Protective Mechanisms of a Metalloporphyrinic Peroxynitrite Decomposition Catalyst, WW85, in Rat Cardiac Transplants

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ABSTRACT
Nitric oxide (NO) derived from inducible NO synthase has been implicated in cardiac rejection. However, little is known about the role of the reactive nitrogen species peroxynitrite. We examined the protective actions of a peroxynitrite decomposition catalyst, WW85, in an experimental model of acute cardiac rejection. Heterotopic, abdominal transplantation of rat donor hearts was performed. Groups included isografts, allografts, or allografts treated with WW85, cyclosporine, or cyclosporine + WW85. We determined graft survival, histological rejection, and graft function (by in situ sonomicrometry). Intragraft biochemical analysis of cytokines and proapoptotic and antiapoptotic gene expression using reverse transcriptase-polymerase chain reaction were determined. Treatment with WW85 or cyclosporine alone prolonged graft survival, improved graft function, and decreased histological rejection. Graft survival was further significantly (P < 0.001) enhanced by combination treatment. A decrease was also shown in nitrotyrosine, poly(ADP-ribose) polymerase (PARP) activation, and lipid peroxide formation by WW85 that was potentiated when given in combination with cyclosporine. Benefits could not be ascribed to changes in intragraft myeloperoxidase activity. Only combination therapy produced significant decreases in inflammatory cytokine gene expression, suggesting that WW85 acted primarily downstream of these stimuli. In general, WW85 had no direct action on expression of the proapoptotic gene, Fas ligand; however, WW85 given alone or with cyclosporine enhanced expression of antiapoptotic genes Bcl-2 and Bcl-xL. Collectively, these findings suggest a protective action of the peroxynitrite decomposition catalyst WW85 on graft rejection that is independent of any action on leukocyte sequestration and cytokine gene expression. Rather, effects seem to be downstream on decreased protein nitration, decreased lipid peroxidation, and decreased PARP activation.

NO formed by the inducible NO synthase (iNOS) has been implicated in cardiac rejection. However, the nature of this action at the molecular level is not fully understood. Previous attempts to understand the role of iNOS in cardiac rejection have used enzyme inhibitors for iNOS or gene deletion strategies. Another strategy to protect the myocardium in cardiac rejection is to influence downstream actions of NO derived from iNOS rather than to alter iNOS expression or NO bioactivity. Among these potential downstream pathways is that derived via NO-derived peroxynitrite formation. Peroxynitrite is formed by the reaction of NO and superoxide anion radical. Potential biological downstream actions of peroxynitrite include: cardiac depression, lipid peroxidation, nitration of tyrosine residues on proteins, activation of poly-(ADP-ribose) polymerase (PARP), and apoptosis. Peroxynitrite formation has been suggested based upon increased nitrotyrosine staining in experimental cardiac rejection in rats (Sakurai et al., 1999). Apoptosis of cardiac myocytes in acute cardiac rejection was associated with iNOS and nitrotyrosine, suggesting that reactive nitrogen species (i.e., NO or peroxynitrite) may contribute to apoptosis and graft failure (Szabo et al., 1996). Furthermore, in human cardiac transplantation, evidence of increased apoptosis was more likely to be associated with increased cardiac dysfunction (Birks et al., 2000). Clinically, the importance of peroxynitrite formation was also suggested from previous studies showing increased immunostaining for nitrotyrosine in

ABBREVIATIONS: iNOS, inducible nitric-oxide synthase; PARP, poly(ADP-ribose) polymerase; POD, post-transplant day; PCR, polymerase chain reaction; TNFα, tumor necrosis factor α; MPT, mitochondrial permeability transition.
biopsies of human cardiac grafts with grade III rejection scores but not in biopsies with grade 0 histological rejection scores (Szabolcs et al., 1998). In a recent study, we have shown that neutralizing NO directly limits the extent of protein nitration in rat cardiac transplants and prolongs graft survival (Pieper et al., 2004). Despite strategies to limit NO bioactivity to protect cardiac transplants, there has not been to date a direct strategy to evaluate protection of grafts following transplantation by limiting the actions of peroxynitrite.

It has been recognized that water-soluble iron (III) porphyrin derivatives are highly reactive toward peroxynitrite with rate constants as high as 5.0 × 10^7 M^−1 s^−1 (Shimanovich and Groves, 2001). These agents catalyze the isomerization of peroxynitrite, resulting in its decomposition to the less reactive anion, nitrate, thereby, decreasing the levels of the potent oxidizing and nitrating species, peroxynitrite. In previous studies, iron (III) metalloporphyrin-based peroxynitrite decomposition catalysts, including 5,10,15,20-tetakis-[4-sulfoanilophenyl]-porphyrinato-iron[III] and FeCl tetakis-2-(triethylene glycol monomethyl ether)pyridyl porphyrin have been shown to have protection in models of myocardial infarction (Bianchi et al., 2002) and cytokine-induced (Ferdinandy et al., 2000) or doxorubicin-induced (Pacher et al., 2003) or endotoxin-induced (Lancel et al., 2004) cardiac dysfunction. In the present study, we examined the antirejection, anti-inflammatory, and antiapoptotic mechanisms of action of the novel metalloporphyrinic peroxynitrite decomposition catalyst WW85 in experimental cardiac transplantation.

### Materials and Methods

#### Animal Model and Treatments.

Lewis (Lew:RT1^a^) and Wistar-Furth (WF:RT1^a^) rats were chosen to represent genetic disparity at both the major and minor histocompatibility loci for donor-to-recipient combinations of Lew → Lew (isografts) or WF → Lew (allograft). Heterotopic transplantation of donor hearts to the abdominal aorta and vena cava of recipient rats was performed by established microsurgical techniques as described previously (Ono and Lindsay, 1969). Allograft recipients were either untreated or received 0.3 or 1.0 mg/kg WW85 (i.p. three times daily) or 2.5 mg/kg i.p. daily cyclosporine or a combination of WW85 + cyclosporine beginning the day of surgery until the day of harvesting. Graft survival was monitored twice daily for presence or absence of palpable activity and was confirmed upon direct inspection following laparotomy. In another set of experiments, the protocol was terminated on posttransplant day (POD) 6. At this time, cardiac graft function quantitated in situ using sonomicrometry from ultrasonic crystals placed on the external surface of the heart at the mid-level to determine short-axis dimension/graft function (Sonometrics Corp., London, Ontario, Canada). Grafts were then harvested for histological rejections scoring, biochemical analysis, and gene expression analysis. This study conform to the Guide for the Care and Use of Laboratory animals published by the U.S. National Institutes of Health.

#### Histological Rejection Scoring.

Tissue was harvested from grafts at the indicated postoperative days upon arrest and flushing with cold 4°C University of Wisconsin solution and used for either biochemical analysis or histological examination. For histology, tissue was fixed initially in 4% phosphate-buffered formalin. Paraffin-embedded sections were stained with hematoxylin and eosin. Histological rejection was scored blinded using criteria established by the International Society for Heart and Lung Transplantation as modified to a linear score system to allow statistical analysis and as described previously (Pieper et al., 2002; Szabolcs et al., 2002).

### Immunostaining for Nitrotyrosine and Poly(ADP-Ribose).

Tissue sections were deparaffinized and rehydrated by passing through xylene and a graded series of ethanol. Antigen retrieval was performed for 20 min in sodium citrate buffer (pH 6.4) in a microwave oven. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% H_2O_2 for 15 min. After 30 min of incubation in normal goat serum, tissue sections were incubated with chicken polyclonal antibody against poly(ADP-ribose) (1:250; Tulip BioLabs, Inc., West Point, PA) or rabbit polyclonal antibody against nitrotyrosine (2.5 µg/ml; Upstate Biotechnology, Lake Placid, NY) overnight and then incubated with biotinylated secondary antibody and avidin-biotin-peroxidase complex kit (Vector Laboratories, Burlingame, CA). Color was developed using Ni-diaminobenzidine. The section was counterstained with nuclear fast red (Xiao et al., 2004).

#### Malondialdehyde Assay.

To quantify lipid peroxidation of cardiac grafts, we performed assays of thiobarbituric acid-reactive material. Tissues were homogenized as processed as described previously (Mabley et al., 2002).

### Gene Expression.

Grafts were harvested by arresting and flushing with cold University of Wisconsin solution, minced, and frozen in liquid nitrogen. Tissues were stored at −80°C for reverse transcription-PCR. Total RNA was purified from approximately 60 mg of frozen tissue using the Promega SV total RNA isolation kit (Promega, Madison, WI). Total RNA was purified from approximately 60 mg of frozen tissue using the Promega SV total RNA isolation kit (Promega). One microgram of RNA was reverse transcribed using the Invitrogen Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA) with oligo(dT) primers. Briefly, 1 µl of cDNA was mixed with 25 pmol of each primer and Invitrogen supernix to a volume of 25 µl and incubated in an Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA) under the following conditions: for iNOS, 94°C (60 s), 60°C (60 s), and 72°C (60 s) for 30 cycles; for interferon-γ, 95°C (30 s), 60°C (30 s), and 72°C (60 s) for 35 cycling times; for interleukin-6 and interleukin-10 and TNFs, 95°C (30 s), 60°C (30 s), and 72°C (60 s) for 35 cycling times; for Bel-2 and Bel-xL, 95°C for 5 min, 95°C for 30 s, 60°C for 30 s, 72°C for 1 min for 30 cycles and 37 cycles, respectively; and for Pias ligand, 95°C for 5 min, 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min for 31 cycles. The PCR products were resolved on a 1% agarose gel and visualized with ethidium bromide staining followed by densitometry and normalized to a β-actin housekeeping gene antibody. Immunoreactive protein was visualized using enhanced chemiluminescence.

#### Data.

Results were expressed as the mean ± S.E.M. Statistical analysis included analysis of variance with Student Newman-Keuls test for multiple group means or Student's t test for differences between two group means. Significance was set at P < 0.05.

### Results

Cardiac allograft survival was significantly (P < 0.001) enhanced by treatment with both 0.3 or 1.0 mg/kg WW85 doses comparable with that seen with low-dose cyclosporine (Fig. 1). Because there was no difference in the prolongation of graft survival between these two doses, all subsequent studies were performed in recipients treated with the lower dose of WW85. When this dose was given concurrently with
cyclosporine, there was a significant \( P < 0.001 \) further enhancement in graft survival greater than that achieved by low-dose cyclosporine or WW85 given singly (Fig. 1).

In other studies, untreated and treated recipients were examined at POD 6 for in situ graft function using sonomicrometry, and then grafts were harvested for histological rejection scoring. Heart rate was decreased in allografts compared with isograft controls (Fig. 2). In addition, both end diastolic and end systolic transverse segment lengths were enhanced with decreased percentage fractional segment shortening in untreated allografts. Diastolic and systolic dimensions were normalized by WW85 or cyclosporine alone and by combination treatment with WW85 plus cyclosporine. Percentage fractional segment shortening and heart rate were increased by either cyclosporine alone or in combination with WW85.

Using a modified, linearized International Society for Heart and Lung Transplantation rejection scoring system, we found a significant \( P < 0.01 \) increase in histological rejection scores in untreated allografts versus isograft controls (Fig. 3). Both cyclosporine and WW85 significantly \( P < 0.05 \) decreased histological rejection compared with untreated allografts.

Both nitrotyrosine and poly(ADP-ribose) staining were enhanced in cardiac allografts, and nitrotyrosine staining was less frequent in recipients treated with either cyclosporine or WW85 alone and essentially eliminated in recipients receiving combination treatment (Figs. 4 and 5). To show efficacy of WW85 on downstream actions of peroxynitrite on lipid peroxidation in graft tissue, we determined lipid peroxidation by malondialdehyde levels in the various groups. Malondialdehyde levels were significantly \( P < 0.001 \) enhanced in untreated cardiac allografts versus isografts (Fig. 6). Treatment with WW85 produced a significant \( P < 0.001 \) inhibition of malondialdehyde, whereas the increase in malondialdehyde was completely blocked by either cyclosporine alone or in combination with WW85. Myeloperoxidase activity was enhanced in allografts versus isografts. Myeloperoxidase activity in recipients treated with either cyclosporine or cyclosporine plus WW85 was not significantly different from the untreated group (Fig. 6).

**Fig. 1.** Increase in cardiac allograft survival by treatment with either WW85 or low-dose cyclosporine (CsA) alone and with CsA + WW85 (n = 10–12 each group). ¶, \( P < 0.001 \) versus untreated; (¶), \( P < 0.001 \) versus groups B and C.

**Fig. 2.** Improvement by treatment with either WW85 or low-dose CsA alone and with a combination (Comb) of WW85 + CsA in situ graft function at POD 6 (n = 5 each group) determined by sonomicrometry. *, \( P < 0.05 \) versus isograft (iso).
We next examined iNOS and inflammatory cytokine gene expression normalized to β-actin in untreated and treated recipient groups. Expression for iNOS was increased in allografts but was not altered by treatment with the peroxynitrite decomposition catalyst, WW85 (Fig. 7). In contrast, cyclosporine or cyclosporine plus WW85 partially attenuated iNOS gene expression. Likewise, interferon-γ, interleukin-6, and interleukin-10 gene expression was not altered by treatment with WW85 alone, whereas decreases were seen only with combination treatment with cyclosporine plus WW85 (Fig. 7).

To examine proapoptotic gene expression, we examined Fas ligand and TNFα gene expression. The increase in Fas ligand gene expression in allografts was not altered by treatment with WW85 or cyclosporine alone or cyclosporine plus WW85 (Fig. 8). To examine antiapoptotic gene expression, we examined Bcl-xL and Bcl-2. Expression for Bcl-xL was increased in cardiac allografts compared with isograft controls (Fig. 8). However, WW85 or cyclosporine alone increased expression of Bcl-xL, but this action was lost in combination treatment. In contrast, expression of the antiapoptotic gene, Bcl-2, was decreased in cardiac allografts versus isograft controls (Fig. 8). The decrease in Bcl-2 gene expression was unaltered by WW85 or cyclosporine alone, whereas the decrease of Bcl-2 expression was reversed by combination treatment with cyclosporine plus WW85.

**Discussion**

The present study showed that treatment with the peroxynitrite decomposition catalyst WW85 decreased lipid peroxidation, increased antiapoptotic gene expression, decreased PARP activation, decreased nitrotyrosine, and improved allograft survival and function. These findings collectively suggest that peroxynitrite plays a role in the pathology of acute cardiac transplant rejection. Previous studies have
focused on the role of NO derived from iNOS in cardiac transplant rejection but have not examined the potential role of other reactive nitrogen species such as peroxynitrite. Our studies suggest that downstream actions of iNOS-dependent peroxynitrite might explain some of the effects previously attributed to NO.

Peroxynitrite is a potent nitrating species and initiator of lipid peroxidation and apoptosis. Traditionally, immunodetection of nitrotyrosine has been used as evidence of peroxynitrite formation in biological tissue. More recently, it has been shown that protein nitration may, under some conditions, arise independently of peroxynitrite via the action of MPO and nitrite (Eiserich et al., 1998; Sampson et al., 1998; Gaut et al., 2002). Although the precise pathways contributing to nitration in acute cardiac rejection have not yet been determined with certainty, a role of iNOS in nitrotyrosine formation has been indicated.

Protein Nitration and Peroxynitrite in Acute Cardiac Transplant Rejection. Studies using a selective iNOS dimerization inhibitor showed decreased nitrotyrosine levels and apoptosis (Szabolcs et al., 2002). Since this intervention also decreased iNOS expression, it is possible that decreased nitration resulted secondarily from decreased production of NO, a substrate for peroxynitrite formation. Other studies from the same group using iNOS knockout mice showed decreased apoptosis and lack of nitrotyrosine formation when iNOS knockout donor and recipients were used compared with presence of nitrotyrosine when iNOS knockout allografts were used (Szabolcs et al., 2001). These findings are complemented by our studies showing that limitation of NO by two mechanisms, by inhibiting iNOS activity but not expression and decreasing iNOS expression by immunosuppressant therapy, both decreased protein nitration (Pieper et al., 2004). Together, these findings support a role of iNOS in nitrotyrosine formation in acute cardiac allograft rejection.
On the other hand, one cannot exclude the possible nitration of protein via iNOS-dependent but peroxynitrite-independent pathways. Indeed, myeloperoxidase in the presence of nitrite and H₂O₂ can cause nitration of proteins (Sampson et al., 1998). Based upon our current knowledge, it cannot be excluded that nitrite derived from increased iNOS activity could contribute to peroxynitrite-independent protein nitration in our model of cardiac allograft rejection.

We found that treatment with WW85 alone limited nitrotyrosine formation but not myeloperoxidase activity. This contrasts with previous findings using iron-based metalloporphyrinic agents as peroxynitrite decomposition catalysts, which resulted in decreased myocardial myeloperoxidase activity in septic rats (Lancel et al., 2004) and decreased myeloperoxidase activity in lung reperfusion injury (Naidu et al., 2003). These discrepancies are possibly related to differences in the type of catalyst used or differences in experimental models. Nevertheless, our findings using a peroxynitrite decomposition catalyst are significant in providing the first known evidence suggesting that myeloperoxidase-derived nitration is probably not a major source of nitrotyrosine formation in our transplant model. Rather, it is more likely that nitrotyrosine derives predominately from a peroxynitrite-dependent pathway.

**PARP Activation.** PARP is a nuclear enzyme that is activated by single-strand DNA breaks. This enzyme is increased in tissue injury and catalyzes the transfer of ADP-ribose subunits to proteins associated with DNA damage. Peroxynitrite is a potent activator of DNA strand breaks and PARP activation (Virág and Szabó, 2002; Szabó, 2003). Detection of poly(ADP-ribose) is used to indicate PARP activation.

PARP activation has been shown after reperfusion injury in rat cardiac transplants (Szabó et al., 2002) or following alloimmune activation and rejection in rat tracheal (Farivar et al., 2004) and cardiac (Liu et al., 2004) allografts. The observation that 5-aminoisouquinoline, an inhibitor of PARP, attenuated rejection scores and improved graft survival in a rodent model of cardiac allograft rejection suggests the importance of PARP activation (Liu et al., 2004). In our study, we found that WW85 given alone decreased poly(ADP-ribose), suggesting that it acted, in part, by decreasing PARP activation. To our knowledge, this is the first suggestion that PARP activation in acute cardiac rejection may be related, at least in part, to peroxynitrite formation.

**Fas Ligand Expression.** Expression of CD95 antigen ligand or Fas ligand is chemotactic for neutrophils (Ottonello et al., 1999) and is one potential pathway for apoptosis during alloimmune activation in transplanted organs. Up-regulation of Fas ligand has been shown in a variety of studies of cardiac allograft rejection. The precise molecular events in up-regulation of Fas ligand are incompletely understood.

The specific regulatory role of NO or NO-derived species on Fas ligand expression has not been developed in any detail. Studies in iNOS knockout mice suggest that NO does not seemo alter Fas ligand expression in cardiac transplant rejection (Köglin et al., 1999). Whether or not peroxynitrite regulates Fas ligand expression is unknown. We found that Fas ligand expression was dramatically increased in allografts versus isograft controls. Furthermore, we found no action of WW85 used alone or in combination with cyclosporine on Fas ligand expression. Since FasL is a chemoattractant for circulating leukocytes, the finding of unchanged Fas ligand expression after treatment with WW85 is consistent
with our other finding that WW85 did not limit myeloperoxidase activity, an index of intragraft neutrophil sequestration. Taken together, these data suggest that WW85 provides benefits distally to these stimulatory pathways.

**Inflammatory Cytokine Gene Expression.** Alloimmune activation was associated with up-regulation of interferon-γ, interleukin-6, and interleukin-10. Previously, we showed that agents that inhibited inflammatory cytokine gene expression promote increased graft survival and/or improved graft function (Pieper et al., 2003, 2005). In the present study, we found that cytokine gene expression was unaltered by treatment with WW85, suggesting that benefits cannot be ascribed to modulation of inflammatory cytokine gene expression. In contrast, gene expression was decreased or prevented (depending on the specific gene) when WW85 was presented. This anti-inflammatory activity could explain its beneficial actions on graft function and survival used in combination therapy.

**Pro- and Antiapoptotic Gene Expression.** We found that cytokine gene expression and FasL (stimulants for apoptosis) were unchanged by treatment with WW85 alone. Apoptosis is also regulated by antiapoptotic factors. In this context, increases in Bcl-2 and Bcl-xL have been argued to counteract apoptosis in cardiac transplant models. Although oxidants such as H$_2$O$_2$ can induce Bcl-xL expression in cardiac myocytes (Valka et al., 2003), our current understanding of the signaling molecules responsible for regulating expression of these antiapoptotic genes in cardiac rejection is incomplete.

Likewise, the effect of reactive nitrogen species on antiapoptotic genes is not well understood. Deletion of iNOS gene in mouse cardiac transplants increased expression of both Bcl-2 and Bcl-xL (Koñgin et al., 1999). These findings suggest that NO may limit antiapoptotic gene expression. However, it is unclear whether this is a direct consequence of limiting the actions of NO or secondarily to downstream peroxynitrite formation. Our study may shed some new understanding on this possibility.

Specifically, we found that the peroxynitrite decomposition catalyst WW85 had a discriminatory effect on antiapoptotic gene expression depending on the specific gene and/or condition. Alloimmune activation decreased Bcl-2 expression, whereas treatment with WW85 alone did not alter this decreased expression. In contrast, alloimmune activation did not alter expression of Bcl-xL, but treatment with WW85 caused a marked increase in expression of this antiapoptotic gene. The increase in Bcl-xL expression by WW85 was lost in combination therapy with cyclosporine; however, this action was countered by findings that combination therapy prevented the decrease in Bcl-2 expression in allografts. Collectively, these studies indicate that peroxynitrite decomposition catalysts may prove beneficial, in general, by stimulating antiapoptotic gene expression.

High Ca$^{2+}$ induces mitochondrial permeability transition (MPT) pore opening that is enhanced in myocytes isolated from allografts versus isografts (Raisky et al., 2004). Although cyclosporine ex vivo can inhibit MPT pore opening, these authors showed that a nonimmunosuppressive analog of cyclosporine inhibited the MPT pore opening but did not alter rejection. They concluded that acute rejection occurs upstream of cardiomyocyte apoptosis and that inhibiting MPT opening may provide a mechanism to prevent actual graft failure. In addition to high Ca$^{2+}$, reactive oxygen and peroxynitrite trigger MPT pore opening in liver mitochondria ex vivo (Brookes and Darley-Usmar, 2004). Based upon these findings, we cannot exclude the possibility that WW85 might provide cardiac protection by a mechanism involving inhibition of peroxynitrite-mediated MPT pore opening leading to diminished apoptosis.

Overall, our findings demonstrate that neutralization of peroxynitrite, when WW85 is applied together with low-dose cyclosporine therapy, prolongs the life of cardiac allografts and inhibits the activation of many cytotoxic pathways of injury (including tyrosine nitration and PARP activation). This is likely to produce a down-regulation of the subsequent immunological and inflammatory response that may be responsible for the benefits of this approach. By allowing a reduction in the amount of cyclosporine used for transplant therapy, coapplication of a peroxynitrite catalyst at the early stages of transplant rejection may be a useful approach to limit the cyclosporine-induced side-effects.

**References**


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