Low-dose carbon monoxide inhalation prevents development of chronic allograft nephropathy

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Low-dose carbon monoxide inhalation prevents development of chronic allograft nephropathy. Am J Physiol Renal Physiol 290:F324–F334, 2006. First published August 30, 2005; doi:10.1152/ajprenal.00026.2005. — Chronic allograft nephropathy (CAN) is the primary cause for late kidney allograft loss. Carbon monoxide (CO), a product of heme metabolism by heme oxygenases, is known to impart protection against various stresses. We hypothesized that CO could minimize the chronic fibroinflammatory process and protect kidney allografts from CAN. Lewis kidney grafts were orthotopically transplanted into bi-nephrectomized Brown-Norway rats under short-course tacrolimus. Recipients were maintained in room air or exposed to CO at 20 parts/million for 30 days after transplant. Efficacy of inhaled CO was studied at day 30 and day 80. Isografts maintained normal kidney function throughout the experiment with creatinine clearance of ~1.5 ml/min. Renal allograft function in air controls progressively deteriorated, and creatinine clearance declined to 0.2 ± 0.1 ml/min by day 80 with substantial proteinuria. CO-treated animals had significantly better creatinine clearance (1.3 ± 0.2 ml/min) with minimal proteinuria. Histological examination revealed the development of progressive CAN in air-exposed grafts, whereas CO-treated grafts had minimal tubular atrophy and interstitial fibrosis, with negligible collagen IV deposition. In vitro analyses revealed that CO-treated recipients had significantly less T cell proliferation against donor peptides via the indirect allorecognition pathway and less anti-donor IgG antibodies compared with air controls. Intragraft mRNA levels for chemokine receptors (CCR1, CXCR3, CXCR5), IL-2, and intercellular adhesion molecule-1 were significantly decreased in CO-treated than in air-treated allografts. Furthermore, reduction of blood flow in air-injected allografts was prevented with CO. In conclusion, inhaled CO at a low concentration efficiently abrogates chronic fibroinflammatory changes associated with CAN and improves long-term renal allograft function.

kidney transplantation; cytoprotection; anti-inflammatory effect

DESPITE SIGNIFICANT IMPROVEMENTS in the outcomes during early posttransplant periods, chronic allograft nephropathy (CAN), a progressive deterioration of renal allografts with fibroinflammatory changes, is a major cause of late renal allograft loss and remains as a challenge in organ transplantation (22, 38, 52). It is histopathologically defined as atherosclerosis, glomerulosclerosis, interstitial fibrosis, and tubular atrophy (42, 48). This multifactorial process is caused by both immunogenic (anti-gen-dependent) and nonimmunogenic (antigen-independent) factors, including ischemia-reperfusion injury, drug toxicity, hypertension, infection, senescence, and ongoing alloimmune injury (4, 19, 41). 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, prolonged cold storage), and grafts from these donors have a higher incidence of severe cold ischemia-reperfusion injury, in addition to preexisting damages, predisposing earlier development of CAN, in part by accelerating alloantigen specific immune reactions.

Heme oxygenase (HO) is the rate-limiting step in heme degradation, and the oxidation of the α-meso carbon of the protoporphyrin ring leads to the formation of free iron, biliverdin, and carbon monoxide (CO). The inducible isozyme of HO, HO-1, is a ubiquitous heat shock protein (HSP32) that is highly induced by diverse stress-related conditions (35). The HO-1 and HO system have been shown to provide generalized endogenous anti-inflammatory protection against oxidative stress (23, 35, 58). The specific mechanism(s) by which HO-1 can mediate these cytoprotective and anti-inflammatory functions is not clear, but three byproducts generated during the heme catabolism may represent potential protective mediators (10, 20, 30, 34).

We have previously shown in transplant-induced ischemia-reperfusion injury models that exposure to low concentrations of CO [20–250 parts/million (ppm)] has cytoprotective effects equal to that attained by HO-1 induction (29–33). Although CO is known to be toxic at high concentrations due to its ability to interfere with oxygen delivery, CO at low concentrations functions as a signaling molecule that exerts significant cytoprotection due to its anti-inflammatory, vasodilating, and anti-apoptotic properties (8, 17, 30, 33, 34, 44).

We hypothesized that CO inhalation therapy could play a seminal therapeutic role in the success of renal transplantation by inhibiting fibroinflammatory changes. In this study, we examined the potential effects of low-dose CO in preventing CAN by using a rodent model of kidney transplantation (KTx).

MATERIALS AND METHODS

Animals. Inbred male Lewis (LEW; RT11) and Brown-Norway (BN; RT1b) rats, 200–250 g, were purchased from Harlan Sprague-Dawley. Animals were housed in a temperature-controlled room (22°C), with a light-dark cycle (12:12 h), and water and food (LabDiet 5001, PMI Nutrition International, Jackson, MS) were provided ad libitum. Animals were randomly assigned to receive air or CO exposure (20 ± 10 ppm) on posttransplant day 1 and continued for 30 days, with 5 animals per group.

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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 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.

KTx. Orthotopic KTx was performed using a previously described technique (14, 28). In short, after intravenous heparinization (300 U), the donor’s left kidney was removed with the left renal artery in continuity with a short aortic segment and the left renal vein with a patch of vena cava. The excised graft was flushed with 3 ml of University of Wisconsin solution (Viaspan, Du Pont, Wilmington, DE). The kidney graft was orthotopically transplanted into the recipient by end-to-side microvascular anastomoses between graft aorta and recipient infrarenal abdominal aorta, and between graft renal vein and recipient infrarenal vena cava with 10-0 Novafil suture. Recipients received prophylactic antibiotics (Cefotetan Dsodium, 100 mg/kg, im) for 3 days following the transplant.

CO exposure. CO (1%) was mixed with air (21% oxygen) in a stainless steel mixing cylinder and then directed into a 3.70-ℓ, 3 glass exposure chamber at a flow rate of 12 ℓ/min (29, 33). A CO analyzer (Interscan, Chatsworth, CA) was used to continuously maintain the CO level at 20 ppm in the chamber. Animals were exposed to CO (20 ppm) by maintaining in a CO chamber for 30 days following the transplant with a regular diet and water ad libitum. CO inhalation at a concentration of 20 ppm increased COHb in the arterial blood samples to ~10% from 1–2% in the room air, whereas MetHb levels remained below 1% and arterial oxygen saturation was ~100% (32).

Experimental groups. LEW-to-LEW syngenic rat KTx served as the syngenic control (group 1). In group 2, BN recipients received LEW kidney grafts and were maintained in room air either in the chamber or in the regular laminar flow cage, whereas in group 3 BN recipients were placed in the CO chamber (20 ppm) immediately after receiving LEW allografts and maintained for 30 days after KTx. Tacrolimus (FKS06, a gift from Fujisawa Pharmaceutical, Osaka, Japan) was intramuscularly administered to allograft recipients in groups 2 and 3 at a daily dosage of 0.5 mg/kg for 7 days (days 0–6). In addition, separate groups of LEW-to-LEW syngenic KTx were performed, and recipient animals were exposed to 20 ppm CO for 30 days to examine the influence of CO treatment.

Recipients were killed at 30 and 80 days after KTx, and blood and graft kidney samples were obtained. Tissues were fixed in 10% buffered formalin for routine histopathology and in 2% paraformaldehyde for immunohistochemistry. Portions of kidney grafts were also snap-frozen in liquid nitrogen for mRNA and protein extraction. Additional groups of animals were prepared for the measurement of blood pressure and renal graft blood flow.

Renal function. At 30, 60, and 80 days after KTx, recipient animals were placed in individual metabolic cages for 24-h urine collections and total urine volume measurement. Urinary protein excretion was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Munich, Germany) with bovine serum albumin (Sigma Chemical, St. Louis, MO) as a standard. Creatinine levels in serum and urine samples were measured using an autoanalyzer (Beckman Instruments, Fullerton, CA). Glomerular filtration rate was calculated using the formula creatinine clearance (Cs; ml/min) = [(U[Cr] × urine volume)/(P[Cr] × time)], where U[Cr] is urinary creatinine (mg/dl), urine volume is in milliliters, and P[Cr] is plasma creatinine (mg/dl).

Routine histopathology. Formalin-fixed graft tissues were paraffin embedded, cut into 5-μm sections, and stained with hematoxilin and eosin, periodic acid-Schiff, and Masson’s trichrome. Sections were blindly reviewed by a pathologist (M. A. Nalesnik) without the knowledge of treatment groups. Severity of histopathological changes in the allograft was graded according to the Banff criteria as mononuclear cell interstitial inflammation, tubulitis, intimal arteritis, glomerulitis, interstitial fibrosis, and tubulatrophathy (42, 48).

Immunofluorescence staining. Kidneys were immersion fixed with 2% paraformaldehyde in PBS, cryoprotected in 2.3 M sucrose in PBS overnight, frozen in liquid nitrogen-cooled isopentane, and 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 blocking with 20% (vol/vol) normal goat serum in PBG, the tissues were incubated with each primary antibody for 2 h and then exposed to the secondary for 1 h. Hoechst dye (bisBenzimide, 1 μg/ml) was used for 30 s to stain nuclear DNA. The sections were washed and coverslipped with Gelvatol, a water-soluble mounting media (23 g of polyvinyl alcohol, 50 ml of glycerol, 0.01% sodium azide in 100 ml of PBS), visualized with an Olympus BX51 epifluorescence microscope, and digitized with an Olympus color video camera.

Measurement of kidney graft cortical blood flow and arterial blood pressure. Renal cortical tissue blood flow was measured using a laser Doppler flowmeter (BLF 21D, Transonic Systems, Ithaca, NY) at 30 days after KTx. Probe was placed on the upper, middle, and lower surface areas of the kidney graft, and three separate measurements were obtained in each location (total of 9 measurements per animal) by one of the authors (A. Nakao) without knowledge of the experimental groups. Blood flows of the recipient abdominal aorta and graft renal artery were also measured.

For systemic blood pressure monitoring, a polyethylene catheter (PE-50, Becton Dickenson, Sparks, MD) was introduced into the right

Table 1. Primers used in real-time PCR

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primers</th>
<th>Reverse Primers</th>
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<tbody>
<tr>
<td>RANTES</td>
<td>5′-GCTTGTGGCTACATCTCTGCCT-3′</td>
<td>5′-GCACAGATTGGGCTCCTTT-3′</td>
</tr>
<tr>
<td>IP-10</td>
<td>5′-AGAGGCCTGGTACAATGAAC-3′</td>
<td>5′-TTAGGGCGCTCCCTCTGAGC-3′</td>
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<tr>
<td>MIP-1α</td>
<td>5′-ATATGGAGCTGAGCTAGCCCTGAC-3′</td>
<td>5′-CCAGTCGTAATGTAATCTTCTTGA-3′</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5′-GTTGGTCGCCACATCTACG-3′</td>
<td>5′-TGTGACTGATTTGACATCAAG-3′</td>
</tr>
<tr>
<td>CCR1</td>
<td>5′-AGAACTCTACCAAAAGAAGGAC-3′</td>
<td>5′-CTGGCTCTCAAGTATTTTCGTGTTAC-3′</td>
</tr>
<tr>
<td>CCR3</td>
<td>5′-ATTTCTTGCTGTCGCCAGTGTAG-3′</td>
<td>5′-AGGTTGCCATTCTGTGACCAACG-3′</td>
</tr>
<tr>
<td>CXC3R5</td>
<td>5′-GGGGAATTTCTTCGACCAGACAA-3′</td>
<td>5′-CTGGCTCTCAAGTATTTTCGTGTTAC-3′</td>
</tr>
<tr>
<td>CCR7</td>
<td>5′-ACAAGGCGTATCCTCTGATCA-3′</td>
<td>5′-TGTGACTGATTTGACATCAAG-3′</td>
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<tr>
<td>INF-γ</td>
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<td>IL-2</td>
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<td>IL-10</td>
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<td>5′-CTGGCTCTCAAGTATTTTCGTGTTAC-3′</td>
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<td>GAPDH</td>
<td>5′-ATGCCGAGACAATGAGTCTAGCAGA-3′</td>
<td>5′-CCCTGACCTGAGCTAGCTGTTAG-3′</td>
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IP, INF-γ inducible protein; RANTES, regulated on activation normal T cell expressed and secreted; MIP, macrophage inflammatory protein.
femoral artery, and arterial pressure was measured with a pressure transducer (model 90603, SpaceLab, Redmond, WA).

**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 ultraviolet spectrophotometry.

mRNA expression was quantified by SYBR Green two-step, real-time RT-PCR for the chemokines [regulated on activation normal T cell expressed and secreted (RANTES), IFN-γ, IL-2, IL-10], interleukin adhesion molecule-1, and GAPDH. Total mRNA pellets were suspended in RNase-free water, followed by removal of potentially contaminating DNA with DNase I. One microgram of total mRNA from each sample was used for reverse transcription with an oligo (dT) and a Superscript II (all from Life Technologies) to generate first-strand cDNA. PCR reaction mixture was prepared using Gold DNA polymerase, followed by 40 cycles of 95°C for 1 min 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 ΔRn fluorescence signal vs. the cycle number. The threshold cycle was defined as the cycle number at which the ΔRn crosses this threshold. The expression of each gene was normalized to GAPDH mRNA content and calculated relative to control using the comparative cycle threshold method (29, 33).

**Protein extraction and immunoblotting.** Protein was extracted from kidney grafts after homogenization in 0.25 M sucrose at 4°C using a Polytron (Janke & Kunken KG IGA Werk, Staufen, Germany). Protein samples (150 μg) were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. CCR1 protein expression was determined with primary goat anti-CCR1 polyclonal antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), followed by second-ary donkey anti-goat IgG antibody conjugated with horseradish peroxidase (1:10,000, Santa Cruz Biotechnology). Membranes were washed and subjected to chemiluminescence Western blotting (Pierce Chemical). Signal intensities were calculated by densitometric analysis using NIH image.

**Mixed leukocyte reaction.** In vitro alloreactivity was tested in mixed leukocyte reaction (28). TriPLICATE cultures of responder lymph node cells (1.75 × 10^5 cells/well) and of irradiated (2,000 rad) stimulator lymph node cells (3 × 10^5 cells/well) obtained from normal LEW, BN, and ACI rats were placed in round-bottom 96-well plates in a final volume of 0.2 ml RPMI supplemented with 25 mM HEPES buffer, 5 × 10^-5 M 2-mercaptoethanol, 2 mM L-glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml), N2E-monomethyl-L-arginine-HOAc (NMA, Cyclo-pss Biochemical, State Lake, UT), and 10% heat-inactivated normal rat serum. Cultures were incubated in a humidified atmosphere of 5% CO2 for 4 days at 37°C. [3H]thymidine (1 μCi) was added to each well 16 h before termination of cultures. Cultures were harvested, and [3H]thymidine incorporation was determined in a liquid scintillation.

**IFN-γ enzyme-linked immunospot assay.** IFN-γ enzyme-linked immunospot (ELISPOT) assay was conducted using a commercially available kit (R&D System) according to manufacturer’s protocol with some modifications (3). ELISPOT plates (Millipore, Bedford, MA) were coated with the capture antibody (Ab) against rat IFN-γ. Lymph node responder cells (5 × 10^5 cells/well) were prestimulated for 24 h with irradiated (2,000 rad) donor (LEW) and third-party (ACI) stimulator cells (5 × 10^5 cells/well) in 0.2 ml of complete RPMI media containing 10% rat serum in a round-bottom well plate. Prestimulated cells were then transferred to antibody-coated ELISPOT plates and incubated with or without stimulator cells for 5 h at 37°C, in 5% CO2. Next, media were aspirated and 0.2 ml of ice-cold deionized water was added into the wells, followed by incubation for 10 min on melting ice. After washing, biotinylated cytokine-detecting antibodies were added overnight. Labeled anti-biotin antibody (1 h at 37°C in 5% CO2) was used as third reagent. After washing, the plates were developed with manufacturer-provided activator solution. The resulting spots were counted on an immunospot image analyzer (ImmuNoSpot, Cellular Technology).

**Assay of the indirect allosresponse.** Indirect allosresponse was studied using soluble membrane antigens, according to Baker et al. (5), with some modifications (3). Soluble membrane antigens were prepared from splenocytes of BN (recipient), LEW (donor), and ACI (third-party) rat strains. After erythrocyte lysis, isolated splenocytes were suspended on 1 × 10^7 cells/ml in HBSS, sonicated with 10 pulses of 1 s each on ice, frozen in a dry ice/ethanol bath, and then thawed at room temperature. The residual intact cells or cell debris were removed by centrifugation at 1,300 rpm for 10 min (6). Quadruplicate cultures of unfractionated responder splenocytes (5 × 10^5 cells/well) were set up in complete RPMI media in 96-well round-bottom plates with different dilutions of soluble membrane antigens. [3H]thymidine (1 μCi) was added to each well 16 h before the termination of the 5-day cultures. [3H]thymidine incorporation was determined in a liquid scintillation counter.

**Allantibody detection.** Naive LEW (donor) thymocytes (1 × 10^5) were incubated with the recipient serum sample for 40 min at 4°C. Cells were washed three times and then incubated for an additional 40 min with dichlorotrizinyl amino fluorescein (DTAF)-conjugated af-

Fig. 1. Renal graft function of creatinine clearance (Cc; A) and urinary protein excretion (B). A: isograft recipients with (n = 3) and without CO (n = 5) maintained normal Cc levels throughout the study period. Air-treated control animals (n = 8) showed progressive deterioration of renal allograft function, and Cc declined to ~10% of normal value by 80 days after transplantation. Recipients treated with CO (n = 8) had significantly improved graft function than control animals at all time points with nearly normal Cc levels. B: isograft recipients showed minimal proteinuria during the study period (~2 mg/24 h) of 80 days. Air control recipients started to show proteinuria at 60 days. By 80 days, urine protein amounted exceeded 400 mg/24 h. In contrast, CO-treated recipients showed minimal urinary protein. *P < 0.05 (air control vs. CO-treated recipients).
finity pure F(ab′)² fragment goat anti-rat IgG (Accurate Chemical Scientific, Westbury, NY). The samples were fixed in paraformaldehyde and analyzed on a Counter Elite ESP.

Monoclonal and polyclonal antibodies. Fluorochrome-conjugated or unconjugated Abs used in this study included R7.3 (αβTCR, 1:300, Pharmingen, San Diego, CA), goat anti-rat IgM (B cells, 1:250, Pharmingen), rabbit anti-rat NPHN (Nephrin; 1:150, Alpha Diagnostic International, San Antonio, TX), rabbit anti-human TGF-β1 (1:100, Santa Cruz Biotechnology), goat-anti human CCR1 (Santa Cruz Biotechnology), and rabbit anti-mouse collagen IV (1:1,000, Biodesign International, Saco, ME). For the secondary antibodies, Alexa 488-conjugated (Molecular Probes, Eugene, OR) goat anti-mouse IgG, Alexa 488-conjugated goat anti-rabbit IgG, and Cy3-conjugated goat anti-rabbit IgG (Molecular Probes) were used.

Data analysis. Results are expressed as means ± SD. Statistical analysis was performed using Student’s t-test or one-way ANOVA and Fisher’s protected least significant difference test where appropriate. A probability level of $P < 0.05$ was considered statistically significant.

### Table 2. Histopathological changes in kidney allografts

<table>
<thead>
<tr>
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<th>30 Days</th>
<th>80 Days</th>
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<tr>
<td></td>
<td>Isograft</td>
<td>Allograft (Air)</td>
</tr>
<tr>
<td></td>
<td>n = 5</td>
<td>n = 5</td>
</tr>
<tr>
<td>Mononuclear infiltrate</td>
<td>0.3±0.3</td>
<td>2.3±0.5</td>
</tr>
<tr>
<td>Tubulitis</td>
<td>0±0</td>
<td>3.0±0</td>
</tr>
<tr>
<td>Intimal arteritis</td>
<td>0±0</td>
<td>0.6±1.3</td>
</tr>
<tr>
<td>Glomerulitis</td>
<td>0.1±0.3</td>
<td>2.0±1.2</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>0±0</td>
<td>0.3±0.5</td>
</tr>
<tr>
<td>Tubular atrophy</td>
<td>0±0</td>
<td>1.3±0.5</td>
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</table>

Grades are according to Banff criteria (Refs. 42 and 48). *$P < 0.05$ vs. air-treated allograft.

![Fig. 2. Histopathological changes in kidney grafts at 30 days (A and B) and 80 days (C–F) after transplantation. A: at 30 days, air-treated control grafts had prominent changes of interstitial mononuclear cell infiltration, tubulitis, and glomerulitis (inset). Some tubular atrophy and occasional vascu-litis were also noted. B: CO-treated grafts showed less severe changes compared with control allografts. C: at 80 days, air-treated allografts showed changes of chronic allograft nephropathy, including interstitial fibrosis and tubular atrophy (arrows). Active inflammation was also seen, and prominent glomerulopathy with segmental necrosis was observed (inset). D: CO-treated allografts showed significantly reduced features of chronic allograft nephropathy and did not show necrotizing glomerulopathy (inset). Control isografts showed essentially normal histology at 30 and 80 days (not shown). Original magnification, ×20 (×40 for insets). E: control allografts showed cortical fibrosis up to 25% of the kidney parenchyma. F: CO-treated grafts lacked fibrous changes. Original magnification: ×200. A–D: hematoxilin and eosin stain. E and F: Masson’s trichrome stain.](http://ajprenal.physiology.org/)}
RESULTS

Renal allograft function and general animal condition. At 30, 60, and 80 days after transplantation, Ccr and proteinuria were assessed in all groups. In normal unoperated LEW, Ccr was 1.6 ± 0.3 ml/min (Fig. 1A). Isograft recipients with and without inhaled CO treatment maintained Ccr at 1.2–2.2 ml/min at all time points in this study. Air-treated control recipients showed a continuous decrease of glomerular filtration rate, and Ccr declined to 0.16 ± 0.12 ml/min at 80 days posttransplantation. Although these air control allograft recipients survived for 80 days after KTx, they developed pleural effusion and started to lose body weight by this time. In contrast, CO-treated recipients maintained significantly higher Ccr levels (1–1.5 ml/min) than air-treated control recipients.

Proteinuria was scarce (<5 mg/24 h) or absent in unoperated normal rats or isograft recipients, with or without CO treatment (Fig. 1B). Air-treated control allografts started to excrete urinary protein at 60 days, and the amount progressively increased and reached >400 mg/24 h at 80 days posttransplantation. In contrast, CO-treated allograft recipients showed minimal levels of proteinuria (2.0 ± 0.7 mg/24 h) at all time points after transplantation.

CO-treated animals showed normal behavior and displayed no particular gross alterations throughout the experiments. All allograft recipients steadily increased body weight for the first 60 days after kidney transplant surgery. Thereafter, the CO-treated group continued to increase weight, whereas body weight gain in the air control group was held due to the impairment of renal function. Mean body weight gains in the CO- and air-treated groups at 60 days were 23.4 ± 10.1 and 25.6 ± 18.8%, respectively, and they were 38.2 ± 4.3 and 24.7 ± 21.0% at 80 days (n = 8 for each group). The mean arterial blood pressure was 100 ± 4.2 mmHg (air-treated control) and 92.5 ± 7.8 mmHg (CO-treated) at 80 days after allotransplantation. These values were not different from those of naive animals (98.2 ± 4.1 mmHg).

Routine histopathology. Isografts maintained a normal cortical architecture throughout the study period (Table 2). At 30 days after transplantation, air-treated control allografts showed interstitial mononuclear cell infiltration, tubulitis, and glomerular changes. At 80 days, air control grafts showed interstitial collagen accumulation, tubular atrophy, and glomerulosclerosis. In contrast, CO-treated grafts showed minimal interstitial changes and preserved glomerular integrity.

Fig. 3. Immunofluorescent staining of kidney allografts at 80 days for collagen type IV (A–C), TGF-β1 (D–F), and nephrin (G–I). A: isografts showed faint interstitial expression of collagen type IV. B: air control allografts had diffuse collagen IV staining throughout the graft interstitium. C: CO-treated allografts showed limited collagen IV staining. D: isografts did not express TGF-β1. E: there was a marked TGF-β1 upregulation in air control allografts. Staining was localized on the tubular epithelial cells (arrows). F: in CO-treated grafts, tubular epithelial cells were negative for TGF-β1. H: podocytes in isografts were positively stained for nephrin, as seen in normal kidneys. In control allografts, nephrin expression on podocytes was significantly reduced. I: glomerular architecture was preserved with normal nephrin staining in CO-treated allografts. Original magnification: A–F, ×200; G–I, ×400; n = 8 animals per group. G, glomerulus.
ulitis, in addition to early phase of arteritis (Table 2; Fig. 2A). By 80 days, the severity of histopathological changes in air-treated allografts progressively increased with persisting interstitial mononuclear cell infiltration, glomerulitis, and arteritis. Additionally, these allografts had chronic changes, and tubular atrophy was seen in 25–50% and interstitial fibrosis up to 25% of the renal cortex (Table 2; Fig. 2C, arrows). All air-control allografts showed focal, diffuse segmental or global glomerulonecrosis (Fig. 2C, inset).

CO treatment had little effect on infiltrates, tubulitis, and glomerulitis (Table 2; Fig. 2, B and D). However, the development of chronic changes was statistically significantly inhibited with CO; kidney allografts were free from interstitial fibrosis and had minimal tubular atrophy (<13%) (Table 2; Fig. 2D). CO-treated animals had less severe glomerular necrosis than controls (Fig. 2D, inset). Masson’s trichrome staining showed a pattern of parenchymal fibrosis in air-treated kidney allografts (Fig. 2E). CO-treated grafts as well as isografts did not develop interstitial fibrosis (Table 2; Fig. 2F).

Renal allograft fibrosis and glomerular damage. Severity of fibrosis and glomerular damages were evaluated in kidney allografts at 80 days with immunohistostaining for collagen IV, TGF-β1, and nephrin. Air-treated control allografts showed intense collagen IV staining throughout the graft interstitium (Fig. 3B). In contrast, CO-treated allografts had minimal collagen IV stain as seen in control allografts (Fig. 3, A and C). TGF-β1, a key profibrotic growth factor, was not seen in isografts (Fig. 3D). There was a distinctive TGF-β1 expression in the tubular epithelial cells throughout the renal cortex of air-treated control allografts (Fig. 3E). In CO-treated grafts, however, tubular epithelial cells were negative for TGF-β1 (Fig. 3F). Nephrin was seen on podocytes in normal kidney and isografts (Fig. 3G). In air-treated controls, nephrin stain was significantly reduced, indicating an impairment of podocyte viability and existence of glomerulonecrosis seen in routine histopathology (Fig. 3H). In contrast, CO-treated allografts showed nearly normal nephrin stain on podocytes, suggesting well-preserved glomerular architecture with CO inhalation (Fig. 3I).

Molecular analysis of cytokines and adhesion molecules. In air-treated control allografts, mRNA levels for Th1-type cytokines (IL-2, IFN-γ) markedly increased at 30 days after transplantation and subsided by 80 days (Fig. 4). CO treatment inhibited mRNA upregulation for IL-2 at 30 days. Marginal upregulation of intercellular adhesion molecule-1 mRNA in air-treated allografts was continuously seen for 80 days after transplantation. CO treatment had little effects in inhibiting intercellular adhesion molecule-1 expression. mRNA for IL-10 was upregulated in both groups with higher levels at 30 than at 80 days. Isografts with and without CO treatment (20 ppm for 30 days) showed baseline mRNA levels (0.5- to 1.5-fold increases compared with normal kidney) for these cytokines both with and without CO treatment. CO-treated isograft samples were analyzed for IFN-γ and IL-2. N = 5 for air-treated isografts and air- and CO-treated allografts at day 30 (d30). N = 8 for air-treated isografts and air- and CO-treated allografts at day 80 (d80). N = 3 for CO-treated isografts at d30 and d80. *P < 0.05 (air control vs. CO-treated allografts).

Analyses of chemokines and chemokine receptors. Analysis of chemokines (RANTES, IP-10, MIP-1α) and chemokine receptors (CCR1, CCR3, CCR5, CCR7, CXCR3, CXCR5) revealed a similar tendency as seen in cytokine analysis (Fig. 5, A and B). In air-treated controls, mRNA upregulation for chemokines/chemokine receptors was more prominent at 30 than 80 days, except for CCR1, which showed a continuous increase after transplantation. CO-treated animals tended to show lower mRNA levels for these chemokines/chemokine receptors than controls at 30 days; and statistical significance was achieved for RANTES, MIP-1α, CCR1, and CXCR3. At 80 days, mRNA levels were similar between air- and CO-treated grafts. Interestingly, however, CCR1 mRNA was continuously upregulated in air-treated animals, and CO treatment significantly inhibited CCR1 upregulation after transplantation. In correlation with mRNA results, CCR1 protein expression in kidney allografts was significantly inhibited with CO treatment at 80 days (Fig. 6). Isografts with and without CO treatment showed baseline mRNA levels (~1.5-fold increases compared with normal kidney) for these chemokines and their receptors.

In vitro cellular responses in direct and indirect mixed leukocyte reaction and ELISPOT. In one-way mixed leukocyte reaction, recipient lymphocytes obtained from CO-treated and air control recipients at 80 days showed significantly reduced anti-donor proliferation compared with naive BN rats, whereas proliferation in response to third-party cells was similar in all groups (Fig. 7A). Frequencies of IFN-γ-producing cells in response to donor cells in ELISPOT were slightly less in allograft recipients than in naive BN, but no statistical difference was observed between groups (Fig. 7B).
Recipient lymphocyte proliferation via the indirect allore cognition pathway was enhanced in both groups after KTx. Air-treated recipients showed significantly increased proliferation with donor peptides compared with CO-treated recipients (Fig. 7C).

**Alloantibody development.** In air-treated control recipients, anti-donor IgG antibodies already increased at 30 days. The antibody binding to donor antigens increased with time, and there were significantly higher anti-donor IgG antibodies at 80 days. In contrast, CO-treated recipients showed a marginal increase of anti-donor antibodies (Fig. 7D).

**Blood flow in the kidney grafts.** CO plays a role in regulating vasomotor tone by promoting vasorelaxation through soluble guanylyl cyclase activation (15, 45). The effect of CO inhalation on renal graft blood flow was analyzed using a laser Doppler flowmeter. Blood flows in the recipient aorta were not different among the three groups and were 100–125 laser Doppler units (LDU). Renal arterial flow was not statistically different among groups; however, there was a tendency of reduction in the air-treated control group compared with the CO-treated group (air, 79.0 ± 3.6 LDU vs. CO, 85.3 ± 1.0 LDU).

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**Fig. 5.** mRNA levels for chemokines (A) and chemokine receptors (B). A: increases of mRNA levels for regulated on activation normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein (MIP)-1α in control allografts at 30 days were significantly inhibited with CO. At 80 days, mRNA levels in both air control and CO-treated allografts declined, and there was no significant difference. CO did not affect upregulation of IFN-γ inducible protein (IP)-10 in control grafts. B: chemokine receptors CCR1, CCR3, CCR5, CCR7, CXCR3, and CXCR5 were all upregulated in air control allografts after transplantation, and CO tended to inhibit upregulation. mRNA levels of all chemokine receptors, except for CCR1, were higher at 30 than 80 days. CCR1 mRNA continued to increase with time, and CO significantly inhibited the upregulation at 30 and 80 days. Both air- and CO-treated isografts showed baseline mRNA levels (~1.5-fold increases compared with normal kidney). CO-treated isografts were analyzed for RANTES, CCR-1, and CXCR3. Same numbers of samples were used as in Fig. 4. *P < 0.05 (air control vs. CO-treated allografts).

**Fig. 6.** Western blot for CCR1. In correlation with CCR1 mRNA levels, CCR1 protein levels significantly increased in air control allografts. CO inhibited intragraft CCR1 expression at 80 days. *P < 0.01 (air control vs. CO-treated allografts).
The renal cortical blood flow in normal unoperated animals was 63.2 ± 4.1 LDU. At 30 days after KTx, renal cortical blood flow in isograft was 62.0 ± 5.5 LDU. Graft blood flow of air-treated control allografts significantly decreased to 44.3 ± 8.6 LDU at 30 days after transplantation. In contrast, CO-treated kidney allografts maintained graft blood circulation with a normal flow rate of 66.4 ± 5.2 LUD (Fig. 8).

**DISCUSSION**

Upregulation of the HO system has been shown to provide cytoprotection in a variety of experimental models of tissue injury, most likely via the enzymatic catalysis of heme and generation of byproducts that are believed to exert actual protection. Allograft injury caused by alloimmune responses is also ameliorated with the modification of the HO system. Several studies have shown that HO-1 overexpression with viral gene transfer (7, 9, 25, 55), injection of cobalt-protoporphyrin (56, 57), or addition of byproduct CO (12, 36, 49) or biliverdin (60) improves survival of a variety of allografts. Furthermore, heart allograft survival is prolonged in transgenic mice overexpressing HO-1 (2). Using HO-1 knockout mice, upregulation of HO-1 on the graft vascular endothelial cells is shown to be a survival factor for xenografts in the mouse-to-rat heart transplant model (46, 47). These studies support the beneficial effects of HO-1 pathway activation in promoting allograft and xenograft survival.

Using the rodent kidney allotransplantation model, the present study demonstrates that inhaled CO provides protection against CAN development. Exogenous low-dose CO gas (20 ppm) significantly improves renal allograft function, reduces the severity of histopathological changes, and downregulates the activation of intragraft Th1-type cytokines and chemokines/chemokine receptors. CO-treated recipients also demonstrate reduced in vitro proliferative responses against donor peptides via the indirect allorecognition pathway of allograft peptides increased in air control animals compared with naive animals and CO-treated recipients. CO-treated recipients showed marginally increased anti-donor proliferation. *P < 0.05 vs. air control. D: development of anti-donor IgG antibodies in air controls was inhibited with CO. MFI, mean fluorescence intensity.

Several in vitro studies have shown that CO is capable of directly inhibiting T cell proliferation and IL-2 secretion (37, 51). We also have observed that CO suppresses anti-CD3 MAb-stimulated mouse T cell proliferation and Th1-type cytokine production without affecting T cell viability (unpublished observations). Anti-T cell proliferative effect of CO is mediated via the selective inhibition of ERK-MAPK pathway in human CD4+ T cells (37). Using p21 knockout mouse T cells, CO is also shown to inhibit T cell proliferation via the expression of p21Cip1 and inhibition of caspase activity (51). A recent study by Kapturczak et al. (21) shows that HO-1 knockout mice generate high levels of proinflammatory Th1-type cytokines (e.g., IL-1, IFN-γ), suggesting a potential role for HO-1 in modulating T cell responses. CO activates HO-1 on endothelial cells, which in turn produces CO, thus creating a feedback loop that amplifies the protective effects of HO-1. This mechanism is also demonstrated by the increased expression of HO-1 in CO-treated grafts compared with air controls. CO provides protection against CAN development and reduces anti-donor IgG antibodies in air controls.
IFN-γ, TNF-α, IL-6) in response to mitogen stimulation. These studies stress the active role of the HO-CO system in immune responses, and CO in this study, in part, might function by directly downregulating alloreactive immune responses. In addition, CO is well known to exert anti-inflammatory, anti-apoptotic, and vasodilative activities (44, 53). These cytoprotective actions could be involved as well in this study in reducing allograft inflammation and leukocyte recruitment.

The most striking finding in the study, however, is the lack of chronic histopathological changes, such as interstitial fibrosis and tubular atrophy, in kidney allografts of the CO-treated group. CAN has features of chronic inflammation, tissue repair, and uncontrolled tissue remodeling, involving increased matrix deposition and fibroblasts as the final common mediator. CO might be merely delaying the chronic stage of these degenerative changes. However, in our ongoing experiments, late CO exposure for 30–100 days is effective in ameliorating CAN, suggesting that CO treatment may have beneficial effects in inhibiting CAN development.

In fact, the HO-CO system has been shown to be involved in cell growth regulation by positively and negatively controlling cell cycle progression of a variety of cell types. The HO-CO system stimulates the proliferation of epidermal keratinocytes and vascular endothelial cells (11, 13), while it exerts a potent antiproliferative effect in vascular and airway smooth muscle cells (27, 39, 50, 54) via the regulation of p21. These opposing effects of the HO-CO system, depending on cell types and stress status, may suggest that the HO-CO system has critical roles in tissue protection and repair. Further study will be required to explore the roles of the HO system and its byproducts in protecting vascular endothelial cells and renal tubular cells during steady and stressed conditions.

The patient with HO-1 deficiency and HO-1 knockout mice have been reported to develop advanced tubulointerstitial injury, in addition to systemic vascular endothelial cell injury due to progressive inflammatory reactions (40, 59). Renal injury associated with HO-1-deficient status includes tubular dilatation and/or atrophy, interstitial fibrosis, and inflammatory cell infiltration (24, 59), suggesting that renal tubules receive protection against oxidative injury with the HO system (61). Induction of HO-1 on cultured tubular epithelial cells is shown to provoke cell cycle arrest, inhibit cell growth, and confer resistance to apoptosis (1). It is tempting to speculate that the HO-CO system protects tubular epithelial cells from excess proliferation by controlling cell cycle progression and prevents tissue remodeling by forestalling the exhaustion of their progenitor compartment.

In the present study, we used a laser Doppler flowmeter for the assessment of renal graft blood flow. This method uses a low-intensity beam of monochromatic light and measures the shift in frequency between the emitted light and the reflected signal spectrum from the illuminated tissue. Because the wavelength shifts are mostly imparted by moving red blood cells in the tissue, the measured values are believed to correspond to the velocity of the moving red blood cells. The hematocrit of the blood in this study averaged 27–29% in normal as well as air- and CO-treated recipients, and we estimate, based on the sampled tissue size (1 mm³) with a laser Doppler flowmeter, that CO treatment in this study increased renal allograft blood flows to the nearly normal levels of 66.4 ± 5.2 from 44.3 ± 8.6 LDU (ml·min⁻¹·100 g wt⁻¹) in air-treated grafts. Although these values are relevant to those in the previous studies using the same method (16, 43), these estimates of renal flow are nearly 10-fold lower than values determined by other techniques (e.g., microsphere technique, thermodilution) (18, 26, 62).

The discrepancy regarding the estimates of the absolute value of renal blood flow (in ml·min⁻¹·g wt⁻¹) between the laser Doppler flowmeter in this study and other techniques may result from the small sampled area, tissues types (difference in volume fraction of erythrocytes), and heterogeneity of tissue perfusion. Several previous studies have examined whether the laser Doppler flowmeter could still be useful to assess blood flow in vivo, by correlating laser Doppler blood flow results to other methods. These studies found that the laser Doppler flow signal was linearly related and highly correlated to capillary blood flow estimates (43). Thus, although the discrepancy concerning the absolute value of renal graft blood flow measured with laser Doppler flowmeter and other techniques will require further investigation, the use of laser Doppler flowmeter for relative measurement appears to be reliable. Regardless of the absolute value of renal graft blood flow, the results in this study showed that CO inhalation resulted in increased renal allograft blood flow.

In summary, the present rodent study demonstrates that the treatment of renal allograft recipients with low-dose CO inhalation prevents the development of chronic fibroinflammatory changes associated with CAN and improves long-term renal allograft function. Although the determination of exact mechanisms and efficiency of the protective actions of inhaled CO awaits further intensive investigation, potential therapeutic application of inhaled CO in this study will serve for a better understanding to ameliorate CAN, which is one of the major problems in current clinical renal transplantation.

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REFERENCES

5. Baker RJ, Hernandez-Fuentes MP, Brookes PA, Chaudhry AN, Cook HT, and Lechler RI. Loss of direct and maintenance of indirect allore-


