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

Hajime Fujimoto, Minoru Ohno, Seiji Ayabe, Hisae Kobayashi, Nobukazu Ishizaka, Hiroko Kimura, Ken-ichi Yoshida, Ryozo Nagai

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)-

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 \( \beta \) 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 Motternili et al reported that CO-releasing molecules could reduce the cardiac muscle damage induced by ischemia–reperfusion via activation of mitochondrial \( K_{\text{ATP}} \) 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: intraarterial IP injection of 50 μg zinc–protoporphyrin (ZnP) (Funakoshi, Tokyo, Japan), a selective HO-1 inhibitor, or vehicle (0.2 mL DMSO) twice, at 48 hours and at 24 hours before ischemia–reperfusion surgery, with or without pre-inhalation of 100 ppm CO for 24 hours.

**Ischemia–Reperfusion Procedure**

Ischemia–reperfusion procedure was performed as described previously. 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 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 excision, 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/monocyte antibody (ED-1; Dainippon Pharmaceutical, Osaka, Japan) was performed as described previously. 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 000g for 60 minutes. The pellet was mixed with 0.5 mL of STE buffer and centrifuged at 10 000g 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.

**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/mL atripin, 1 mmol/L PMSF, and 10 μg/mL leupeptin, pH 7.5). After centrifugation at 15 000g 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 Cell Signaling Technology, Beverly, Mass). The membranes were incubated in 0.1% (500 ppm), and 30.1% (250 ppm), 24.9% (500 ppm) and 0.7% (1000 ppm) CO for 24 hours. The concentration of carboxyhemoglobin (HbCO) in blood of the rats pretreated with 250 ppm, 500 ppm, and 1000 ppm CO for 24 hours were 19.8 ± 0.9%, 24.9 ± 1.0%, and 30.1 ± 0.9%. In the Myers laboratory, 1849 CO Prevents Cardiac Ischemia–Reperfusion Injury 1849 CO Prevents Cardiac Ischemia–Reperfusion Injury 1849 CO Prevents Cardiac Ischemia–Reperfusion Injury.
ppm), respectively. 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 (Figure 1B).

Even under the inhibition of HO-1 by pre-administrating 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).

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** A and B, R/T ratio and I/R ratio with pre-exposure to CO or low barometric pressure hypoxia. A, R/T ratio was not changed by CO inhalation or low barometric pressure hypoxia. B, I/R ratio was significantly lower in rats that had inhaled 1000 ppm CO for 24 hours before ligation than in the rats without CO pretreatment, or those 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).

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** A, Histology of myocardium: a, d, and g show control areas of myocardium of a rat without ischemia-reperfusion. b, e, and h show the ischemic areas of myocardium of a rat without CO pretreatment. c, f, and i show the ischemic areas of a rat pretreated with 1000 ppm CO. a through c, HE-staining; d through f, ED-1 staining (original magnification: a through f, × 20; g through i, × 60). Inflammatory cell infiltration after ischemia-reperfusion was reduced in myocardium of the rats pretreated with CO. B, Percentage of ED-1 positive cells. *P < 0.05. C, TNF-α expression in the hearts: a shows TNF-α expression in the risk area (n = 5), b shows TNF-α expression in the non-risk area (n = 5). In both the risk areas and the non-risk areas, TNF-α was significantly decreased by pretreatment with 1000 ppm CO (*P < 0.05).
TNF-α Protein Expression

Reactive oxygen species promote the secretion of TNF-α, a proinflammatory cytokine that induces dysfunction and apoptosis in the myocardium. 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. Akt is a serine–threonine kinase known as a survival signal and protects cardiomyocytes against apoptosis induced by ischemia–reperfusion. Activation of Akt leads to eNOS phosphorylation, and low concentration of NO produced by eNOS reduces cell apoptosis. Inhalation of 1000 ppm CO activated all of these kinases in the myocardium (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, 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 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. 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. 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

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.
Finally, our data suggest that CO may be an important player in myocardial protection by induction of HO-1.

**References**


Carbon Monoxide Protects Against Cardiac Ischemia—Reperfusion Injury In Vivo via MAPK and Akt—eNOS Pathways
Hajime Fujimoto, Minoru Ohno, Seiji Ayabe, Hisae Kobayashi, Nobukazu Ishizaka, Hiroko Kimura, Ken-ichi Yoshida and Ryozo Nagai

Arterioscler Thromb Vasc Biol. 2004;24:1848-1853; originally published online August 12, 2004;
doi: 10.1161/01.ATV.0000142364.85911.0e

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/24/10/1848

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/