Carbon Monoxide Protects Against Cardiac Ischemia–Reperfusion Injury In Vivo via MAPK and Akt–eNOS Pathways

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Background—Carbon monoxide (CO) is postulated to protect tissues against several types of injuries. We investigated the role of CO in amelioration of cardiac ischemia–reperfusion injury in vivo and the mechanisms involved in it.

Methods and Results—Rats inhaled CO (250 ppm, 500 ppm, or 1000 ppm) for 24 hours in a chamber after myocardial ischemia–reperfusion induced by occluding the left anterior descending coronary artery for 30 minutes. Pre-exposure to 1000 ppm of CO significantly reduced the ratio of infarct areas to risk areas and suppressed the migration of macrophages and monocytes into infarct areas, and the expression of tumor necrosis factor (TNF–H9251 in the heart; however, 250 ppm, 500 ppm of CO, or low barometric pressure hypoxia (0.5 atm) did not affect them. Exposure to 1000 ppm CO resulted in the activation of p38 mitogen-activated protein kinase (p38MAPK), protein kinase B (Akt), endothelial nitric oxide synthase (eNOS), and cyclic guanosine monophosphate (cGMP) in the myocardium. Inhibition of p38MAPK, PI3kinase, NO, and soluble guanylate cyclase with SB203580, wortmannin, N(G)-nitro-l-arginine methyl ester (l-NAME), and methylene blue, respectively, attenuated the cytoprotection by CO.

Conclusion—CO has beneficial effects on cardiac ischemia–reperfusion injury; this effect is mediated by p38MAPK pathway and Akt–eNOS pathway, including production of cGMP. (Arterioscler Thromb Vasc Biol. 2004; 24:1848-1853.)

Key Words: ischemia ■ ischemic heart disease ■ nitric oxide synthase ■ pharmacokinetics ■ reperfusion injury

When acute myocardial infarction occurs, revascularization therapy should be performed as soon as possible to minimize the myocardial damage. However, reoxygenation of ischemic heart induces ischemia–reperfusion injury. Oxidative stress seems to play important roles in ischemia–reperfusion injury.1–3

Heme oxygenase-1 (HO-1) is an endogenous antioxidant enzyme that catabolizes heme to free iron, carbon monoxide (CO), and biliverdin.4 HO-1 acts protectively against hypoxia5,6 or ischemia–reperfusion injury.7,8 Biliverdin, a byproduct of heme catabolism, is endogenously converted to bilirubin and protects tissues.9 CO, another product of heme catabolism, is usually regarded as a toxic species that disrupts cellular respiration. However, recently, CO has been shown to be an important signaling molecule and protects tissues against injuries induced by several types of stress. HO-derived CO reduces vasoconstriction, thus lowering blood pressure and increasing tissue blood flow.10,11 Exogenous CO was reported to reduce ischemia–reperfusion injury of lung and apoptosis of pancreatic β cells via activating soluble guanylate cyclase,12,13 and to reduce hepatic ischemia–reperfusion injury, apoptosis of endothelial cells, and inflammation through p38 mitogen-activated protein kinase (p38MAPK) pathway.14–16 Motterlini et al reported that CO-releasing molecules could reduce the cardiac muscle damage induced by ischemia–reperfusion via activation of mitochondrial K_ATP channel in an ex vivo model;17 however, the precise mechanism of tissue protection by CO is still controversial. In this study, we investigated whether exogenous CO acts protectively against cardiac ischemia–reperfusion injury in vivo and studied the mechanisms involved in it.

Methods

Animal Models
All animal experimentation protocols in the study were approved by the Animal Research Committee in accordance with the Guidelines on Animal Experimentation of the University of Tokyo and the Japanese Government Animal Protection and Management Law.

Inhalation of CO
CO was blended with air at 250 ppm, 500 ppm, and 1000 ppm by a commercial vendor (Suzusho Medical Co, Tokyo, Japan). Gases were delivered into specially designed chambers in which female
Sprague-Dawley rats (180 to 250 grams) were kept with access to chow and water for 24 hours. The concentrations of O₂ and CO in the chambers were monitored; O₂ was kept at 20% by volume and CO was kept at 250 ppm, 500 ppm, or 1000 ppm. Because CO was expected to induce tissue hypoxia, we exposed another group of rats to low barometric pressure hypoxia (0.5 atm) for 24 hours before surgery. The temperature in the chambers was kept at 25 ± 3°C. Rats were subsequently taken out of the chambers and ischemia–reperfusion surgery was immediately started. The concentration of carboxyhemoglobin (HbCO) in blood was measured by a hemoximeter (model OSM3; Radiometer Trading Co), using 100 μL of arterial blood. Other rats were randomized to receive 1 of the following treatments: intraperitoneal (IP) injection of 50 ppm CO, protoporphyrin – IV (ZnPP) (Funakoshi, Tokyo, Japan), or vehicle (0.2 mL DMSO) twice, at 48 hours and at 24 hours before ischemia–reperfusion surgery, with or without pre-inhalation of 1000 ppm CO for 24 hours.

Ischemia–Reperfusion Procedure
Ischemia–reperfusion procedure was performed as described previously.5 Rats were anesthetized and artificially ventilated with a respirator (model 683: Harvard Apparatus). The proximal portion of the left anterior descending coronary artery was occluded with a suture. After occlusion for 30 minutes, the suture was loosened and the myocardium was reperfused. To keep the concentration of HbCO in blood at approximately the same level before and during ischemia–reperfusion, all the animals with CO pretreatment were ventilated with 250 ppm CO during ischemia and during 120 minutes of reperfusion. Control rats were ventilated with room air during ischemia–reperfusion.

Assessment of Infarct Area
After 120 minutes of reperfusion, the coronary artery was again briefly occluded, and 1% Evans Blue solution (Wako, Osaka, Japan) was infused through the left jugular vein to map the risk areas for ischemia. The hearts were excised and divided into 4 to 5 serial cross-sections. The infarct areas were detected by incubating the cross-sections in a 1% triphenyl tetrazolium chloride solution (Wako) for 10 minutes at 37°C. Left ventricular areas, risk areas, and infarct areas were measured using the image analysis software, NIH Image (National Institutes of Health, Research Service Branch, Bethesda, Md).

Immunohistochemistry
For immunohistochemistry, the chest walls of the animals were closed after reperfusion, and hearts were excised after 24 hours of reperfusion. Immediately after incision, hearts were flushed with phosphate-buffered saline pH 7.4, fixed in 10 formalin overnight at 4°C, and embedded in paraffin. Three-micron sections were cut from the paraffin embedded tissue and stained with hematoxylin and eosin. Immunohistochemical staining with mouse–antirat macrophage/macropage monoclonal antibody (ED-1; Dainippon Pharmaceutical, Osaka, Japan) was performed as described previously.7 The number of ED-1 positive nuclei was scored in 6 sequentially cut sections of the ischemic lesion. Apical, mid-ventricular, and basal sections were examined for a total of 18 measurements per heart. The percentage of ED-1 positive nuclei of 3 hearts in each experimental group was recorded.

Assay of Tumor Necrosis Factor–α in the Myocardium
Other hearts were excised after dying with Evans Blue, dissected into risk area blocks and nonrisk area blocks, and frozen at −80°C as soon as possible. Each frozen sample was homogenized in 5 volume of STE buffer (320 mMOL/L sucrose, 10 mMOL/L Tris-HCl; pH 7.4, 1 mMOL/L EGTA, 10 mMOL/L β-mercaptoethanol, 50 mMOL/L NaF, 10 mMOL/L NaVO₃, 0.2 mMOL/L PMSF, 20 μMOL/L leupeptin, and 0.15 μMOL/L pepstatin), and centrifuged at 100 000 g for 10 minutes. The pellet was mixed with 0.5 mL of STE buffer and centrifuged at 10 000 g for 10 minutes. The quantity of protein in the supernatant (membrane fraction) was determined using a fluorescence assay and the fluorescence was measured with a Spectra Thermo machine (SLT Labinstruments, Bremen, Germany). Tumor necrosis factor (TNF)–α assay was performed using time-resolved fluoroimmunoassay as described previously by Kimura et al.18

Protein Purification and Western Blotting
For Western blot analysis, each frozen heart sample was homogenized in the lysis buffer (50 mMOL/L HEPES, 5 mMOL/L EDTA, and 50 mMOL/L NaCl, 10 μG/L atropin, 1 mMOL/L PMSF, and 10 μG/L Leupeptin, pH 7.5). After centrifugation at 15 000 g for 10 minutes, the supernatant was used for Western blotting. Antibodies against p38MAPK, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), protein kinase B (Akt), endothelial nitric oxide synthase (eNOS), and phosphorylated forms of these kinases (P-p38MAPK, P-JNK, P-ERK, P-Akt, P-eNOS) were used (all antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 dilution. Horseradish peroxidase-conjugated goat antimouse IgG antibody (Pierce, Rockford, Ill) was used at 1:1000 dilution. The ECL Western blotting system (Amersham Biosciences) was used for detection. Bands were visualized and quantified using a lumino-analyzer (LAS-1000; Fuji Photo Film).

Effects of Various Inhibitors on the Tissue Protection by CO
A number of rats were randomly assigned to receive one of the following treatments: Pi-3 kinase inhibitor (wortmannin; α-Aldrich), which was administered twice at 1.5 mg/kg IP injection, at 24 hours and at 30 minutes before surgery; NO antagonist [N(G)-nitro-l-arginine methyl ester (L-NAME); Wako], which was administered twice at 30 mg/kg IP injection, at 24 hours and at 30 minutes before surgery; p38MAPK inhibitor (SB203580; TOCRIS, Ellisville, MO), which was administered twice at 1 mg/kg IP injection, at 48 hours and at 24 hours before surgery; and vehicle (0.2 mL 5% DMSO) administered by IP injection 24 hours before surgery. We investigated the effect of these inhibitors on the phosphorylation of each marker and on the cytoprotection by CO.

Measurement of cGMP in Myocardium
To investigate whether CO could upregulate NO production in the myocardium, cGMP levels in the hearts of the rats treated with 1000 ppm CO for 24 hours and without CO treatment were measured using a cGMP ([35S] assay system (Amersham Biosciences) according to the manufacturer’s instructions. Another group of rats was administered a soluble guanylate cyclase inhibitor (methylene blue; Funakoshi, Tokyo, Japan) or 0.2 mL phosphate-buffered saline twice at 30 mg/kg IP injection, at 24 hours and at 30 minutes before ischemia–reperfusion surgery. The effect of the inhibitor on cytoprotection by CO was investigated.

Statistical Analysis
Results were expressed as mean ± SD. An unpaired Student t test was used for comparisons of the means of 2 groups. P<0.05 was considered to be statistically significant.

Results
Time Course of the Concentration of HbCO During CO Inhalation
The concentration of HbCO in blood increased in time-dependent and CO dose-dependent manner. The concentrations of HbCO in blood of the rats pretreated with 250 ppm, 500 ppm, and 1000 ppm CO for 24 hours were 19.8 ± 0.7% (250 ppm), 24.9 ± 1.0% (500 ppm), and 30.1 ± 0.9% (1000 ppm).
The maximum concentration of HbCO observed in our study (30.1 ± 0.9%) was far less than the lethal level in human beings (generally between 60% and 80%).

**Effect of CO on Ischemia–Reperfusion Injury**

Pretreatment with CO did not affect the arterial blood pressure, pulse rate, and body temperature of the animals before, during, and 120 minutes after the coronary ligation. There were no significant differences in the ratio of risk areas to total left ventricular areas (R/T ratio) between the rats pretreated with CO and those without CO pretreatment (Figure 1A). But the ratio of infarct areas-to-risk areas (I/R ratio) was significantly lower in the rats pretreated with 1000 ppm CO for 24 hours than in the rats without CO pretreatment and those that had been exposed to 250 ppm CO, 500 ppm CO, or low barometric pressure hypoxia for 24 hours; R/T ratio and I/R ratio were not affected by ZnPP (n=5 per treatment group). *P<0.01. C, Representative heart sections. Infarct area was reduced in the heart with preinhalation of 1000 ppm CO (right) compared with control (room air; left).

Even under the inhibition of HO-1 by pre-administering ZnPP as mentioned, 1000 ppm CO reduced the I/R ratio almost to the same level as that without ZnPP (Figure 1B).

**Histology**

Hematoxylin and eosin staining of the hearts showed that pretreatment with 1000 ppm CO reduced the accumulation of mononuclear cells in the risk areas (Figure 2A a through c). As shown in Figure 2A d through i, abundant ED-1 staining cells were seen in the risk areas of the hearts of the rats without CO pretreatment (percentage of ED-1 positive cells was 36.9 ± 14.3%). In contrast, in the rats pretreated with 1000 ppm CO for 24 hours, only a small number of ED-1 staining cells were detected in the risk areas (percentage of ED-1 positive cell was 12.3 ± 7.6%, *P<0.05) (Figure 2B).
**TNF-α Protein Expression**

Reactive oxygen species promote the secretion of TNF-α, a proinflammatory cytokine that induces dysfunction and apoptosis in the myocardium.\(^{19,20}\) After ischemia–reperfusion procedure, there was a significant increase in the quantity of TNF-α in the myocardium in the risk areas and the nonrisk areas (\(P<0.05\), ischemia–reperfusion versus control) (Figure 2C). In the animals with 24-hour 1000 ppm CO pretreatment, the concentration of TNF-α in myocardium was significantly reduced in the risk areas and the nonrisk areas (\(P<0.05\), without CO pretreatment versus with 1000 ppm CO pretreatment).

**The Role of MAPK Superfamily, Akt, and eNOS in the Tissue Protection by CO**

We investigated the role of MAPK and Akt–eNOS pathways in the tissue protection by CO. MAPK superfamilies are known to be activated in response to reactive oxygen species and regulate cell death.\(^{21}\) Akt is a serine–threonine kinase known as a survival signal and protects cardiomyocytes against apoptosis induced by ischemia–reperfusion.\(^{22,23}\) Activation of Akt leads to eNOS phosphorylation,\(^{24}\) and low concentration of NO produced by eNOS reduces cell apoptosis.\(^{25}\) Inhalation of 1000 ppm CO itself activated all of these kinases (Figure 3A through 3E). Peak increases in these kinases were seen 4–12 hours after the animals had begun inhaling 1000 ppm CO (\(P<0.05\), maximal fold increase versus baseline level). In contrast, neither exposure to 500 ppm CO nor low barometric pressure hypoxia (0.5 atm) activated these kinases.

We confirmed that the dose of SB203580 used in our study blocked CO-induced phosphorylation of p38MAPK, but did not affect the phosphorylation of Akt and eNOS (Figure 4A). Wortmannin completely blocked CO-induced phosphorylation of Akt and eNOS, but not that of p38MAPK. Moreover, \(\text{L-NAME} \) did not affect the phosphorylation of p38MAPK, Akt, and eNOS (data not shown). The I/R ratios in the hearts pretreated with each inhibitor and CO were higher than those pretreated with vehicle and CO, but lower than those pretreated with each inhibitor but without CO pretreatment (\(P<0.001\), respectively; Figure 4B). When both SB203580 and wortmannin were administered to rats before CO pretreatment, the CO-induced activation of p38MAPK, Akt, and eNOS was completely blocked and the tissue protection by CO was completely attenuated.

**The Role of cGMP in the Tissue Protection by CO**

Inhalation of 1000 ppm CO activated cGMP in the heart (Figure 5A). Administration of wortmannin or \(\text{L-NAME}\) previous to CO inhalation disturbed the CO-induced activation of cGMP. When methylene blue was administered to rats previous to CO inhalation, cardiac tissue protection by CO was significantly attenuated (Figure 5B).

**Discussion**

In our study, pre-inhalation of 1000 ppm CO for 24 hours significantly reduced the cardiac ischemia–reperfusion injury. CO seemed to replace the action of endogenous HO-1 in protection of cardiomyocytes against ischemia–reperfusion injury.

Thus far, several pathways have been reported through which CO protects tissues against injuries. Several studies suggest that tissue protection by CO is via the MAPK signaling pathway.\(^{14–16,26}\) As expected, our data also demonstrated that 1000 ppm CO activated MAPK in the heart, and blocking of p38MAPK activation partially inhibited the tissue protection by CO. Therefore, CO protects cardiomyocytes partially via p38MAPK pathway. CO also activated JNK and ERK in the heart, but the contribution of these kinases was not addressed in our study.

Inhalation of 1000 ppm CO also activated Akt, eNOS, and cGMP in the hearts. Administration of wortmannin abrogated the activation of Akt, eNOS, and cGMP, and cardiac tissue protection by CO. \(\text{L-NAME}\) and methylene blue also altered the degree of tissue protection by CO. Our data suggest that CO ameliorates cardiac ischemia–reperfusion injury partially via Akt–eNOS pathway, including production of NO, soluble guanylate cyclase, and cGMP. To our knowledge, a close relation-
ship between the Akt–eNOS pathway and the tissue protection by CO has not been reported so far. In some previous studies, tissue protection by CO seemed to be independent of NOS pathway. Endothelial cells are especially abundant in the heart. Therefore, it is possible that eNOS plays a much more important role in tissue protection by CO in the heart than in other organs. Regarding the relationship between CO and NO, Suematsu et al reported that micromolar CO increases the basal activity of soluble guanylate cyclase if local concentration of NO is low, whereas CO can serve as a partial antagonist of NO-induced activation of the soluble guanylate cyclase. From these studies, endogenous CO seems to compete with NO in activating soluble guanylate cyclase. However, the results of these previous studies do not necessarily contradict our results because NO and CO may play a role as signaling molecules to protect tissues from injury. Recently, several studies have suggested a coordinated physiological role for NOS and HO in cytoprotection. Thus, it does not seem surprising if CO, a byproduct of heme catabolism by HO, induces NO, which in turn may act to reduce cardiac ischemia–reperfusion injury. But much more investigation may be necessary to clarify the precise relationship between CO and NO.

Inhalation of CO may induce tissue level hypoxia, which may also activate MAPKs, Akt, and eNOS. However, in this study, the I/R ratio of the rats exposed to low barometric pressure hypoxia was significantly higher than that of the rats pretreated with 1000 ppm CO. And the activation of MAPKs, Akt, and eNOS induced by hypoxia was significantly weaker than that induced by 1000 ppm CO. In addition, I/R ratio was significantly lower in the rats pretreated with 1000 ppm CO than in those pretreated with 500 ppm CO, although the concentrations of HbCO in blood were not significantly different between the 2 groups (30.1% and 24.9% at 24 hours of inhalation, respectively). Thus, tissue protection by CO cannot be explained only by a hypoxic preconditioning effect.

In our study, pre-inhalation of 1000 ppm CO for 24 hours significantly reduced the cardiac ischemia–reperfusion injury. According to other studies about the tissue protective role of CO, the most efficacious concentration of CO ranges from 250 ppm to 1000 ppm. The most beneficial concentration of CO may be specific for each species and each tissue. We cannot extrapolate the most efficacious condition of CO administration for the treatment of myocardial infarction in human beings from our data alone. It seems likely that a higher dose of exogenous CO may be necessary to reduce an invasive injury such as cardiac ischemia–reperfusion injury than to reduce other types of tissue injuries. However, when the concentration of HbCO

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**Figure 4.** A, The effects of inhibitors of p38MAPK, Akt, and eNOS. SB203580 blocked the activation of p38MAPK by CO, but not that of Akt or eNOS. Wortmannin completely blocked the activation of Akt and eNOS by CO, but not that of p38MAPK. Administration of both SB203580 and wortmannin blocked the activation of all of Akt, eNOS, and p38MAPK. B, The myocardial protection by CO was inhibited by SB203580, wortmannin, and L-NAME. *P<0.01, n=5 for each. Inhalation of 1000 ppm CO for 24 hours before ligation and 250 ppm CO during ischemia-reperfusion surgery, # vehicle; 0.2 ml DMSO.

**Figure 5.** A, Activation of cGMP in myocardium by CO; n=4 for each. B, Attenuation of tissue protective effect of CO by soluble guanylate inhibitor, methylene blue. *P<0.05, n=5 for each. †Inhalation of 1000 ppm CO for 24 hours.
increases to $\approx 30\%$, CO can induce several noxious symptoms. Therefore, the safety of the use of 1000 ppm CO in human beings is still questionable, and much more experimentation using other species with higher numbers of animals is necessary to assess the clinical use of CO. Even so, we believe that these data suggest a possible therapeutic use for CO in cardiac ischemia–reperfusion injury.

**Conclusion**

CO inhalation decreases ischemia–reperfusion injury in vivo. CO ameliorated cardiac ischemia–reperfusion injury via MAPK pathway and Akt–eNOS pathway including production of cGMP. The heart is much more susceptible to hypoxia than other organs, and CO has the property of inhibiting cellular respiration. Thus, it was remarkable that CO inhalation ameliorated ischemia–reperfusion injury in the heart. Finally, our data suggest that CO may be an important player in myocardial protection by induction of HO-1.

**References**

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Arterioscler Thromb Vasc Biol. 2004;24:1848-1853; originally published online August 12, 2004;
doi: 10.1161/01.ATV.0000142364.85911.0e
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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