how its properties of a "slow" CO releaser could be exploited in a therapeutic context.

MATERIALS AND METHODS

Preparation of chemicals and solutions

CORM-A1 was prepared as described previously by Alberto and coworkers (33). CORM-3 was synthesized as recently reported by our group (30, 32). Stock solutions of CORM-3 and CORM-A1 (10–100 mM) were freshly prepared before the experiments by dissolving the compounds in pure distilled water. In our preliminary tests, we noticed that acidic pHs significantly accelerate the spontaneous release of CO from CORM-A1. We therefore took advantage of this specific property of CORM-A1 and generated a CO-depleted inactive form (iCORM-A1) to be used as a negative control by initially dissolving CORM-A1 in 0.1 M HCl and then bubbling pure N_2 through the solution for 10 min in order to remove the residual CO gas. The solution of iCORM-A1, which is probably predominantly sodium borate (33), was finally adjusted to pH 7.4 and tested with the myoglobin assay (see below) before each experiment to verify its inability to liberate CO. 1H-(1,2,4)oxadiazole(4,3-a)quinoxalin-1-one (ODQ), glibenclamide, and 3-(5′-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) were from Alexis Corporation (Nottingham, UK). All other chemicals used in the study were from Sigma (St. Louis, MO), unless otherwise specified.

Detection of CO release using the myoglobin assay

The release of CO from CORM-A1 was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO) as previously reported by our group (24, 29, 30). A small aliquot of concentrated CORM-A1 solution was added to 1 ml deoxy-Mb solution in phosphate buffer (final concentrations: CORM-A1 60 μM; deoxy-Mb 53 μM), and changes in the Mb spectra were recorded over time. The amount of MbCO formed was quantified by measuring the absorbance at 540 nm (extinction coefficient = 15.4 M^{-1} cm^{-1}). To examine the effect of pH on the rate of CO liberation from CORM-A1, we

conducted experiments using solutions of myoglobin in 0.04 M phosphate buffer prepared at different pHs (7.4, 7.0, 6.5, and 5.5). The amount of MbCO formed was plotted over time, and the half-life of CORM-A1 at different pHs and temperatures was calculated from the fitted curves.

Detection of CO release using an amperometric CO sensor

The release of CO from CORM-A1 was also detected using a prototype electrode purchased from World Precision Instrument (WPI, Stevenage, Herts, UK). This CO electrode is a membrane-covered amperometric sensor that has been designed on a basic operating principle similar to the nitric oxide (NO) sensor. The CO sensor can be connected to the WPI ISO-NO Mark II meter for detection of the current signals, providing that the poise potential is set to a different value (900 mV for CO as opposed to 860 mV for NO). In principle, CO diffuses through the gas-permeable membrane and is then oxidized to CO_2 on the working electrode. This oxidation will create a current whose magnitude can be related directly to the concentration of CO in solution. The CO sensor was used to generate standard curves and calculate the rates of CO release from CORM-A1 at different pHs and temperatures. The electrode was immersed into the solutions at different pHs and equilibrated for 30 min before addition of CORM-A1. For the experiments conducted at 37, 30, 25, and 20°C, the solutions were maintained at the desired temperature using a Grant W6 thermostat (Cambridge).

Measurement of carboxyhemoglobin (HbCO) levels in human blood exposed to CORM-A1

Blood from a healthy male volunteer was collected in tubes containing EDTA, aliquoted in four Eppendorff tubes, and transferred to a water bath at 37°C. Levels of carboxyhemoglobin (HbCO) were measured 15 or 45 min after addition of increasing concentrations of CORM-A1 (0, 4.5, 8, and 18 mM) using a blood gas analyzer (Omni Modular System, Roche, Lewes, UK). In a different set of experiments, the formation of HbCO was also evaluated in a time-dependent manner after addition of 18 mM CORM-A1.

Aortic ring preparation

Transverse ring sections prepared from aortas of male adult Sprague Dawley rats (350 g) were suspended under 2 g tension in oxygenated Krebs Henseleit buffer as described previously (4, 29, 32). To establish the potential vasorelaxant effects of CORM-A1, we precontracted aortic rings with phenylephrine (1 μM) before addition of the compound at different concentrations (40–160 μM). The extent of vasorelaxation over time was determined and compared with the effect produced by CORM-3. In another set of experiments, the inactive form (iCORM-A1) and sodium borohydride (NaBH$_4$) were used as negative controls to exclude the possibility that vasorelaxation was a nonspecific effect of boron compounds or borate. Additional experiments were conducted to examine the involvement of cGMP and ATP-dependent potassium channels (K_{ATP}) in the relaxation mediated by CORM-A1. For this, aortic rings were incubated with the inhibitor of guanylate cyclase ODQ (30 μM) or the K_{ATP} blocker glibenclamide (10 μM) before addition of phenylephrine, respectively. To investigate the possible involvement of NO in the relaxation elicited by CORM-A1, a separate group of experiments was performed by treating aortic rings with the NO synthase inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME, 100 μM) 30 min before addition of phenylephrine. CORM-A1 was also tested in endothelium-denuded

rings. For this, the internal lumen of rings was subjected to gentle rubbing with a fine wooden stick and failure to dilate upon addition of acetylcholine was taken as a proof of successful endothelium removal. The effect of CORM-A1 on vascular tone was also assessed in the presence of the stimulator of soluble guanylate cyclase, YC-1 (1 μM), which was added 30 min before phenylephrine. Previous experiments from our group have established that 1 μM YC-1 does not significantly affect the response of aortic rings to phenylephrine (32). The relaxation responses were expressed as percentage of the maximal contraction elicited by phenylephrine.

Blood pressure monitoring in vivo

Anesthetized adult Lewis rats (290–330 g) were chronically catheterized, and blood pressure was continuously monitored as previously reported (3, 29). Animals were anaesthetized using enfluorane as an inhalation anesthetic. Surgical anesthesia was maintained throughout the operation using oxygen in combination with enfluorane. Changes in mean arterial pressure (MAP) were recorded over time following an intravenous injection of CORM-A1 (30 μmol/kg). Similar experiments were performed by administering YC-1 (1.2 μmol/kg, i.v.) to animals 5 min before the injection of CORM-A1 or the inactive compound (iCORM-A1).

Statistical analysis

Statistical analysis was performed using one-way or two-way ANOVA combined with Bonferroni test. Differences were considered to be significant at $P < 0.05$.

RESULTS

CORM-A1 liberates CO in a pH- and temperature-dependent manner

The spectrophotometric assay that detects the formation of carbonmonoxy myoglobin (MbCO) from deoxy-Mb has been shown to be a reliable method for assessing the extent and kinetic of CO liberation from CO-RMs (24, 29, 30). The conversion of deoxy-Mb to MbCO can be followed over time by measuring the changes in the absorption spectra of this protein. As shown in Figure 1A (see curve with filled square), the addition of 60 μM CORM-A1 ($Na_2[H_3BCO_2]$) to a phosphate buffer solution containing Mb at 37°C and pH 7.4 resulted in the gradual change of the deoxy-Mb spectrum, which has a maximal absorption peak at 560 nm, into spectra typical of MbCO. The Mb appears to be fully saturated 2 h after addition of CORM-A1 (see curve with filled diamond in Fig. 1A). Interestingly, the time required to fully saturate Mb with CO liberated from CORM-A1 gradually decreased by lowering the pH to 7.0, 6.5, and 5.5 (Fig. 1B–D), indicating that the rate of CO release from CORM-A1 is pH-dependent.

From the curves fitted to the spectral data (Fig. 2A) we can calculate that the half-lives of CORM-A1 at 37°C are as follows: 21 min at pH 7.4, 7.1 min at pH 7.0, 3.3 min at pH 6.5, and 2.5 min at pH 5.5. Predictably, iCORM-A1 did not generate any MbCO (see line with open square in Fig. 2A). We have also found that the rate of MbCO formation from CORM-A1 decreases by gradually lowering the temperature of the solutions (data not shown). Because CO is promptly liberated to Mb at pH 5.5, we used these conditions to generate a standard curve that clearly indicates that the conversion of the carboxyl group from $Na_2[H_3BCO_2]$ to CO goes to completion as one mole of CO is formed per mole of CORM-A1 (Fig. 2B, 2C).

CORM-A1 was also tested for its ability to liberate CO using an amperometric electrode sensitive to CO. Figure 3 reveals that the results obtained with the CO sensor are in good accordance with the ones found with the myoglobin assay. From an initial test (see Fig. 3A), it can be observed that the rate of CO release from 100 μM CORM-A1 at 37°C is much faster at pH 5.5 ($t_{1/2}$=2.01 min) than pH 7.4 ($t_{1/2}$=27.06 min), and the calculated half-lives are comparable to the ones obtained with the myoglobin assay. As expected, the CO electrode was completely insensitive to iCORM-A1, which does not release CO (Fig. 3A). A standard curve generated at pH 5.5 using CORM-A1 in a range between 10 and 50 μM indicated a good linear correlation (R^2=0.998) between the concentrations used and the electrode response (Fig. 3B). Therefore, a concentration of 20 μM CORM-A1 was subsequently used to calculate the rate of CO dissociation from CORM-A1 at different pHs and temperatures. From the curves shown in Figure 3C, we can calculate that the dissociation rate constants of CO and half-lives of CORM-A1 at 37°C are as follows: 0.55×10^{-3} s^{-1} and 21.06 min at pH 7.4; 5.8×10^{-3} s^{-1} and 1.96 min at pH 5.5; 11.0×10^{-3} s^{-1} and 1.02 min at pH 4.0. In addition, we found that the rate of CO generation from CORM-A1 is strictly temperature-dependent as already indicated by the myoglobin assay. Specifically, from the curves shown in Figure 3D, we can calculate that the initial rate of CO release at pH 5.5 is 6.84 μmol/min at 37°C, 3.83 μmol/min at 30°C, 2.16 μmol/min at 25°C, and 1.22 μmol/min at 20°C. Thus, the spontaneous liberation of CO from CORM-A1 in aqueous solutions is accelerated by decreasing the pH and increasing the temperature.

Formation of carboxyhemoglobin (HbCO) in isolated human blood incubated with CORM-A1

The average concentration of human hemoglobin in male subjects is 15 g/dl, which is equivalent to 9.6 mM heme. This justifies the choice of mM concentrations of CORM-A1 used in order to properly evaluate the kinetics of HbCO formation. As shown in Figure 4A, incubation of human blood with CORM-A1 at room temperature resulted in a concentration-dependent increase in the levels of HbCO over time. The prolonged release of CO from CORM-A1 is emphasized in the data showing that the HbCO levels measured 15 min after addition of any concentration of CORM-A1 used are much lower than those measured after 45 min. In fact, when HbCO levels were determined at regular intervals over a 2-h period after addition of 18 mM CORM-A1, a gradual and time-dependent formation of HbCO was observed (Fig. 4B), with a kinetic very similar to the ones observed using the Mb assay. Note that the concentrations of CORM-A1 needed to produce substantial increases in HbCO are in the mM range, while the pharmacological effects in terms of vessel relaxation and decrease in MAP (see results reported below) are observed when CORM-A1 is used at low micromolar concentrations.

Vasodilatory effects of CORM-A1 in isolated aortic rings

CORM-3 has been shown to promote a rapid and significant relaxation in isolated vessels, and this effect has been demonstrated to be mediated by CO (24, 32). Figure 5A shows typical isometric recordings from aortic rings precontracted with phenylephrine (Phe) and treated with either CORM-3 or CORM-A1. It can be observed that CORM-A1 (80 μM) caused a profound but much slower dilatory effect compared with CORM-3 (80 μM). Specifically, CORM-3 (solid line) promoted >50% relaxation within 4–5 min whereas CORM-A1 (dashed line) caused a gradual vasorelaxation, which was maximal (96%) 33 min following addition of the compound

to the organ bath. Precontracted aortic rings were also treated with increasing concentrations of CORM-A1 (40, 80, and 160 µM), and vasorelaxation was measured at different time points. As shown in Figure 5B, CORM-A1 caused a significant relaxation over time in a concentration-dependent manner. For instance, the extent of relaxation elicited by the different concentrations of CORM-A1 after 10 min was as follows: $21.0 \pm 2.3\%$ with 40 µM CORM-A1, $40.2 \pm 3.4\%$ with 80 µM CORM-A1, and $74.9 \pm 1.8\%$ with 160 µM CORM-A1. The maximal relaxation mediated by CORM-A1 was reached after 30 min; the rings spontaneously recontracted again thereafter, but no vasodilatory effect was observed if a new addition of CORM-A1 was performed immediately after recontraction (data not shown). Precontracted aortic rings were also treated with 80 µM iCORM-A1 (the inactive compound) or sodium borohydride ($NaBH_4$), which was used as an additional negative control to exclude any effect of boron on vessel tone. As shown in Figure 6A, 6B, CORM-A1 promoted a gradual and profound vasorelaxation, whereas both iCORM-A1 and $NaBH_4$ were totally ineffective. Moreover, the addition of myoglobin (100 µM) to the organ bath significantly attenuated the vasorelaxation mediated by CORM-A1 (Fig. 6C). Collectively, these results strongly suggest that CO liberated from CORM-A1 is directly responsible for the observed pharmacological effect.

CO-mediated vasorelaxation by CORM-A1: possible mechanisms of action

It is well-established that CO, either endogenously generated from HO-1, exogenously applied as CO gas, or delivered by CO-RMs to vascular tissues, activates guanylate cyclase to increase the production of cGMP (4, 29, 32). A recent report suggested the involvement of ATP-dependent (K_{ATP}) potassium channels as part of the dilatory mechanisms exerted by CORM-3, a ruthenium-based water-soluble CO carrier (32). In this study, the vasodilatation caused by CORM-A1 was partially inhibited in the presence of a guanylate cyclase blocker (ODQ, 30 µM) but was not affected by glibenclamide (GLI, 10 µM), an inhibitor of K_{ATP} potassium channels (see Fig. 7A). Aortic rings were also pretreated with L-NAME (100 µM) to examine the contribution of the NO synthase pathway in CORM-A1-mediated vasorelaxation. Apart from an initial $17 \pm 2.4\%$ attenuation of the vasodilatory response to CORM-A1 in the first 10 min, the presence of L-NAME did not considerably change the effect of CORM-A1 thereafter (Fig. 7B). Similarly, removal of the endothelium did not alter the potency of relaxation responses to CORM-A1 (Fig. 7C). These data together suggest that NO and/or factors derived from the endothelium barely contribute to the action of CORM-A1 and cooperate with CO in the regulation of aortic vessel tone.

YC-1 potentiates the vasodilatory action of CORM-A1 both in vitro and in vivo

The benzylindazole derivative, YC-1, is known to sensitize guanylate cyclase to activation by CO (34). When rings were preincubated with YC-1 at 1 µM, the vasoactivity of CORM-A1 was markedly intensified at all concentrations tested (Fig. 8A), further sustaining the direct contribution of guanylate cyclase to the effect elicited by CORM-A1-derived CO. Specifically, 20 µM CORM-A1 caused $13 \pm 4.9\%$ relaxation after 20 min, but a more pronounced and significant relaxation response ($61\pm6.2\%$, $P<0.01$) was detected after pretreatment of vessels with YC-1 (1 µM). Control vessels pretreated with YC-1 alone and contracted with phenylephrine also exhibited a degree of relaxation over time ($14.1\pm1.1\%$ after 20 min). Interestingly, the rings treated with CORM-A1 in combination with YC-1 did not spontaneously recontract after reaching the maximal relaxation. The relaxation response of vessels pretreated

with YC-1 was also markedly potentiated ($P<0.01$) in the presence of 1 µM or 10 µM CORM-A1 (35±9.8% and 51±3.3%, respectively). These data are in line with previous reports showing that YC-1 amplifies the vasorelaxation mediated by CORM-3 (32). The effect of YC-1 and CORM-A1 was also examined on mean arterial pressure (MAP) in vivo. The compounds were injected intravenously as a bolus at a final dose of 30 µmol/kg for CORM-A1 (or iCORM-A1) and 1.2 µmol/kg for YC-1. When the two compounds were used in combination, YC-1 was administered 10 min before CORM-A1. As shown in Figure 8*B* (see line with solid circles), CORM-A1 produced a gradual and sustained decrease in MAP over time; specifically, 60 min after CORM-A1 injection, MAP decreased by 6.3 ± 1.5 mmHg from the initial baseline value. Administration of YC-1 alone (see line with solid square) or in combination with the negative control iCORM-A1 (see line with solid diamonds) produced only a transient decrease in MAP, reaching a maximum of 5.5 ± 1.0 mmHg after 10 min and returning to basal levels 50 min after injection. Interestingly, the combination of CORM-A1 and YC-1 (see line with solid triangles) produced a synergistic effect, resulting in a rapid and profound hypotension. In fact, MAP significantly decreased by 16.1 ± 5.6 mmHg after 10 min ($P<0.05$ vs. baseline or CORM-A1 alone) and remained at this level thereafter.

DISCUSSION

Certain transition metal carbonyls exert interesting biological activities in so far as they reproduce many of the pharmacological effects mediated by CO gas and thus can be used as CO-RMs for experimental purposes (28). Our early investigations revealed that manganese and ruthenium-containing carbonyl complexes (CORM-1 and CORM-2), which are soluble only in organic solvents (e.g., DMSO), can be safely used at low concentrations to promote relaxation of blood vessels and mitigate acute hypertension in vivo (28, 29). Other laboratories have subsequently confirmed the bioactive properties of CORM-1 and CORM-2 in mediating cerebral vasodilatation (35–37), smooth muscle relaxation of internal anal sphincter (38), suppression of smooth muscle cell proliferation (39), angiogenesis (40), inhibition of apoptosis (41, 42) and cardiac hyperthrophy (43), improved renal function (44), and anti-inflammatory and immunological responses (45, 46). More recently, the first prototype of a water-soluble carbonyl complex that contains the amino acid glycine covalently bound to a ruthenium metal (CORM-3) has been described. This compound has been reported to promote cardioprotection in both in vitro and in vivo models of myocardial infarction and in cardiac transplantation (30, 31), and its use has provided important information on how some of the pharmacological and vasoactive properties of CO-RMs could be exploited therapeutically (24, 25, 32).

In this study, we report on sodium boranocarbonate, $Na_2[H_3BCO_2]$, as a newly identified CO-RM that spontaneously liberates CO with a different rate compared with CORM-3. The idea of testing boranocarbonate originated from an interesting publication reporting the need of a CO source to synthesize a transition metal carbonyl ($[^{99m}Tc(OH_2)_3(CO)_3]^+$) specifically designed for radioimaging technology (47). The preparation of this carbonyl requires the use of CO gas, which is impractical for use in commercial radiopharmaceutical kits (48). The solution to this problem has been presented in a recent study reporting the preparation of $[^{99m}Tc(OH_2)_3(CO)_3]^+$ in physiological media using a boron-based carbonylating agent, potassium boranocarbonate, which acts as a CO source and a reducing agent at the same time (33). In the present study, we confirm the ability of sodium boranocarbonate, here termed CORM-A1, to liberate CO in aqueous solution and elicit pharmacological effects that are typical of CO gas. CORM-A1 does not

contain a transition metal but a boron atom to which a carboxyl group (COO⁻) is covalently bound. At room temperature, it is known that aqueous solutions of CORM-A1 are alkaline and very stable, but in the presence of hydrogen ions, the compound starts to decompose and liberate CO (33). CORM-A1 is involved in two acid/base equilibria as shown in Equation 1.

The constant pK_{a1} is ~10, which means that CORM-A1 is fully monoprotonated at physiological pH. The direct precursor for CO release is the highly unstable borane-carbonyl H_3B-CO formed after a second protonation and water cleavage, which represents the rate-limiting step. The more acidic the solution, the higher is the "active" concentration of double protonated CORM-A1 and the faster the CO release.

By using an established myoglobin assay as well as an amperometric CO sensor, we found that the kinetic of CO formation from CORM-A1 is strictly pH- and temperature-dependent. Specifically, the rate of CO release from CORM-A1 at 37°C is much slower at pH 7.4 (K_d=0.55 × 10^{-3} s^{-1}) than pH 5.5 (K_d=5.8 × 10^{-3} s^{-1}) and, most notably, slower than the rate of CO release previously reported for the ruthenium-based carbonyl CORM-3. Indeed, it is known from our recent studies that the liberation of CO from CORM-3 ([Ru(CO)$_3$Cl(glycinate)]) in biological systems occurs very rapidly (within 1–5 min) (24, 30); as a consequence, CORM-3 elicits a correspondingly rapid relaxation in isolated vessels (24, 32). In the case of CORM-A1, its half-life at physiological pH and temperature is ~21 min. In line with this slower release, CORM-A1 added to isolated aortic rings precontracted with phenylephrine promoted a gradual and sustained vasorelaxation that was maximal after 33 min, whereas CORM-3 caused a profound relaxation within 4–5 min only after addition to the organ bath (see Fig. 5A). Similarly, the rate of change in MAP observed in vivo after injection of rats with CORM-3 or CORM-A1 is in agreement with the different CO-release characteristics of these two compounds. Indeed, the gradual decrease in MAP over time after infusion of CORM-A1 (6.3 mmHg drop within 60 min) is in contrast with the prompt hypotensive effect (9.4 mmHg drop within 10 min) reported recently for CORM-3 (see ref 32). These results suggest a strong correlation between the distinct rate of CO release from CORM-A1 and CORM-3 and their specific pharmacological activities, which appear to be dictated by the rate of CO liberation. This idea is further corroborated by the measurements of HbCO levels following exposure of human blood to CORM-A1, where the gradual and time-dependent formation of HbCO indicates a slow and controlled release of CO from CORM-A1 even in a complex biological fluid such as blood. It is worth highlighting that while micromolar concentrations of CORM-A1 are sufficient to elicit pharmacological effects both in isolated vessels and in vivo, millimolar concentrations are needed to cause significant increases in HbCO levels in human blood. The measurements on HbCO were performed in isolated blood, thereby eliminating all the other potential cellular targets that could be affected by CO in an in vivo situation. These considerations suggest that CO liberated from CORM-A1 influences intra- and/or extracellular targets and modulates physiological functions at far lower concentrations than those required to change HbCO levels.

There is little doubt that the biochemical and pharmacological effects elicited by CORM-A1 are mediated by CO. First, linear correlations can be obtained between the levels of MbCO formed (R^2=0.99) or the amounts of CO detected with the electrode (R^2=0.98) and the concentrations of CORM-A1 used. Second, the extent of vasorelaxation elicited by CORM-A1 over time was concentration-dependent. Third, when the compound was depleted of CO (iCORM-A1) by adding acid to the solution, no CO was detected with either the Mb assay or the CO sensor and

no vasorelaxation could be observed. In addition, NaBH$_4$, which was used as another negative control to verify any possible contribution of boron in the bioactivity mediated by CORM-A1, was equally ineffective. The extent of vasodilatation exerted by CORM-A1 was also significantly attenuated by addition of myoglobin to the organ bath, confirming the direct involvement of CO in the observed biological effects. The experiments conducted to assess changes in MAP and vessel contractility by the combined administration of CORM-A1 and YC-1, a well-known benzylindazole derivative that activates the guanylate cyclase/cGMP pathway, further supported the role of CO as a potential pharmaceutical agent. While YC-1 alone or iCORM-A1 plus YC-1 slightly and only temporarily decreased MAP, the combination of CORM-A1 and YC-1 produced a significant and sustained hypotensive effect in vivo. Similarly, the presence of YC-1 markedly amplified the extent of vasodilatation caused by CORM-A1 in isolated aortas.

The effect of YC-1 indicates that guanylate cyclase is a target for CO in the vasorelaxation mediated by CORM-A1. This is also supported by the data showing that an inhibitor of guanylate cyclase (ODQ) significantly decreased the extent of dilatation caused by CORM-A1, thus confirming the results previously obtained with the metal carbonyl complex CORM-3 (32). However, blockade of potassium channels with glibenclamide, which is also known to partially attenuate the vasorelaxation by CORM-3 (32), did not have any effect on the vasoactivity elicited by CORM-A1. Notably, inhibition of NO synthase activity by L-NAME or removal of the endothelium, which significantly inhibited CORM-3-mediated vessel relaxation (32), failed to prevent the pharmacological effects of CORM-A1, suggesting that the action of CO exerted through the reactivity of boranocarbonate is independent of the endothelial function. At present, we do not have a precise answer for this discrepancy, and future experiments will examine these aspects more in detail. However, we know that the intrinsic chemical nature of the two compounds is very different since CORM-A1, unlike CORM-3, possesses some reducing capacities in addition to its ability to liberate CO (33) and thus may differently affect the response of the vessels. Collectively, these data reveal that CORM-A1 promotes an endothelium-independent relaxation response that is partially mediated by activation of guanylate cyclase and emphasize the distinct nature of this compound, which liberates CO into aqueous solutions with a kinetic that leads to a gradual relaxing and hypotensive effect.

Thus, the data of our present and previous studies (24, 29, 32) confirm that both transition metal carbonyls and sodium boranocarbonate meet the criteria of pharmacologically active CO-RMs. Our attempt to diversify the portfolio of CO-RMs that possess a variety of chemical characteristics (i.e., water-soluble vs. lipid-soluble, slow vs. fast releasers) will help to elucidate the biological function of cellular targets that are responsive to CO and will facilitate the therapeutic delivery of CO in a safe, measurable, and controllable fashion (49). For instance, irrespective of their solubility in DMSO or aqueous solutions, the metal carbonyls tested so far have been demonstrated to promptly liberate CO upon addition to biological systems, as ligands present in the extracellular or plasma environment (i.e., phosphate, glutathione) appear to accelerate dissociation of CO from the metal center (24). In the case of the water-soluble CORM-3, the release of CO is further accelerated when this compound is added to a solution containing myoglobin (30). Therefore, CORM-3 would fall into a category of compounds that release CO very rapidly ("fast releasers") in biological systems, which would be ideal for those therapeutic applications where CO acts as a prompt signaling mediator. However, chemicals that release CO with a slow kinetic ("slow releasers"), as in the case of CORM-A1, could be more

versatile in the treatment of certain chronic diseases. To this extent, CORM-A1 might mimic more closely the natural function of heme oxygenase, which is expected to generate endogenous CO in a sustained manner particularly in vascular and inflammatory disease states typified by up-regulation of the inducible HO-1 (5, 10).

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Equation 1

$$[H_3BCOO]^{2-} \underset{\substack{-H^+ \\ K_{a1}}}{\overset{+H^+}{\rightleftharpoons}} [H_3BCOOH]^- \underset{\substack{-H^+ \\ K_{a2}}}{\overset{+H^+}{\rightleftharpoons}} [H_3BCOOH_2]$$

$$\updownarrow\, -H_2O$$
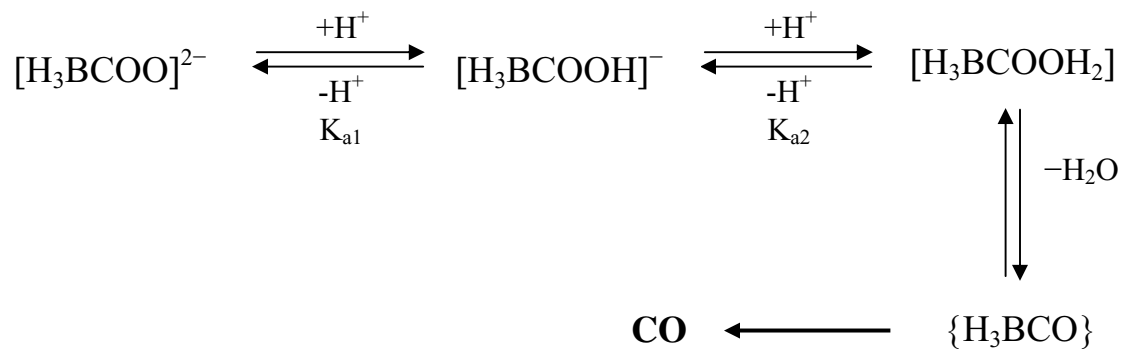
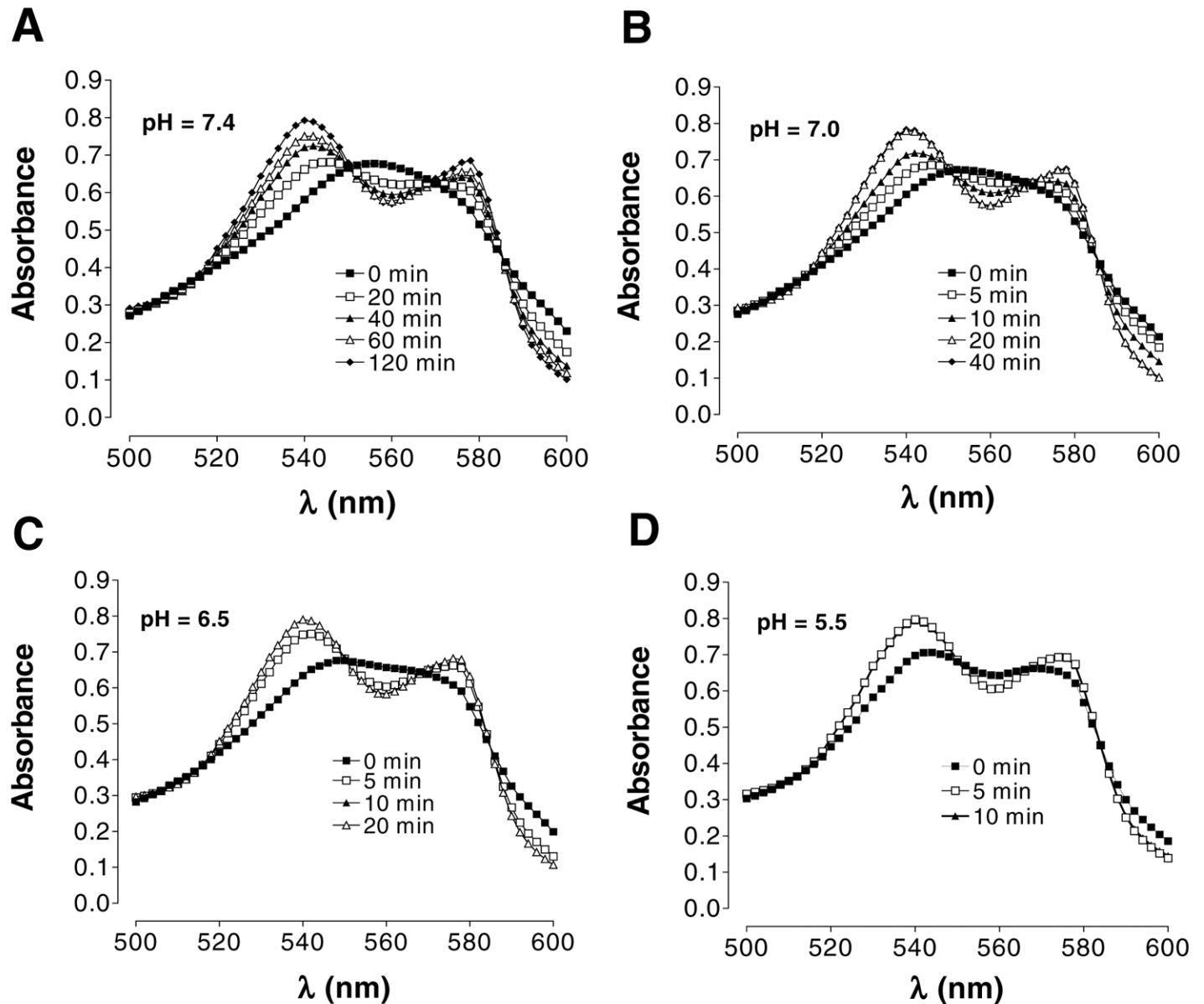$$\mathbf{CO} \longleftarrow \{H_3BCO\}$$

Fig. 1

Figure 1. The rate of conversion of deoxy-myoglobin (Mb) to carbon monoxide myoglobin (MbCO) by CORM-A1 is pH-dependent. The conversion of Mb to MbCO was followed over time by measuring the changes in the absorption spectra of this protein at pH 7.4 (*A*), 7.0 (*B*), 6.5 (*C*), and 5.5 (*D*) after addition of 60 µM CORM-A1. The solutions of Mb were prepared in phosphate buffer and the spectra run at 37°C as reported in Materials and Methods. The maximal absorption peak of Mb at 560 nm (see **Figure 1***A*, curve with filled square) is gradually converted over time by CORM-A1 to spectra typical of MbCO, with two maximal absorption peaks at 540 and 578, respectively (see curve with filled diamond).

Fig. 2
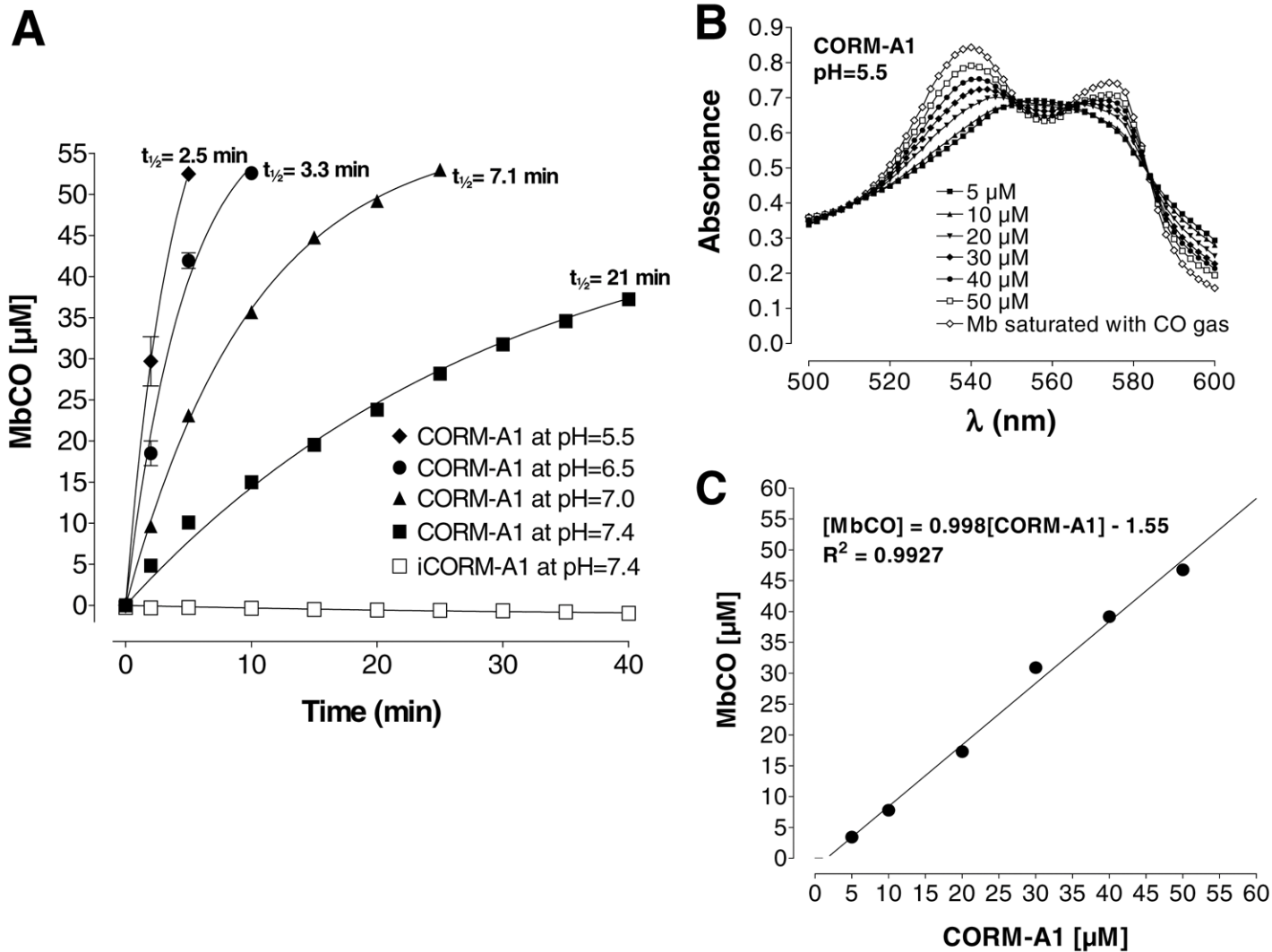
Figure 2. Quantification of CO liberated from CORM-A1 using the myoglobin assay. *A)* The amount of MbCO formed over time at 37°C was measured after addition of CORM-A1 (60 µM) to the Mb solution, and the half-lives ($t_{1/2}$) of this compound at different pHs were calculated from the fitted curves. Note that the inactive compound (iCORM-A1) did not generate any MbCO. *B, C)* Spectra of MbCO formation after addition of different concentrations of CORM-A1 to Mb at pH 5.5 and relative standard curve. The data indicate that the reaction favoring the release of CO from CORM-A1 goes to completion as approximately one mole of CO per mole of CORM-A1 is formed. The data are represented as mean ± SE of five independent experiments.
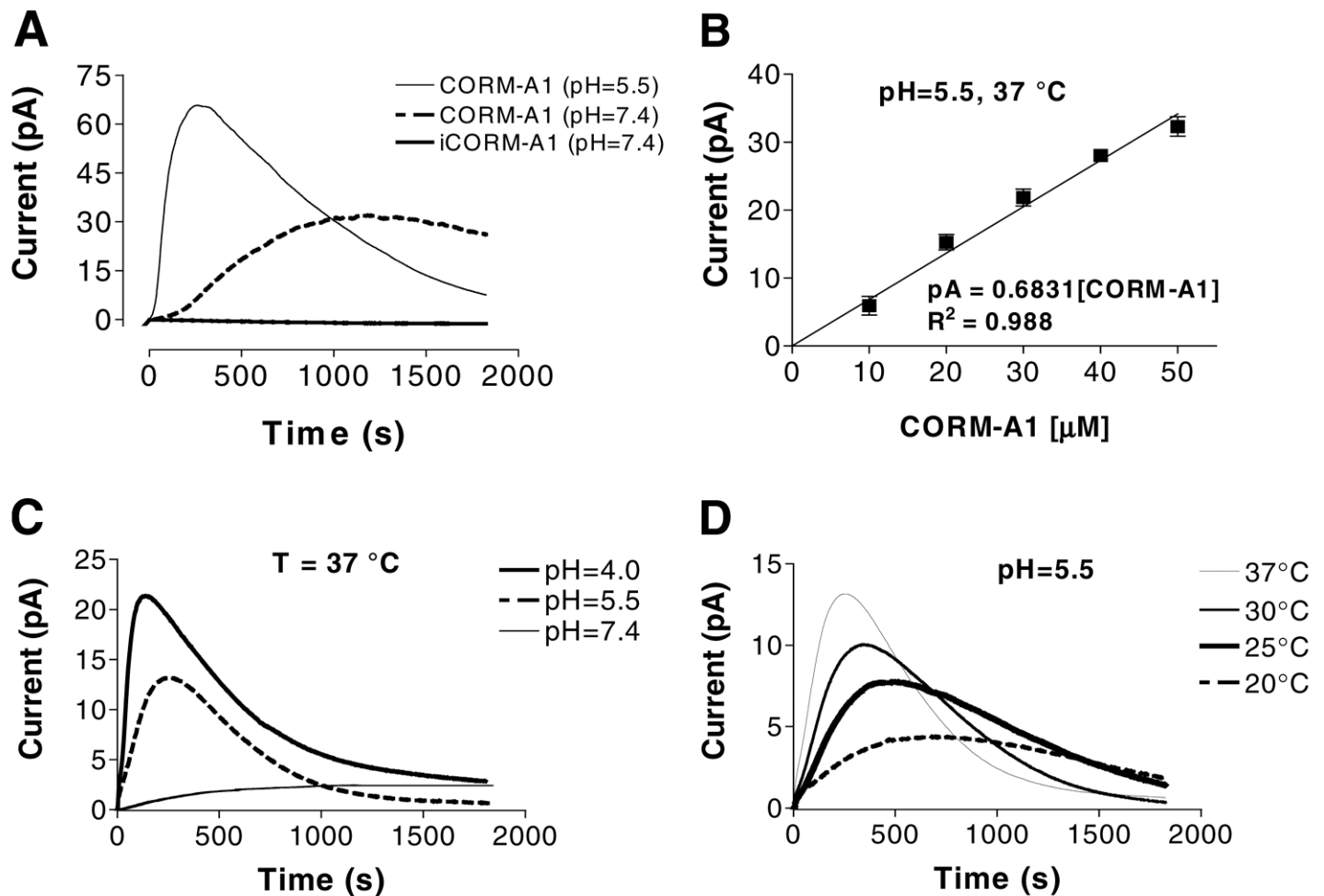
Fig. 3

Figure 3. Quantification of CO liberated from CORM-A1 using an amperometric CO sensor. *A)* Typical tracings showing the current measured over time by the CO electrode upon addition of 100 µM CORM-A1 at 37°C. Note that the liberation of CO from CORM-A1 is much faster at pH 5.5 than at pH 7.4 and that no CO is detected after addition of the inactive compound (iCORM-A1) *B)* Calibration of the CO electrode by plotting the maximal currents measured with increasing concentrations of CORM-A1 at pH 5.5 and 37°C. The data are represented as mean ± SE of five independent experiments. *C, D)* Typical tracings of the CO electrode showing that the rate of CO release over time from CORM-A1 (20 µM) is strictly dependent on pH (*C*) and temperature (*D*). See Materials and Methods as well as Results for more technical details.
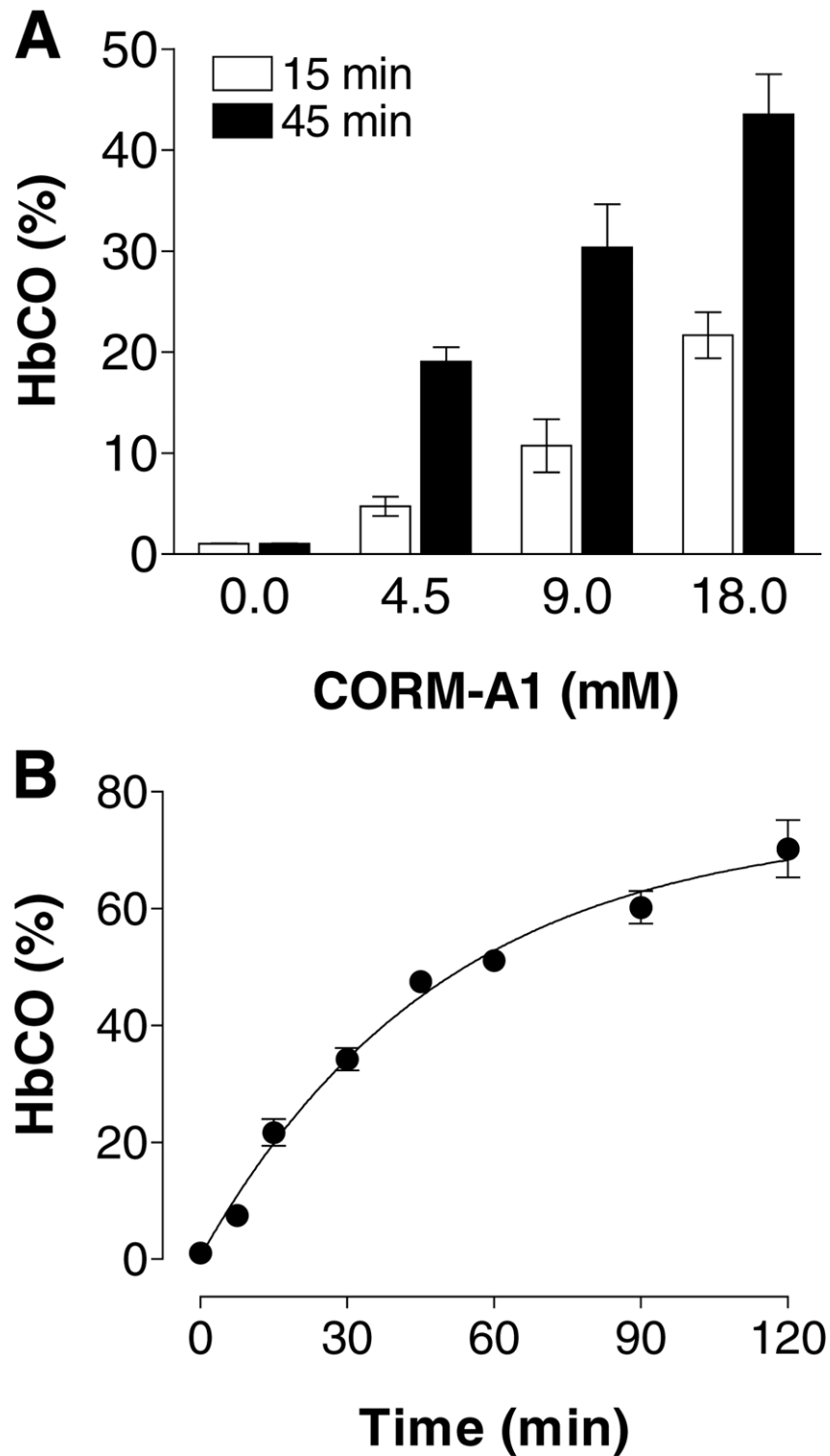
Fig. 4

Figure 4. Formation of carboxyhemoglobin (HbCO) in isolated human blood incubated with CORM-A1. *A)* Freshly collected human blood was incubated with different concentrations of CORM-A1 at 37°C and HbCO levels detected after 15 or 45 min using a blood gas analyzer. *B)* Time course of HbCO formation after incubation of human blood (37°C) with 18 mM CORM-A1. In both graphs, data are expressed as mean ± SE of four independent experiments.
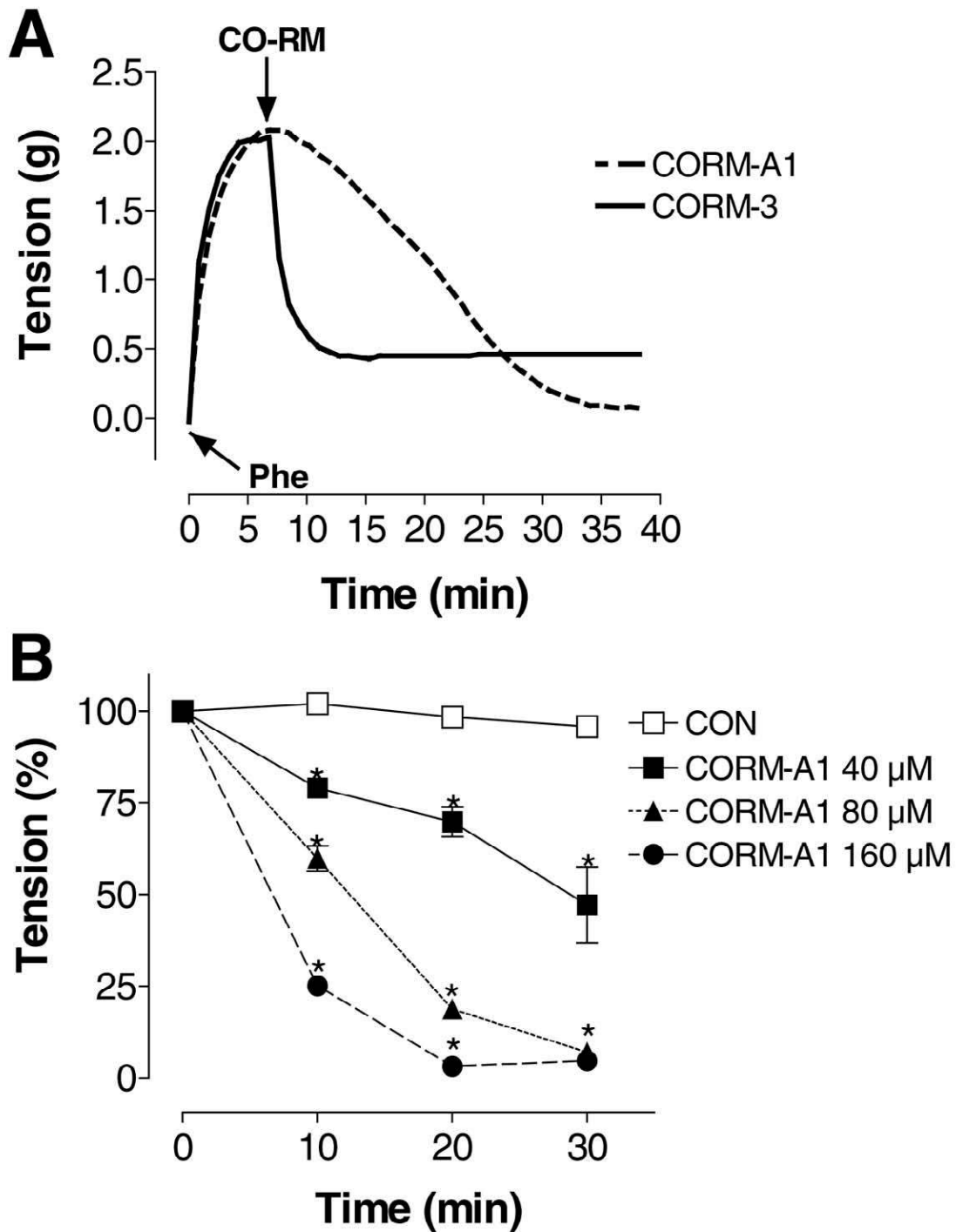
Fig. 5

Figure 5. Vasodilatation elicited by CORM-A1: a comparison with CORM-3. *A)* Representative recording of isometric tension in aortic rings precontracted with phenylephrine (Phe, 1 µM) and subsequently subjected to a bolus addition of CORM-3 or CORM-A1 (100 µM). Typically, CO-RMs were added to the water bath containing aortic rings once phenylephrine had produced a stable contraction; CORM-3 caused profound relaxation within a few minutes of addition, whereas CORM-A1 required ~33 min to elicit maximal vasorelaxation. *B)* Concentration-dependent relaxation responses to CORM-A1 in aortic rings precontracted with phenylephrine. The vasodilatation is expressed as percentage of the maximal precontraction. Data represent the mean ± SE of six independent experiments. *$P < 0.05$ compared with control (CON).
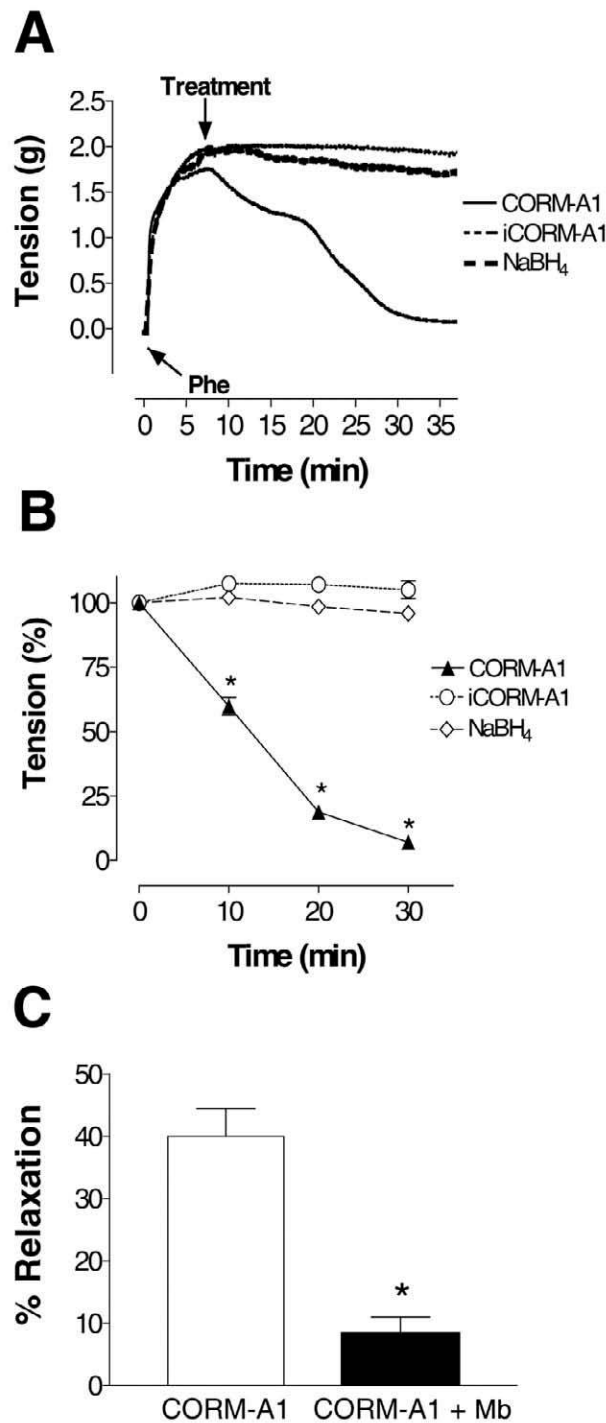
Fig. 6

Figure 6. The vasorelaxation by CORM-A1 is mediated by CO. *A)* Representative recording of isometric tension in aortic rings precontracted with phenylephrine (Phe, 1 µM) and subsequently subjected to a bolus addition of 80 µM CORM-A1, iCORM-A1, or sodium borohydride (Na_2BH_4). iCORM-A1, which does not release CO, and Na_2BH_4 were used as negative controls (see Materials and Methods as well as Results for details). *B)* Vasodilatory responses to 80 µM CORM-A1, iCORM-A1, and Na_2BH_4 in aortic rings precontracted with phenylephrine. The vasodilatation is expressed as percentage of the maximal precontraction. Data represent mean ± SE of six independent experiments. *$P < 0.05$ compared with iCORM-A1 and Na_2BH_4. *C)* The relaxation mediated by CORM-A1 (80 µM) in aortic isolated rings is attenuated by Mb (100 µM). The relaxation is expressed as a percentage of the maximal precontraction, and the values represent relaxation measured 10 min after addition of CORM-A1. Data represent mean ± SE of six independent experiments. *$P < 0.05$ compared with CORM-A1.
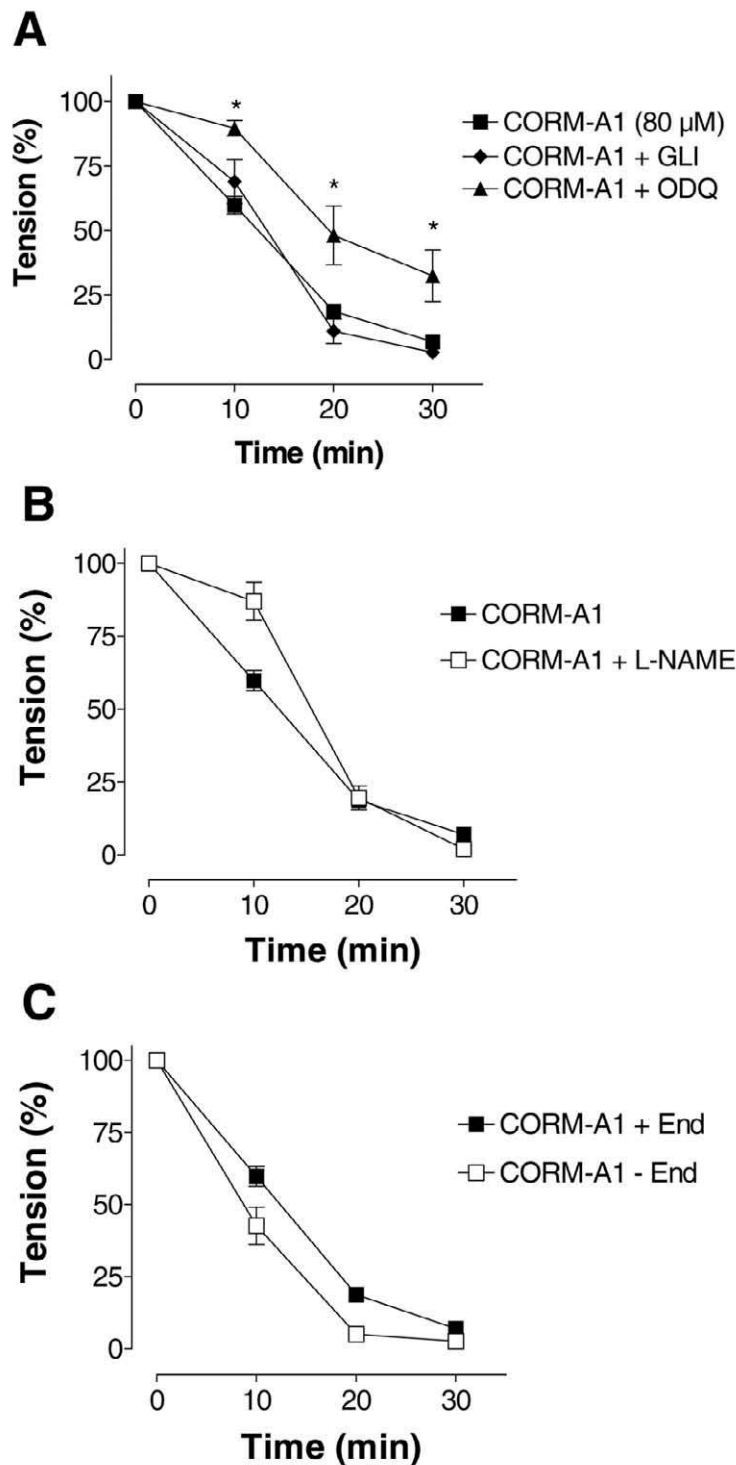
Fig. 7

Figure 7. Mechanisms of vasorelaxation mediated by CORM-A1. *A)* Vasodilatory responses to CORM-A1 in the presence of a potassium channel blocker (glibenclamide, GLI) or a guanylate cyclase inhibitor (ODQ). Aortic rings were pretreated with GLI (10 µM) or ODQ (30 µM) before contraction with phenylephrine, and then CORM-A1 was added. $*P < 0.05$ compared with CORM-A1. *B)* Vasodilatory responses to CORM-A1 in the presence of a NO synthase inhibitor (N^G-nitro-L-arginine-methyl ester, L-NAME). Aortic rings were incubated with L-NAME (100 µM) for 30 min before contraction with phenylephrine, and then CORM-A1 (80 µM) was added. *C)* Effect of CORM-A1 on endothelium-denuded vessels. The endothelium of aortic rings was gently removed (-End) as described in Materials and Methods, and the vasodilatory response to CORM-A1 (80 µM) was compared with that of intact rings (+End). In all graphs, the relaxation is expressed as percentage of the maximal precontraction. Data represent mean ± SE of six independent experiments.
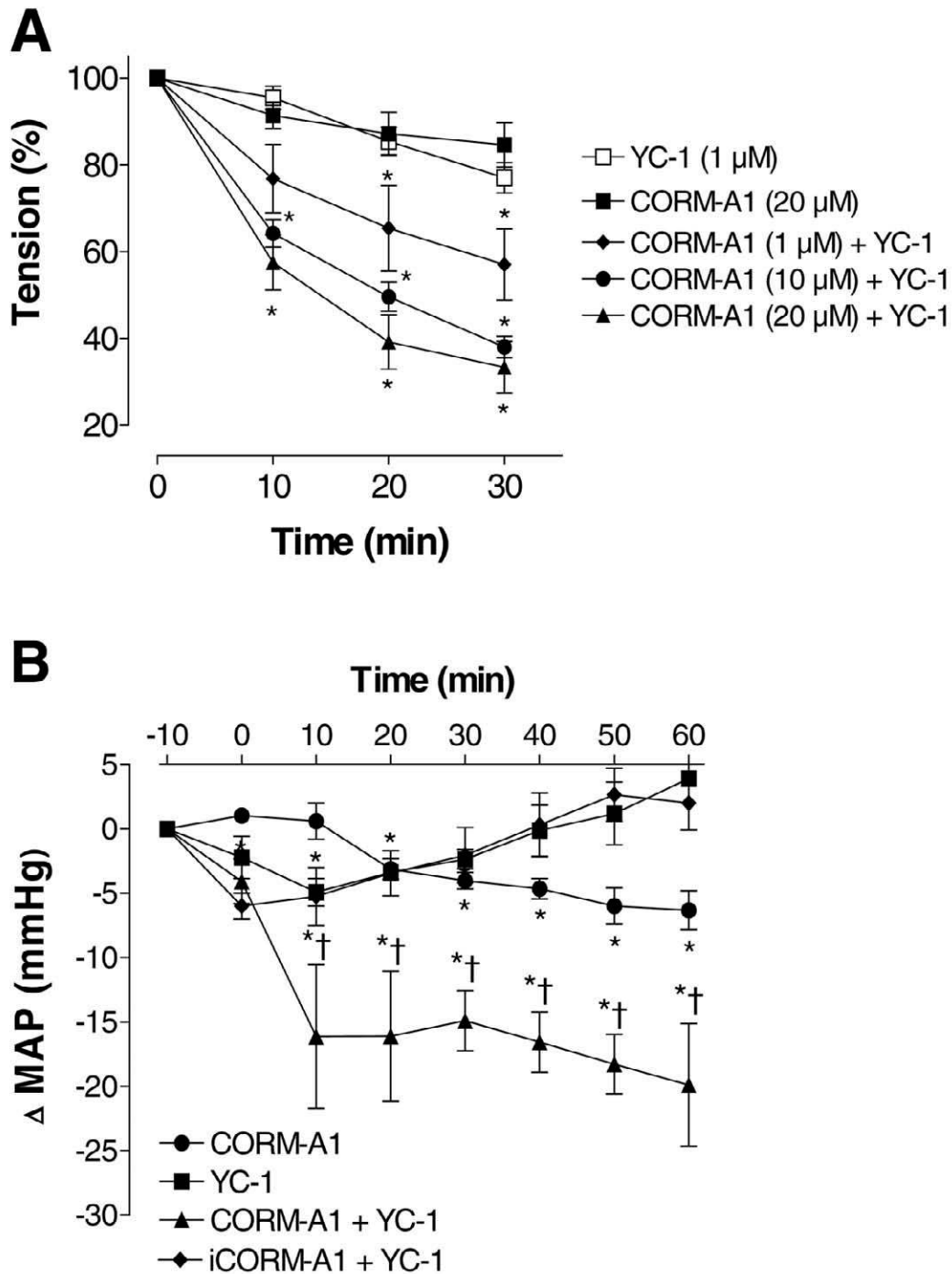
Fig. 8

Figure 8. YC-1 potentiates CORM-A1-mediated vasodilatation in vitro and in vivo. *A)* Effect of YC-1 on CORM-A1-mediated vasorelaxation in isolated vessels. Aortic rings were pretreated with 1 µM YC-1 (30 min before contraction with phenylephrine) and then subjected to a bolus addition of CORM-A1 (1, 10, and 20 µM, respectively). The relaxation is expressed as a percentage of the maximal precontraction. Data represent mean ± SE of six independent experiments. *$P <$ 0.05 compared with YC-1 alone. *B)* Effect of YC-1 on mean arterial pressure (MAP) in vivo. Rats were anesthetized and chronically catheterized as described in Materials and Methods. CORM-A1 (30 µmol/kg, i.v.) or the inactive compound (iCORM-A1) was injected alone or in combination with YC-1 (1.2 µmol/kg, i.v.) and mean arterial pressure (MAP) was measured over time. Data represent mean ± SE of four to five independent experiments. *$P < 0.05$ compared with baseline values (time, −10 min); †$P < 0.05$ compared with YC-1, CORM-A1, or iCORM-A1 plus YC-1.