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Administration of a CO-releasing molecule at the time of reperfusion reduces infarct size in vivo

Yiru Guo[1,*], **Adam B. Stein**[1,*], **Wen-Jian Wu**[1], **Wei Tan**[1], **Xiaoping Zhu**[1], **Qian-Hong Li**[1], **Buddhadeb Dawn**[1], **Roberto Motterlini**[2], and **Roberto Bolli**[1]

[1]Division of Cardiology and The Institute of Molecular Cardiology, University of Louisville, Louisville, Kentucky 40292

[2]Vascular Biology Unit, Northwick Park Institute for Medical Research, Harrow HA1 3UJ, United Kingdom

Abstract

Although carbon monoxide (CO) has traditionally been viewed as a toxic gas, increasing evidence suggests that it plays an important homeostatic and cytoprotective role. Its therapeutic use, however, is limited by the side effects associated with CO inhalation. Recently, transition metal carbonyls have been shown to be a safe and effective means of transporting and releasing CO groups in vivo. The goal of the present study was to test whether a water-soluble CO-releasing molecule, tricarbonylchloro (glycinato) ruthenium (II) (CORM-3), reduces infarct size in vivo when given in a clinically relevant manner, i.e., at the time of reperfusion. Mice were subjected to a 30-min coronary artery occlusion followed by 24 h of reperfusion and were given either CORM-3 (3.54 mg/kg as a 60-min intravenous infusion starting 5 min before reperfusion) or equivalent doses of inactive CORM-3, which does not release CO. CORM-3 had no effect on arterial blood pressure or heart rate. The region at risk did not differ in control and treated mice (44.5 ± 3.5% vs. 36.5 ± 1.6% of the left ventricle, respectively). However, infarct size was significantly smaller in treated mice [25.8 ± 4.9% of the region at risk (n = 13) vs. 47.7 ± 3.8% (n = 14), $P < 0.05$]. CORM-3 did not increase carboxyhemoglobin levels in the blood. These results suggest that a novel class of drugs, CO-releasing molecules, can be useful to limit myocardial ischemia-reperfusion injury in vivo.

Keywords

carbon monoxide-releasing molecules; myocardial ischemia; reperfusion injury; transition metal carbonyls

Mammalian tissues continually produce carbon monoxide (CO) as a result of the breakdown of heme by heme oxygenase (HO) (13). Although CO has been traditionally regarded as toxic, recent evidence has revealed that this gas exerts pleiotropic homeostatic effects. Specifically, CO has been shown to promote vasorelaxation (12, 17) and to inhibit proliferation of smooth muscle cells (21), apoptosis (2), transplant rejection (4), inflammation (14, 15), platelet aggregation, microvascular thrombosis (3), cytokine production (9, 18), and oxidative stress (16). CO, delivered either as a gas or via CO-releasing molecules (CORMs), has also been shown to alleviate hypoxia/reoxygenation

Address for reprint requests and other correspondence: R. Bolli, Div. of Cardiology, Univ. of Louisville, Louisville, KY 40292 (rbolli@louisville.edu).

*Y. Guo and A. B. Stein contributed equally to this study.

injury in isolated cells and ischemia-reperfusion injury in isolated hearts (4) and in the liver (1). Although the mechanism(s) underlying the cytoprotective actions of CO has not been elucidated, evidence suggests that this gas exerts some of its effects via activation of the guanylate cyclase/cGMP pathway (10, 22) and the p38 MAPK-dependent pathway (15).

In view of the mounting evidence supporting a salubrious role of CO in a variety of pathophysiological conditions, much interest has focused on harnessing the actions of this molecule for therapeutic purposes. Thus far, most studies in vivo have utilized inhalation of CO to deliver this gas to tissues. However, administering CO gas is a very nonspecific approach, as most of the free CO reaching the bloodstream reacts rapidly with hemoglobin and other heme proteins before reaching the target tissue. This can potentially result in toxic effects. Carriers of CO that can transport and deliver this gas to a target tissue would clearly enhance both the clinical feasibility and the specificity of CO therapy. Recently, Motterlini and colleagues (4, 11) have reported that transition metal carbonyls are good candidates for this purpose as they function as CORMs in biological systems. These compounds contain a heavy metal surrounded by carbonyl (CO) groups as coordinated ligands (11). The first CORM to be characterized was tricarbonyldichlororuthenium (II) dimer, a lipid-soluble molecule that was found to liberate CO and reproduce the effects of HO-1-derived CO both in vitro and in vivo (11). A new CORM has been subsequently developed, namely, tricarbonyldichloro (glycinato)ruthenium (II) (CORM-3), which is water soluble (4). Clark et al. (4) have demonstrated that CORM-3 effectively delivers CO to tissues under physiological conditions and limits anoxia/reoxygenation or ischemia-reperfusion injury in isolated rat hearts and cardiomyocytes.

The water-soluble properties of CORM-3 suggest that this compound may have clinical utility. Accordingly, the goal of the present study was to determine whether the protective effects of CORM-3 demonstrated in vitro are also present in vivo. To this end, we utilized a well-established murine model of myocardial infarction in which fundamental physiological variables that modulate myocardial ischemia were carefully monitored and controlled (8). The results demonstrate that intravenous administration of CORM-3 started at the time of reperfusion effectively limits infarct size in vivo.

METHODS

Animal preparation

The experimental procedures and protocols used in this study were reviewed and approved by the Animal Care and Use Committee of the University of Louisville School of Medicine (Louisville, KY) and conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 86-23). The experimental preparation has been described in detail previously (7, 8). Briefly, the study was performed in male ICR mice weighing 35.0 ± 0.6 g (age, 9.4 ± 0.3 wk). Mice were anesthetized with pentobarbital sodium (60 mg/kg iv), intubated, and ventilated with room air supplemented with oxygen at a rate of 105 strokes/min and with a tidal volume of 0.6 ml using a small rodent ventilator. These respiratory settings result in physiological values of arterial pH (7.39 ± 0.01) and adequate oxygenation (7, 8). Body temperature was carefully monitored with a rectal probe and maintained as close as possible to 37.0°C. To maintain blood pressure in the normal range during the open-chest procedure, blood from a donor mouse was given intravenously at 40 ml/kg divided into three equal doses (8). With the aid of a dissecting microscope and a microcoagulator, the chest was opened, an 8-0 nylon suture was passed under the left anterior descending coronary artery, and a nontraumatic balloon occluder was applied on the artery. After the coronary occlusion/reperfusion protocol, the chest was closed in layers and the mice were allowed to recover.

Experimental protocol

Myocardial infarction was produced by a 30-min coronary occlusion followed by 24 h of reperfusion. Mice were assigned to five groups. *Group I* (control) received no treatment or manipulation. To compare the effects of CORM-3 to the early phase of ischemic preconditioning (PC), *group II* (early ischemic PC) was subjected to six cycles of 4-min coronary occlusion/4-min reperfusion (8) followed, 10 min later, by 30 min of occlusion and 24 h of reperfusion. To compare the effect of CORM-3 to the late phase of ischemic PC, mice in *group III* (late ischemic PC) underwent six cycles of 4-min coronary occlusion/4-min reperfusion on *day 1*. The chest was then closed, and the mice were allowed to recover. Twenty-four hours later (*day 2*), the mice were reanesthetized, the chest was reopened, and the mice were subjected to a 30-min occlusion followed by 24 h of reperfusion (8). In *group IV* (inactive CORM-3), mice received inactive CORM-3 at the same dose used in *group V*. CORM-3 was inactivated by dissolving it in PBS (0.35 mg/ml) and leaving it at room temperature for 24 h; under these conditions, one mole of CO per mole of compound is released in the solution and, as a result, no additional CO is liberated upon administration of the drug (4). *Group V* (active CORM-3) received a 60-min intravenous infusion of CORM-3 starting 5 min before reperfusion and ending 55 min after reperfusion (total dose, 3.54 mg/kg). CORM-3 was dissolved in PBS (pH 7.4).

Postmortem tissue analysis

At the conclusion of the study, the heart was excised and perfused with Krebs-Henseleit solution through an aortic cannula. To delineate infarcted from viable myocardium, the heart was then perfused with 1% triphenyltetrazolium chloride in phosphate buffer. To delineate the occluded/reperfused bed, the coronary artery was tied at the site of the previous occlusion and the aortic root was perfused with 10% Phthalo blue dye (7, 8). As a result of this procedure, the region at risk was identified by the absence of blue dye, whereas the rest of the left ventricle (LV) was stained dark blue. The LV was cut into five to seven transverse slices, which were fixed in 10% neutral buffered formaldehyde, weighed, and photographed under a microscope. The corresponding areas were measured by computerized videoplanimetry, and from these measurements infarct size was calculated as a percentage of the region at risk (7, 8).

Statistical analysis

Data are reported as means ± SE. Measurements were analyzed with a one-way or two-way repeated-measures ANOVA, as appropriate, followed by unpaired Student's *t*-tests with the Bonferroni correction. The relationship between infarct size and risk region size was compared between groups with analysis of covariance, with the size of the risk region as the covariate. The correlation between infarct size and risk region size was assessed by linear regression with the least-squares method.

RESULTS

A total of 75 mice was used. Ten mice were excluded for the reasons specified in Table 1.

Pilot studies

Pilot studies were conducted to identify a dose of CORM-3 that would not affect heart rate or arterial blood pressure. Mean arterial pressure and heart rate were determined at baseline, during a 60-min infusion of CORM-3, and for 20 min postinfusion. The doses of CORM-3 that were studied included 3.54, 5.31, 7.08, and 14.2 mg/kg. At the lowest dose (3.54 mg/kg), there was no change in heart rate or mean blood pressure at baseline, during infusion, or after infusion. The three other doses produced a dose-dependent decrease in arterial blood

pressure (9%, 12%, and 36%, respectively) that began ~30 min into the CORM-3 infusion. Therefore, we selected 3.54 mg/kg as the highest dose of CORM-3 that does not alter blood pressure or heart rate.

Carboxyhemoglobin levels

To determine whether the dose of CORM-3 used in this study increases carboxyhemoglobin (COHb), blood COHb levels were measured with an AVOXimeter 4000 Whole Blood Oximeter in mice receiving an intravenous infusion of CORM-3 (3.54 mg/kg over 1 h) or inactive CORM-3 (same dose) and in control mice (anesthetized for 1 h). Blood samples were taken immediately after the 1-h infusion. Percent COHb levels were similar in the CORM-3-treated, inactive CORM-3-treated, and control groups: 0.65 ± 0.3%, 0.58 ± 0.4%, and 0.6 ± 0.3%, respectively (n = 4 in each group). To further investigate the properties of CORM-3 in blood, in vitro dose-response studies were performed. CORM-3 was added to blood from a healthy human volunteer in vitro to achieve final concentrations of 20, 50, 100, 200, 300, and 400 μM, and COHb was measured after 30 min. As shown in Fig. 1, COHb levels were virtually undetectable (<1.0%) until a concentration of at least 200 μM of CORM-3 was achieved. For comparison, the dose of CORM-3 given to the mice in our study (3.54 mg/kg) resulted in estimated blood concentrations of 20 μM. These data imply that CORM-3 can deliver CO to tissues at concentrations that do not increase blood COHb.

Body temperature and heart rate

By experimental design (7, 8), rectal temperature remained within a narrow, physiological range (36.8–37.3°C) in all groups. Five minutes before the 30-min coronary occlusion, the average heart rate in *groups I*, *II*, *IV*, and *V* ranged from 540 to 549 beats/min (P = not significant; Table 2). In *group III*, the average heart rate 5 min before occlusion was higher (636 ± 33 beats/min, $P < 0.05$ vs. other groups), likely due to the trauma from the surgery that had been performed 24 h previously (8). During the 30-min occlusion and ensuing reperfusion, heart rate did not differ significantly among *groups I*, *II*, *IV*, and *V* but was elevated in *group III* compared with the other groups (Table 2).

Infarct size

There were no significant differences among the five groups with respect to the heart weight-to-body weight ratio or weight of the region at risk (Table 3). In untreated control mice (*group I*), infarct size averaged 47.7 ± 3.8% of the region at risk (Fig. 2). As expected (8), both *group II* (early PC) and *group III* (late PC) exhibited smaller infarct sizes (15.6 ± 2.0% and 27.0 ± 2.9% of the region at risk, respectively). In the inactive CORM-3-treated mice (*group IV*), infarct size was similar to the control group (50.8 ± 2.7% of the region at risk), indicating that the chemical, in itself, does not affect the extent of cell death. However, in the CORM-3-treated group (*group V*), infarct size (25.8 ± 4.9% of the region at risk) was significantly ($P < 0.05$) smaller than that measured in *groups I* and *IV* (Fig. 2). Infarct size in *group V* was similar to that in *groups II* and *III*, indicating that administration of the CO donor 5 min before reperfusion resulted in a protective effect that was comparable to that induced by early and late PC. The size of infarction in *group IV* (inactive CORM-3 treated) and *group V* (CORM-3 treated) was positively and linearly related to the size of the region at risk (r = 0.70 and 0.54, respectively). The regression line, however, was shifted to the right in *group V* compared with *group IV* ($P < 0.05$; Fig. 3), indicating that for any given size of the region at risk, the resulting infarction was smaller in CORM-3-treated mice than in inactive CORM-3-treated mice.

DISCUSSION

This study demonstrates that CORM-3, a water-soluble CORM, decreases myocardial infarct size when given just before reperfusion in vivo. The magnitude of the infarct size reduction was similar to that afforded by early or late ischemic PC. Because inactive CORM-3, which is chemically identical to CORM-3 but does not liberate CO (4), had no effect, the reduction in infarct size observed in CORM-3-treated mice must be ascribed to the actions of CO (as opposed to potential nonspecific actions of the CORM-3 moiety). The cardioprotection observed with CORM-3 cannot be ascribed to hemodynamic effects, because the drug produced no appreciable changes in heart rate or arterial pressure. Because CORM-3 infusion was started 5 min before release of coronary occlusion, the reduction in infarct size indicates that CO acted by alleviating the component of tissue injury that is associated with reperfusion, as opposed to the injury inflicted by ischemia itself. The infusion of CORM-3 did not produce any appreciable increase in COHb levels in the blood. Taken together, these results support the potential usefulness of a new class of drugs, CORMs, for limiting myocardial infarct size.

A previous study by Clark et al. (4) has shown that CORM-3 protects isolated myocytes against hypoxia/reoxygenation injury and isolated rat hearts against ischemia-reperfusion injury. The present results corroborate these previous findings and extend them to the more complex in vivo setting. Because CORM-3 was started at the time of reperfusion and did not raise blood COHb levels, the experimental protocol employed herein has clinical relevance for the treatment of patients with acute myocardial infarction.

As shown in Fig. 2, infarct size in the CORM-3 treated group was variable. The most plausible explanation for this unexpected variability is the short half-life of CORM-3 in PBS (18 min). To test this theory, we performed pilot studies in two mice in which CORM-3 was given as in *group V* except that it was dissolved in water instead of PBS. [The half-life of CORM-3 in water is 98.3 h (4).] In these two mice, infarct size was very small (8.1% and 5.1% of the region at risk), supporting the idea that the variability in infarct size is probably related to the short half-life of CORM-3 dissolved in PBS in combination with the variable time that elapsed between preparation of the CORM-3 solution and the start of the intravenous infusion.

Compared with inhalation of CO gas, the use of CO donors offers important advantages. In the present study, a brief (1 h) exposure to low (3.54 mg/kg) doses of CORM-3 was sufficient to elicit powerful protection, equivalent to that elicited by both early and late PC. In contrast, CO gas needs to be used in much higher amounts for prolonged periods of time to produce an effect, which makes it impractical (11). In virtually all in vivo studies reported thus far, animals have been exposed to an atmosphere of 100–250 ppm CO for several hours or days before a stressful event; this corresponds to millimolar concentrations of CO in the inhaled air and raises the levels of COHb from <1% to >10%, implying that local and possibly chronic hypoxia were likely contributors to the response observed with CO gas exposure in these experiments (11). In contrast, the doses of CORM-3 used by Clark et al. (4) and in the present study liberate significantly less CO per kilogram body weight over time, as demonstrated by the fact that no change in blood COHb levels were observed. Therefore, the therapeutic approach used herein appears to be safe and mimics the kinetics of endogenous CO generation, which is in the range of micro-moles rather than millimoles (5, 6, 20).

The fact that CORM-3 did not increase COHb levels in the blood may appear surprising. For this reason, we performed dose-response studies in vitro. As Fig. 1 shows, a concentration of 200 μM of CORM-3 was required to elevate COHb to levels >1%; this concentration is

much higher than that (~20 μM) which we estimate was achieved in the blood in vivo with our dose (3.54 mg/kg). The chemistry of transition metal carbonyls, such as CORM-3, and their rate of reaction with tissue targets vis-à-vis blood Hb are still incompletely understood. Nevertheless, our in vitro data indicate that the doses of CORM-3 that are cardioprotective in vivo are well below the threshold needed to raise blood COHb levels, implying that at least some of the CO released by CORM-3 in vivo escapes the reaction with Hb in the blood and is delivered to tissues.

The mechanisms underlying CO-induced cardioprotection remain to be elucidated. As mentioned above, CO modulates many different signaling pathways that could potentially lead to cardioprotection. One plausible hypothesis is that CO may alleviate the ischemia-reperfusion injury by activating mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels. This is supported by the finding by Clark et al. (4): that the protective effects of CORM-3 in cardiac cells and isolated hearts were abrogated by 5-hydroxydecanoic acid. Another pathway that may potentially mediate CO donor-induced cardioprotection is the p38 MAPK signaling pathway, which has been previously implicated in the protective effects of ischemic PC (19). Activation of p38 MAPK has been shown to underlie CO-dependent alleviation of hepatic ischemia-reperfusion injury (1) and CO-dependent inhibition of apoptosis during lung ischemia-reperfusion injury (24). Further studies will be needed to address the mechanism of CORM-3-induced cardio-protection.

Extensive evidence now indicates that HO-1 plays a pivotal role in protecting tissues against various types of injury (2, 12, 13, 23). However, to date, this ubiquitous defensive role of HO-1 has not been exploited for therapeutic purposes. Taken together with previous studies (4), the present results provide a rationale for the development of novel anti-ischemic therapies predicated upon the supplementation of exogenous CO to patients with acute myocardial infarction.

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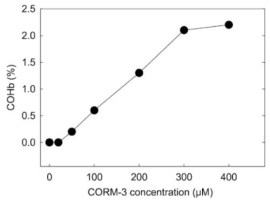

Fig. 1.

Dose-response curve showing blood carboxyhemoglobin (COHb) levels (%) generated by different concentrations of tricarbonylchloro(glycinato)ruthenium (II) (CORM-3) in in vitro human blood samples.

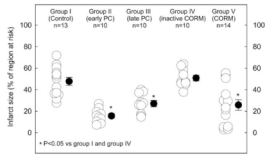

Fig. 2.

Myocardial infarct size in *groups I–V*. Infarct is expressed as a percentage of the region at risk of infarction. PC, preconditioning. ○, Individual mice; ●, mean ± SE for respective groups.

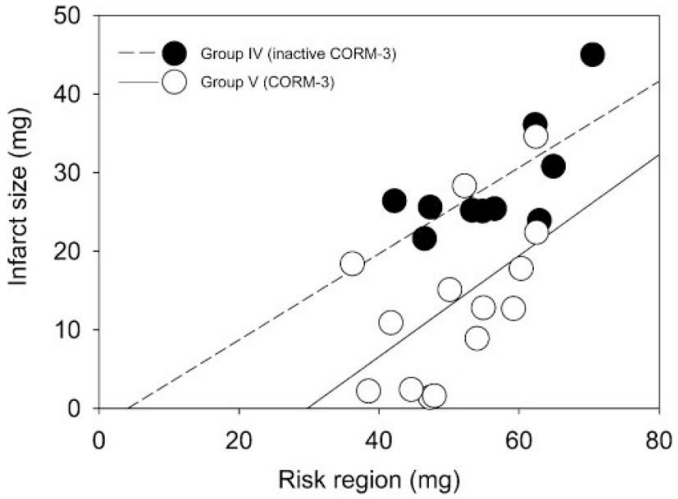

Fig. 3.

Relationship between size of the region at risk and size of the infarction in *groups IV* and *V*. This graph shows individual values and regression lines obtained by linear regression analysis. In both groups, infarct size was positively and linearly related to risk region size. The linear regression equations were as follows: *group IV*, $y = -2.27 + 0.55x$, $r = 0.70$; and *group V*, $y = -19.12 + 0.64x$, $r = 0.54$. Analysis of covariance demonstrated that the regression line for *group IV* was significantly different from *group V* ($P < 0.05$), indicating that for any given risk region size, infarct was smaller in CORM-3-treated mice than in inactive CORM-3-treated mice.

Table 1

Reasons for excluding mice from study

	AMI (*Group I*)	EPC (*Group II*)	LPC (*Group III*)	Inactive CORM-3 (*Group IV*)	Active CORM-3 (*Group V*)	Pilot Study	Total
Death	1	0	2	1	2	0	6
Technical problems	1	0	1	0	0	0	2
Poor postmortem staining	1	0	1	0	0	0	2
Mice excluded	3	0	4	1	2	0	10
Mice instrumented	16	10	14	11	16	8	75
Mice included in study	13	10	10	10	14	8	65
Mice included in study, %	81	100	71	91	88	100	87

AMI, acute myocardial infarction; EPC, early preconditioning; LPC, late preconditioning; CORM-3, tricarbonylchloro(glycinato)ruthenium (II).

Table 2

Heart rate on the day of the 30-min coronary occlusion

		Occlusion, min		Reperfusion, min		
	Preocclusion	**5**	**30**	**5**	**15**	**60**
Group I	540±13	583±16	589±19	546±12	552±11	582±11
Group II	545±22	558±21	535±16	537±12	528±13	558±21
Group III	636±33*	675±25*	689±17*	679±11*	662±13*	670±15*
Group IV	548±18	562±20	543±23	570±22	540±25	532±18
Group V	549±13	556±20	539±20	533±20	529±20	529±20

Data are means ± SE (in beats/min). Measurements of heart rate were taken 5 min before the 30-min coronary occlusion (preocclusion), at 5 and 30 min into the 30-min occlusion, and at 5, 15, and 60 min after reperfusion. The experimental protocols for the 5 groups of mice are specified in METHODS. Note that the heart rate in *group III* (measured on *day 2*) was higher than that in groups *I, II, IV,* and *V* (measured on *day 1*), possibly reflecting the effect of surgical trauma 24 h earlier.

*$P < 0.05$ vs. *groups I, II, IV,* and *V*.

Table 3

Size of LV, risk region, and infarct

	Age, wk	Body Weight, g	Heart Weight, mg	LV Weight, mg	Heart Weight/Body Weight	Risk Region Weight, mg	Infarct Weight, mg	Risk Region, % of LV	Infarct, % of risk region	Infarct, % of LV
Group I	8.6±0.6	32.4±0.8	162.9±5.3	117.6±4.6	0.50±0.01	51.8±4.2	25.2±3.0	44.5±3.5	47.7±3.8	21.732.7
Group II	7.2±0.5	30.3±1.0	146.2±4.6	107.3±2.8	0.49±0.02	51.7±4.3	8.9±1.7*	49.0±4.8	15.6±2.0*	8.131.7*
Group III	8.1±0.5	34.3±0.8‡	164.1±5.8	122.3±4.1	0.48±0.01	53.8±4.9	14.1±1.7*	44.0±3.9	27.0±2.9*	11.431.1*
Group IV	10.1±0.6‡	38.5±1.4†‡§	180.0±8.0‡	127.9±6.0	0.47±0.01	56.1±2.9	28.5±2.2	43.9±1.2	50.8±2.7	22.331.3
Group V	10.4±0.3‡§	38.9±0.5†‡§	198.0±7.0†‡§	140.5±5.8†‡	0.51±0.01	50.8±2.3	13.5±2.7*	36.5±1.6	25.8±4.9*	9.932.0*

Data are means ± SE. Heart weight, total heart weight (ventricles and atria); LV, left ventricle.

*$P < 0.05$ vs. *groups I* and *IV;*

†$P < 0.05$ vs. *group I;*

‡$P < 0.05$ vs. *group II;*

§$P < 0.05$ vs. *group III.*