Protection of transplant-induced renal ischemia-reperfusion injury with carbon monoxide

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Submitted 30 April 2004; accepted in final form 26 July 2004

Neto, Joao Seda, Atsunori Nakao, Kei Kimizuka, Anna Jeanine Romanosky, Donna B. Stolz, Takashi Uchiyama, Michael A. Nalesnik, Leo E. Otterbein, and Noriko Murase. Protection of transplant-induced renal ischemia-reperfusion injury with carbon monoxide. Am J Physiol Renal Physiol 287: F979–F989, 2004. First published August 3, 2004; doi:10.1152/ajprenal.00158.2004.—Carbon monoxide (CO), a product of heme metabolism by heme oxygenases, is known to impart protection against oxidative stress. We hypothesized that CO would protect ischemia-reperfusion (I/R) injury of transplanted organs, and the efficacy of CO was studied in the rat kidney transplantation model. A Lewis rat kidney graft, preserved in University of Wisconsin solution at 4°C for 24 h, was orthotopically transplanted into syngeneic rats. Recipients were maintained in room air or exposed to CO (250 ppm) in air for 1 h before and 24 h after transplantation. Animals were killed 1, 3, 6, and 24 h after transplantation to assess efficacy of inhaled CO. Rapid upregulation of mRNA for IL-6, IL-1β, TNF-α, ICAM-1, heme oxygenase-1, and inducible nitric oxide synthase was observed within 3 h after transplantation in the control grafts of air-exposed recipients, associating with histopathological evidences of acute tubular necrosis, interstitial hemorrhage, and edema. In contrast, the increase of inflammatory mediators was markedly inhibited in kidney grafts of CO-treated recipients, which correlated with improved renal cortical blood flow. Further detailed morphological analyses revealed that CO preserved the glomerular vascular architecture and podocyte viability with less apoptosis of tubular epithelial cells and less ED1+ macrophage infiltration. CO inhalation resulted in improved serum creatinine levels and clearance, and animal survival was significantly improved with CO to 60.5 from 25 days in untreated controls. The study demonstrates that exposure of kidney graft recipients to CO at a low concentration can impart significant protective effects against renal I/R injury and improve function of renal grafts.

Kidney transplantation; heme oxygenase; oxidative stress; cold preservation

ISCHEMIA-REPERFUSION INJURY (I/R) of the kidney graft has been considered one of the major deleterious factors of successful renal transplantation. In the immediate posttransplant period, it causes an increased risk of delayed or primary nonfunction of transplanted grafts and complicates posttransplant recipient management, associating with high morbidity and mortality (40). In addition, in clinical and experimental studies, I/R injury has been identified as a key risk factor in predisposing earlier development of chronic allograft nephropathy and short graft life, in part, by accelerating alloantigen-specific immune reactions (12, 16). Because of the current shortage of organs for transplantation, the donor pool has been expanded with the use of marginal donors (e.g., old donors, non-heart-beating donors, grafts with prolonged cold storage), and grafts from these donors have a higher incidence of severe cold I/R injury. I/R injury is a complex sequelae of events, resulting in pathophysiological features of persistent intrarenal vasoconstriction, injury of microvascular endothelial cells and tubular epithelial cells, and activation of inflammatory cascades. It is initiated by the lack of oxygen during cold preservation and ATP depletion, followed by an alteration in intracellular calcium and sodium concentrations and activation of cytotoxic enzymes (e.g., proteases, phospholipases) (8, 18). Subsequent warm reperfusion of grafts initiates a rapid increase in the generation of reactive oxygen species, which further promotes cell damage and activates inflammatory cascades (14). Vascular endothelial cell injury and upregulation of adhesion molecules are also implicated during renal I/R injury (36, 39, 52) and result in vasoconstriction, platelet activation, and increased leukocyte extravasation, which subsequently lead to further inflammatory injury.

Carbon monoxide (CO), a byproduct of heme catalysis by heme oxygenases (HO-1, HO-2, HO-3), has been shown to have cytoprotective and anti-inflammatory effects (6, 23, 30–32). It exerts biological actions by inhibiting proinflammatory cytokines (e.g., TNF-α, IL-1β) and chemokines, preventing vascular constriction, and inhibiting platelet aggregation and plasminogen activator inhibitor (15). Additionally, CO is able to inhibit apoptosis in vitro in a variety of cell types including pancreatic β cells, fibroblasts, and endothelial cells and in vivo in models of lung injury (6, 19, 33, 51).

We hypothesized in this study that CO at low concentrations would minimize I/R-induced renal injury and improve graft function following transplantation. The current study examined the efficacy of recipient CO inhalation at 250 ppm on renal cold I/R injury in a model of syngeneic rat kidney transplantation with prolonged cold storage.

MATERIALS AND METHODS

Animals. Inbred male Lewis (LEW, RT1+) rats weighing 200–250 g were purchased from Harlan Sprague Dawley (Indianapolis, IN) and maintained in laminar flow cages in a specific pathogen-free animal facility at the University of Pittsburgh. Animals were fed a standard diet and water ad libitum. All procedures in this experiment were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

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performed according to the guidelines of the Council on Animal Care at the University of Pittsburgh and the National Research Council’s Guide for the Humane Care and Use of Laboratory Animals.

**Kidney transplantation.** Orthotopic kidney transplantation (KTxs) was performed using a previously described technique (13). In short, after intravenous heparinization (300 U), the donor left kidney was removed with the left renal artery (RA) and a short aortic segment, and the left renal vein with a patch of vena cava. The excised graft was flushed with 3 ml University of Wisconsin (UW) solution (Viaspan, DuPont, Wilmington, DE) and preserved in UW solution at 4°C for 24 h. After recipient left nephrectomy, the kidney graft was orthotopically transplanted into the syngeneic recipient by end-to-side microvascular anastomoses between the graft aorta and recipient infrarenal abdominal aorta, and between the graft renal vein and recipient infrarenal vena cava with 10–0 Novafil sutures. End-to-end ureteral anastomosis was performed using 10–0 Novafil sutures. Efficacy of CO on animal survival and renal graft function was examined after the remaining host right kidney was removed 10 days after KTxs.

**CO exposure.** Animals were exposed to CO at a concentration of 250 ppm. CO (1%) was mixed with air (21% oxygen) in a stainless steel mixing cylinder and then directed into a 3.70-ft³ glass exposure chamber at a flow rate of 12 l/min. A CO analyzer (Interscan, Chatsworth, CA) was used to continuously measure CO levels in the chamber. CO concentration was maintained at 250 ppm. Animals were maintained in a CO chamber for the duration of CO exposure with regular diet and water ad libitum.

**Experimental groups.** Syngeneic KTxs was performed in LEW rats following 24-h preservation of kidney graft in UW solution without immunosuppression. Three groups of animals were examined: unoperated normal LEW control (group 1), control recipients maintained in room air either in the chamber or in the regular laminar flow cage (group 2), and recipients placed in the CO chamber for 1 h before and for 24 h after KTxs (group 3). Recipients were killed at 1, 3, 6, and 24 h after KTxs. Blood and graft kidney samples were obtained for routine and immunohistopathology and for mRNA and protein extraction.

Separate groups of animals were studied for cortical blood flow at 1 h after KTxs. Kidney grafts were then excised, fixed, and prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

**Kidney function.** Serum was obtained from blood samples, and creatinine levels were measured using a Beckman analyzer (Beckman Instruments, Fullerton, CA) using standard methods. Protein and creatinine levels in 24-h urine were determined after KTxs using the metabolic cage system.

**Measurement of kidney graft cortical blood flow.** Renal cortical microvascular blood flow was measured by a laser Doppler flowmeter (BLF 21D, Transonic Systems, Ithaca, NY) on the upper, middle, and lower surface areas of the kidney graft. Three measurements were made in each location (9 measurements per animal) by one of the authors (T. Uchiyama) without knowledge of the experimental groups. Blood flows of the recipient abdominal aorta and graft RA were also measured.

**Total RNA extraction and SYBR green real-time RT-PCR.** Total RNA was extracted from the kidney grafts using the TRIzol reagent (Life Technologies, Grand Island, NY), and RNA content was determined using 260/280 UV spectrophotometry.

mRNA expression was quantified by SYBR green two-step, real-time RT-PCR for ICAM-1, IL-6, IL-1β, TNF-α, inducible nitric oxide synthase (iNOS), HO-1, Bax, Bcl-2, and GAPDH. Total mRNA pellets were suspended in RNAse-free water, followed by removal of potentially contaminating DNA with DNase I (Life Technologies, Rockville, MD). One microgram of total mRNA from each sample was used for reverse transcription with oligo dT (Life Technologies) and Superscript II (Life Technologies) to generate first-strand cDNA. PCR reaction mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA). Each sample was analyzed in duplicate using the conditions recommended by the manufacturer. The following primers were used: IL-6 sense primer, 5′-CAAGGCCAGTGTTTCAACGG-3′, antisense primer, 5′-GTTCCTGAATTCTCCCTTGT-3′, iNOS sense primer, 5′-GGAGAAGTTTTCTCAGACCC-3′, antisense primer, 5′-CCATGCTAAATTGTGGTCGGA-3′, HO-1 sense primer, 5′-CCAGAGAGCAGAGTCCTCAG-3′, antisense primer, 5′-AAATTCACCTGCAAGG-3′, ICAM-1 sense primer, 5′-CGTGGCTCATTACACC-3′, antisense primer, 5′-TATGGGCCTCTCCTGAC-3′, TNF-α sense primer, 5′-GGTATCTGGCCCTCACCAGGA-3′, antisense primer, 5′-CAGCTGTCGCTACACTGC-3′, IL-1β sense primer, 5′-CACCTCTAACAGGACACAG-3′, antisense primer, 5′-GGTTCATGGTGAATGACTAC-3′, Bax sense primer 5′-GCAACACAACTGAGGC-3′, antisense primer, 5′-AGCCCATGATTGGTCGTAC-3′, bcl-2 sense primer, 5′-CATGCAGACCTGTTG-3′, antisense primer, 5′-GTITCATGTTGACCTCTTTG-3′, and GAPDH sense primer, 5′-ATGCCAGCATAAGGCAGTA-3′, antisense primer 5′-CGTCTCTGGAATGTTGAT-3′. Thermal cycling conditions were 10 min at 95°C to activate the AmpliTaq Gold DNA polymerase, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Using the manufacturer’s software, real-time PCR data were plotted as the ΔΔCt fluorescence signal vs. the cycle number. The threshold cycle was defined as the cycle number at which the ΔΔCt crosses this threshold. The expression of each gene was normalized to GAPDH mRNA and the relative fold change was determined relative to using the comparative cycle threshold method (25).

**Serum IL-6 and nitrite/nitrate.** Serum IL-6 concentrations were determined using a rat ELISA kit (R&D, Cambridge, MA). The serum nitrite/nitrate levels, the stable end products of NO metabolism, were measured using a commercially available test kit (Cayman, Ann Arbor, MI).

**Protein extraction and immunoblotting.** Protein was extracted from kidney grafts after homogenization in 0.25 mol/l sucrose at 4°C using a Polytron (Janke & Kunke KG IGA Werk, Staufen, Germany). Protein samples (20 μg) were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. HO-1 protein expression was determined with primary rabbit anti-HO-1 polyclonal antibody (1:1,000, Stressgen, Victoria, British Columbia), followed by secondary goat anti-rabbit IgG (H+L) antibody conjugated with horseradish peroxidase (1:1,000, Pierce Chemical, Rockford, IL). Membranes were washed and subjected to chemiluminescence Western blotting (Pierce Chemical).

**Routine histopathology.** Formalin-fixed graft tissues were paraffin-embedded, cut into 5-μm sections, and stained with H&E. Sections were blindly reviewed by a pathologist without the knowledge of treatment groups. The severity of renal I/R injury was graded according to the severity of acute tubular necrosis (ATN) (0 = minimal changes, 1 = 5 to 25% ATN, 2 = 26 to 50% ATN, and 3 = > 50% ATN).

**Immunohistochemical staining.** Formalin-fixed, paraffin-embedded graft tissue was cut into 5-μm sections for immunohistochemical staining using the avidin-biotin-peroxidase complex method after antigen retrieval. Endogenous peroxidase activity was blocked with superblock (Sey Tek Laboratories, Logan, UT) with 10% horse serum. For the detection of cleaved caspase-3, sections were incubated with polyclonal rabbit anti-cleaved caspase-3 antibody (1:100, Cell Signaling Technology, Beverly, MA) overnight at 4°C, followed by biotinylated goat anti-rabbit IgG (Dako, Glostrup, Denmark) for 1 h at room temperature. To evaluate macrophage infiltration, sections were incubated for 2 h with monoclonal mouse anti-rat ED1 antibody (1:200, Serotec, Raleigh, NC), followed by LSAB+ horseradish peroxidase (Dako, Carpenteria, CA) for 30 min.

The immune complex was visualized with 3-amin-9-ethyl carbazole and hematoxylin counterstaining. Positively stained cells were counted in blinded fashion in five random cortical high-power fields (HPF: ×400) per section.

For CD31 immunofluorescence stain, kidneys were cleared of blood with lactated Ringer solution, perfusion-fixed with 2% parafor-
maldehyde in PBS, cryoprotected in 2.3 M sucrose in PBS overnight, frozen in liquid nitrogen-cooled isopentane, then cut into 6-μm sections onto gelatin-coated slides. Sections were washed with PBS with 0.5% BSA and 0.15% glycine (PBG buffer). After being blocked with 20% (vol/vol) normal goat serum in PBG, the tissue was stained with mouse anti-rat CD31 (PE-CAM, 1:100, Serotec) for 1 h, and then incubated for 1 h with Alexa 488 (1:500, Molecular Probes, Eugene, OR) conjugated goat anti-mouse IgG. F-actin was visualized by staining with rhodamine-phalloidin (1:250, Molecular Probes) for 30 min, then with Hoechst dye (bisBenzimide, 1 μg/100 ml) for 30 s to stain nuclear DNA. The sections were washed and coverslipped with Gelvatol, a water-soluble mounting media (23 g polyvinyl alcohol, 50 ml glycerol, 0.01% sodium azide in 100 ml PBS), and visualized with an Olympus BX51 epifluorescence microscope and digitized with an Olympus color video camera.

TEM. Kidneys were perfusion-fixed via the abdominal aorta with 2.5% glutaraldehyde, then stored overnight in fixative at 4°C. Samples were cut into 1-mm³ size, washed in PBS, then postfixified in aqueous 1% OsO₄, 1% K₃Fe(CN)₆ for 1 h. After three PBS washes, the tissue was dehydrated through a graded series of 30–100% ethanol, 100% propylene oxide, and then infiltrated in 1:1 mixture of propylene oxide:Polybed 812 epoxy resin (Polysciences, Warrington, PA) for 1 h. After several changes of 100% resin over 24 h, tissue was embedded in molds, cured at 37°C overnight, followed by additional hardening at 65°C for 2 more days. Ultrathin (70 nm) sections were collected on 200 mesh copper grids, stained with 2% uranyl acetate in 50% methanol for 10 min, followed by 1% lead citrate for 7 min. Sections were photographed using a JEOL JEM 1210 transmission electron microscope (Jeol USA, Peabody, MA) at 80 kV onto electron microscope film (Kodak, ESTAR thick base, Rochester, NY). Electron micrographs were digitized on a flatbed scanner at 400 ppi (StudioStar, Agfa, Ridgefield Park, NJ). Digitized images were assembled into montages using Adobe Photoshop 6.1.

Vascular corrosion casting. The abdominal aorta was cannulated for 1 h, carboxyhemoglobin (COHb) levels in arterial blood samples elevated to 25.3 ± 2.0% from 2.3 ± 1.4% in room air. During transplant surgery in room air, COHb levels decreased to 13.7 ± 0.1% before the return to the CO chamber. Peak COHb levels observed during the 24-h posttransplant CO exposure were 24.5 ± 0.5%. Methemoglobin (MetHb) levels were below 1% at all time points. Oxygen saturation (SaO₂) was ~100% in both CO chamber and room air.

Inflammatory mediators. Renal I/R injury resulted in a rapid increase of mRNA levels for inflammatory cytokines (IL-1β, IL-6, TNF-α) and the stress-induced molecule iNOS in air-treated control kidney grafts, peaking at 1–3 h following reperfusion. Perioperative recipient exposure to CO significantly decreased peak levels of mRNA for these inflammatory mediators after 24-h cold preservation (Fig. 1, A–D).

Serum IL-6 and nitrite/nitrate levels. In correlation with an increase of mRNA expression, serum IL-6 elevated in air-treated control animals and peaked at 6 h following transplantation (Fig. 2A). CO significantly decreased serum IL-6 levels at 3 and 6 h after reperfusion. In addition, renal I/R injury caused an increase in serum nitrite/nitrate in air-treated con-

Fig. 1. Intragraft mRNA expression for inflammatory mediators. mRNA levels of TNF-α (A), IL-6 (B), IL-1β (C), and inducible nitric oxide synthase (iNOS; D) were upregulated during ischemia-reperfusion (I/R) injury in air-treated controls with peaks at 1 (IL-1β) and 3 h (TNF-α, IL-6, and iNOS). Carbon monoxide (CO) treatment significantly reduced mRNA expression for these cytokines. Real-time PCR analysis of kidney graft samples obtained at 1 (n = 3 for each group), 3 (n = 5), 6 (n = 4), and 24 h (n = 4) after kidney transplantation (KTx). ∗P < 0.05 (CO vs. air control).
controls, which was significantly decreased at 3, 6, and 24 h in recipients treated with CO inhalation (Fig. 2B).

Endogenous HO-1 expression. In kidney grafts from air-treated control animals, I/R injury resulted in a rapid upregulation of HO-1 mRNA to a 30-fold increase at 3 h after reperfusion, compared with normal kidney. In the CO-treated group, HO-1 mRNA upregulation was delayed and peaked at 6 h to ~15-fold increase (Fig. 3A). Although HO-1 mRNA (~15-fold increase) was continuously upregulated in control grafts for 24 h, they decreased in CO-treated grafts to normal levels by 24 h. Further analysis with Western blot showed that I/R injury increased HO-1 protein levels in control kidney grafts at 6 and 24 h after reperfusion. CO-treated kidney grafts had a minimum increase of HO-1 protein expression (Fig. 3B).

Routine histopathology. Cold preservation for 24 h and subsequent reperfusion induced interstitial hemorrhage, peritubular capillary congestion, and edema in kidney grafts. The most significant changes were tubular epithelial cell death, apoptotic debris and protein casts inside the tubular lumen, and dilatation of tubules. These changes were less severe in CO-treated grafts, and there was a significant early decrease in the ATN score in CO-treated grafts (Fig. 3B).

Apoptosis of tubular epithelial cells. Several previous studies have demonstrated the antiapoptotic function of CO (6, 19, 33, 51), and the efficacy of CO on tubular epithelial cell death, in particular tubular cell apoptosis, was further studied by cleaved caspase-3 immunohistochemistry. Although normal kidney cortex did not express activated caspase-3, activated caspase-3-positive cells rapidly increased and peaked at 6 h after reperfusion during I/R injury in air-treated control grafts (Figs. 5, A–C, and 6A). Positive staining was seen in isolated tubular epithelial cells and cells/debris in the tubular lumen. CO inhalation reduced tubular cell apoptosis, and the peak number of caspase-3-positive cells was decreased to <50% (11.3 ± 5.5 cells/HPF) at 6 h after reperfusion.

Macrophage infiltration. ED1+ macrophages represent the predominant infiltrating cells during I/R injury (12), and the efficacy of CO in ED1+ macrophage recruitment was studied by immunohistochemistry. In the normal renal cortex, a small number of ED1+ cells were present in peritubular, periglomerular and intraglomerular spaces (17.0 ± 3.7/HPF; Figs. 5, D–F, and 6B). As previously demonstrated (11), in air-treated control grafts, ED1+ infiltrates increased after reperfusion with a peak at 24 h to 59.0 ± 35.2/HPF. CO significantly (P < 0.05) decreased the number of ED1+-infiltrative macrophages to <56% in kidney grafts.

Molecular analysis of apoptosis and adhesion molecules. Proapoptotic Bax signal was not changed at 1 h after reperfusion. At 3 h, Bax mRNA increased 15-fold in control grafts. In contrast, Bax was downregulated in CO-treated grafts; levels of Bax mRNA were lower than those of normal kidney (Fig. 7A). There was no significant difference in mRNA levels for Bcl-2 (Fig. 7B). ICAM-1 mRNA levels in air-treated control grafts rapidly elevated to 14-fold compared with normal kidney with a peak at 3 h after reperfusion (Fig. 7C). CO significantly ablated ICAM-1 mRNA expression in the grafts, and peak value was decreased to 6.3 ± 2.3-fold.
Blood flow in the kidney graft. Because CO has been shown to play an important role in regulating vasomotor tone by promoting vasorelaxation through soluble guanylyl cyclase activation (15, 35), the effect of CO on renal graft blood flow was analyzed by a laser Doppler flowmeter. Blood flows in the recipient aorta and graft RA were not different among normal unoperated and transplanted kidneys, regardless of CO inhalation, and were $116 \pm 126$ ml·min$^{-1}$·100 g$^{-1}$ for the aorta and $83 \pm 87$ ml·min$^{-1}$·100 g$^{-1}$ for the RA. Graft cortical blood flow in normal kidney ($63.3 \pm 4.2$ ml·min$^{-1}$·100 g$^{-1}$) decreased to $20.8 \pm 7.1$ ml·min$^{-1}$·100 g$^{-1}$ in air-treated control kidney grafts at 1 h after reperfusion. In contrast, CO-treated kidney grafts maintained graft blood circulation with a flow rate of $39.7 \pm 11.8$ ml·min$^{-1}$·100 g$^{-1}$ (Fig. 8).

CD31 and F-actin stain. Immunofluorescent analysis of normal kidney revealed rhodamine-phalloidin (F-actin)-positive stain of arterial smooth muscles, microvilli of proximal tubular epithelial cells, and glomerular epithelial cells (podocytes) (Fig. 9). Renal I/R injury in control grafts resulted in the disappearance of F-actin at 1 h after reperfusion, indicating disruption of the cellular actin cytoskeleton. In contrast, CO-treated animals showed increased preservation of glomerular podocyte architecture and viability. Arterioles and proximal tubular cells also maintained F-actin positivity; however, significant dilatation of tubules was noticed compared with the F-actin stain pattern observed in normal kidney. Double immunofluorescent stain with F-actin and CD31 (PE-CAM) demonstrated CD31-positive vascular endothelial cells in the peritubular arterioles, and glomerular and peritubular capillary endothelium of normal kidney. CD31 stain, as seen with F-actin stain, was lost in air-treated control grafts, indicating severe endothelial injuries. The CO-treated group showed slightly faint CD31 stain in glomerular capillary endothelial cells. The CD31 stain on the peritubular capillaries was lost in both groups, indicating significant endothelial cell dysfunction in peritubular capillaries during ischemic renal injuries (3, 5).

Vascular casting and SEM. To evaluate detailed microvascular architectural changes associating with I/R injury and CO treatment, three-dimensional visualization of the fine microvascular architecture of the glomerular tuft was obtained using...
vascular casting and SEM. In a normal kidney, as depicted in Fig. 10A, the afferent arteriole divides dichotomously and gives rise to four to eight capillaries, each of which subdivides into an anastomosing network of capillaries. After running toward the urinary pole, the capillaries merge to form the efferent arteriole (1, 47, 48). Renal I/R injury seen in air-treated control grafts at 1 h after reperfusion resulted in an exudation and pooling of casting resin, vasoconstriction and a significant number of filling defects in the capillary network, indicating interruptions in vascular continuity (Fig. 10B). Castings of kidney grafts from recipients exposed to CO showed the preserved glomerular tuft architecture, with protection against capillary leakage, microcircular interruption, and vasoconstriction, although slight capillary dilatation within the glomerulus was observed (Fig. 10C).

TEM. The ultrastructure of cells in the kidney was studied with TEM. Normal kidneys showed the characteristic filtration apparatus of the glomerulus, including fenestrated endothelium, uninterrupted basement membrane, and healthy podocytes (Fig. 10D). Air-treated control kidney grafts at 1 h after reperfusion showed platelet accumulation in the capillary lumen of the glomerulus. Decreased cytoplasmic density and swelling were also observed in the podocytes with disruption and detachment of the characteristic foot processes from the glomerular basement membrane (Fig. 10E). In contrast, kidneys from CO-treated animals showed amelioration of these changes, with viable podocytes and preservation of foot processes (Fig. 10F). TEM of normal proximal tubular epithelial cells showed characteristic microvilli and mitochondrial apparatus (Fig. 10G). In air-treated control grafts, loss of microvilli, numerous vacuolization, and mitochondrial breakdown in tubular epithelial cells indicated extensive damage (Fig. 10H). CO-treated grafts showed less frequent vacuolization and maintenance of internal cellular architecture (Fig. 10I).

Kidney function and survival. Effects of CO treatment on renal function were analyzed in recipients at 25 days after KTx. Impairment of graft function due to I/R injury was evident: mean serum creatinine levels were 4.5 ± 2.9 mg/dl and glomerular filtration rate (GFR) decreased to a mean of 0.10 ± 0.09 ml/min in untreated controls (Fig. 11A). CO significantly improved GFR to 0.41 ± 0.29 ml/min with mean creatinine levels of 1.7 ± 0.7 mg/dl compared with untreated controls; however, rates were considerably lower than those of normal unoperated rats (1.7 ± 0.2 ml/min, n = 4) and recipients of nonpreserved kidney grafts at the same posttransplant time point (1.52 ± 0.06 ml/min, n = 5). Progressive deterioration of renal function in control animals was reflected by the weight loss of −8.0 ± 10.3% by 25 days after KTx compared with a mean body weight gain of 4.1 ± 2.1% in the CO-treated group. Median survival in the air-treated control group was 25 days. Survival was significantly improved in the CO group with a median survival of 60.5 days (Fig. 11B).

DISCUSSION

The byproducts of heme degradation, biliverdin, iron, and CO are believed to be the effector molecules underlying the potent cytoprotection observed with the heme oxygenase system (2, 6, 23, 30–32). In particular, CO has been shown to ameliorate injuries in several experimental models of oxidative stress, endotoxemia, hyperoxic lung injury, and organ allograft rejection (6, 23, 30–32). Using the kidney transplantation

![Fig. 6. Numbers of cells positive for activated caspase-3 and ED1. A: numbers of activated caspase-3-positive cells per high-power field (HPF; ×400) in air-treated grafts increased to 24.0 ± 11.9/HPF compared with 11.3 ± 5.5/HPF cells in CO-treated grafts (n = 4 per group, 6 h after reperfusion). B: ED1+ cells in normal kidney cortex were 17.0 ± 3.7/HPF (×400). ED1+ cells increased to 59.0 ± 35.2/HPF in air-treated control kidney grafts. CO significantly reduced the number of ED1+ macrophage infiltration (26.3 ± 10.5/HPF; n = 4 per group at 24 h after reperfusion). *P < 0.05 (CO vs. air control).](http://ajprenal.physiology.org/)

![Fig. 7. mRNA levels for Bax (A), Bel-2 (B), and ICAM-1 (C). A: Bax signal was not changed at 1 h after reperfusion. At 3 h, Bax mRNA increased 1.5-fold in air-treated control grafts, whereas Bax was downregulated in CO-treated grafts to the level lower than normal kidney. B: no difference was observed in Bel-2 mRNA levels. C: I/R injury-induced increase of ICAM-1 mRNA in air-treated controls was inhibited in CO-treated grafts. Real-time RT-PCR analysis of kidney graft samples obtained at 1 (n = 3 for each group), 3 (n = 5), 6 (n = 4), and 24 h (n = 4) after KTx. *P < 0.05 (CO vs. air control).](http://ajprenal.physiology.org/)
model, the present study demonstrates that short-term recipient exposure to a low concentration of CO ameliorates I/R injury-induced in kidney grafts following 24-h cold preservation and subsequent transplantation. These data illustrate the potent anti-inflammatory, antiapoptotic, and vasodilative capacities of CO in mediating protection against renal I/R injury. CO inhalation at 250 ppm in this protocol increased recipient blood COHb levels to ~25% with MetHb levels <1% and SaO₂ ~100% during the exposure, as seen in the previous study (26). COHb levels returned to the normal value within 4 h after completing treatment, and there was no evident toxicity associating with a short-term CO treatment in this study. COHb levels 10~20% have been known to induce clinical toxicity including mild headache and shortness of breath on exertion. Severe fatal toxicity is seen with COHb levels of 50~80% (46).

Several previous studies have shown that CO inhibits the generation of proinflammatory mediators, in part, via the select activation of the p38 MAPK signaling pathway (2, 6, 7, 23, 30~32). In accordance with these studies, this study showed a significant inhibition of I/R injury-induced mRNA upregulation for proinflammatory mediators such as TNF-α, IL-1β, IL-6, and iNOS, as well as a reduction of circulating IL-6 and nitrate/nitrate levels. In addition, CO was able to downregulate the proinflammatory cascade by inhibiting expression of adhesion molecules and decreasing circulating inflammatory cell recruitment into injured tissues. There was a significantly reduced infiltration of monocytes/macrophages, the key effector cells mediating injury, in CO-treated kidney grafts (11, 42).

HO-1, an extremely pleiotropic molecule, is typically upregulated in response to a variety of stimuli (e.g., bacterial endotoxins, cytokines, and reactive oxygen intermediates), and in the majority of conditions, HO-1 is able to provide potent cytoprotection (41, 49). Indeed, renal cold I/R injury seen in the control group of this study resulted in a rapid upregulation of HO-1 in the kidney graft. Interestingly, however, both mRNA and protein levels of HO-1 were only marginally increased with a delayed mRNA peak in kidney grafts from CO-treated recipients. In addition, renal I/R injury in the...
control group provoked upregulation of iNOS, another stress-inducible enzyme, responding to similar stimuli that induce HO-1 (27, 44). iNOS induction in kidney grafts was also suppressed with CO inhalation. These data suggest that CO inhibited endogenous generation of stress-inducible enzymes following renal I/R injury. Because NO is one of the most potent inducers of HO-1 (4, 28), these results may illustrate the remarkable interrelationship amongst these two gas-producing enzymes. Although roles of these molecules during I/R injury remain to be determined, downregulation of these "stress-

Fig. 10. Scanning electron microscopy of kidney vascular casts (A–C) and transmission electron microscopy of glomerular (D–F) and tubular (G–I) ultra-structure. A: normal glomerular tuft. B: in air-exposed control grafts, there is a loss of vascular continuity, severe leakage shown by resin extravasation (arrows), filling defects in the vasculature network (arrowhead), and vasoconstriction. C: CO-treated grafts showing preservation of the glomerular tuft architecture. D: filtration apparatus of the normal glomerulus, with fenestrated endothelium, basement membrane, and podocyte foot processes facing the urinary space of the Bowman’s capsule. E: control kidney grafts show decreased cytoplasmic density and swelling of the podocyte with disruption and detachment of foot process from basement membrane. The capillary lumen is filled with platelets (arrows). F: podocyte and foot process viability is preserved in kidney grafts treated with CO. G: normal proximal tubular epithelial cells show characteristic microvilli and mitochondrial apparatus. H: loss of microvilli and numerous vacuolization (arrowhead) in tubular epithelial cells of air-exposed control grafts suggests mitochondrial breakdown and irreversible degeneration. I: CO-treated grafts show considerably less vacuolization, preservation of microvilli, and maintenance of internal cellular architecture. Representative picture of 3 animals per group at 1 h after reperfusion (original magnification ×900 for A–C, ×4,000 for D–F, ×5,000 for G–I). BM, basement membrane; C, capillary lumen; P, podocyte; U, urinary space.

Fig. 11. Kidney graft function and survival. A: in air-treated control recipients, creatinine clearance (CCr) at 25 days after KTx was reduced to ~10% of normal unoperated animals (1.7 ± 0.2 ml/min) or recipients of kidney grafts with minimum cold preservation (~30 min, 1.5 ± 0.1 ml/min). CO significantly improved CCr to 0.41 ± 0.29 ml/min. B: median animal survival after KTx in air-treated control group was 25.0 days (n = 7). CO treatment significantly improved survival up to 60.5 days (n = 8). *P < 0.05 vs. air-treated controls.
induced” gene products by CO in our study suggests that these enzymes may be induced in response to tissue damage, probably to accommodate the repair process, and may simply reflect the severity of injury. Some increases of HO-1 or iNOS in the CO-treated group in this study may indicate the existence of I/R injury in the treated group, although the extent of injury is reduced.

The antiapoptotic function of CO was shown in several experimental models (6, 19, 33, 51), and we previously showed in the intestinal transplantation model that CO exposure reduces cold I/R injury-induced apoptosis of capillary endothelial cells in the laminar propria (25). The current study demonstrates that apoptosis of tubular epithelial cells seen during cold renal I/R injury is considerably reduced with recipient CO inhalation. It is well documented that renal tubular cells are the primary targets of I/R injury. Tubular epithelial cells are sensitive to oxygen deprivation because of their high rates of solute transport activity and mitochondrial respiration. As the term “acute tubular necrosis” is commonly used to describe renal I/R injury, the predominant response to injury in the rat kidney is necrosis; however, apoptosis of tubular cells also has been shown to represent an established feature of renal I/R injury (17, 29, 50). Apoptosis can be triggered by a variety of stimuli and is defined by highly characteristic morphological features, including condensation of nuclear heterochromatin, cytoplasmatic condensation, and cell shrinkage with retention of organelles (37). Apoptosis requires energy and has been considered as a physiological cell deletion mechanism that allows elimination of unnecessary cells with minimum tissue reactions (e.g., inflammation) via a variety of internal signaling pathways, involving proteolytic activation of caspases (43).

Interestingly, despite this paradigm and general view that apoptosis and inflammation are two largely independent processes, recent studies by Daemen et al. (10) suggest that apoptotic cell death is a crucial event in initiating inflammation and subsequent tissue injury. Based on their finding that abrogation of early I/R-induced apoptosis can prevent the development of inflammation and organ dysfunction, suppression of apoptosis with CO may also play a part in this study in reducing inflammation during renal I/R injury.

Although the involvement of tubular cells during I/R injury has been well documented, podocytes (glomerular visceral epithelial cells) that are critical in regulating the GFR also are the target of I/R injury (21). Podocytes float within Bowman’s space and are attached to glomerular basement membrane (GBM) by their processes. Alteration in foot processes is a common response of podocytes to almost every kind of stress, in part, as an adaptive change to improve their attachment to the GBM. Podocytes have been shown to undergo flattening and spreading of major processes in early postischemic renal failure (34). CO in this study showed protection of podocytes by maintaining cell integrity and tight foot process attachment to GBM. Because the cell replication capability of podocytes is limited in the adult and cannot be replaced once they undergo degeneration (20, 22, 38), prevention of podocyte damage with CO likely leads to sustained renal function.

Several studies have shown that CO exerts potent protective effects against ischemic injury by promoting soluble guanylyl cyclase/cGMP-dependent activities and thereby inhibiting platelet aggregation and smooth muscle relaxation (9, 15, 24, 35, 45). Indeed, renal cortical blood flow after cold renal I/R injury was significantly improved in this study with CO inhalation. Although mechanisms of CO effects in this study involved downregulation of the inflammatory cascade, inhibition of apoptosis, and restoration of graft microcirculation, these do not appear to be independent pathways. CO preserved the overall glomerular vascular architecture of the kidney, allowing for improved graft microcirculation and ultimate improved viability of tubular cells, endothelial cells, and podocytes. It is premature to say that it is the anti-inflammatory effects that lead to the antiapoptotic effects or visa versa and whether ultimately it is the effects of improved vasomotor tone that prevent the initiation of these processes. Further studies will be required to determine the detailed mechanisms of CO in preventing destructive inflammatory reactions seen in renal I/R injury.

It is noteworthy to point out that the study shows some degrees of incongruity in CO efficacy between in vitro assays and actual in vivo renal function/survival. CO was effective in inhibiting I/R injury-induced activation of proinflammatory mediators, neutrophil extravasation, and apoptosis. However, the improvement with CO inhalation of GFR and definite animal survival was modest, even though it was statistically significant. The finding may suggest that complete inhibition of early proinflammatory mediators may be required for the protection of the kidney from I/R injury. The proinflammatory cascade is an efficiently augmenting system with positive feedback amplification loops, and incomplete inhibition of the initial activation step can nevertheless result in significant damage. An increase in ATN score at later time points and subsequent impairment of renal function in CO-treated recipients suggest that although CO is able to mitigate renal I/R injury in this study, damages are not entirely prevented.

In summary, inhalation of CO at a low concentration provides protection of kidney grafts against cold I/R injury and preserves overall improved kidney graft functioning following transplantation. Mechanisms mediating cytoprotective effects of CO in this study included improvement of blood flow, decrease in proinflammatory mediators, and inhibition of apoptosis. Taken together, these data suggest that CO might be a beneficial clinical modality for kidney transplantation where preservation of organs may need to be extended.

ACKNOWLEDGMENTS

We thank E. Ifedigbo for the maintenance of the CO chamber, M. Tabacek, L. Chedwick, and F. Liu for excellent technical support, and C. Forsythe for the preparation and organization of the manuscript.

GRANTS

This study was supported by National Institutes of Health Grants DK-54232, CA-76541, HL-42365, HL-55330, and American Heart (160332U) and Atofrvastatin Pfizer Research Award.

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