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ORIGINAL ARTICLE

# Peritransplant treatment with cobalt protoporphyrin attenuates chronic renal allograft rejection

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## Summary

Allogen-independent injury contributes to chronic rejection in renal allografts and heme oxygenase-1 (HO-1) has been shown to be protective in a number of settings. This study evaluated the effect of renal allograft recipient HO-1 up-regulation on chronic rejection in a rat model. Rat (F344 to Lewis) renal transplantation recipients were grouped: (i) cyclosporine (CsA) alone (0.75 mg/kg s.c. × 10 day; $n = 5$); (ii) CsA + low dose cobalt protoporphyrin (CoPP) an HO-1 inducer (0.5 mg/kg i.p. on days −5,0,5; $n = 13$) and (iii) CsA + high dose CoPP (5.0 mg/kg i.p. on days −5,0,5; $n = 8$). Renal function was assessed by serum creatinine levels on day 140. Histopathologic changes in allografts were graded. Morphometric analyses performed to objectively quantify the vascular changes and glomerulosclerosis. HO-1 expression quantified by Western blot and both HO-1 and endothelin (ET-1) localized using immunohistochemistry. Recipients treated with CsA + high dose CoPP had significantly decreased cortical scarring, vascular hyalinization and intimal thickness. They also had a significant, dose dependant, reduction in luminal obliteration and glomerulosclerosis by morphometric analyses. This freedom from chronic rejection in recipients treated with CoPP translated into quiescent grafts at postoperative day 140 with immunostaining and Western blot demonstrating decreased level of HO-1 versus controls ($P = 0.012$). In summary, the peritransplant up-regulation of HO-1 in renal allograft recipients significantly attenuates chronic rejection in rat renal allografts by inhibiting transplant vasculopathy.

## Introduction

Chronic rejection is the most common cause of renal allograft loss after the first post-transplant year. It has an insidious onset and is associated with increasing proteinuria and hypertension in the clinical setting [1]. Unfortunately, there are no pathognomonic lesions of chronic rejection on renal biopsy but it is associated with varying degrees of tubular and glomerular atrophy and new onset intimal plaque formation. Current immunosuppressive protocols, while drastically reducing the episodes of acute rejection, have made little inroads in the prevention of chronic rejection as demonstrated by the stable 7–8 year median graft survival [2].

The concept of chronic allograft dysfunction has emerged to better describe the series of events occurring in long-term surviving allografts (reviewed in 2). In this model both allogen-dependant and allogen-independent factors contribute to graft injury and the subsequent changes seen in failing renal allografts [3]. Clinical

evidence supporting the importance of allogen-independent injury in the pathogenesis of chronic rejection is inferred from studies showing that cadaveric renal allografts have a lower survival than either living related or unrelated grafts [4]. Experimental evidence supporting this concept is found in small animal studies which have shown that a 50% reduction in renal mass in association with an ischemia-reperfusion (IR) injury can produce the lesions typically associated with chronic rejection [5]. These chronic changes can be reduced with the early administration of soluble P-selectin glycoprotein ligand (sPSGL), which helps limit the damage secondary to IR [6]. Furthermore, the combination of sPSGL and low dose cyclosporine (CsA) produces indefinite survival of renal allografts in rats [7]. These studies underline the important role of allogen-independent injury and endothelial activation in the pathogenesis of chronic renal allograft dysfunction.

The role of protective genes in the settings of IR injury and transplantation has generated much attention in recent literature. One of these genes encodes for the heme oxygenases (HO), which are the first and rate limiting enzymes in the catabolism of heme into equimolar portions of iron (Fe), biliverdin and carbon monoxide (CO). To date, three isoforms of the enzyme have been described including: the inducible heme oxygenase-1 (HO-1), the constitutively expressed HO-2 and the recently described HO-3 isoform [8]. HO-1 has been identified as the heat shock protein 32 (HSP 32) and therefore plays an integral cytoprotective role in the cellular stress response [9]. This highly conserved response to a wide variety of noxious stimuli is characterized by a rapid and dominant change in gene expression and leads to a protective state [9]. Specifically, HO-1 is induced by such varied stimuli as heavy metals, endotoxin, hormones, hyperthermia and hyperoxia [10]. It has also been demonstrated that HO-1 is up-regulated by oxidative stress and, more specifically, by IR injury [11].

The role of HO-1 expression in renal homeostasis and protection is rapidly being described. Zou *et al.* [12] have shown that the greatest expression of renal HO-1 was in the medulla and that it played an important role in maintaining the medullary blood flow to an area of the kidney which, even under basal conditions, is relatively underperfused. Investigators have demonstrated that renal HO-1 is induced by IR injury and that its up-regulation prior to this type of insult was associated with protection from oxidative injury [11,13,14]. Furthermore, HO-1 induction by a rationally designed peptide (RDP 1258) was associated with a lower Banff score at day 7 post-transplant in an orthotopic rat renal transplant model and studies evaluating the protective role of HO-1 in a chronic renal model appear confirmatory [14–16].

This study was designed to further examine the link between injury and chronic rejection, more specifically, whether recipient HO-1 induction prior to rat renal transplantation could attenuate the long-term changes seen in rejected allografts. We demonstrate that the induction of HO-1 in renal allograft recipients during the peritransplant period by CoPP significantly attenuates chronic rejection by preventing transplant vasculopathy.

## Materials and methods

### Animals

Adult Fisher 344 rats ($RT^{1v1}$) and Lewis rats ($RT^{1}$) weighing 250–300 g were used as donors and recipients respectively. Animals were housed in conventional conditions at the Animal Care Facility, The University of Western Ontario and protocol approved by the Animal Care and Veterinary Services Committee. Animals were cared for in accordance with the guidelines established by The Canadian Council on Animal Care [17].

### Experimental groups

Lewis rat recipients were randomly allocated to one of three groups: (i) CsA alone [0.75 mg/kg/day s.c. postoperative day (POD) 0–10; $n = 5$]; (ii) CsA + low dose cobalt protoporphyrin (CoPP; 0.5 mg/kg i.p. on days −5,0,5; $n = 13$) a known HO-1 inducer [14,18]; and (iii) CsA + high dose CoPP (5.0 mg/kg i.p. on days −5,0,5; $n = 8$). CoPP was purchased from Porphyrin Products Inc. (Logan, UT, USA). The CoPP was dissolved in 0.2 M NaOH, its pH adjusted to 7.4 and diluted in 0.85% NaCl. All recipients were treated with a brief course of CsA (0.75 mg/kg s.c. × 10 days) to reverse the initial acute rejection. All animals were followed until death or study end point on POD 140.

### Preoperative care and anesthesia

Food and water were not restricted for either donors or recipients. Atropine (0.04 mg/kg s.c.) and buprenorphine (0.1 mg/kg s.c.) were given subcutaneously prior to the induction of anesthesia. The animals were anaesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). Recipients were kept on a warming blanket and under a heating lamp during surgery.

### Surgical procedure

Orthotopic renal transplantations were performed as previously described by [19]. Briefly, the renal grafts were harvested in standard fashion. After bilateral recipient nephrectomies, the graft was revascularized with end-to-side anastomoses between the donor renal artery and recipient abdominal aorta and the donor renal vein and

recipient inferior vena cava. Subsequently, an end-to-end ureteric anastomosis was fashioned. All anastomoses were performed with 11–0 nylon sutures.

### Postoperative care

The animals were given food and water *ad libitum* as well as analgesia (buprenorphine 0.1 mg/kg s.c. prn). They were followed until study termination on POD 140 or sacrificed prior if evidence of >10% weight loss. Serum CsA levels were taken at POD 7 and serum creatinine at POD 140.

### Histopathology

At necropsy, all allografts from animals surviving to study endpoint were removed, fixed in 10% buffered formaldehyde and embedded in paraffin for analysis. They were then sectioned for hematoxylin-eosin and Masson's trichrome staining. The sections were graded by a blinded pathologist (B.G.) and graded for severity of chronic rejection changes as follows: 0 = no change, 1 = minimal change, 2 = mild change, 3 = moderate change and 4 = severe change. These semiquantative assessments were used based on the modified Banff Score [20]. For example, 0 (no changes), refers to normal renal histology; 1 (mild changes), refers to focal lesions of tubular/glomerular atrophy or scarring; 2 (moderate changes), refers to <25% of areas involved; 3 (moderate changes), refers to 25–50% areas involved and 4 (severe changes), refers to more 50% areas involved. Grading for vascular changes was based on extent of fibrous intimal thickening in the most involved vessel. As well, glomerulosclerosis was graded using the Masson's trichrome stained slides where a minimum of one hundred individual glomeruli were assessed per renal allograft for five grafts per group using a grading scale of 0 = normal to 4 = severe fibrosis.

### Morphometric analysis

Intimal plaque formation was quantified using the five arteries with the largest detectable area of atherosclerotic plaque in each renal allograft using the Empix Northern Eclipse trace application program (Empix Imaging Inc., ON, CA). A Sony Power HAD 3CCD color video camera was attached to the microscope and calibrated to the microscope objective used. The total average luminal obliteration and plaque-to-vessel wall ratio were calculated for all animals.

### Immunohistochemistry

The tissue was embedded in optimum cutting temperature gel, snap-frozen in liquid nitrogen, and stored at −80 °C until 4-μ sections were prepared and mounted on gelatin-coated glass slides. A standard indirect avitin-biotin immunoperoxidase staining method was performed using an Elite Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA). Specimens were stained for HO-1 using a rabbit anti-rat HO-1 polyclonal antibody (StressGen, Victoria, BC, Canada) and developed by using secondary biotinylated goat anti-rabbit IgG antibody (Vector Laboratories Inc., Burlingame, CA, USA). Sections from each allograft were blindly graded (0 = no stain to 4 = intense stain).

### Western blot studies

Frozen allografts from each animal were homogenized in lysis buffer (0.1 mM ethylenediaminetetraacetic acid, 10 μg/ml leupeptin, 0.1 mM PMSF (phenylmethylsulphonylfluoride), 1% SDS, and 10 mM Tris-HCl, pH 7.4). The homogenate protein concentrations were obtained using the Lowry method for protein quantification (Waterborg J.H., & Matthews H.R. 1994). A total of 40 μg of kidney protein was used for HO-1 detection. Equal volumes of SDS-sample buffer (0.5 M Tris-HCl, pH 6.8; 10% SDS; 20% glycerol; 0.05% Bromophenol blue) were added and samples boiled for 5 min. Protein were electrophoretically resolved on SDS-polyacrylamide gels (12.5%; Bio-Rad Laboratories, CA, USA) and transferred onto polyvinylidene fluoride membranes (Immobilon, Millipore, MA, USA). The membranes were then blocked for 1 h at room temperature in 5% nonfat powdered milk containing 1X Tris-buffered saline and 1% polyoxyethelene sorbitan monolaurate (TTBS). The membranes were then incubated for 2 h with mouse monoclonal antibody against rat HO-1 (1:1000 dilution; Stressgen, BC, Canada). After three washes with TTBS, the membranes were incubated with goat anti-mouse IgG antibody (Stressgen, BC, Canada) for 2 h. The membranes were then washed three times in TTBS followed by detection of signal using an ECL detection kit (Amersham, IL, USA).

### Statistical analysis

The data are reported as mean ± SD except for the glomerulosclerosis data which is expressed as a percentage of glomeruli with specified level of injury and the pathologic grading which is expressed as the median score given that it is original data. Recipient survival between experimental groups was compared using Kaplan–Meier analysis and the log-rank test. Histological and immunostaining findings were compared using the Mann–Whitney *U*-test. The glomerulosclerosis data was an ordered categorical data set and, therefore, groups were compared with the proportional odds model and expressed as odds

ratio where a value of 1 indicates no effect, a value of less than one indicates less injury and a value of more than 1 indicates more injury with their associated 95% confidence intervals. Otherwise, differences with $P$ values <0.05 were considered significant.

## Results

### Overall survival and serum studies

Animal survival to study end-point (POD 140) was 80% for the CsA alone group versus 92% and 88% for the low and high dose CoPP groups respectively. The difference in survival did not achieve significance by Kaplan–Meier analysis. The CoPP treatment did not have a significant effect on CsA therapy as demonstrated by similar serum CsA levels on POD 7, which were 77, 78 and 55 ng/ml for groups 1–3. The serum creatinine measured at sacrifice on POD 140 was 67 mg/l in the CsA alone group versus 66 and 77 mg/l in the low and high dose CoPP treated animals ($P$ = not significant).

### Histological changes and glomerulosclerosis in renal allografts

The histopathological changes seen in renal allografts are summarized in Table 1. The animals treated with low dose CsA alone had evidence of moderate to severe glomerular and tubular atrophy (Table 1, group 1; Fig. 1a). They also had frequent, deep cortical scars (Fig. 1a) and severe transplant vasculopathy (Fig. 1c). In contrast, animals treated with CoPP had attenuated changes of chronic rejection. The group 3 animals had a marked reduction in tubular atrophy ($P < 0.07$ vs. group 1, Fig. 1b), glomerular atrophy ($P < 0.1$ vs. group 1) and a significant decrease in vascular hyalinization ($P < 0.04$ vs. group 1), intimal thickness ($P < 0.01$ vs. group 1) and cortical scarring ($P < 0.04$ vs. group 1, Fig. 1d).

Animals treated with CsA alone had a significantly greater amount of glomerulosclerosis (Table 2) shown by 71% of glomeruli having 2+ or greater fibrosis versus only 25% of glomeruli in group 3 (high dose CoPP) and 7% in group 2 (low dose CoPP). This translates into an important protective effect from glomerulosclerosis caused by the CoPP treatment with an odds ratio of 0.082 (95% confidence interval 0.062–0.109) for more fibrosis versus group 1.

### Morphometric quantification of intimal plaque in renal allografts

A dose dependent decrease in transplant vasculopathy was observed when objective morphometric measurements were used to quantify intimal plaque present in the renal allografts (Fig. 2). Recipients treated with CsA alone had a mean luminal obliteration rate of 53.7% vs. 23.7% for those in the low dose CoPP group ($P < 0.0001$ vs. group 1), and 17.5% for those animals receiving high dose CoPP ($P < 0.0001$ vs. group 1). The animals in group 1 also had a significantly greater intimal plaque to vessel wall thickness ratio versus those treated with CoPP (1.63 vs. 0.76 and 0.30 for low and high dose CoPP groups, $P < 0.001$).

### Immunohistological changes in renal allografts

The renal allografts from animals treated with high dose CoPP during the peritransplant period had significantly less staining for HO-1 at necropsy versus those receiving CsA alone ($P = 0.01$; Fig. 3a,b). In control animals, the HO-1 was mainly localized to proximal tubular cells and the smooth muscle of the vasculature.

### Western blot for heme oxygenase-1

The renal allografts from recipients receiving CoPP in the peritransplant period appeared to have a dose-dependant decrease in HO-1 protein at POD 140 as determined by Western blot (Fig. 4). The rats receiving the high dose CoPP had very low levels of HO-1 expression at POD 140 correlating with the results obtained at immunohistochemistry.

## Discussion

Alloantigen-independent injury has gained acceptance as a critical mechanism in the pathogenesis of chronic renal

**Table 1.** Histopathologic grading of renal allografts at necropsy.

| Group | Tubular atrophy | Glomerular atrophy | Vascular hyalinization | Vascular intimal thickness | Cortical scarring |
|---|---|---|---|---|---|
| (1) CsA alone ($n = 5$) | 2.0 | 3.0 | 2.0 | 3.0 | 3.0 |
| (2) CsA + low dose CoPP ($n = 13$) | 0.0* | 1.0* | 2.0 | 0.0 | 1.0* |
| (3) CsA + high dose CoPP ($n = 8$) | 0.0 | 0.0 | 0.0* | 0.0* | 0.0* |

Data presented as median injury scores: 0, no change; 1, minimal change; 2, mild change; 3, moderate change; 4, severe change.
*$P < 0.05$ versus CsA alone.

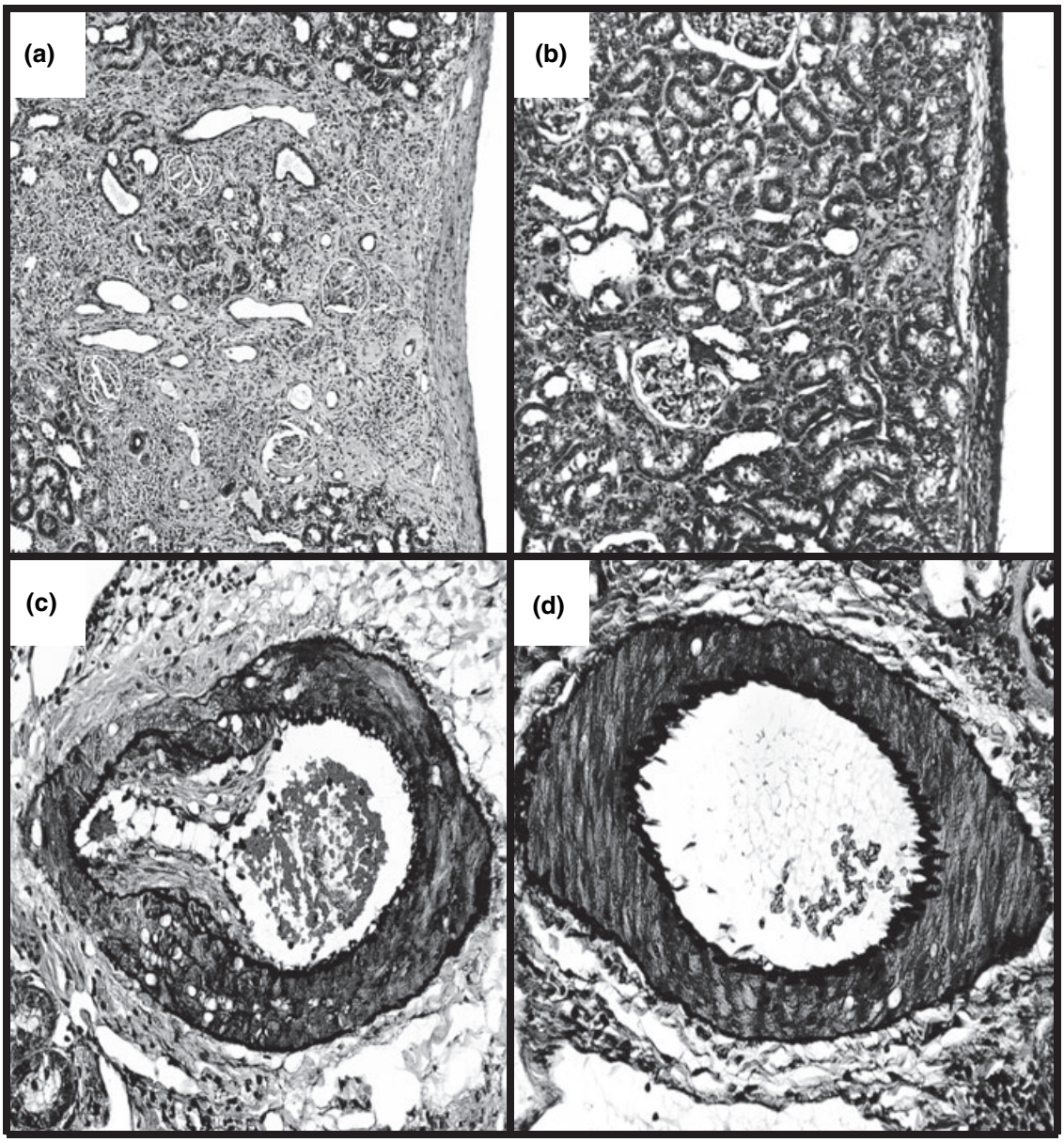


**Figure 1** Representative photomicrographs of renal allografts stained with Masson's trichrome stain. The CsA alone treated animals showed typical features of chronic rejection with widespread evidence of tubular and glomerular atrophy associated with interstitial fibrosis, as well as, frequent and deep cortical scars (a; magnification ×10). Renal allografts recipients treated with high dose CoPP had a preserved renal architecture, little interstitial fibrosis and few, shallow cortical scars (b; magnification ×10). The HO-1 induction caused a significant inhibition of transplant vasculopathy as seen by decreased arteriosclerosis in CoPP treated (d; magnification ×25) versus control animals (c; magnification ×25).

allograft rejection. This broad category includes factors such as: poor pretransplantation graft quality, donor kidney variables, recipient diabetes or hypertension, post-transplantation infection and delayed graft function [3]. IR injury associated with the transplantation process is recognized as an underlying factor in the development of delayed graft function and its importance is inferred from clinical studies showing that kidneys from living donors (related or unrelated) have a better long-term outcome compared with those from cadaveric donors [2,4]. This observation along with the consideration of marginal donor use because of organ shortages has renewed interest in research attempting to describe novel, clinically applicable methods to decrease peritransplant graft injury.

**Table 2.** Grading of glomerulosclerosis.

| Group | Grading | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| (1) CsA alone ($n = 5$) | 2 | 27 | 40 | 11 | 20 |
| (2) CsA + low dose CoPP ($n = 13$) | 25 | 68 | 5 | 1 | 1 |
| (3) CsA + high dose CoPP ($n = 8$) | 48 | 27 | 17 | 8 | 0 |

Grading: 0, normal; 1, minimal change; 2, mild change; 3, moderate change; 4, severe change. Data are presented as the percentage of glomeruli counted in each grading category with a minimum of 100 glomeruli counted in five animals per group.

Many strategies aimed at attenuating renal IR injury have previously been described, however many of these methods target various recipient end-effector molecules such as selectin blockade [6,7], complement inhibition [21] and free radical scavenging [22]. While these methods have expanded our understanding of IR injury and have all been shown to possess important effects in laboratory models we believe the optimal approach would be to modulate the inflammatory cascade more proximally in the recipient and/or donor.

Heme oxygenase-1 has been shown to be up-regulated by various causes of oxidative injury including IR [10,11]. Its induction, by pharmacologic or transfection means, has been shown to be protective in several *in vivo* rodent models including: heart allograft [23], small bowel allograft [24], liver allograft [25], renal IR [13], and renal allograft [14,15,18]. However, there is little data examining the long-term beneficial effects of HO-1 induction in renal allografts. Our experiments have shown that peritransplant HO-1 induction in allograft recipients caused a striking dose-dependant decrease in transplant vasculopathy. As well, there was an attenuation of the typical parenchymal changes seen in chronically rejected renal allografts with significantly less cortical scarring and glomerulosclerosis in treated animals. Interestingly, in contrast to allografts from HO-1 induced recipients, which were relatively quiescent at study end, allografts from control animals had significantly increased active inflammation and rejection. HO-1 levels in these control allografts at the day 140, which were measured by both immunohistochemistry and Western blot were higher than those in perioperatively CoPP treated allografts. Such up-regulation of HO-1 represents an anti-inflammatory response, as there has been ongoing injury in those control allografts not initially protected by HO-1 induction via peritransplant CoPP administration. Our findings are supported by previous observation by Nath and his colleagues [26]. In their chronic renal inflammation rat model, HO-1 was up-regulated.

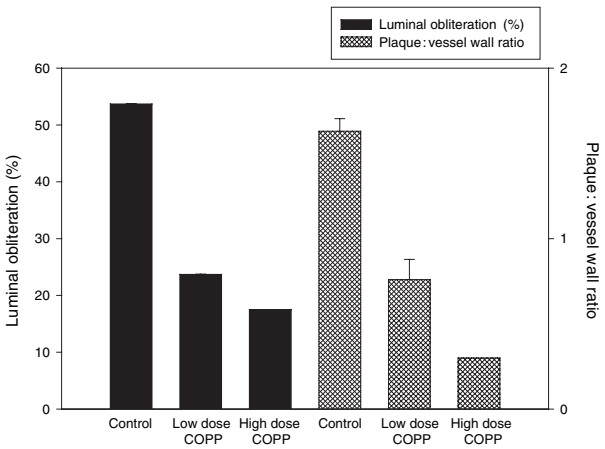


**Figure 2** Morphometric measurement of intimal plaque in renal allografts. The animals treated with CoPP demonstrated a dose dependant decrease in the mean rate of luminal obliteration on morphometry ($P < 0.0001$ for both CoPP treated groups versus CsA alone animals). As well, the renal allografts from CoPP treated animals showed a decreased intimal plaque to vessel wall thickness ratio compared with CsA alone controls ($P < 0.001$ versus for both CoPP treated groups).

The data presented in this study extends the observations of Magee *et al.* [15] who demonstrated lower Banff scores on POD 7 in kidneys from animals treated with HO-1 induction and CsA and parallel those of Hancock *et al.* [27] in their landmark demonstration of the protective effects of HO-1 induction in a chronic rat cardiac allograft model. It is important to note that our findings also complement those of Tullius *et al.* who showed that *donor* kidney allograft HO-1 up-regulation prevented IR injury and was associated with improved term graft function [14,16,18]. We appear to have achieved a similar result via the peritransplant induction of HO-1 strictly in allograft recipients. It is possible that, in the future, interventions to induce HO-1 in both donors and recipients will be necessary to allow the safe use of marginal donors, ensure minimal IR injury and allow long-term graft survival.

The results presented in this study, along with those from other groups, demonstrate that HO-1 induction is protective, however the question of how this occurs remains to be answered. Several different mechanisms attributable to products of the HO-1 catalyzed degradation of heme may be responsible for the protective effect seen in the transplant setting. The degradation of intracellular heme compounds eliminates them as a source of oxidative injury and each end product of its degradation is purported to have potential beneficial effects. The iron released during the reaction results in a rapid up-regulation of ferritin to act as an iron 'sink' thus preventing their role in the Fenton reaction propagation of free radical mediated injury [28]. The protective roles of biliverdin/bilirubin lie in their anti-oxidant, anti-complement

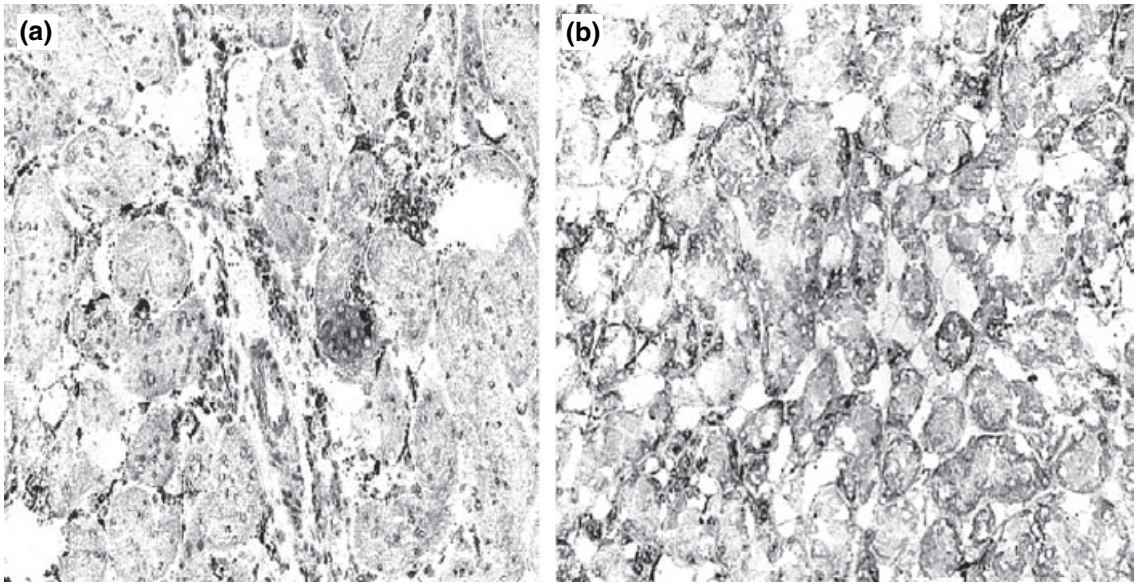


**Figure 3** Immunohistopathology of renal allografts. Control animals had high levels of HO-1 at sacrifice on POD 140 (a; magnification ×25). The HO-1 was localized throughout the renal parenchyma with higher concentrations in the proximal tubular cells and vascular wall smooth muscle cells. There was very little HO-1 staining in the high dose CoPP treated recipients (b; magnification ×25).

and lymphocyte inhibitory functions [29–31]. Furthermore, a recent study showed that HO-1 derived bilirubin attenuated myocardial dysfunction after IR injury [32].

The reactions catalyzed by the HO isoforms constitute the largest endogenous source for CO [10]. In the past, it was well known that CO possessed vasoregulatory properties, but it was not until the last decade that its full physiological role was appreciated. CO directly induces its vasorelaxation effects via activation of both smooth muscle soluble guanylate cyclase (sGC) and high conductance potassium channels [33]. The smooth muscle cell derived CO also has a paracrine affect on endothelial cells by inhibiting the production of known vasoconstrictors and growth factors [endothelin-1 (ET-1) and platelet derived growth factor], and therefore indirectly affects vascular tone [34]. CO also decreases hypoxia stimulated smooth muscle cell proliferation via inhibition of ET-1 and PDGF production in both endothelial and smooth muscle cells [34,35]. These vasoregulatory actions of CO are complimented by the localization of HO-1 in atherosclerotic lesions and the recent hypothesis of an anti-atherogenic role of the HO-1/CO signaling pathway because of bilirubin and CO [36,37]. The combined vasodilatory and anti-proliferative effects of CO represent a potentially important role in the modulation of cardiovascular diseases including transplant arteriosclerosis.

Endothelin-1, a known potent vasoconstrictor, has been shown to have important mitogen and pro-fibrotic roles, and may act as a key effector in the development of transplant vasculopathy. The expression of ET-1 was shown to induce rolling and sticking of PMNs to the endothelium and experiments using ET-1 receptor blockade resulted in a decreased intra-graft accumulation of PMNs and attenuation of its vasoconstrictive effects seen post-IR injury [38,39]. Further studies have documented the presence of elevated levels of vascular (neointimal) and tubular epithelial ET-1 in chronic renal allograft rejection models [40,41]. Transplantation models using either the inhibition of ET-1-converting enzyme or endothelin A receptor antagonism have shown attenuation of transplant vasculopathy [42,43].

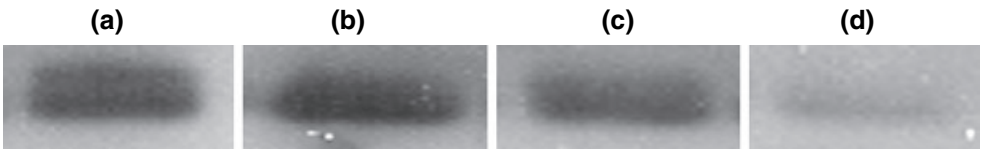


**Figure 4** Representative HO-1 western blot in renal allografts on POD 140. Lane (a) +ve control (rat spleen), (b) CsA alone group, (c) low dose CoPP group, (d) high dose CoPP group.

In summary, there is little doubt of the importance of allogen-independent injury in the development of chronic renal allograft rejection. We have shown that peritransplant induction of HO-1 in recipients alone effectively inhibits the development of chronic rejection in rat renal allografts via prevention of transplant vasculopathy. There may be important clinical implications for HO-1/CO derived allograft protection for all solid organs given the potentially easy administration of inhaled of CO to both graft donor and recipient allowing the safe use of marginal organs.

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