SMALL INTESTINE

Immunomodulatory effects of inhaled carbon monoxide on rat syngeneic small bowel graft motility


Background: Intestinal transplantation provokes an intense inflammatory response within the graft muscularis that causes intestinal ileus. We hypothesised that endogenously produced anti-inflammatory substances could be utilised as novel therapeutics. Therefore, we tested the protective effects of inhaled carbon monoxide (CO) and an endogenous haeme oxygenase 1 (HO-1) anti-inflammatory mediator on transplant induced inflammatory responses and intestinal ileus in the rat.

Methods: Gastrointestinal transit of non-absorbable FITC labelled dextran and in vitro jejunal circular muscle contractions were measured in controls and syngeneic orthotopic transplanted animals with and without CO inhalation (250 ppm for 25 hours). Inflammatory mRNAs for interleukin (IL)-6, IL-1β, tumour necrosis factor α (TNF-α), intercellular adhesion molecule 1 (ICAM-1), inducible nitric oxide (iNOS), cyclooxygenase 2 (COX-2), and IL-10 were quantified by real time reverse transcriptase-polymerase chain reaction and HO-1 by northern blot. Histochemical stains characterised neutrophil infiltration and enterocyte apoptosis.

Results: Transplantation delayed transit and suppressed jejunal circular muscle contractility. Transplantation induced dysmotility was significantly improved by CO inhalation. Transplantation initiated a significant upregulation in IL-6, IL-1β, TNF-α, ICAM-1, iNOS, COX-2, and HO-1 mRNAs with the graft muscularis. CO inhalation significantly decreased expression of IL-6, IL-1β, iNOS, and COX-2 mRNAs. CO also significantly decreased serum nitrite levels (iNOS activity).

Conclusions: CO inhalation significantly improved post-transplant motility and attenuated the inflammatory cytokine milieu in the syngeneic rat transplant model. Thus clinically providing CO, the end product of the anti-inflammatory HO-1 pathway, may prove to be an effective therapeutic adjunct for clinical small bowel transplantation.

Materials and Methods

Experimental groups

Inbred male LEW (RT1’’) 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’ cubic 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 exposures.

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.
(n=4–6 each). Intestinal transit was measured in controls and transplanted animals 48 hours postoperatively by evaluating the gastrointestinal distribution of orally fed fluorescein labelled dextran (Molecular Probes, Eugene, Oregon, USA), and intestinal transit was statistically analysed using the calculated geometric centre, as previously described.46

Intestinal tissues were harvested 24 hours postoperatively, the time point at which transplant induced in vitro dysmotility peaks.46 Circular muscle mechanical activity was measured and analysed as previously described.46

Morphological studies
Histopathological
Small intestinal grafts at four hours and 24 hours were fixed in 10% buffered formalin and embedded in paraffin (n=4 each). Sections were cut at a thickness of 4 µm and stained with haematoxylin and eosin. Mucosal apoptotic cells with routine histopathology characteristics were counted in haematoxylin and eosin cross sections. Slides were blindly reviewed by one of the authors (MAN), a pathologist, without knowledge of the experimental groups.

Myeloperoxidase histochemistry
Muscularis whole mounts were prepared from the mid small intestine collected 24 hours postoperatively and Hanker-Yates reagent was used for detection and quantification of polymorphonuclear neutrophils (PMNs) exhibiting myeloperoxidase (MPO) activity.46

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 was dissected and immediately snap frozen in liquid nitrogen and analysed as previously described.

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) followed by removal of potential contaminating DNA by treatment with DNase I (DNA-Free Kit, Ambion Inc.). Equal aliquots (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.

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, four times, at 65°C followed by 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 1 Primer summary

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ to 3’</th>
<th>Source</th>
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<tr>
<td>GAPDH</td>
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<td>CACCTCTACGAGGAGGAGCA</td>
<td>Li and Wang (Brain Res Brain Res Protoc 2000;5:211–17)</td>
</tr>
<tr>
<td>iNOS</td>
<td>GTCACAGCTTTCGAGAGACTGGAA</td>
<td>AF233596</td>
</tr>
<tr>
<td>COX-2</td>
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<td>NM_012611</td>
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</tbody>
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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;
subcloned into pBluescript vector, and a HindIII/EcoRI digestion was performed to isolate the 0.9 kb HO-1 cDNA insert out of the pBluescript vector. To control for variation in either the amount of RNA in different samples or loading errors, blots were hybridised with an oligonucleotide probe corresponding to the 18S rRNA. A 24 base pair oligonucleotide (5′-ACGGTATCTGATCGTCTTCGAACC-3′) complementary to the 18S RNA was synthesised using a DNA synthesiser (Applied Biosystems, Foster City, California, USA). HO-1 cDNA was synthesised using a DNA synthesiser (Applied Biosystems, Foster City, California, USA). All oligonucleotide probes were labelled with [32P] CTP or [32P] ATP at the 3′ end with terminal deoxynucleotidyltransferase (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 immunosassay 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

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**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. Data represent averaged per cent distribution of fluorescence intensity from four animals (st, stomach). *p<0.05.

**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). However, CO treatment demonstrated significantly greater spontaneous contractile activity compared with untreated transplants.
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 caecum. 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/s (fig 2A). Control animals that breathed CO in the inhalation chambers for 24 hours demonstrated no change in their spontaneous circular muscle contractile activity (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/s) (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/s) and CO treated (3.2 (0.5) g/mm²/s) 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-

ras were characterised 24 hours after SITx. MPO activity, as determined by Hanker-Yates histochemistry, was used to quantify the PMN infiltrate in tissues from control and transplanted animals, with and without CO treatment. Photomicrographs of muscularis whole mounts showing MPO positive cells which extravasated into muscularis in response to transplantation.
were rare (fig 5A). SITx resulted in a significant recruitment of MPO positive cells infiltrating the muscle layers in unoperated control and CO treated control rats, MPO positive cells; however, this reduction did not achieve statistical significance (p=0.08, n=6). 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 cyclooxygenase 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 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 cyclooxygenase 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

RPAs 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 (2.55)-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 engraftment and the serum IL-6 increase was significantly less in recipients that had received CO inhalation therapy (1096 (206) pg/ml). However, unlike IL-6 and IL-1β, CO inhalation did not significantly change the upregulation of TNF-α or ICAM-1 caused by transplantation (2.8 (0.44)-fold and 12.5 (2.29)-fold, respectively). CO inhalation by control animals also did not alter basal mRNA expression of any of the cytokines.

NO and prostanoids play a significant role in causing intestinal dysmotility in other models of intestinal inflammation. Therefore, gene expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) were quantified by real time RT-PCR. The results showed that both iNOS and COX-2 were significantly upregulated 28.4-fold and fivefold, respectively, in the graft muscularis (fig 7D, 7E). Mean relative mRNA expression of both enzymes was reduced by approximately 50% in CO treated rats (p=0.060 and p=0.26, n=6 each). CO inhalation by unoperated control animals did not alter mRNA expression of iNOS or COX-2. We followed the molecular expression of iNOS with measurement of nitric oxide metabolites in the serum of transplanted animals. In air and CO treated controls, mean serum nitrite/nitrate (NO) levels determined by the Griess reaction were 7.2 (2.1) and 6.1 (1.9) µM. As predicted by the molecular upregulation of iNOS mRNA, SITx resulted in a significant increase compared with controls. Therefore, gene expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2)

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 nitrate 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 haeme oxygenase, HO-1, is a ubiquitous heat shock protein (HSP32) that is highly induced by diverse stress related conditions.\textsuperscript{11, 12, 29–31} 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,\textsuperscript{13} as well as ischaemia/reperfusion injury in the lung.\textsuperscript{14}

Post-transplant gastrointestinal motility is markedly suppressed due to a variety of injurious stimuli, including harvest, preservation, manipulation, and sepsis, as well as rejection.\textsuperscript{15}–\textsuperscript{16} We have previously shown that syngeneic 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 neutrophilic infiltration into the muscularis.\textsuperscript{17, 18} The data presented above demonstrate a similar finding of suppressed gastrointestinal transit, decreased in vitro circular muscle contractility, and leucocyte recruitment into the transplanted intestinal muscularis. As hypothesised, CO inhalation therapy significantly prevented the transplant induced delay in gastrointestinal transit and suppression in muscle 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 leucocyte 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.\textsuperscript{28–30} Muscularis macrophages play a key autocrine role in regulating smooth muscle contractility through their proloicity to produce NO and prostanooids.\textsuperscript{31} 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.\textsuperscript{32–34} 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.\textsuperscript{47, 48} 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.\textsuperscript{49} 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.\textsuperscript{50}

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

**DISCUSSION**

The unique immunological characteristics of the gastrointestinal tract have made the realisation of successful clinical small bowel transplantation a Sisyphean task. Unfortunately, the proinflammatory and anti-inflammatory events within the specific structures of the gut wall that are initiated by transplantation and rejection of the bowel are poorly understood. We and others have observed that proinflammatory molecular and cellular events play a vital role in damaging the bowel following syngeneic transplantation,\textsuperscript{17, 21, 22} acute rejection,\textsuperscript{18} and chronic rejection.\textsuperscript{12, 13} However, the potential of specific anti-inflammatory molecular events, which could be therapeutically exploited to limit inflammation and avert functional changes of the intestinal graft, are completely unexplored. We hypothesised that pharmacologically providing the end products of anti-inflammatory pathways will significantly prevent the development of detrimental molecular and functional alterations within the intestinal graft following transplantation.

The HO-1 gene has not been sequenced in the rat for RT-PCR experiments but northern blot analyses clearly showed a transplant induced increase in HO-1 in muscle extracts. HO-1 mRNA expression was not detected by northern blot in the intestinal muscularis of unoperated animals. However, dense bands of HO-1 were visible four hours after SITx in animals intestinal muscularis of unoperated animals. However, dense bands of HO-1 were visible four hours after SITx in animals.
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 Siphenan challenges of clinical small bowel transplantation.

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