Treatment with CO-RMs during cold storage improves renal function at reperfusion

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Low concentrations of carbon monoxide (CO) can protect tissues against ischemia–reperfusion (I–R) injury. We have recently identified a novel class of compounds, CO-releasing molecules (CO-RMs), which exert important pharmacological activities by carrying and delivering CO to biological systems. Here, we examined the possible beneficial effects of CO liberated from CO-RMs on the damage inflicted by cold storage and I–R in isolated perfused kidneys. Hemodynamic and biochemical parameters as well as mitochondrial respiration were measured in isolated perfused rabbit kidneys that were previously flushed with CO-RMs and stored at 4°C for 24 h. Two water-soluble CO-RMs were tested: (1) sodium boranocarbonate (CORM-A1), a boron-containing carbonate that releases CO at a slow rate, and (2) tricarbonylchloro(glycinato)ruthenium(II) (CORM-3), a transition metal carbonyl that liberates CO very rapidly in solution. Kidneys flushed with Celsior solution supplemented with CO-RMs (50 μM) and stored at 4°C for 24 h displayed at reperfusion a significantly higher perfusion flow rate (PFR), glomerular filtration rate, and sodium and glucose reabsorption rates compared to control kidneys flushed with Celsior solution alone. Addition of 1*H*-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ), a guanylate cyclase inhibitor, prevented the increase in PFR mediated by CO-RMs. The respiratory control index from kidney mitochondria treated with CO-RMs was also markedly increased. Notably, renal protection was lost when kidneys were flushed with Celsior containing an inactive compound (iCO-RM), which had been deliberately depleted of CO. CO-RMs are effective therapeutic agents that deliver CO during kidney cold preservation and can be used to ameliorate vascular activity, energy metabolism and renal function at reperfusion.

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Kidney transplantation is the treatment of choice for end-stage renal disease.[1] Most kidneys prior to transplantation are exposed to a period of cold storage (CS), which can limit but not completely avoid tissue injury and graft dysfunction in transplanted patients.[2] Therefore, new strategies for mitigating cold ischemic damage and novel therapies for successful kidney transplantations are required. CS procedures are widely used for preserving cadaveric kidneys prior to transplant; these techniques involve intravascular flushing of the isolated organ using a hypothermic solution followed by storage at low temperatures for the time required to transfer the graft to the surgery unit. In addition to the injury imposed by CS, kidneys are subjected to further damage at reperfusion when warm oxygenated blood (37°C) is reintroduced into the transplanted graft. The pathophysiological consequences of CS followed by warm reperfusion involve cellular edema and the generation of reactive oxygen species, which trigger acute inflammatory responses and promote apoptosis once the graft is transplanted.[3,4] Mitochondria are a key contributor to cell survival, as mitochondrial respiration and oxidative phosphorylation are essential for keeping the adenosine 5′ triphosphate demands and restoring cellular energy after adenosine 5′ triphosphate depletion caused by ischemia.[5]

Several approaches have been used to counteract the damaging mechanisms of CS-mediated injury and consequently ameliorate renal function at transplantation. Perhaps one of the most surprising and unforeseen strategies involves the use of carbon monoxide (CO), which has recently attracted attention as a fundamental cell signalling mediator and cytoprotective agent against apoptosis and ischemia–reperfusion (I–R) injury.[6] Low levels of CO are produced endogenously in mammalian tissues by heme oxygenase (HO), the first and rate-limiting step in heme catabolism.[7,8] Different types of HO enzymes have been characterized, including constitutive (HO-2) and inducible (HO-1) isoforms. HO-1 is a stress protein that possesses the peculiar feature of being finely upregulated by stimuli or pathological events that trigger oxidative and nitrosative stress.[9,10] The induction of HO-1 and the consequent increase in endogenous CO production play important roles in vasorelaxation,[11,12] inhibition of cell proliferation,[13,14] blockade of apoptotic pathways,[15] suppression of inflammation,[16]

protection against organ rejection,[17] and I–R injury.[18] In essence, both the use of potent HO-1 inducers and administration of low doses of CO gas have been employed to evaluate and sustain a therapeutic role of CO in I–R injury in the kidney.[18–22]

As a novel approach to deliver CO, our group has recently identified a class of compounds, termed CO-releasing molecules (CO-RMs), which are able to transport and release CO both *in vivo* and *in vitro* in a controllable manner under physiological conditions.[23,24] Different types of CO-RMs have been characterized and their biological activities assessed. Two water-soluble CO-RMs have recently shown promising results in our studies: tricarbonylchloro(glycinato)ruthenium(II) (CORM-3), a metal carbonyl complex that rapidly liberates CO in physiological buffers,[24,25] and sodium boranocarbonate (CORM-A1), a newly identified generator of CO that does not contain a transition metal and liberates CO at a much slower rate under physiological conditions.[26] The specific effect of CO liberated from CO-RMs in modulating important physiological effects has been confirmed by the parallel use of specific inactive compounds (iCO-RMs), which do not liberate CO in the cellular environment.[23,25,26] Previous data from our own laboratory and results provided by collaborators support a critical role for CO-RMs in vasorelaxation,[23,26,27] suppression of inflammation,[28] protection against hypoxia–reoxygenation, and oxidative stress as well as mitigation of I–R injury,[25] allograft rejection,[25] and myocardial infarction.[29,30] Thus, the hypothesis that low concentrations of CO released from CO-RMs could protect against cold ischemia-mediated injury and be used as a novel therapeutic strategy for organ preservation and transplantation is tantalizing. This study was designed to assess how cold preservation of kidneys in the presence of CO-RMs could affect renal biochemical and physiological functions at reperfusion using an *ex vivo* isolated kidney preparation.

RESULTS

Effects of CO-RMs on renal function in fresh isolated kidneys

To decide upon the effective dose range to be used in the cold preservation protocols, the effect of 0, 10, 25 and 50 μM CORM-A1 was examined over a 45 min perfusion time on the isolated perfused kidney (Figure 1). Addition of 50 μM CORM-A1 significantly increased perfusion flow rate (PFR) compared to controls (Figure 1a). On the other hand, there was no detectable increase in PFR with 10 and 25 μM CORM-A1; notably, iCORM-A1 (50 μM), which does not release CO, was also totally ineffective (Figure 1b). CORM-A1, but not

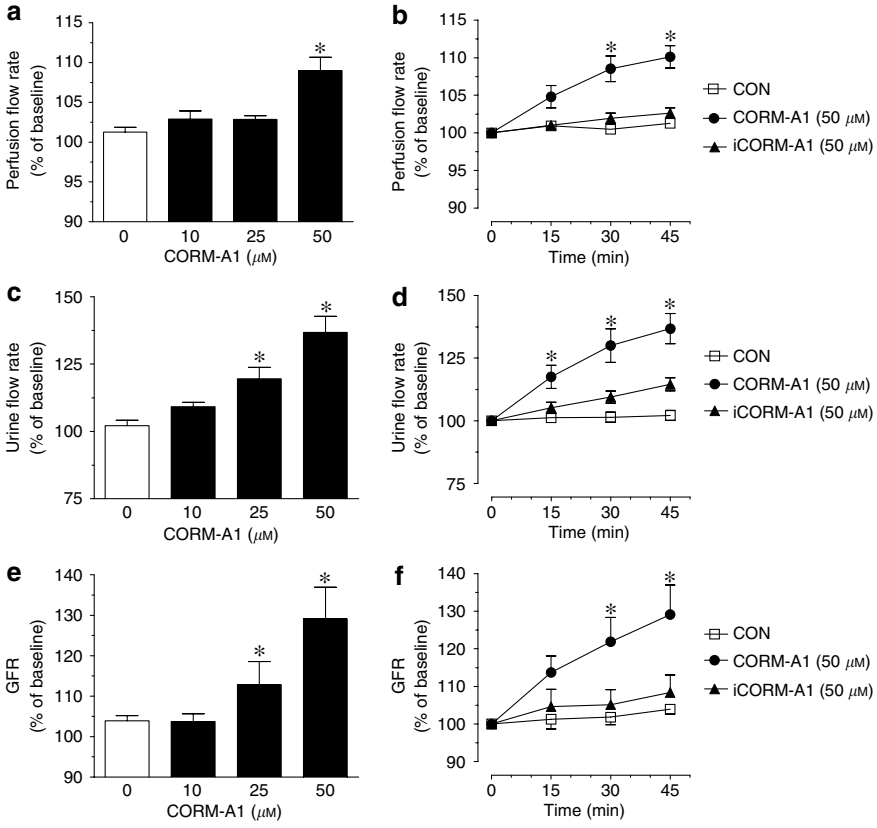

Figure 1 | Effect of CORM-A1 on renal hemodynamics in freshly isolated kidneys. (**a**) Perfusion flow rate, (**c**) urine flow rate, and (**e**) glomerular filtration rate (GFR) were measured in freshly isolated rabbit kidneys perfused for 45 min in the presence of increasing concentrations of CORM-A1 (see Materials and Methods for details). Changes in these hemodynamic parameters were also followed over time after the addition of 50 μM CORM-A1 (see graphs **b**, **d**, and **e**, respectively). iCORM-A1, which does not release CO, was used as a negative control. Each bar or line represents the mean $\pm$ s.e.m. of six independent experiments. *$P<0.05$ vs 0 μM CORM-A1 or control (CON).

iCORM-A1, also significantly increased urine flow rates (UFR) ($P<0.05$) in a time- and concentration-dependent manner when compared to the control group (Figure 1c and d). Similarly, perfusion of isolated kidneys in the presence of CORM-A1 resulted in a marked concentration- and time-dependent increase in glomerular filtration rate (GFR), but no significant changes were observed with the negative control, iCORM-A1 (Figure 1f and e). Interestingly, another water-soluble CO carrier (CORM-3) did not cause any change in the renal hemodynamics of freshly isolated kidneys (data not shown).

Detection of CO release from CO-RMs in Krebs perfusion buffer (37°C) and Celsior solution (4°C)

The rate and amount of CO liberated from CO-RMs were measured in the solutions used for kidney perfusion as well as organ preservation. We found that the rate of CO release by CORM-A1 in Krebs solution at 37°C directly correlates with the concentrations used (Figure 2a). Specifically, the calculated rates of CO release were 6.16 ± 0.12, 14.50 ± 0.66, and 30.16 ± 1.33 nmol/h for 10, 25, and 50 μM CORM-A1, respectively. Predictably, iCORM-A1 did not release any detectable CO in the Krebs solution at 37°C (Figure 2a). Given the fact that 50 μM CO-RMs was chosen as the ideal concentration for preserving kidneys at low temperatures, we measured the kinetics of CO release from CORM-A1 and CORM-3 in Celsior solution at 4°C. As observed with Krebs solution, we found that the addition of iCO-RMs to deoxymyoglobin dissolved in Celsior solution at 4°C did not produce any detectable carbonmonoxy myoglobin over a 24 h period (data not shown). Addition of CORM-3 (50 μM) increased carbonmonoxy myoglobin formation over time, reaching a maximal level after 4 h; the rate of CO release in the first 2 h was calculated as 14.0 ± 1.0 nmol/h (see Figure 2b). Interestingly, and in contrast to CORM-3, the increase in carbonmonoxy myoglobin after the addition of 50 μM CORM-A1 to the cold Celsior solution was slower and the calculated rate of CO release was only 1.39 ± 0.05 nmol/h (see Figure 2b).

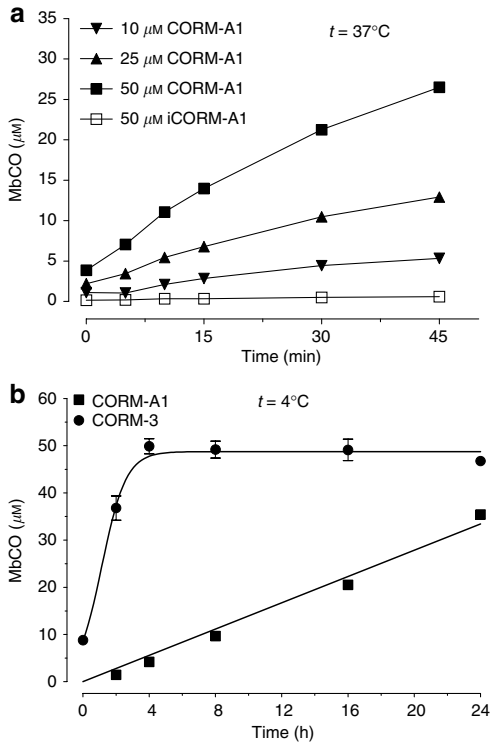

Figure 2 | Detection of CO released from CO-RMs at 37°C and 4°C. (**a**) Time course of CO released from CORM-A1 (10, 25, and 50 μM) after incubation in Krebs perfusion buffer (pH = 7.4) at 37°C. iCORM-A1, which does not release CO, was used as a negative control. (**b**) Time course of CO released from 50 μM CORM-A1 and CORM-3 after incubation in Celsior solution (pH = 7.4) at 4°C. In both experiments, the amount of CO released was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin to carbonmonoxy myoglobin (MbCO). Each line represents the mean ± s.e.m. of two independent experiments. The rate of CO release was calculated from the fitted curves as reported in the Results section.

CS of kidneys in the presence of CO-RMs increases renal function at reperfusion

Kidneys flushed in the presence of CORM-A1 and CORM-3 prior to 24 h CS in Celsior solution (CS + CO-RMs) produced a significantly higher ($P<0.05$) PFR on the isolated system at reperfusion (Figure 3a and b). In addition, the use of both CO-RMs during the flushing procedure of kidneys prior to CS significantly increased UFR (Figure 4a and b) and GFR (Figure 5a and b) ($P<0.05$). Interestingly, all the renal parameters evaluated (PFR, UFR, and GFR) were not affected when kidneys were flushed with Celsior solution

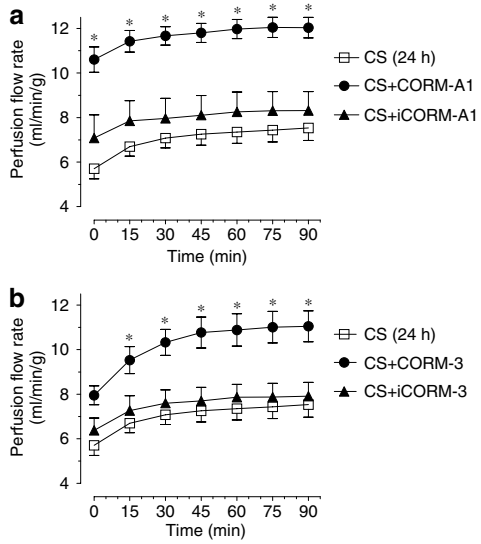

Figure 3 | Effect of CO-RMs on perfusion flow rate in isolated kidneys after cold storage. Rabbit kidneys were initially flushed with Celsior solution alone or supplemented with (**a**) 50 μM CORM-A1 or (**b**) CORM-3 and then subjected to a 24 h cold storage (CS) (see Materials and Methods for details). Perfusion flow rate was then measured in isolated kidneys over 90 min after a period of initial equilibration. iCORM-A1 and iCORM-3, which do not release CO, were used as negative controls. Each line represents the mean ± s.e.m. of $n=6$ for each group. *$P<0.05$ vs CS group.

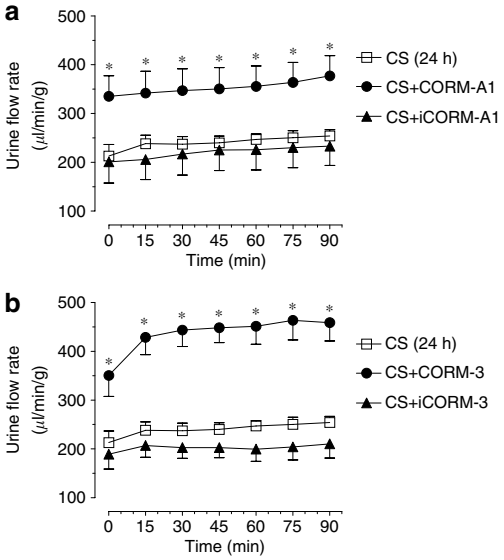

Figure 4 | Effect of CO-RMs on urine flow rate in isolated kidneys after cold storage. Rabbit kidneys were initially flushed with Celsior solution alone or supplemented with (**a**) 50 μM CORM-A1 or (**b**) CORM-3 and then subjected to a 24 h cold storage (CS) (see Materials and Methods for details). Urine flow rate was then measured in isolated kidneys over 90 min after a period of initial equilibration. iCORM-A1 and iCORM-3, which do not release CO, were used as negative controls. Each line represents the mean ± s.e.m. of $n = 6$ for each group. *$P < 0.05$ vs CS group.

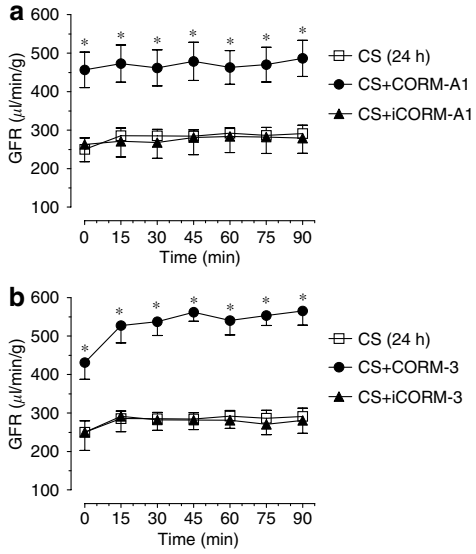

Figure 5 | Effect of CO-RMs on glomerular filtration rate (GFR) in isolated kidneys after cold storage. Rabbit kidneys were initially flushed with Celsior solution alone or supplemented with (**a**) 50 μM CORM-A1 or (**b**) CORM-3 and then subjected to a 24 h cold storage (CS) (see Materials and Methods for details). GFR was then measured in isolated kidneys over 90 min after a period of initial equilibration. iCORM-A1 and iCORM-3, which do not release CO, were used as negative controls. Each line represents the mean ± s.e.m. of $n = 6$ for each group. *$P < 0.05$ vs CS group.

supplemented with the negative controls (iCO-RMs), indicating that CO liberated from CO-RMs was directly involved in the improved renal function at reperfusion.

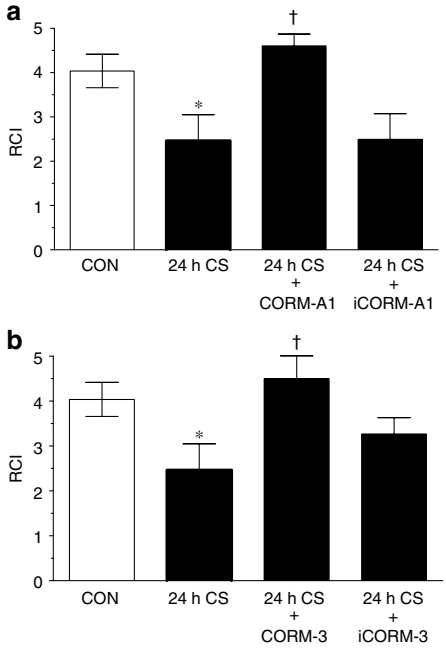

Figure 6 | Effect of CO-RMs on respiratory control index (RCI) in isolated renal mitochondria. Kidneys were freshly isolated (CON) or subjected to a 24 h cold storage (CS) period in the presence or absence of (**a**) CORM-A1 or (**b**) CORM-3 (see Materials and Methods for details). Mitochondria were then isolated and the RCI was assessed in the presence of malate and glutamate as substrates and ADP. iCORM-A1 and iCORM-3, which do not release CO, were used as negative controls. Each bar represents the mean ± s.e.m. of $n = 5$–6 for each group. *$P < 0.05$ vs control (CON); †$P < 0.05$ vs CS group.

CO-RMs improve mitochondrial respiration at reperfusion after 24 h CS

As mitochondrial function contributes significantly to the outcome of I–R injury in organ transplantation,[5] we assessed the influence of CO liberated from CO-RMs during CS on mitochondrial oxygen consumption and viability (Figure 6). Cold preservation (24 h) followed by 2 h reperfusion resulted in a significant decrease in the renal mitochondria respiratory control index (RCI) from 4 (control) to 2.5. Remarkably, the use of CO-RMs during CS resulted in a statistically significant ($P < 0.05$) improvement of RCI values (RCI = 4.6 with CORM-A1; RCI = 4.5 with CORM-3).

ODQ, a guanylate cyclase inhibitor, abolishes the vasodilatory effects of CO-RMs

Figure 7 shows the effect of 1*H*-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ), an inhibitor of soluble guanylate cyclase,[27] on PFR in freshly isolated kidneys perfused with CORM-A1 (Figure 7a) and in kidneys subjected to CS + CORM-A1 and reperfusion (Figure 7b). In both conditions, the effect of CORM-A1 treatment on PFR was completely abolished by the presence of ODQ (30 μM). PFR was not affected at reperfusion when kidneys were flushed with Celsior containing ODQ alone (data not shown). A similar decrease in PFR by ODQ was observed in kidneys stored in the presence of CORM-3 (data not shown).

CO-RMs improve tubular function at reperfusion after CS

As shown in Table 1, glucose (R_{GLU}) and Na^+ (R_{Na}) reabsorption were found to be significantly increased in kidneys that were previously flushed with CORM-A1. Both R_{GLU} and R_{Na} levels were unchanged in kidneys treated with iCORM-A1. Notably, both iCORM-3 and iCORM-3 did not significantly affect R_{GLU} and R_{Na} levels (Table 1). The urinary release of gamma-glutamyltransferase (GGT), a marker of tubular injury, was increased at reperfusion after CS and treatment with CORM-A1 or CORM-3 (but not iCO-RMs) reduced these values by more than two-fold. Urinary nitrite was significantly reduced when CORM-3 was used, but was unchanged after treatment with CORM-A1.

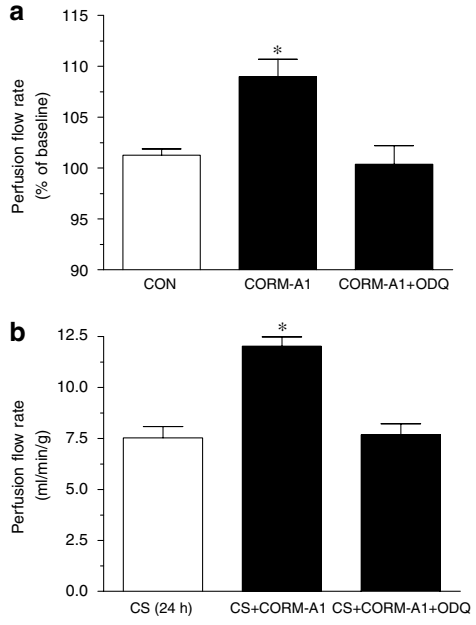

Figure 7 | Effect of ODQ, a guanylate cyclase inhibitor, on perfusion flow rate in freshly isolated kidneys and after cold storage. (**a**) ODQ (30 μM), a soluble guanylate cyclase inhibitor, was perfused 30 min before the addition of CORM-A1 in freshly isolated kidneys at 37°C. (**b**) ODQ (30 μM) was added to the flushing Celsior solution (4°C) in combination with CO-RMs prior to 24 h cold storage (CS). Changes in perfusion flow rate were then measured in the isolated perfused rabbit kidney system (see Materials and methods for details). Each line represents the mean ± s.e.m. of $n = 5$ for each group. *$P < 0.05$ vs control (CON) or CS alone.

DISCUSSION

CO-RMs are a novel class of bioactive agents that have been recently identified to substantiate the important biological function of CO in mammals.[23–25] We have proposed that their pharmacological properties could be exploited for the therapeutic delivery of CO in the treatments of a variety of pathophysiological states that affect the cardiovascular and immune systems.[25,27,28,31] Here, we show that CO liberated from water-soluble CO-RMs exerts significant beneficial effects on renal vascular function of fresh isolated kidneys as well as kidneys stored at low temperatures (4°C) in Celsior solution, a clinical strategy commonly used to preserve organs for transplantation. The fact that CO-RMs were used only during the flushing procedure prior to CS indicates the feasibility of utilizing these CO carriers as adjuvants of preservation solutions to greatly limit the damage of donor organs.

Impairment of renal blood flow during I–R plays a significant role in the exacerbation of tissue injury in a number of kidney diseases.[32] It has been shown that a persistent reduction in renal blood flow can be attributed to a decreased GFR observed in renal allografts following an ischemic event.[33] In addition, intense vasoconstriction and endothelial damage are prominent features of CS-mediated injury.[34] In the present study, we initially found that freshly isolated kidneys perfused with CORM-A1, a boron-containing CO generator, resulted in a significant increase in PFR. Moreover, PFR markedly increased in reperfused isolated kidneys previously flushed with either CORM-A1 or CORM-3 and subjected to a 24 h CS procedure. The fact that iCO-RMs were ineffective clearly demonstrates that CO is directly responsible for the observed pharmacological effects. Since the perfusion pressure in our isolated model was kept constant (100 mmHg), we conclude that the increase in PFR by CO-RMs is mainly due to a decrease in vascular resistance. We also found that the beneficial vascular effects mediated by CORMs were lost in the presence of ODQ, a guanylate cyclase inhibitor. These results are supported by data showing that endogenous CO promotes renal vasodilatation in chronically hypoxic rats[35] and are consistent with previous studies from our laboratory demonstrating that both CORM-3 and CORM-A1 exert vasorelaxation of precontracted aortas as well as systemic hypotension through stimulation of the cyclic 3′, 5′ guanosine monophosphate pathway.[26,27] We cannot exclude *a priori*

Table 1 | Effect of CO-RMs on tubular function and other biochemical parameters at reperfusion after cold storage (CS)

	$\%R_{GLU}$	$\%R_{Na}$	GGT (U/l)	Nitrite (μM)	% Protein leakage
CS	54.8 ± 6.3	16.4 ± 2.8	18.0 ± 9.2	3.8 ± 0.5	7.0 ± 0.2
CS+CORM-A1 (50 μM)	72.7 ± 3.9*	29.3 ± 1.9*	8.7 ± 2.1	3.4 ± 0.4	7.7 ± 0.3
CS+iCORM-A1 (50 μM)	54.3 ± 7.0	18.1 ± 2.8	30.0 ± 12.8	3.4 ± 0.6	7.6 ± 1.1
CS+CORM-3 (50 μM)	69.8 ± 6.9	25.3 ± 2.8	6.4 ± 2.7	2.2 ± 0.4*	7.3 ± 0.5
CS+iCORM-3 (50 μM)	71.3 ± 5.0	26.0 ± 3.4	15.8 ± 5.5	5.8 ± 0.5	8.0 ± 0.6

R_{GLU}=glucose reabsorption; R_{Na}=sodium reabsorption; GGT=gamma-glutamyltransferase. [2]Isolated rabbit kidneys were flushed with Celsior solution (4°C) alone or Celsior supplemented with CORM-A1 or CORM-3. Kidneys were then stored at 4°C for 24 h and then reperfused *ex vivo* as discussed in the Materials and Methods section.
*$P < 0.05$ vs CS.

that other mechanisms, particularly activation of K^+ channels[36] and modulation of the vasoconstrictor endothelin,[37] could participate in CO-mediated renal vasorelaxation as these two pathways have been shown to be potential targets for CO-RMs in cardiac and smooth muscle cells.[25,38] Thus, the present study emphasizes that water-soluble CO-RMs exert a positive effect on the vascular activity of the kidney and confirm that CO is crucial in maintaining renal hemodynamics after cold ischemia.

The increase in GFR and UFR mediated by CO-RMs was accompanied by a markedly improved glucose and sodium reabsorption as well as reduced GGT activity, suggesting that CO also protects tubular function against the detrimental effect of cold ischemia and reperfusion. Our data are consistent with a previous report by Arregui and colleagues showing that $Mn_2(CO)10$ (CORM-1), a light-sensitive non-water-soluble CO-RM firstly identified by our group and found to possess biological activities,[23,24] plays an important role in the control of renal hemodynamics and function *in vivo*. The authors reported that an intrarenal administration of CORM-1 in Sprague–Dawley rats increases renal blood flow (+54%), GFR (+38%) and urinary cyclic 3′, 5′ guanosine monophosphate excretion (+128%), and that inhibition of HO activity progressively compromises renal hemodynamics leading to acute renal failure, an effect that was completely reversed by CORM-1.[39] Similarly, Vera *et al.*[40] reported that CORM-3 significantly decreased the levels of plasma creatinine and limited renal damage in a mouse model of ischemia-induced acute renal failure. Collectively, these and our present results suggest that CO synthesis and activation of guanylate cyclase are dynamically involved in counteracting renal dysfunction in a variety of stress conditions that affect the physiology of the kidney.

In addition to its well-established beneficial effects on vascular activity, CO promotes anti-inflammatory[16] and antiapoptotic effects[15] that can contribute to the defence against tissue injury. The molecular mechanisms underlying these cytoprotective actions by CO are not fully understood at present, but emerging evidence highlights the pleiotropic properties of this gaseous molecule. Administration of CO gas *in vivo* has been shown to improve kidney graft function in part by decreasing the levels of proinflammatory mediators and inhibition of apoptosis.[6] Recent studies from our group confirm the anti-inflammatory action of CO by showing that CORM-3 inhibits the increase in tumor necrosis factor alpha and nitric oxide in endotoxin-stimulated macrophages.[28] Moreover, CORM-2 appears to modulate the production of reactive oxygen species generated by nicotinamide adenine dinucleotide phosphate oxidase and the mitochondrial respiratory chain in human airway smooth cells.[41] This is in line with the data presented in this study showing that the use of CORM-3 during the CS procedure reduced urinary nitrite levels and improved kidney mitochondrial respiration at reperfusion. Since CO preferentially binds to metal-centered proteins, heme-dependent cytochromes present in the mitochondria could potentially serve as a possible target for CO to confer protection against oxidant-mediated injury. Indeed, mitochondria play a fundamental role in I–R injury in organ transplantation[5] by controlling apoptotic events[42] and the generation of reactive oxygen species.[43] In this context, uncoupling has been suggested as an important mechanism for reducing reactive oxygen species generation,[44] and the concept that a continuous mild production of reactive oxygen species from mitochondria is essential for life is fully supported by experimental evidence.[45] Notably, in pig hearts subjected to cardiopulmonary bypass with cardioplegic arrest, treatment with CO gas showed significantly higher adenosine 5′ triphosphate and phosphocreatine levels, less interstitial edema, and reduced apoptosis of cardiomyocytes, suggesting that CO can improve the energy status of the cell.[46]

The ability of CO-RMs to confer significant protection against renal cold ischemia and reperfusion damage is in agreement with the beneficial effects mediated by CO gas and HO-1-derived CO against kidney dysfunction in various models of disease. Kidney grafts preserved in University of Wisconsin solution at 4°C for 24 h and orthotopically transplanted into syngeneic rats previously treated with CO gas displayed an increased renal cortical blood flow and was accompanied by enhanced serum creatinine clearance and improved animal survival.[6] Treatment of organ donors and long-term recipients with methylene chloride, a CO-generating chemical, ameliorated renal function and reduced histological signs of deterioration in a rat model of kidney allograft rejection.[47] Similarly, induction of HO-1 preserved kidney graft function and prevented postreperfusion apoptosis after cold ischemia, an effect that was completely reversed by inhibition of HO activity.[48] Moreover, overexpression of HO-1 either by hemin or gene transfer results in protection against renal CS-mediated damage and renders transplanted kidneys more resistant to I–R injury.[49,50]

It is intriguing to note that in freshly isolated kidneys perfused at 37°C, CORM-A1 promoted a significant increase in PFR, UFR, and GFR, while CORM-3 did not change renal hemodynamics and function under this condition. This is rather surprising since both agents were equally effective in preventing renal dysfunction when used during the flushing procedure at 4°C prior to CS and reperfusion. The reason for this apparent discrepancy may lie in the different chemical features and rate of CO release from the two drugs used. CORM-3, a ruthenium-based carbonyl complex, is known to release CO very rapidly in physiological buffers and plasma at 37°C with a half-life ranging from 1 to 5 min.[24,25] In contrast, CORM-A1 liberates CO slowly and the rate of CO release is strictly dependent on pH and temperature.[26] In fact, the half-life of CORM-A1 in phosphate buffer at 37°C is approximately 21 min[26] and is similar that one found in this study using Krebs buffer perfusion solution. The rate of CO release from CO-RMs in Celsior at 4°C is dramatically reduced despite being still 10 times faster for CORM-3 (14.0 ± 1.0 nmol/h) than CORM-A1 (1.39 ± 0.05 nmol/h). Given the large volume of recirculating perfusate used in

the isolated system, which is open to atmospheric exchange, the CO rapidly liberated from CORM-3 in the perfusate at 37°C may be lost from the system before it reaches the renal circulation at effective concentrations. This technical problem would be avoided with CORM-A1, which effectively delivers CO to the kidney circulation by virtue of its features as a slow releaser at 37°C. However, at low temperatures (4°C), the rate of CO release from CORM-3 and CORM-A1 is significantly decreased and CO liberated more gradually from both compounds during the 24 h CS period could exert a stronger pharmacological activity that is manifested at reperfusion. This indicates that the kinetics of CO release from CO-RMs under different conditions plays a crucial role in determining and maximizing the beneficial effects of these CO carriers. Although both CORM-3 and CORM-A1 afford the same degree of renoprotection when used at low temperatures, our data show that during the first 30 min of reperfusion both vascular activity (PFR) and renal function (GFR and UFR) are better in kidneys previously treated with CORM-A1, suggesting that slow CO releasers may be the preferred choice for organ preservation.

In summary, our results emphasize that CO liberated from CO-RMs has a protective vasodilatory effect, improves renal function and increases mitochondrial respiration after cold ischemia and reperfusion. These findings suggest that CO-RMs could be used therapeutically in preservation solutions as an efficacious strategy to prevent the injury sustained by organs during CS prior to transplantation.

MATERIALS AND METHODS

Chemicals, reagents and solutions

CORM-3 and its inactive counterpart (iCORM-3) as well as CORM-A1 and iCORM-A1 were synthesized and prepared as previously reported by us.[25,26] Adenosine diphosphate was from Boehringer Mannheim (Lewes, UK) and all other reagents were obtained from Sigma Aldrich. Celsior, a universal CS solution for preservation of organs,[51] was from Imtix-Sangstat (Lyon, France).

Surgical procedure

Male New Zealand White rabbits (2.5–4 kg) were supplied by Harlan UK Ltd. All experiments were performed under Home Office licenses (UK) under the Animal (Scientific Procedures) Act, 1986. After anesthesia, the renal arteries were cannulated and kidneys were flushed with 30 ml perfusion solution at 37°C.

Isolated perfused rabbit kidney

The kidney was perfused using a closed perfusion circuit built from nylon tubing based on a system described previously.[52] Oxygenated Krebs solution was perfused at constant pressure (100 mmHg) through the renal artery while the renal vein was kept open to allow the perfusate to run through the organ chamber and then recirculated again. Sampling of perfusate for biochemical analysis and measurement of hemodynamic parameters were undertaken at set intervals.

Experimental protocols

Isolated kidneys were divided in two major protocols. In protocol A ($n = 6$ for each group), kidneys were not subjected to CS but were immediately perfused on the circuit at 37°C; CO-RMs (0, 10, 25, and 50 μM) or iCO-RMs (50 μM) were added to the perfusion solution in the last 45 min of the 2 h perfusion period to investigate their effects on kidney function. In protocol B ($n = 6$ for each group), kidneys were flushed with 60 ml cold Celsior solution alone or supplemented with 50 μM CO-RMs or iCO-RMs and bathed at 4°C for 24 h in Celsior prior to evaluating their hemodynamic functions on the isolated system. In an additional set of experiment, ODQ (30 μM), a soluble guanylate cyclase inhibitor, was perfused 30 min before the addition of CORM-A1 in freshly isolated kidneys at 37°C or added to the flushing Celsior solution (4°C) in combination with CO-RMs prior to CS.

Functional and biochemical parameters

PFRs and UFRs were measured every 15 min after 30 min of initial stabilization and expressed as ml/min/g tissue. Perfusate and urine samples from renal vein and ureter were collected at the same time points for biochemical study. The samples were kept at −80°C until further analysis. GFR was calculated by measuring urine and perfusate creatinine levels according to the following formula: GFR = UFR × urine creatinine/perfusate creatinine. Glucose, Na^+, creatinine, and GGT levels were measured using a biochemical analyzer. Nitrite levels were determined in the urine using the Griess method as described previously.[53] Protein leakage was assessed by measuring the protein concentration (Lowry method) appearing in the urine.

Detection of CO release

The release of CO from CO-RMs in Krebs buffer perfusion (37°C) and in Celsior (4°C) solutions was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin to MbCO using a method previously described by us.[23,26] Since both CORM-3 and CORM-A1 liberate 1 mol of CO/mol of compound and because we used 1 ml of solution containing myoglobin, the maximum amount of CO that can be released from 50 μM CO-RMs into the solution is 50 nmol. Therefore, the rate of CO release was expressed as nmol/h.

Isolation of rabbit kidney mitochondria

At the end of the reperfusion studies on the isolated system, cortical mitochondria from each kidney were isolated by homogenization and differential centrifugation as reported before.[54]

Measurement of oxygen consumption in isolated mitochondria

All experiments were carried out using a 2 ml capacity incubation chamber maintained at 30°C and a Clark electrode attached to an oxygen monitoring system (Yellow Springs Instruments, Yellow Springs, OH). Incubation was carried out using approximately 1 mg protein of isolated mitochondria resuspended in 1 ml respiratory buffer supplemented with 0.5 mg bovine serum albumin. Mitochondrial respiration was measured using malate (5 mM) + glutamate (5 mM) as substrates. State 3 respiration was initiated by the addition of adenosine diphosphate and state 4 respiration was measured after all the ADP was converted to adenosine 5′ triphosphate. The RCI, which indicates the tightness of the coupling between respiration and phosphorylation, was calculated as the ratio between states 3 and 4.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance combined with the Bonferroni test for multiple comparisons. When

differences were established between groups, an unpaired two-tailed *t*-test was used to assess the statistical significance between two groups at each time point. Data were presented as mean $\pm$ s.e.m. and differences were considered to be statistically significant at $P<0.05$.

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