Small intestine transplantation (SITx) necessitates ex-trinsic denervation, surgical manipulation, preservation, and ischaemia/reperfusion of the graft, all of which potentially contribute to intestinal dysfunction and paralytic ileus. As early transplantation induced intestinal dysfunction is an important cause of endotoxaemia, the systemic inflammatory response syndrome, and multiorgan failure, these sequelae of transplantation compromise the host and increase the susceptibility of the graft to secondary failure.

Haeme oxygenase 1 (HO-1) has been shown to provide protection against oxidative stress via degradation products of haeme. The specific mechanism(s) by which HO-1 can mediate these cytoprotective functions are only now beginning to be elucidated but appear to be related to HO-1 catalysis of haeme, which produces carbon monoxide (CO), iron, and bilirubin. Although CO is known to be toxic at high concentrations due to its ability to interfere with oxygen delivery, low CO concentrations provide cytoprotection due to its anti-inflammatory properties. In this study, we examined the ability of exogenous CO to blunt isograft muscularis inflammation and prevent graft motility.

Materials and Methods

Experimental groups

Inbred male LEW (RT.1) rats weighing 200–300 g were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana, USA) and maintained in a laminar flow animal facility at the University of Pittsburgh under a standard diet and water supplied ad libitum. Specific experimental groups of animals were exposed to CO at a concentration of 250 ppm. Briefly, 1% CO in air was mixed with air (21% oxygen) in a stainless steel mixing cylinder and then directed into a 3.70 ft glass exposure chamber at a flow rate of 12 l/min. A CO analyser (Interscan, Chatsworth, California, USA) was used to measure CO levels continuously in the chamber. CO concentrations were maintained at 250 ppm at all times. Animals were supplied food and water ad libitum during the experiments.

Four groups of 4–6 animals each were constructed for this study, two of which received a small intestinal transplant: (1) unoperated control rats, (2) unoperated controls exposed to CO for 24 hours, (3) recipients receiving SITx that were exposed to inhaled room air, and (4) recipients receiving a SITx graft which were exposed to CO (250 ppm) for one hour before transplantation and then again for a period of 24 hours following surgery. SITx with caval drainage was performed using a previously described procedure.

Functional studies

The effect of CO treatment on intestinal motility in controls and transplanted grafts was assessed both in vivo and in vitro.
the time point at which transplant induced in vitro dysmotility occurred, interferon γ, IL-6, IL-10, tumour necrosis factor α and β cytokines (interleukin (IL)-1, IL-2, IL-3, IL-4, IL-5, IL-16), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). Protected probes were loaded onto a 40% polyacrylamide gel electrophoresis and autoradiography was performed using a PhosphorImager system (Molecular Dynamics, Krefeld, Germany). Quantification of radioactivity of mRNA bands was done with NIH Image, normalised to GAPDH, and expressed as a ratio of cytokine/GAPDH (n=3-4).

**SYBR green real time RT-PCR**

The effects of CO inhalation on transplant induced proinflammatory and anti-inflammatory gene expression was assessed in muscularis extracts by reverse transcriptase- polymerase chain reaction (RT-PCR) (n=4-6 each group). Muscularis externa was collected from control intestine and transplanted grafts four hours following reperfusion, and snap frozen in liquid nitrogen. This time point falls within the range of maximum inflammatory mediator expression that occurs between three and six hours following reperfusion, based on RPA results. The extracted RNA pellets were resuspended in RNA secure resuspension solution (Ambion Inc., Austin, Texas, USA) and aliquots of 5 µg of total RNA from each sample were quantified by spectrophotometry (wavelength 250 nm) and aliquoted at a concentration of 40 ng/µl. Primers were taken from the literature or designed according to published sequences (table 1).

**Molecular biological studies**

**RNAse protection assay (RPA)**

To investigate the sequential analysis of cytokine mRNA expression in the graft, RNAse protection assays (RPAs) were performed with the Riboquant kit (Pharmingen) according to the manufacturer's protocol. A jejunal segment of the graft (Parhamingen, San Diego, California, USA). Protected probes were loaded onto a 40% polyacrylamide gel electrophoresis and autoradiography was performed using a PhosphorImager system (Molecular Dynamics, Krefeld, Germany). Quantification of radioactivity of mRNA bands was done with NIH Image, normalised to GAPDH, and expressed as a ratio of cytokine/GAPDH (n=3-4).

**SYBR green real time RT-PCR**

The effects of CO inhalation on transplant induced proinflammatory and anti-inflammatory gene expression was assessed in muscularis extracts by reverse transcriptase-polymerase chain reaction (RT-PCR) (n=4-6 each group). Muscularis externa was collected from control intestine and transplanted grafts four hours following reperfusion, and snap frozen in liquid nitrogen. This time point falls within the range of maximum inflammatory mediator expression that occurs between three and six hours following reperfusion, based on RPA results. The extracted RNA pellets were resuspended in RNA secure resuspension solution (Ambion Inc., Austin, Texas, USA) and aliquots of 5 µg of total RNA from each sample were quantified by spectrophotometry (wavelength 250 nm) and aliquoted at a concentration of 40 ng/µl. Primers were taken from the literature or designed according to published sequences (table 1). Peak mRNA expression was quantified in duplicate by SYBR green two step real time RT-PCR and quantification of mRNA expression was normalised to GAPDH and calculated relative to control using the comparative CT method.16,25

**HO-1 analysis**

Northern blot analysis was performed as previously described. Briefly, 10 µg of total RNA extracted from the tissue, as described above, was electrophoresed on a 1% agarose gel and transferred to nylon membranes by capillary action. Nylon membranes were then prehybridised in buffer (1% bovine serum albumin (BSA), 7% sodium dodecyl sulphate (SDS), 0.5 M PO4 buffer, pH 7.0, and 1 mM EDTA) at 65°C for two hours followed by hybridisation with 32P labelled rat HO-1 cDNA at 65°C for 24 hours. Nylon membranes were then washed in buffer A (0.5% BSA, 5% SDS, 40 mM PO4 buffer, pH 7.0, and 1 mM EDTA) for 15 minutes, three times, at 65°C. Following washes in buffer B (1% SDS, 40 mM PO4 buffer, pH 7.0, and 1 mM EDTA) for 15 minutes, three times, at 65°C.

**HO-1 cDNA probe**

A full length rat cDNA (pHO1) was generously provided by Dr S Shibahara of Tohoku University (Sendai, Japan). pHO1 was

<table>
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<th>Table 1</th>
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GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COX-2, cyclooxygenase 2; iNOS, inducible nitric oxide; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; TNF-α, tumour necrosis factor α;
Figure 1 Transit histograms for distribution of non-absorbable FITC labelled dextran along the gastrointestinal tract two hours after oral administration. In unoperated animals and control animals exposed to carbon monoxide (CO), most of the fluorescent marker accumulated in the last two segments of the small bowel (sb6, 7, and 8), which was similar to unoperated animals. The calculated transit geometric centre measurements summarised in (C) demonstrated that CO inhalation significantly improved gastrointestinal transit after SITx. In contrast, small intestinal transplantation (SITx) resulted in a significant delay in the transit of the fluorescence (B). In unoperated control intestine exhibited robust spontaneous activity (A, B). Small intestinal transplantation (SITx) resulted in a significant decrease in spontaneous circular muscle contraction (C). However, CO treatment demonstrated significantly greater spontaneous contractile activity compared with untreated transplants.

Figure 2 Representative traces of spontaneous jejunal circular muscle contractility are shown. Circular muscle strips from unoperated control intestine and carbon monoxide (CO) treated control intestine exhibited robust spontaneous activity (A, B). Small intestinal transplantation (SITx) resulted in a significant decrease in spontaneous circular muscle contraction (C). In contrast, CO inhalation significantly improved gastrointestinal transit after SITx. Data represent averaged per cent distribution of fluorescence intensity from four animals (st, stomach). *P<0.05.

were hybridised with an oligonucleotide probe corresponding to the 18S rRNA. A 24 base pair oligonucleotide (5'-ACGGTATCTGATCGTCTTCGAACC-3') complementary to the 18S rRNA was synthesised using a DNA synthesiser (Applied Biosystems, Foster City, California, USA). HO-1 cDNA was labelled with [32P] CTP using the random primer kit from Boehringer Mannheim (Mannheim, Germany). All oligonucleotide probes were labelled with [32P] ATP at the 3' end with terminal deoxynucleotidyl transferase (Bethesda Research Laboratories, Gaithersburg, Maryland, USA). Autoradiograph signals were compared with 18S rRNA obtained from the same blot.

Detection of serum mediators
Serum samples were taken at four hours after reperfusion and stored at −80°C until evaluation (n=4 from each group). Serum cytokine concentrations, including IL-6 and IL-10, were determined using rat enzyme linked immunoassay kits, as described by the manufacturer (R&D, Cambridge, Massachusetts, USA). Nitric oxide (NO) secretion (serum nitrite/nitrate) was measured four hours after engraftment using a commercially available test kit (Cayman, Ann Arbor, Michigan, USA).

Data analysis
Results are expressed as mean (SEM). Statistical analysis was performed using the Student’s t test or analysis of various (ANOVA) where appropriate. Statistical analysis for multiple
CO and small bowel transplantation injury

Transplantation.

Bethanechol stimulated contractions (D). SITx, small intestinal transplanted animals demonstrated a significant improvement in concentrations (A, B). The response to bethanechol was significantly showed large phasic and tonic responses to bethanechol at higher contractile activity recorded in response to bethanechol (100 μM).

Figure 3 Representative in vitro jejunal circular smooth muscle contractile activity recorded in response to bethanechol (100 μM) from the four groups of animals. Unoperated circular smooth muscles showed large phasic and tonic responses to bethanechol at higher concentrations (A, B). The response to bethanechol was significantly suppressed in circular smooth muscles harvested from untreated transplanted animals (C). Carbon monoxide (CO) inhalation of transplanted animals demonstrated a significant improvement in bethanechol stimulated contractions (D). SITx, small intestinal transplantation.

Comparisons used the Student–Neuman–Keul's test. A probability level of p<0.05 was considered statistically significant.

RESULTS

Functional studies

The effects of SITx and CO inhalation on intestinal function was investigated by measuring the intestinal distribution of orally fed fluorescein labelled dextran (that is, gastrointestinal transit for a period of two hours) in controls and transplanted animals 48 hours postoperatively. The average gastrointestinal transit distribution histograms of the fluorescence signal contained in each bowel segment from the stomach to the colon are plotted in fig 1A and 1B for unoperated controls, controls receiving CO inhalation, SITx animals, and SITx animals receiving CO inhalation. As shown in fig 1A, CO inhalation did not alter gastrointestinal transit distribution histograms measured from control animals, with the majority of the fluorescent label being localised to the end of the small intestine and cecum. Statistical analyses of the geometric centre calculations were similar between the two groups (see fig 1C).

Intestinal transplantation caused a significant delay in gastrointestinal transit (fig 1B). The intestinal anastomosis sites in segments 3 and 11 did not appear to influence transit. In sharp contrast, recipient transplanted animals treated with CO displayed a markedly increased more normal gastrointestinal transit with the fluorescein labelled dextran progressing down to the distal segments of the small intestine. The calculated transit geometric centre measurements summarised in fig 1C demonstrate that CO inhalation therapy significantly increased gastrointestinal transit in rats undergoing SITx.

Secondly, the effects of SITx with and without CO inhalation treatment were investigated on spontaneous and bethanechol stimulated jejunal circular muscle contractility using in vitro organ bath experiments. Tissues were harvested 24 hours after transplantation of the intestinal graft, a time point when intestinal motility associated with SITx is known to be maximally suppressed. Representative traces of spontaneous jejunal circular muscle contractility are shown in fig 2. Circular muscle strips from unoperated control intestine generated regular contractions with a mean contractile area of 0.96 (0.16) g/mm² (fig 2A). Control animals that breathed CO in the inhalation chambers for 24 hours demonstrated no change in their spontaneous circular muscle contractile activity (1.02 (0.15) g/mm²) (fig 2B). As we have previously demonstrated, SITx results in a significant decrease in spontaneous circular muscle contractile activity (fig 2C). Interestingly, jejunal circular muscle strips harvested from the graft, which had been transplanted into the recipient animals that received CO for 24 hours, demonstrated significantly greater spontaneous contractile activity compared with untreated transplants (0.73 (0.10) g/mm²) (fig 2D).

Addition of bethanechol (0.3–300 μM) to the bathing superfusate elicited a concentration dependent increase in circular muscle contractility. Representative 100 μM bethanechol stimulated contractility traces recorded from jejunal circular muscle strips for the four groups of animals are shown in fig 3. Control circular muscles from untreated (3.5 (0.7) g/mm²) and CO treated (3.2 (0.5) g/mm²) animals exhibited similar robust phasic and tonic contractions to bethanechol (100 μM), while circular muscles from the untreated transplanted intestine generated significantly less...
contractility (51%) in response to bethanechol (1.7 (0.4) g/mm²/s). However, bethanechol stimulated circular muscle contractility generated by CO treated animals was significantly improved over the untreated graft circular muscles (3.6 (0.7) g/mm²/s). These observations were reflected throughout the generation of the complete integrated contractile bethanechol dose-response curves for each of the four groups of animals.

As shown in fig 4, CO inhalation therapy completely prevented the transplant induced suppression in circular muscle contractility, restoring the response of circular muscle to pretransplant levels.

Enterocyte histopathology
In unoperated controls and control CO treated rats, apoptotic cells were rarely found in crypts (<5 apoptotic cells/10 crypts). Intestinal transplantation significantly increased apoptosis in crypts and villous lamina propria after four hours of graft reperfusion. Microscopic examination of the effects of CO inhalation did not show any significant change in enterocyte apoptosis (table 2).

Leucocyte recruitment
Cellular inflammatory events in the small intestinal musculi-

Table 2 Micrscopic visual examination of mucosal crypts (n=10 each) for apoptotic enterocytes. CO inhalation did not significantly change enterocyte apoptosis caused by small intestinal transplantation

<table>
<thead>
<tr>
<th></th>
<th>Erosion</th>
<th>Villous congestion</th>
<th>Re-epithelialisation</th>
<th>Apoptosis/10 crypts</th>
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<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.0 (2.3)</td>
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CO, carbon monoxide; SITx, small intestinal transplantation.

Figure 5 Typical histochemically stained full thickness muscularis whole mounts for the presence of myeloperoxidase positive (MPO) polymorphonuclear neutrophils (PMNs) extravasated into muscularis. (A) Typical muscularis whole mount from an unoperated control animal which was virtually devoid of MPO positive leukocytes. (B) Presence of numerous extravasated PMNs within the graft muscularis taken from the animal 24 hours after small intestinal transplantation (SITx). (C) Histogram quantifying the number of extravasated neutrophils within full thickness whole mounts of jejunal muscularis externa from the four groups of animals (n=4 each). As reflected in the histological analysis, SITx resulted in a significant cellular inflammatory response within the muscularis. Carbon monoxide (CO) inhalation decreased the number of MPO positive cells which extravasated into muscularis in response to transplantation.

Figure 6 Sequential analysis of cytokine mRNA measured by RNase protection assay demonstrated that small intestinal transplantation (SITx) resulted in significant upregulation of both interleukin (IL)-6 and IL-1β mRNAs, which peaked between three and six hours within the graft wall. Data points in (A) at 1, 3, 6, and 12 hours and data points in (B) at 1, 3, and 6 were significantly different (p<0.05) over control extracts by multiple comparisons (Student-Neuman-Keul’s test). Values are mean (SEM), n=4 each.

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
PMNs into the intestinal muscularis (fig 5B). Cell counts per were rare (fig 5A). SITx resulted in a significant recruitment of generated control and CO treated control rats, MPO positive cells infiltrating the muscle layers are shown in fig 5. In unop-
erated control and CO treated control rats, mean expression of IL-6 and IL-1β mRNA, but not ICAM-1, were significantly reduced by greater than 40%. Inducible nitric oxide (iNOS) and cyclo-
oxogenase 2 (COX-2) gene expression were also significantly upregulated in the muscularis of the transplanted intestine (D, E). CO exposure tended to reduced expression of iNOS (p<0.08) and significantly decreased COX-2 mRNA in the graft muscularis extracts. IL-10 expression was upregulated over 100-fold by SITx but CO inhalation did not affect IL-10 expression four hours after reperfusion (F) [*p<0.05].

Molecular inflammatory responses

RPs demonstrated that SITx caused significant upregulation of both IL-6 and IL-1β mRNAs, which peaked 3–6 hours within the transplanted graft (fig 6). Based on these findings, we chose four hours following reperfusion as a time point for quantitative analysis of mRNA levels of various prototypical inflammatory mediators.

Real time RT-PCR analysis revealed a significant increase in mRNA expression for the proinflammatory cytokines IL-6 and IL-1β in graft muscularis externa extracts four hours after reperfusion compared with unoperated controls (fig 7A, 7B). Intercellular adhesion molecule 1 (ICAM-1) gene expression, an adhesion molecule that plays an important role in the recruitment of circulating inflammatory cells into inflamed tissues, was also significantly increased 14.6 (2.35)-fold compared with controls (fig 7C). But at the four hour time point studied, TNF-α was not significantly upregulated (2.9 (1.94)-fold).

In graft muscularis extracts of recipient rats treated with CO, mean comparative expression of IL-6 and IL-1β was reduced on average by 40% (p=0.084, n=6) and 50% (p=0.046, n=6), respectively, compared with the untreated transplanted and reperfused graft at four hours (fig 7A, 7B). The decreased expression of IL-6 mRNA was also reflected in protein production. As shown in fig 8A, in untreated and CO treated controls, serum IL-6 concentrations were low. Transplantation caused a significant increase in serum IL-6 protein concentrations (1758 (77.8) pg/ml) four hours after engraft-

Figure 7 Real time reverse transcriptase-polymerase chain reaction analysis revealed a significant increase in mRNA expression of interleukin (IL)-6, IL-1β, and intercellular adhesion molecule 1 (ICAM-1) in graft muscularis four hours after small intestinal transplantation (SITx) compared with unoperated animals (A, B, C). In recipients treated with carbon monoxide (CO), mean expression of IL-6 and IL-1β mRNA, but not ICAM-1, were significantly reduced by greater than 40%. Inducible nitric oxide (iNOS) and cyclo-
oxogenase 2 (COX-2) gene expression were also significantly upregulated in the muscularis of the transplanted intestine (D, E). CO exposure tended to reduced expression of iNOS (p<0.08) and significantly decreased COX-2 mRNA in the graft muscularis extracts. IL-10 expression was upregulated over 100-fold by SITx but CO inhalation did not affect IL-10 expression four hours after reperfusion (F) [*p<0.05].

Figure 8 Serum interleukin 6 (IL-6) levels in the small intestinal transplantation (SITx) group were significantly higher than those in unoperated animals. Carbon monoxide (CO) inhalation significantly reduced serum IL-6 levels in transplanted animals (A). SITx resulted in elevation of serum nitric oxide products four hours after reperfusion. CO treated transplanted animals showed significantly lower levels than animals exposed to room air (B). *p<0.05.
The inducible isofrom of haem oxygenase, HO-1, is a ubiquitous heat shock protein (HSP32) that is highly induced by diverse stress related conditions.11–13 We hypothesised that CO inhalation therapy could play a seminal therapeutic role in the success of SITx, as it was shown to prevent rejection in a heart model of mouse to rat xenotransplantation,14 as well as ischaemia/reperfusion injury in the lung.15

Post-transplant gastrointestinal motility is markedly suppressed due to a variety of injurious stimuli, including harvest, preservation, manipulation, and sepsis, as well as rejection.15–17 We have previously shown that syngenic SITx, as well as the isolated events of intestinal manipulation and sepsis, result in a significant delay in intestinal transit, a decrease in circular muscle strip contractility, and massive neutrophil infiltration into the muscularis.18–20 The data presented above demonstrate a similar finding of suppressed gastrointestinal transit, decreased in vitro circular muscle contractility, and leukocyte recruitment into the transplanted intestinal muscularis. As hypothesised, CO inhalation therapy significantly prevented the transplant induced delay in gastrointestinal transit and the suppression of muscularis contractility in vitro. Interestingly, the improvement in motility occurred in the absence of a significant decrease in PMN infiltration which appeared to be mechanistically confirmed based on the fact that post-transplant ICAM-1 mRNA levels were not altered by CO inhalation. It would appear that in this model, CO is primarily modulating macrophage function to improve motility.

The data above reconfirmed that intestinal transplantation induces a complex molecular and cellular inflammatory response within the graft muscularis. Our results showed that CO significantly decreased the transplant induced upregulation of the inflammatory cytokines IL-6 and IL-1β. These cytokines have important immune/inflammatory functions if their downregulation may also have a significant impact directly on motility because of their ability to modulate neuromuscular transmission.21–24

Muscularis macrophages play a key autocrine role in regulating smooth muscle contractility through their prolocity to produce NO and prostanooids.25 Here we showed significant upregulation of iNOS and COX-2 within the graft muscularis. A likely mechanism for improved motility in CO treated animals is the finding that the mean relative mRNA expression of both enzymes was reduced by approximately 50% in CO treated rats. Also, this decrease in iNOS mRNA correlated with a significant decrease in nitrite levels. Previous studies have shown that decreased production of NO and prostanooids is associated with improved circular muscle function,26–28 Thus CO inhalation selectively decreased the intensity of the proinflammatory milieu within the graft intestinal muscularis by blunting the post-transplant levels of IL-6, IL-1β, iNOS, and COX-2. However, no significant change was observed in the transplant induced upregulation of TNF-α. Two anti-inflammatory pathways that have been shown to be important in the gut are IL-10 and HO-1.29–31 As HO-1 and CO have previously been linked to IL-10 and HO-1 expression. The data above demonstrated that both IL-10 and HO-1 are activated as potential anti-inflammatory mechanisms following transplantation. Coinduction of these two pathways appears to be important because HO-1 mediates the inhibitory effects of IL-10 on lipopolysaccharide induced TNF-α.32 In addition to CO, antioxidant HO-1 products bilirubin and ferritin may also contribute to decreasing the detrimental effects of the transplant induced anti-inflammatory milieu.23

This study shows that CO attenuates the post-transplant inflammatory milieu by selectively decreasing induction of IL-6 and IL-1β. Functionally, CO was shown to improve post-transplant motility by limiting the autocrine effects of iNOS and COX-2 produced nitric oxide and prostanooids and by
restraining the immune and potential enteric neuronal effects of IL-6 and IL-1β. The results of these experiments suggest that clinically providing CO, a product of the anti-inflammatory HO-1 pathway, may prove to be an effective therapeutic adjunct to overcome the Siymphic challenges of small bowel transplantation.

ACKNOWLEDGEMENTS

This work was supported by GM-58241 and GM-53789 to AJB, DK5232 to NM, HL5234, AI4265, and HL55530 to MCG, and American Heart 160352U and Atorvorstatin Pfizer Research Award to LEO.

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REFERENCES


