Carbon Monoxide Protects Against Ischemia-reperfusion Injury in Vitro via Antioxidant Properties

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List of non-standard abbreviations: ANOVA, analysis of variance; AU, arbitrary units; +dP/dt, left ventricular maximal pressure development; −dP/dt, left ventricular minimal pressure development; HR, heart rate; IU, international units; L, Langendorff mode; LDH, lactate dehydrogenase; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; W, working mode

ABSTRACT

Carbon monoxide (CO) is believed to mediate many of the cytoprotective effects attributed to the activation of heme oxygenase (HO-1), the enzyme responsible for CO production. Recently, the study of CO-releasing molecules (CO-RMs) has provided a new approach for the delivery of CO. In the present study, we examined whether the cardioprotective properties of CO-RM2 in isolated rat hearts subjected to an ischemia-reperfusion (I/R) sequence were associated with the presence of CO. In addition, the antioxidant properties of CO-RM2 were evaluated.

In hearts pretreated with CO-RM2, the improvement in contractile function at the end of the reperfusion period after 20 min of global total ischemia was significantly greater than in controls. These beneficial effects were accompanied by a reduction in 1) LDH activity release 2) infarct size 3) ventricular superoxide production. The improvement in myocardial function and the reduction in oxidative stress were not observed when hearts were pretreated with inactivated CO-RM2 (iCO-RM2). Additionally, CO-RM2, but not iCO-RM2, was found to exert antioxidant properties.

These results suggest that the production of CO is a necessary factor in the cardioprotective and antioxidant actions of CO-releasing compound. These results may open up new ground for a novel class of cardioprotective compounds.

Keywords: Carbon monoxide, CO-RM2, Antioxidant, Heart, Ischemia, Rats

INTRODUCTION

Carbon monoxide (CO) has been described as a “silent killer” as a result of its high affinity for reduced iron-heme in hemoglobin, which is essential for oxygen delivery to tissues [1]. In mammalian cells, CO is produced by a family of enzymes called heme oxygenases (HO), which catalyze the degradation of heme with the coproduction of biliverdin and iron. Both the inducible and constitutive forms of heme oxygenase, namely heme oxygenase-1 (HO-1) and heme oxygenase-2 (HO-2), contribute to the endogenous production of CO in the cardiovascular system [2]. The expression of HO-2 in the endothelial and smooth muscle cell layers of arterial and venous blood vessels has been reported to generate CO, which intrinsically modulates vascular tone. HO-1 expression can induce cytoprotection in many models of vascular injury and disease [3, 4]. This effect is related to the formation of CO as the end-product. The biological actions of HO-derived CO are corroborated by the pharmacological effects of CO itself, observed at concentrations ranging from 10 to 500 ppm, which promoted protection against ischemic injury [5].

It has recently been shown that CO-releasing molecules (CO-RMs) also deliver CO, and these compounds have since emerged as potential therapeutic agents for the treatment of various cardiovascular disorders [6-10].

In the field of ischemia-reperfusion injury, several ex vivo and in vivo experimental models have highlighted the cardioprotective properties of CO carriers [9-12]. However, the cardioprotective mechanisms mediated by CO-RMs remain controversial [8]. CO is recognized as a physiological signaling molecule capable of regulating a variety of proteins such as membrane channels. Recently, it has been shown that CO-RM2 was an inhibitor of voltage-activated potassium channels [13], and it has been suggested that the interaction of K^+ channels with metal centers may determine the sensitivity of membrane channels to CO [14]. The hypothesis that mitochondria may serve as targets in transducing the beneficial signaling properties of CO has also been proposed [15]. In the mitochondria, CO has been

shown to inhibit cytochrome-c-oxidase. Like nitric oxide (NO), CO exerts this inhibitory effect by competing with oxygen for binding to the reduced form of the enzyme. CO can displace NO from heme sites, thereby increasing free NO levels. Therefore, the interactions of competing pathways can further modulate reactive oxygen species (ROS) [16]. Few studies have been conducted on the impact of CO-RMs on the generation of ROS and the results are inconsistent. In some, CO was shown to promote a transient and subtle increase in mitochondrial ROS production [17, 18], a role that may appear paradoxical given the described cardioprotective effects of CO-RMs. Recently, Lo Iaconono et al. [12] suggested that CO may act as both a stimulus for the generation of physiologically relevant levels of ROS in the mitochondria and an inhibitor in situations in which excessive ROS are produced by these organelles.

In the present study, we examined whether the cardioprotective properties of CO-RM2 in isolated rat hearts subjected to a sequence of ischemia-reperfusion were associated with the presence of CO, and evaluated with two different methods the potential antioxidant properties of this molecule. The isolated working heart model was used in order to avoid possible indirect cardiac effects of CO-RM2, such as autonomic nervous and hormonal influences, as well as preload and afterload factors.

MATERIALS and METHODS

Chemicals

All of the chemicals were bought from Sigma (Saint Quentin Fallavier, France). CO-RM2 ($[Ru(CO)_3Cl_2]_2$) was purchased from Sigma-Aldrich Chemical Co (Saint Quentin Fallavier France) and the LDH assay kit from Promega (Charbonnières, France).

CO-RM2 was initially dissolved in DMSO at a concentration of 10^{-3} mol/L, and then diluted in 0.9% NaCl solution in order to reach a final concentration of 10^{-4} mol/L. Inactive CO-RM2 (iCO-RM2) was prepared by dissolving CO-RM2 in similar conditions, but the solution was left for 18 h at 37°C. The iCO-RM2 solution was finally bubbled with nitrogen gas to remove the residual CO present in the solution.

Working heart model

The local ethics committee approved the experimental protocol and the investigators complied with authorization 5605 from the French government, which agrees with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes for Health. Male Wistar rats (280-320 g) were purchased from IFFA Credo (France). The rats were anesthetized with sodium thiopental (60 mg/kg, i.p.) and heparin was intravenously injected (500 IU/kg). After 1 minute, the hearts were excised and placed in a cold (4°C) perfusion buffer bath until contractions ceased. Once the aorta had been isolated, the hearts were cannulated and retrograde perfusion (Langendorff mode) was immediately initiated with warmed perfusion liquid at a constant hydrostatic pressure of 55 mmHg. The hearts resumed beating almost immediately on rewarming. The total time from excision of the hearts to initiation of Langendorff perfusion did not exceed 3 minutes. While in the Langendorff mode, a cannula (PE-50) was passed through a pulmonary vein into the left ventricle and pulled through the ventricular wall. It was then connected to a pressure transducer by a fluid-filled line. The left atrium was then cannulated with a cannula through the same pulmonary vein,

and the pulmonary veins were then collectively ligated around the cannula. After 10 minutes of equilibration, the hearts were switched to the working heart model; liquid was then delivered via the left atrial cannula and ejected via the aortic cannula against a hydrostatic fluid column. Hearts were excluded if their aortic flow (AF) was under 30 mL/min and/or if heart rate was less than 270 beats per minute (bpm) and/or if dP/dt_{max} was under 2,500.

Perfusion medium

The perfusion buffer consisted of a modified Krebs-Henseleit bicarbonate buffer (KH) (concentrations in mmoles/L: NaCl 118, $NaHCO_3$ 25, $MgSO_4$ 1.2, KCl 4.5, glucose 11, $CaCl_2$ 1.5). The perfusion fluid was filtered through a 0.45 µM Millipore filter in order to remove any particulate contaminants and gassed with 95% oxygen and 5% carbon dioxide (pH 7.3 – 7.4 at 37°C). Software developed by the laboratory was used to assess left ventricular end-diastolic pressure (LVEDP) and left ventricular end-systolic pressure (LVESP). Left ventricular developed pressure (LVDP = LVESP – LVEDP) and heart rate (HR), dP/dt_{max} were then calculated by the software. Coronary flow (CF) and aortic flow (AF) were measured, and cardiac output was calculated as AF + CF. An index of contractile function was calculated as the rate pressure product (RPP) = LVDP $\times$ HR.

Perfusion protocols

After a stabilization period of 10 min (Figure 1), the isolated hearts were perfused aerobically for 10 min in the Langendorff mode prior to 20 min of the working heart mode; CF, AF, cardiac output, HR, LVEDP, LVESP were measured and used as pre-ischemic values. The hearts were not paced. Global normothermic ischemia was induced by clamping aortic inflow for 20 min, during which a thermoregulated chamber maintained the heart temperature at 37°C. Vehicle (NaCl 0.9% containing 10% DMSO; 1:100 dilution), CO-RM2 (10^{-6} mol/L) or iCO-RM2 (10^{-6} mol/L) were added to the perfusate 10 min before the onset of ischemia and

the concentration was maintained during the first 5 min of reperfusion. After ischemia, the Langendorff mode was maintained during the first 10 min of reperfusion, after which the hearts were switched to the working mode for 30 min. Samples of coronary flow were collected during reperfusion in order to measure the release of lactate dehydrogenase (LDH), a global marker of cell injury.

Determination of lactate dehydrogenase (LDH) release

LDH release was measured using a kit (Promega, France). Samples of coronary effluents were stored at 4°C until assay (within 6 hours) following the manufacturer's instructions. The accumulated amount of LDH released was obtained by integrating the area under the time course curve during the reperfusion. Results are expressed as arbitrary units per gram of tissue per milliliter (AU/g/mL).

Oxidative fluorescence histology

Dihydroethidium (DHE), an oxidative fluorescent probe, was used to localize superoxide anion ($O_2^{\bullet -}$) in ventricular tissue slices. The hearts were harvested at the end of the reperfusion period. Frozen heart tissues (middle area) were fixed in acetone for 10 min. The slices were incubated in light-protected humidified chambers at room temperature with DHE (5 µmol/L) for 5 min, then immediately analyzed with a computer-based digitizing image system (Microvision, Evry, France) using a microscope equipped with a fluorescent lamp (Eclipse 600, Nikon, Champigny-Sur-Marne, France) and connected to a video camera (Tri CCD, Sony, Paris). Fluorescence was detected with a 510-560 nm excitation and 590 nm emission filters. Nuclei were counted using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) which selectively binds to nuclei. Automatic computer-based analysis was performed with the same threshold for all sections (x500 magnification). Results were expressed as the fluorescence intensity/nuclear number.

Tissue injury

The frozen hearts were cut into 4 slices of approximately equal thickness, and were incubated in 10% triphenyl-tetrazolium chloride (TTC, Sigma, France) in phosphate buffer at 37°C for 20 minutes. The viable tissue was TTC-positive and stained red, whereas the necrotic tissue was TTC-negative. After staining, the heart slices were fixed with 10% formaldehyde overnight and tissue injury was measured by computer morphometry (Histolab, Microvision). The extent of injury was evaluated and expressed as a percentage of the total right + left ventricle area.

Antioxidant capacity

In order to further characterize the antioxidant effects of CO-RM2, two different approaches were used: (1) Electron Paramagnetic Resonance (EPR) spectroscopy with spin-trapping techniques to determine if CO-RM2 substances can scavenge superoxide anion radical [19]; (2) Protection of allophycocyanin (a fluorescent protein) against oxidation, induced by a peroxyl radical generator, for the measurement of the peroxyl radical scavenging capability of CO-RM2.

For EPR spectroscopy, xanthine oxidase (XO) was used as a source of oxyradicals, and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (Alexis) was the spin-trap chosen. For the experiments where superoxide radical anion ($O_2^{\bullet -}$) was generated, the solutions contained xanthine (500 µM), xanthine-oxidase (50 mIU/ml), catalase (500 IU/ml), deferrioxamine (1 mM), DMPO (50 mM) and various concentrations of CO-RM2 in phosphate buffer (50 mM, pH 7.4). Due to the insolubility of the CO-RM2, dimethylsulfoxide (DMSO) was used as the solvent and buffers containing equal concentrations of DMSO were used as control (2%).

Spectra were recorded at 310K with a Bruker EMX-X band spectrometer (Bruker, Wissembourg, France), using a HS cavity and a capillary quartz cells. The following parameters were selected for detection of DMPO adducts: microwave power, 20 mW; microwave frequency, 9.44 Ghz; amplitude modulation, 0.8 G; frequency modulation, 100 kHz; gain, 1.10^6, time constant 164 ms. Relative radical concentrations, in Arbitrary Units (AU), were determined by measurement of line intensities on spectra recorded with identical spectrometer settings as described previously [20].

For protection of fluorescent protein against oxidation, the method employed was that described by [21]. This technique is based on the ability of antioxidant components to protect an indicator protein, allophycocyanin (APC), whose fluorescence is altered when it is oxidized. Oxidative stress was induced in vitro by a peroxyl radical generator, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). APC fluorescence was measured at 37°C and at 652 nm, after excitation at 598 nm, every minute from the addition of AAPH until the end of the oxidation reaction. The results are expressed as a comparison between the net protection offered by the tested compounds and that provided by a reference antioxidant: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid or trolox, 1 µM, a water-soluble vitamin E analogue. For all of the assays there was 10 % DMSO in the cuvettes. The antioxidant properties of CO-RM2 and iCO-RM2 were evaluated; the antioxidant properties of different concentrations of these compounds were compared to that of 1 µmol/L of Trolox. The results were expressed in ORAC (oxygen radical absorbing capacity) units, where 1 ORAC unit represents the net protection provided by 1 µM trolox.

Statistical analysis

All data are expressed as means ± SEM. Statistical analyses were performed with the two-factor analysis of variance (ANOVA) test (SigmaStat). ANOVA was followed by inter-group pair-wise comparisons with Tukey HSD multiple comparisons. For histology, statistical

analyses were performed with a nonparametric Mann-Whitney rank sum test (SigmaStat). Significance was established at a value of $P < 0.05$.

RESULTS

Effect of CO-RM2 on myocardial post-ischemic recovery

Functional parameters

All of the hearts were stable and showed similar CF during the pre-ischemic period, around 11 mL/min during the Langendorff mode, and around 15 mL/min during atrial perfusion. After 20 min of global ischemia, reperfusion induced only partial recovery of coronary flow in the control group (8.92 ± 0.37 mL/min at the end of reperfusion in Langendorff) whereas the administration of CO-RM2 during pre and post-ischemic period led to better recovery of CF (10.6 ± 0.45 mL/min; $p<0.05$).

Cardiac output was identical (about 55 mL/min) in all groups before ischemia (Figure 2). During reperfusion, the control group showed very poor recovery (around 8%; 4.17 ± 3.38 mL/min) of cardiac output while treatment with CO-RM2 led to improved post-ischemic recovery (around 40%; 22.70 ± 4.47 mL/min; $p<0.05$).

All of the hearts showed sinus rhythm and a heart rate of around 305 bpm before ischemia in both the Langendorff and working mode. Post-ischemic recovery at the end of the reperfusion period was similar in all groups (around250 bpm). However, heart rate recovery was faster in hearts treated with CO-RM2 (10 min to reach 250 bpm vs. 25 min in the control group).

The rate pressure product (RPP), corresponding to the product of LVDP and HR, is considered an index of myocardial contractility (Figure 3). During ischemia, there was no myocardial contraction and the RPP was therefore zero. During the final minute of reperfusion, the RPP in hearts treated with CO-RM2 was significantly greater than that in control hearts ($p<0.05$) .

When the isolated hearts were perfused with iCO-RM2, there was no improvement in myocardial function (cardiac output, rate pressure product) after the ischemia reperfusion sequence (Table 1).

Tissue injury:

The administration of CO-RM2 during the pre-ischemic and post-ischemic periods led to a significantly smaller ($p<0.05$) infarct size (27.8 ± 3.5% in the control group vs. 16.9 ± 3.2% in the CO-RM2 group; Table 1). The administration of iCO-RM2 did not reduce infarct size.

LDH release

Coronary effluent was analyzed for LDH, an intracellular enzyme released during post-ischemic reperfusion, in order to assess the degree of cardiac injury (Table 1). The release of LDH during reperfusion was significantly lower in hearts pre-treated with CO-RM2 than in controls (101.34 ± 10.68 AU/mL/g vs. 75.95 ± 6.89 AU/mL/g; $p<0.05$), whereas the administration of iCO-RM2 did not lessen LDH release.

In situ measurement of superoxide anion

Dihydroethidium (DHE) reacts with superoxide anion radical to form ethidium bromide, which intercalates in nuclear DNA, inducing intra-cellular nucleus fluorescence. The assessment of oxidative stress with DHE in ventricular tissues harvested after the ischemia reperfusion sequence showed 1) significantly higher superoxide production due to the ischemia reperfusion protocol and 2) significantly lower DHE staining ($p<0.05$) in heart samples from the CO-RM2 group than in those from the control group ($0.736 \pm 0.026\ 10^{-3}$ vs. $0.885 \pm 0.052\ 10^{-3}$ % fluorescence/nuclei (Table 1).

Antioxidant capacity

Figure 4 shows the characteristic spectra of the adduct of DMPO with superoxide anion radical (DMPO-OOH). Several spectra were obtained with increasing concentrations of CO-RM2 in the presence of 2 % DMSO. At this dilution, DMSO had no effect on DMPO-OOH signal intensity, but 0.5 IU/mL of superoxide dismutase were able to decrease the signal by about 50%. However, the intensity of DMPO-OOH signals were not modified by any of the concentrations of CO-RM2 tested.

When CO-RM2 was added to APC and AAPH, a concentration-dependent decay in fluorescence was observed. The ORAC values were calculated for the different concentrations of CO-RM2 and the dose-dependent antioxidant properties of CO-RM2 were observed for concentrations ranging from 1 to 12 µM (Figure 5). A regression line was drawn. The concentration of CO-RM2 that led to a protection corresponding to one ORAC unit was 4 µM. Thus, CO-RM2 could protect APC from AAPH-induced oxidative stress at a concentration 4 times greater than the reference antioxidant Trolox. However, solutions of CO-RM2 left at 37°C showed a time-dependent loss of their antioxidant capacity, losing 50% in 6 hours. Consequently, solutions of iCO-RM2 were not able to exert any antioxidant effect in the same experimental conditions.

DISCUSSION

The first aim of our study was to describe the effects of CO-RM2 on the functional parameters of isolated perfused rat hearts during an ischemia-reperfusion sequence. The administration of CO-RM2 during the pre and post-ischemic period led to better recovery of coronary flow at the end of the reperfusion. The rate pressure product (RPP), an index of myocardial contractility, in the two groups of hearts was different. In hearts pretreated with CO-RM2 contractility during the final minute of reperfusion was significantly greater than that in control hearts ($p<0.05$) These beneficial effects were accompanied by a reduction in LDH release during reperfusion. Additionally, the administration of CO-RM2 induced a significant reduction in infarct size.

There was no improvement in myocardial function in hearts perfused with iCO-RM2 and subjected to ischemia-reperfusion thus confirming the critical role of CO in this cardioprotection.

Several studies support the hypothesis that CO-RMs play a crucial role in the suppression of inflammation, in cellular protection and in the prevention of cardiovascular disorders. Our results agree with those of Motterlini *et al.* [7] who found that the recovery of function in hearts reperfused in the presence of CO-RM3 was significantly greater than that in controls; this effect was not observed in the presence of iCO-RM3. The *ex vivo* and *in vivo* experiments conducted with models of cardiac ischemia highlighted the protective pharmacological properties of CO carriers. In a mouse model of myocardial infarction by coronary artery occlusion, intravenous infusion of CO-RM3 before reperfusion reduced infarct size, fibrillation and tachycardia [9, 10, 12]. These cardioprotective mechanisms mediated by CO-RMs probably involve a direct effect of CO, probably through its interactions with potassium channels [15]. Calcium-activated potassium channels are indeed important in regulating several physiological phenomena, including oxygen sensing and vasodilatation. It

has been shown that CO-RM2 is an inhibitor of voltage-activated K^+ channels [13] and that this compound could be a useful tool to help understand the mechanisms of electromechanical uncoupling.

In vivo, endogenous heme oxygenases can generate CO, bilirubin and iron at the time of heme breakdown. Both heme oxygenase-1 and heme oxygenase-2 contribute to the endogenous production of carbon monoxide in the cardiovascular system [2]. Heme oxygenase-2 expressed in the endothelial and smooth muscle layers of arterial and venous blood vessels has been reported to generate carbon monoxide that intrinsically modulates vascular tone. Low doses of CO appear to be important in the homeostatic control of cardiovascular functions that provide cardioprotection, but exposure to higher exogenous levels of CO has, either acutely or chronically, profound deleterious effects on cardiac function and can exacerbate outcomes in cardiovascular diseases [18]. Heme-containing proteins are very widely distributed, and a very large number of reactive oxygen and nitrogen species can interact, either directly or indirectly, with their iron center. The number of signaling pathways and potential targets are therefore very high [3, 15].

In our study, after a cardiac ischemia-reperfusion sequence, a significant increase in superoxide production, assessed with DHE, was observed in myocardial tissue, a result that is in keeping with previous works [22, 23]. There is growing evidence that reactive oxygen species and lipid peroxidation are of major importance in the development of cardiac injury associated with reperfusion. It is well documented that free radicals are generated during reperfusion [19, 24, 25] and that various compounds that can either inhibit their formation or scavenge them may protect the heart against reperfusion dysfunction [26].

In our experimental conditions, the cardioprotective effect of CO-RM2 was associated with a reduction in tissue oxidative stress. Superoxide levels were determined on frozen sections of hearts harvested at the end of reperfusion, and can therefore be considered a marker of delayed oxidative stress. [19]. Our results are in agreement with spin-trapping

experimentation showing a biphasic free radical profile with one peak of production occurring during the first minute of reperfusion and a second peak, related to secondary radicals, after 20 minutes [19]. Thus, it seems plausible that during an ischemia-reperfusion sequence, CO-RM2 was able to modify one step in the oxidative stress process,, this effect being associated with a beneficial impact on the recovery of function and cellular injury. In addition, no improvement in myocardial function was seen in ischemic-reperfused hearts in the presence of iCO-RM. These results are consistent with the hypothesis that CO is directly implicated in cardiac protection.

The current study was designed to determine whether CO released from CO-RM2 exhibited direct effects on the heart, independently not only of neuronal and humoral interactions but also of dynamic effects due to changes in preload and afterload pressures., We therefore used the isolated perfused heart model. Our results support the view that CO exerts its effects at myocardial sites. In our model, we clearly showed that CO-RM2 in the perfusion medium has beneficial effects on functional parameters.

Many criteria must be considered when evaluating the antioxidant potential of a compound: such as specificity of free radical scavenging, metal chelating activity, interaction with other antioxidants, concentration in the cellular compartment and induction of proteins involved in antioxidant protection. In our study, we evaluated the specificity of free radical scavenging. In the present work, EPR spectroscopy was used to determine if CO-RM2 could scavenge superoxide radical. However, CO-RM2 was found ineffective in directly buffering this radical. High concentrations of antioxidants are usually required in this test to scavenge the large amount of superoxide radical anion produced. For instance, the antioxidant capacity of superoxide dismutase was 0.5 IU/mL and this of Trolox was measured as an IC_{50} of 0.117 mM.

In our in vitro study, CO-RM2 appeared to have dose-dependent antioxidant properties. This effect was evaluated using the allophycocyanin model in the presence of carbon-centered

free radicals generated by AAPH. In our experiments, the peroxyl radicals were produced by temperature-dependent unimolecular decomposition of AAPH. The carbon radicals derived from this azo-compound react with oxygen to produce peroxyl radicals [27]. Our study showed that CO-RM2 and Trolox are potent peroxyl radical scavengers in vitro. These in vitro experiments with AAPH, though not clinically relevant, may help to understand the specific role played by antioxidants [28, 29]. An important point in our results is that we found a considerable loss of the antioxidant properties of CO-RM2 with time, underlining the role of CO in this protective effect. This property could be explained by the free radical scavenging ability of CO. It can be hypothesized that CO released from CO-RM2 interacts with and scavenges oxygen free radicals. The chief mechanisms underlying the ability of CO to scavenge ROS have yet to be explored. CO could appear to act via oxidative stress since it reduced the activity of several antioxidant enzymes [30]. This effect on antioxidant enzymes, however, is not always observed [18]. It appears that the effects of CO on cardiac and metabolism functions are dependent not only on the doses but also on the tissues. In addition, given the great number of effects of endogenous gases and gas transducing systems, the interactions are complex. Numerous studies have tried to analyze the relationship between CO and NO [31]. Recently, Marazioti et al. [32] showed that CORM did not activate purified soluble gluanyl cyclase but were able to elevate cGMP levels in cells, causing vasorelaxation through the direct scavenging of NO. Surprisingly, it is worth noting that in this study, CO-RMs were shown to increase superoxide production. It is clear in our study that CO-RM2 exerts antioxidant properties. Thus, in biological systems, it appears that CO-RMs have a high affinity for free radicals and scavenge various radicals such as NO both directly and indirectly through a range of mechanisms.

Some data suggest that CO-RM2-released CO can directly interfere with the production of intracellular ROS. The potential role CO-RM2-released CO on the production of intracellular ROS has been studied. Oxidant production in HUVEC was assessed by measuring the oxidation of intracellular dihydrorhodamine-123 an oxidant-sensitive

fluorochrome. Stimulation of HUVEC with LPS resulted in increased production of ROS. Administration of CO-RM2 to HUVEC significantly attenuated LPS-induced ROS production in a dose-dependent manner [33]. In this context, it is not possible to identify the ROS associated with the oxidative stress. However, recent findings indicate that CO derived from enhanced HO-1 activity or from CO-RM2 inhibits activity of NADPH oxidase and therefore suppresses superoxide overproduction and the accumulation of ROS in LPS-stimulated macrophages [34].

In vivo, CO-RM2 exerts beneficial cardioprotective effect in vivo against DXR-induced cardiotoxicity by reducing oxidative stress measured by ABTS (2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate]) assay [35]. Recently, a study reports that two CO-RMs (CO-RM2 and ALF062) stimulate the production of ROS in Escherichia coli, an effect that is abolished by addition of antioxidants [36]. Importantly, it is showed that CO-RMs generate hydroxyl radicals in a cell-free solution, a process that is abolished by scavenging CO.

Finally, it appears that the antioxidant or pro-oxidant potential of CO is a subject of controversy. Indeed, the chemical reactivity of oxyradicals such as superoxide, hydroxyl and peroxyls radicals towards biological targets is different; accordingly, the relative efficiency of an antioxidant can vary depending on the oxidant [37]. For instance, it has been demonstrated that ergothioneine, a low-molecular-mass thiol, was a protective against oxidation in tissues, scavenging singlet oxygen and hydroxyl radicals [38]. By contrast, this compound did not react with superoxide or hydrogen peroxide and it did not inhibit microsomal lipid peroxidation in the presence of iron [39]. The specificity of free radical scavenging has been described with several other agents. Studies indicated that lipoic acid scavenges hydroxyl radicals, hypochlorous acid and singlet oxygen. In our Laboratory, where we have developed several projects in the field of oxidative stress associated with cardiovascular disease [40-44], we also observed that lipoic acid was able to diminish the superoxide-driven oxidation of a sensitive spin probe, in a manner comparable to that of superoxide dismutase; this effect being not noted with all the antioxidant agents [45]. It can

be stated that the sites of reaction of antioxidant agents are not always the same. It depends on the nature of the reacting free radicals and is also modulated by the environment.

Unlike the highly reactive NO, which by itself is a free radical, CO does not contain free electrons. Given its stable chemical nature, CO should not by itself generate reactive oxygen species. However, CO may be involved in oxidative stress indirectly, especially under pharmacological and toxicological conditions. Toxic and presumed sub-toxic exposure to CO is associated with significant oxidative and nitrosative stress. CO-dependent lipid peroxidation was prevented or reduced by the inhibition of xanthine oxidase or superoxide dismutase and iron chelators. It has also been shown that intracellular H_2O_2 production in the brain was increased by high concentrations of CO, accompanied by increases in hydroxyl radical production and decreases in the reduced to oxidized glutathione (GSH/GSSG) ratio in mitochondria [46].

Like for the majority of pharmacological substances, the potentially harmful or beneficial effects of CO are dependent on its concentration. Low concentrations of CO, equivalent to these released by the local activity of HO, can affect various deleterious processes such as inflammatory reactions. However, CO in higher concentrations is able to aggravate oxidative stress.

In conclusion, it appears that CO released from low concentrations of CO-RM2 is able to exert cardioprotective effects, probably through its antioxidant properties, in a situation of ischemia reperfusion injury. Therefore, it is important to point out that the local effects of CO may derive from its interaction with other reactive species such as NO and that this can influence the activity of several modulatory factors [47]. Many technical advances are needed to detect physiological levels of “biological gases”: O_2, NO, H_2S and CO in a real-time manner with spatial information. Compounds such as CO-RM2 should be useful tool to help understand the mechanisms of CO via an integrated approach.

Authorship Contributions

Participated in Research design: Rochette

Conducted experiments: Berne, Lauzier, Vergely

Performed data analysis: Berne, Rochette, Vergely

Wrote or contributed to the writing of the manuscript: Vergely, Rochette

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Figure Legends

Figure 1: Perfusion protocol of isolated perfused rat hearts.

L: Langendorff mode; W: Working mode

Horizontal arrows show time of perfusion for vehicle, CO-RM or inactivated-CO-RM (iCO-RM); Vertical arrows show sampling time for lactate dehydrogenase (LDH) activity in coronary effluents

Figure 2: Evolution of cardiac output (mL/min) in isolated rat hearts treated with 10^{-6} mol/L CO-RM2 (n= 10, white circles) or 10 % DMSO vehicle (controls, n= 12, black circles) during the protocol of perfusion in Langendorff (L) or working (W) mode, before and after 20 min of global total normothermic ischemia.

All values represent means ± SEM. *: P<0.05 vs Control

Figure 3: Evolution of rate pressure product (bpm.mmHg) in isolated rat hearts treated with 10^{-6} mol/L CO-RM2 (n= 10, white circles) or 10% DMSO vehicle (controls, n= 12, black circles) during the protocol of perfusion in Langendorff (L) or working (W) mode, before and after 20 min of global total normothermic ischemia.

All values represent means ± SEM. *: P<0.05 vs Control

Figure 4: Representative EPR spectra of the adduct of the spin trap DMPO with superoxide anion (DMPO-OOH) generated by xanthine (500 µM)/xanthine oxidase (50 mIU/mL) system at 310K, in the presence of 2% of DMSO. The addition of 0.5 UI/mL of superoxide dismutase (SOD) decreased the signal by about 50 % whereas the highest concentration of CO-RM2 did not.

Figure 5: ORAC values for increasing concentrations of CO-RM2

Table 1. Myocardial post-ischemic recovery and biochemical parameters at the end of reperfusion periods

	Control	CO-RM2 (10^{-6} M)	iCO-RM2 (10^{-6} M)
Cardiac output (mL/min)	4.17 ± 3.38 a n = 12	22.70 ± 4.47 b n = 10	6.6 ± 1.50 a n = 5
Rate Pressure Product (mmHg.beats/min)	16 198 ± 1 870 a n = 12	25 527 ± 3 563 b n = 10	12 160 ± 1 570 a n = 5
Tissue injury (%)	27.8 ± 3.5 a n = 12	16.9 ± 3.2 b n = 10	33.8 ± 2.8 a n = 5
LDH release (AU/mL/g)	101.34 ± 10.6 a n = 8	75.95 ± 6.89 b n = 10	118.80 ± 15.09 a n = 5
Myocardial superoxide ($\times 10^{-3}$ %)	0.885 ± 0.052 a n = 5	0.736 ± 0.026 b n = 5	0.974 ± 0.044 a n = 5

Means with a different letter differ with $p<0.05$

Tissue injury: Infarct size is expressed as a percentage of the total right + left ventricle area

LDH: Lactate dehydrogenase in coronary effluent during reperfusion

Myocardial superoxide was measured using the DHE method

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Figure 1

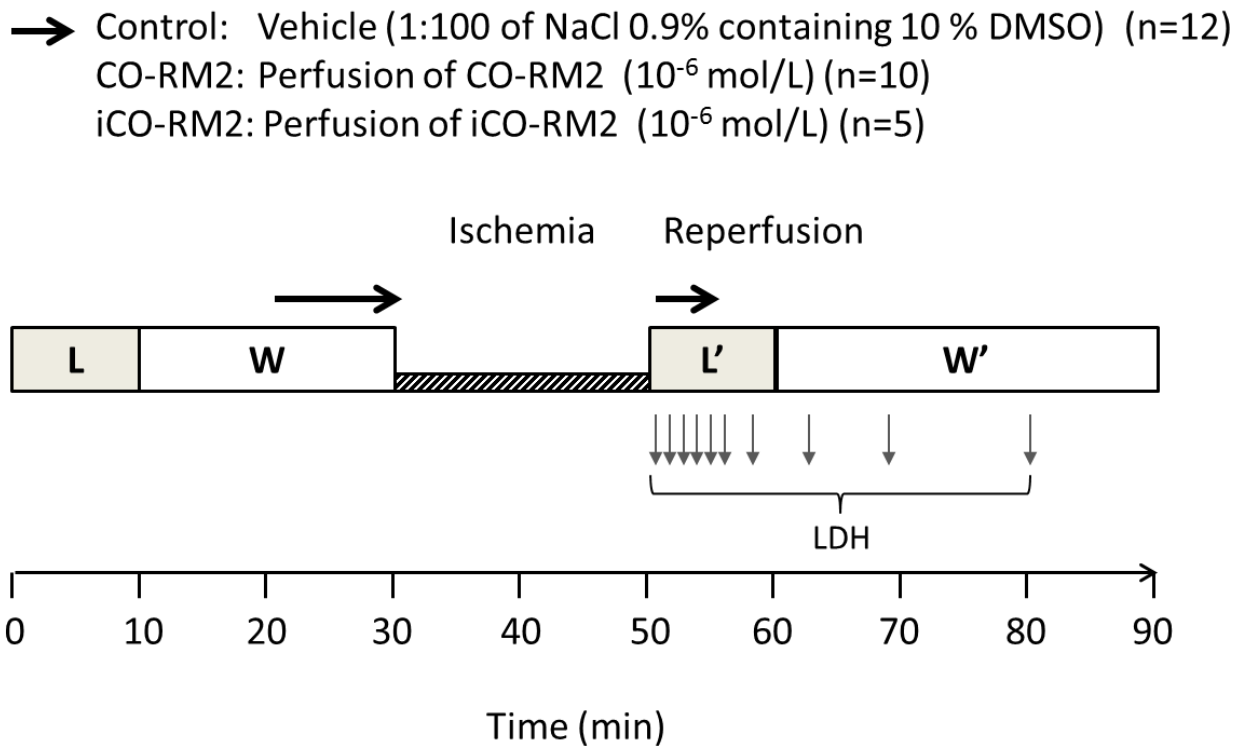

Control: Vehicle (1:100 of NaCl 0.9% containing 10 % DMSO) (n=12)
CO-RM2: Perfusion of CO-RM2 (10^{-6} mol/L) (n=10)
iCO-RM2: Perfusion of iCO-RM2 (10^{-6} mol/L) (n=5)
Ischemia
Reperfusion
L
W
L'
W'
LDH
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 2

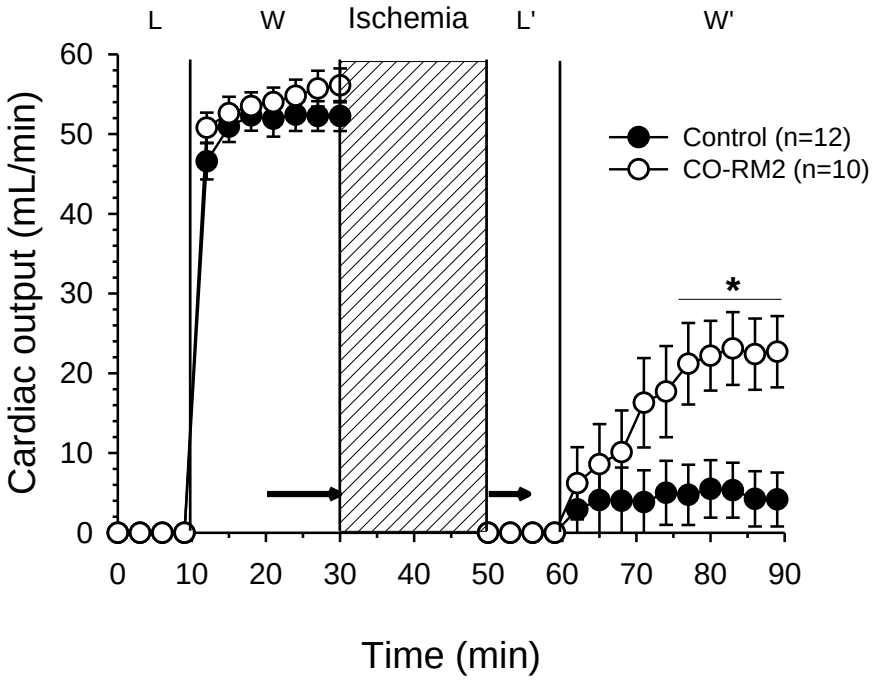

L
W
Ischemia
L'
W'
Cardiac output (mL/min)
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
Time (min)
Control (n=12)
CO-RM2 (n=10)
*

Figure 3

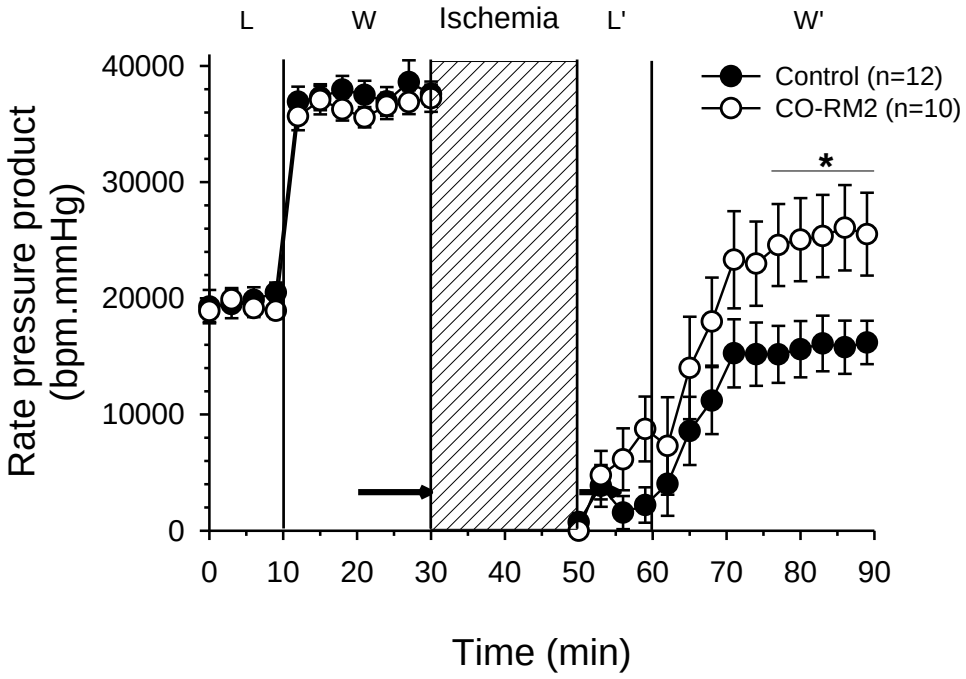

L
W
Ischemia
L'
W'
Control (n=12)
CO-RM2 (n=10)
*
Rate pressure product
(bpm.mmHg)
40000
30000
20000
10000
0
0
10
20
30
40
50
60
70
80
90
Time (min)

Figure 4

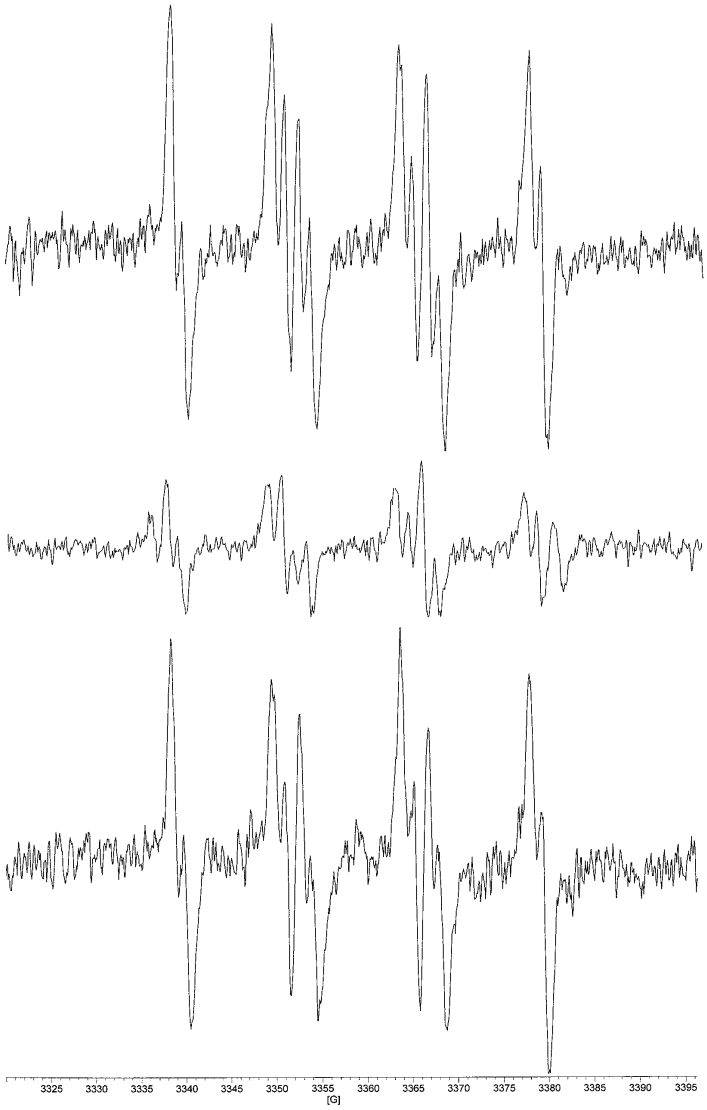
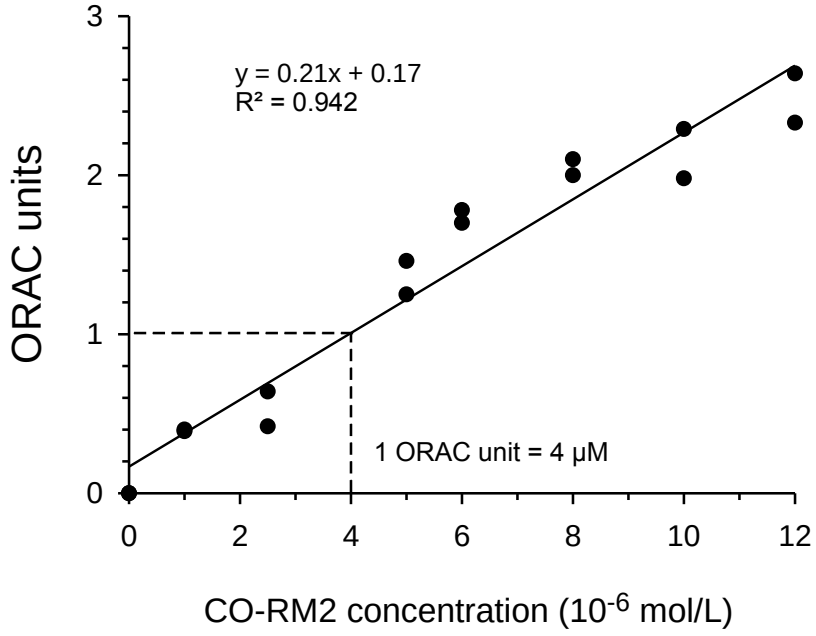

Xa + XOD +DMPO + 2% DMSO
Xa + XOD +DMPO + 2% DMSO
+ SOD 0.5 UI/mL
Xa + XOD +DMPO + 2% DMSO
+ CO-RM2 3.8 10-5M
3325 3330 3335 3340 3345 3350 3355 3360 3365 3370 3375 3380 3385 3390 3395
[G]

Figure 5


y = 0.21x + 0.17
R² = 0.942
ORAC units
CO-RM2 concentration (10-6 mol/L)
1 ORAC unit = 4 µM
0
1
2
3
0
2
4
6
8
10
12