Carbon monoxide potently prevents ischemia-induced high-mobility group box 1 translocation and release and protects against lethal renal ischemia–reperfusion injury

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High-mobility group box 1 (HMGB1) is a chromatin-binding nuclear molecule that has potent proinflammatory effects once released by damaged cells. In some disease models, carbon monoxide (CO) exhibits anti-inflammatory and protective properties. Here, we investigated whether the protective effect of CO on renal ischemia–reperfusion injury is associated with the inhibition of HMGB1 translocation and release. A renal ischemia–reperfusion injury model was established with a 100% mortality rate in untreated mice. Pretreatment with the CO–releasing molecule-2 (CORM-2) resulted in 100% survival, maximal preservation of renal function, a marked reduction in pathological damage, and blunted upregulation of TLR4, RAGE, TNF-α, IL-1β, IL-6, and MCP1 mRNA. Interestingly, CORM-2 pretreatment almost completely inhibited ischemia-induced HMGB1 nucleocytoplasmic shuttling and release. This inhibition was associated with a decrease in nuclear histone acetyltransferase activity. Indeed, CORM-2 pretreatment inhibited the acetylation and release of HMGB1 during hypoxic culture of primary mouse renal tubular epithelia cells *in vitro*. Using the same renal ischemia–reperfusion injury model, neutralization of HMGB1 was protective, and administration of exogenous HMGB1 largely reversed the protective effect of CORM-2 on kidney ischemia–reperfusion injury. Thus, CORM-2-delivered CO protects against lethal renal ischemia–reperfusion injury. This protection is correlated with the prevention of HMGB1 nuclear-cytoplasmic translocation and release.

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Ischemia–reperfusion injury (IRI) is a major deleterious factor limiting the success of transplantation, particularly in the current era of organ shortage and the increasing use of 'marginal' organs, which are more susceptible to IRI.[1,2] As an unavoidable process that occurs during renal transplantation, IRI negatively affects both early graft function and long-term graft survival.[3–5] The pathophysiology of renal IRI includes initial direct cellular damage caused by ischemia, as well as delayed renal injury, resulting from inflammatory responses following reperfusion.[6] A key 'trigger' generated from ischemic renal cells may contribute critically to the propagation of the inflammatory responses and subsequent exacerbation of renal injury after renal reperfusion.

High-mobility group box 1 (HMGB1) has been recognized as an essential damage-associated molecular pattern (DAMP) molecule that, once present in the extracellular milieu, can activate proinflammatory signaling pathways by interacting with some pattern recognition receptors, such as Toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE).[7,8] Recent evidence in animal models indicates that HMGB1 is an early mediator of injury and inflammation in IRI in the liver, brain, and heart.[9–11] In a mouse model of renal IRI, HMGB1 was found to translocate rapidly from its normal site of residence in the nucleus to the cytoplasm and out of cells after stimulation by ischemia.[12] In addition, administration of neutralizing antibodies against HMGB1 provided significant protection against renal IRI damage.[12,13] Taken together, these findings strongly suggest that HMGB1 is a key factor that has a vital role in the early

stages of damage signal propagation following renal reperfusion. A therapeutic tool that prevents HMGB1 nuclear-cytoplasmic translocation and release from ischemic cells may have a more potent and efficient protective effect on renal IRI than those therapies that directly target HMGB1's downstream inflammatory mediators.

During the past decade, carbon monoxide (CO) has received great attention as a cytoprotective molecule that may serve as a therapeutic tool to prevent clinical IRI. Therapeutic upregulation of CO tissue levels can be achieved by exogenous application of CO, including direct inhalation of CO gas or administration of CO–releasing molecules (CORMs).[14,15] Exogenously delivered CO has been shown to provide potent protection in various IRI models through its anti-inflammatory, vasodilating, and antiapoptotic activity.[16–21] However, the molecular mechanisms underlying the protective effect of CO on IRI have not been fully elucidated. It has been proposed that the *in vivo* functions of CO might be mediated by the binding to heme-containing enzymes (such as cytochrome *P*450, cytochrome *c* oxidase, and guanylyl cyclase) and regulation of the mitogen-activated protein kinases.[20,22–26] Thus far, whether the protective effect of CO on IRI shows any correlation with the key DAMP molecule HMGB1 is not known. As a potent cytoprotective gaseous molecule and biological regulator, exogenously delivered CO may effectively protect against ischemia-induced renal cell injury and block nuclear-cytoplasmic translocation and release of HMGB1, preventing the propagation of the subsequent inflammatory responses that follow renal reperfusion. In the present study, we tested this hypothesis using CORM-2 in a lethal renal warm IRI model in mice.

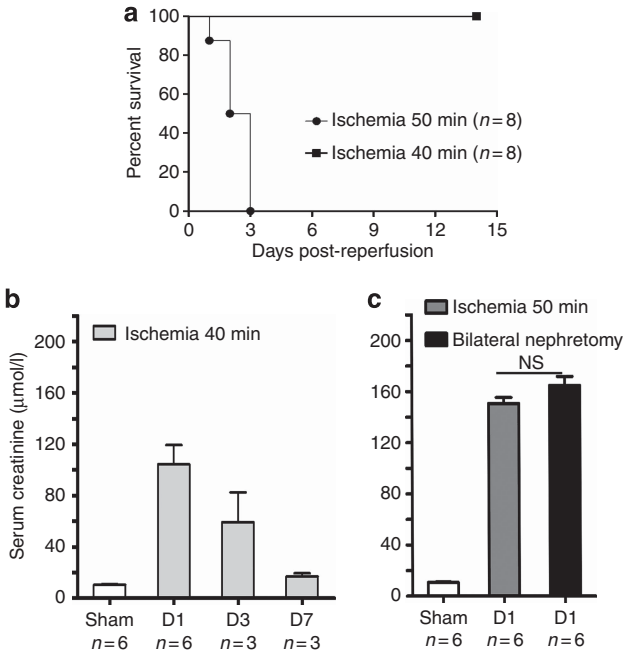

Figure 1 | Establishment of a lethal renal ischemia–reperfusion injury (IRI) model in mice. (**a**) Survival of mice that had undergone 40 or 50 min of renal ischemia and were observed for 14 days. (**b**) Serum creatinine (Cr) levels of mice that had undergone 40 min of renal warm ischemia, on days 1, 3, or 7 after reperfusion. (**c**) Serum Cr levels of mice that had undergone 50 min of renal ischemia at 24 h after reperfusion were similar to those of mice at 24 h after bilateral nephrectomy ($P > 0.05$). NS, nonsignificant.

RESULTS

Establishment of a severe lethal renal IRI model

The left renal pedicle of each untreated mouse was clamped for either 40 or 50 min, and then the contralateral kidney was removed. None of the mice subjected to 40 min of renal ischemia died within the 2-week observation period (Figure 1a). The serum creatinine (Cr) levels moderately increased at 24 h after reperfusion, and then returned to near normal at 7 days after reperfusion (Figure 1b), indicating that this is a reversible model of ischemic acute renal failure. With 50 min of renal ischemia, all the mice died within 3 days after reperfusion (Figure 1a). The serum Cr levels at 24 h after reperfusion were significantly elevated, to a level similar to that of mice at 24 h after bilateral nephrectomy (Figure 1c), indicating that this model is a lethal renal IRI model.

Effects of CORM-2 on COHb and O_2Hb levels

The carboxyhemoglobin (COHb) and oxyhemoglobin (O_2Hb) levels in the arterial blood of mice were measured at various times after an intravenous injection of CORM-2 (20 mg/kg). No significant changes were observed in either COHb or O_2Hb levels up to 6 h after CORM-2 administration (Supplementary Figure S1 online).

CORM-2 pretreatment almost completely protects against lethal kidney IRI

Using the established lethal renal IRI model, we first assessed the survival of CORM-2-treated mice versus inactive CORM-2 (iCORM-2)–treated or phosphate-buffered saline (PBS)–treated mice. Pretreatment with a single dose of CORM-2 (20 mg/kg) 1 h before renal ischemia markedly improved survival after ischemia/reperfusion (I/R) (Figure 2a). All mice in this group survived more than 14 days, as compared with 1 of 8 mice in the iCORM-2–treated group and 0 of 8 mice in the PBS–treated control group ($P < 0.001$).

We next measured blood urea nitrogen (BUN) and serum Cr levels to assess the degree of renal dysfunction in each group. After 24 h of I/R, both PBS– and iCORM-2–treated control mice exhibited significantly increased serum Cr (Figure 2b) and BUN levels (Figure 2c) ($P < 0.0001$ vs. the sham-operated group). In contrast, pretreatment with CORM-2 almost completely prevented the abrupt increase in serum Cr and BUN values after 24 h of I/R ($P < 0.0001$ vs. the iCORM-2 and PBS control groups; Figures 2b and c). Furthermore, the serum Cr and BUN levels in CORM-2-treated mice were still normal when measured at days 3 and 7 after I/R (Figures 2b and c).