Nitric oxide formation in acutely rejecting cardiac allografts correlates with GTP cyclohydrolase I activity

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Inducible nitric oxide synthase (iNOS) is a prominent component of the complex array of mediators in acute graft rejection. While NO production is determined by iNOS expression, BH₄ (tetrahydrobiopterin), a cofactor of iNOS synthesized by GTP cyclohydrolase I, has been considered critical in sustaining NO production. In the present study, we examined time-dependent changes in iNOS and GTP cyclohydrolase I in rat cardiac allografts. The increase in iNOS protein and mRNA in allografts was similar at POD4 (post-operative day 4) and POD6. However, the peak increase in intragraft NO level at POD4 was not sustained at POD6. This disparity could not be explained by any decrease in iNOS enzyme activity measured *ex vivo* with optimal amounts of substrate and cofactors. Lower iNOS activity could be explained by changes in total biopterin levels in allografts at POD4 that was decreased to baseline at POD6. Changes in biopterin production correlated with lower GTP cyclohydrolase I

protein levels but not by any change in GTP cyclohydrolase I mRNA. Functionally, allografts displayed bradycardia and distended diastolic and systolic dimensions at POD6 but not at POD4. Likewise, histological rejection scores were increased at POD4 but with a secondary increased stage at POD6. It is hypothesized that the dissimilar amounts of NO at early and later stages of rejection is due to uncoupling of iNOS arising from disproportionate synthesis of BH₄. These findings provide insight into a potential pathway regulating NO bioactivity in graft rejection. Such knowledge may potentially assist in the design of newer strategies to prevent acute graft rejection.

Key words: biopterin, cardiac allografts, GTP cyclohydrolase I, inducible nitric oxide synthase (iNOS), tetrahydrobiopterin, transplantation.

INTRODUCTION

NO derived from iNOS (inducible nitric oxide synthase) together with cytokines and lymphokines constitute a complex array of potential mediators that are stimulated upon interaction of antigen-presenting cells and lymphocytes in acute cardiac transplant rejection. A number of studies conducted in animal models have indicated a deleterious role for iNOS in acute graft rejection. Experimental evidence has been provided from studies showing that iNOS inhibitors improved graft survival and decreased histological rejection [1,2]. Similar findings have been demonstrated in our laboratory by using agents that scavenge NO [3–5]. At the molecular level, NO production, via iNOS, can be induced by increased transcription regulated by NF- κ B (nuclear factor κ B), a major transcription factor that is activated in inflammatory disorders. Active NF- κ B dimers bind to the promoter regions of genes such as inflammatory cytokines and iNOS that are believed to be important mediators of alloimmune response induced by foreign histoincompatible alloantigens in graft rejection. We have found that agents that inhibit NF- κ B DNA binding activity decreased iNOS gene expression, prolonged graft survival and decreased histological evidence of acute graft rejection [5–7].

In addition to transcriptional regulation of iNOS, there is growing evidence from cultured cell models that NO production from iNOS is also regulated via the co-ordinated expression of GTP cyclohydrolase I (EC 3.5.4.16) [8–12]. GTP cyclohydrolase I

is the first enzyme in the *de novo* biosynthesis of BH₄ (tetrahydrobiopterin), which is an essential cofactor required for NO production by all NOS isoforms. This cofactor function is exerted through several mechanisms. It has been shown that BH₄ influences the electronic state of the haem group, stabilizes the dimeric conformation of iNOS and augments substrate affinity for the enzyme [13]. Also, BH₄ is a key redox cofactor co-ordinating electron transfer from the reductase domain to the haem–dioxygen intermediate complex during catalysis. This last function is probably critical to the control of superoxide release from the enzyme. Thus a deficiency in BH₄ levels may convert iNOS from an NO-producing enzyme to a potential generator of superoxide. In addition, it is anticipated that, depending on the severity of the BH₄ deficiency, iNOS may generate superoxide formation concomitant to NO, thereby producing peroxynitrite, an oxidizing and nitrating agent that may be involved in organ dysfunction.

Despite the known importance of BH₄ in controlling NOS activity, there is little information on how BH₄ levels are controlled in models of acute cardiac allograft rejection. Although it has been proposed that increased GTP cyclohydrolase I activity is co-ordinated to iNOS expression, it is unclear when exactly this up-regulation occurs, the duration of the signal and the levels of BH₄ reached in acutely rejecting cardiac allografts. In the present study, the time-dependent changes in the balance of total biopterin (BH₄ + BH₂ + biopterin), iNOS and GTP cyclohydrolase I gene

Abbreviations used: BH₄, tetrahydrobiopterin; iNOS, inducible nitric oxide synthase; ISHLT, International Society for Heart and Lung Transplantation; NF- κ B, nuclear factor κ B; POD, post-operative day; RT, reverse transcriptase.

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expression in a rat model of acutely rejecting cardiac allografts were examined to assess variations in iNOS bioactivity, i.e. NO production, relative to iNOS expression.

EXPERIMENTAL

Animal model and surgical procedures

All animal methods were approved according to the guidelines of the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. Lewis (Lew: RT1⁺) and Wistar-Furth (WF: RT1⁺) 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 (allografts). Sterile surgery was performed in rats anaesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium. Heterotopic transplantation of donor hearts to the abdominal aorta and vena cava of recipient rats was performed by established microsurgical techniques [14]. Graft function was monitored twice daily for the presence or absence of palpable activity and confirmed on direct inspection after laparotomy. In addition, graft function was monitored *in situ* using sonometric crystals (Sonometrics, London, ON, Canada) placed on the exterior surface of the heart at mid-level to determine short-axis dimension/function.

Harvesting of hearts and histological studies

Tissue was harvested from grafts at the indicated PODs (post-operative days) upon arrest and flushed with cold University of Wisconsin solution (Barr Laboratories, Pomona, NY, U.S.A.). Aorta and atrial tissue were removed and ventricular tissue was used for biochemical analysis and histological examination. For histology, tissue was fixed in 4% phosphate-buffered formalin. Paraffin-embedded sections were stained with haematoxylin and eosin. Histological rejection was scored blind based upon a six-point graded criterion established by the ISHLT (International Society for Heart and Lung Transplantation) as described previously [4].

Assay of NOS activity

NO levels in cardiac allografts were determined as NO metabolites (nitrate + nitrite). Tissue (~100 mg) was homogenized in PBS (pH 7.4) and centrifuged at 10 000 *g* for 20 min to remove cellular debris. The supernatant was ultracentrifuged at 100 000 *g* for 30 min and filtered through 10 kDa molecular mass cut-off filters (Amicon, Danvers, MA, U.S.A.). For analysis of total nitrate + nitrite levels, 40 µl of sample was processed according to the manufacturer's guidelines using a commercial kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) as described previously for heart tissue and normalized to per mg of protein [15]. The iNOS activity was also determined as the calcium-independent NOS activity by measuring ¹⁴C-L-arginine to ¹⁴C-L-citrulline conversion as described previously under calcium-free conditions [16] and confirmed by the ablation of citrulline production in samples treated with the iNOS inhibitor, aminoguanidine [17]. Because of the amount of tissue required, these analyses were performed on separate allografts different from those used for other analyses in this study. However, we independently confirmed by Western blots that iNOS protein levels were similar in the same allograft samples at POD4 and POD6 used for the citrulline assays. Briefly, approx. 600 mg of graft tissue (primarily ventricular tissue excluding aorta and atria) was homogenized and incubated in calcium-free 50 mM Hepes buffer (pH 7.5) containing 20 µM ¹⁴C-L-arginine (313 µCi/µmol), 100 µM EDTA, 100 µM dithiothreitol, 2.5 µM FAD, 2.5 µM FMN, 50 µM BH₄, 500 µM NADPH and 2 mM

EGTA. The reaction was linear for 15 min after which time 50 µl of the reaction mixture was removed and the reaction was stopped with 200 µl of stop solution (200 mM Hepes, pH 5.5, containing 20 mM EGTA and 2 mM L-citrulline). Radiolabelled L-arginine was eluted on Dowex 50 (Na⁺ form) columns with double distilled water and activity was determined after counting in a scintillation counter and normalized to per mg of protein.

Measurements of total biopterin

Quantification of total biopterin (BH₄ + BH₂ + biopterin) in heart tissue was performed by HPLC. Briefly, frozen tissue was pulverized under liquid nitrogen and a known amount (~20 mg) was homogenized directly into 0.1 M HCl containing 0.1 mM EDTA. After centrifugation for 10 min at 14 800 *g* followed by an additional spin of 30 min at 100 000 *g*, the supernatants (450 ml) were incubated with 75 ml of KI/I₂ (2:1) solution for 1 h in the dark. The reaction was stopped by addition of a 5 µl aliquot of 20% ascorbate solution. Tissue debris was centrifuged and supernatants were cleared with a 10 kDa molecular mass cut-off filter. Samples were loaded on to a C-18 Synegi-HR column and eluted with 97% trifluoroacetic acid (0.1%):3% acetonitrile. Under these conditions biopterin eluted at 10 min. Some samples were spiked with authentic biopterin as an internal standard. Biopterin concentrations were determined using authentic biopterin standards (20–75 pg) by fluorescence with excitation at 350 nm and emission at 440 nm. Biopterin concentrations were normalized with respect to the amount of tissue.

Measurement of GTP cyclohydrolase activity

Enzyme activity in grafts was measured by following the conversion of GTP to dihydroneopterin 3'-triphosphate following oxidation/desphosphorylation to neopterin. Typically, tissue homogenates were prepared in 50 mM Tris/HCl buffer (pH 7.4), containing 1 mM MgCl₂, 0.1 M KCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and a protease inhibitor cocktail (Roche, Indianapolis, IN, U.S.A.). Samples were clarified by centrifugation at 14 800 *g* for 10 min followed by a spin at 40 000 *g* for 30 min. Protein homogenates (approx. 150 mg in 100 ml) were incubated with 1 mM GTP (20 ml of a 10 mg/ml stock solution), 50 µg/ml BSA and 1 mM dithiothreitol in 50 mM Tris/HCl buffer. After incubation for 1 h at 37°C, the reaction was stopped by placing in an ice-water bath followed by addition of 8 µl of 5 M HCl. Next, samples were oxidized with 75 ml of KI/I₂ (2:1) solution at room temperature (25°C) for 1 h and protected from light after which the reaction was stopped by addition of ascorbate (2 ml of a 20% solution). After alkalization and treatment with 5 units of alkaline phosphatase followed by centrifugation through a 10 kDa molecular mass cut-off filter, samples were loaded on to a C-18 Synegi-HR column and eluted with trifluoroacetic acid (0.05%). Under these conditions neopterin eluted at approx. 8.5 min. Neopterin content was analysed by HPLC with fluorescence detection at 350 nm excitation and 440 nm emission. Neopterin concentrations were calculated using neopterin standards and normalized with respect to protein content in the homogenates.

Western-blot analysis

Frozen tissues were homogenized in ice-cold PBS with 1% Triton X-100, 1 mM PMSF, 35 ng/ml pepstatin A and 10 ng/ml leupeptin as described in [5]. Samples were resolved on SDS/7.5% polyacrylamide gels and transferred on to membranes. Blots were probed with 1:2000 dilution of rabbit anti-iNOS and 1:100 dilution of β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). For GTP cyclohydrolase I, we used a 1:1000 dilution of a

polyclonal rabbit anti-rat GTP cyclohydrolase I antibody prepared as described in [18]. Immunoreactive protein was visualized using 1:5000 dilution of donkey anti-rabbit IgG horseradish peroxidase conjugated antibody and enhanced chemiluminescence. Blot densities were normalized for protein loading using either β -actin or Ponceau S.

Gene expression

Total RNA was purified from approx. 60 mg of frozen tissue per sample using the Promega SV Total RNA Isolation System (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions and RNA concentration was determined spectrophotometrically. cDNA was synthesized from 1 μ g of total RNA and oligo(dT) primers using the Invitrogen Superscript First-Strand Synthesis System for RT (reverse transcriptase)–PCR (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. For amplification of iNOS and GTP cyclohydrolase I, 1 μ l of cDNA was mixed with 25 pmol of specific forward and reverse primers and PCR Supermix (Invitrogen) to a volume of 25 μ l. Primer sequences were: 5'-CA-CCTTGGAGTTCACCCAGT-3', for iNOS sense; 5'-TGTTGT-AGCGCTGTGGTCCA-3', for iNOS antisense; 5'-GGATAC-CAGGAGACCATCTCA-3', for GTP cyclohydrolase I sense; and 5'-TAGCATGGTAGTGACAGT-3', for GTP cyclohydrolase I antisense. The reaction was incubated in a Bio-Rad iCycler (Bio-Rad, Hercules, CA, U.S.A.) under the following conditions: for iNOS, 94°C (60 s), 60°C (60 s), 72°C (60 s) for 30 cycles; and for GTP cyclohydrolase I, 94°C (60 s), 60°C (60 s), 72°C (1.5 min) for 30 cycles. The amplified product was visualized on a 1% (w/v) agarose gel after ethidium bromide staining and visualized by UV light. Densitometry of specific bands was determined on an Alpha Imager (Alpha Innotech, San Leandro, CA, U.S.A.) and expressed as a ratio to β -actin housekeeping gene control.

Statistical analysis

Results were expressed as means $\pm$ S.E.M. Statistical analysis included ANOVA with Student–Newman–Keuls test for multiple group means or *t* test for differences between two group means. Significance was set at $P < 0.05$.

RESULTS

Time course of iNOS expression in heart grafts

An initial study was performed to determine the time-dependent changes in iNOS after heart transplantation at POD2 through POD6. The latter time point was chosen since all cardiac allografts were observed to fail by POD7. Western blot analysis showed that iNOS was not detectable in any cardiac isograft or allograft on POD2 whereas on POD3, iNOS was only marginally increased in two of the six heart allograft samples (Figure 1). In contrast, high expression of iNOS protein was seen in all ($n = 6$) allograft samples at POD4 through POD6 (Figure 1). To show that this up-regulation was not due to surgery or to systemic infection, we probed for iNOS in isografts or native hearts of allograft recipients from the same animals. iNOS was not detected in any isograft until POD5 (Figure 1) or in native hearts of allograft recipients at any time point studied (results not shown). Up-regulation of iNOS mRNA was not evident at POD2 and POD3 (results not shown). Significant increases in iNOS mRNA were seen at POD4 that was similar in magnitude at POD4 and POD6 (Figure 2).

In situ and ex vivo iNOS activity

NO levels in harvested graft tissues were measured using the Griess reaction in order to assess *in vivo* intragraft NO production.

Figure 1 Time-course detection of iNOS protein in allografts versus isograft controls

(A) Isograft (▼) and allograft (▽) tissues were harvested at POD2 to POD6 and iNOS protein determined by Western blot and (B) blot density normalized to protein loading using Ponceau S. For densitometry, isografts and allografts at various days are represented as I2, I3, I4, I5 and I6 or A2, A3, A4, A5 and A6 respectively. ¶ $P < 0.001$ allograft versus isograft for corresponding day; (¶) $P < 0.001$ allograft for corresponding day versus allograft at POD3.

Figure 2 GTP cyclohydrolase I and iNOS mRNA levels in allografts at POD4 versus POD6

Total RNA was isolated from cardiac graft tissue and cDNA obtained as indicated in the Experimental section. RT–PCR analysis was performed using specific primers for rat GTP cyclohydrolase I, iNOS and β -actin followed by densitometric analysis of both GTP cyclohydrolase and iNOS normalized to β -actin. The results indicate no difference between these two time periods. Values represent the means $\pm$ S.E.M. for $n = 6$ experiments.

Figure 3 Quantification of NO metabolites (nitrate plus nitrite) in heart tissue

Grafts frozen and stored in liquid nitrogen were homogenized and filtered through a 10 kDa molecular mass cut-off filter. Nitrate + nitrite was determined in the filtrate by a Griess reaction. Values represent the means $\pm$ S.E.M. for three or four experiments. $\blacksquare P < 0.001$ allografts at POD4 (Allo4) versus isografts at POD4 (Iso4) and $\blacksquare P < 0.001$ allografts at POD6 (Allo6) versus allografts at POD4 (Allo4).

Figure 4 Total (with calcium) and calcium-independent citrulline formation by cardiac grafts

Tissue freshly frozen in liquid nitrogen was homogenized and NOS activity was determined in isografts, allografts or native hearts of allograft recipients at POD4 and POD6 in the presence of calcium (solid bars) or absence of calcium (open bars). Calcium-independent citrulline formation was defined as being iNOS activity based as described previously [16,17]. Citrulline production was evaluated by addition of 20 μ M of [14 C]-L-arginine in the presence of 50 μ M of BH₄. Values represent the means $\pm$ S.E.M. of $n = 6$ experiments for each group. Isografts, POD4 (bars 1 and 2); allografts, POD4 (bars 3 and 4); isografts, POD6 (bars 5 and 6); allografts, POD6 (bars 7 and 8); and native hearts of allograft recipients, POD6 (bars 9 and 10). $\ddagger P < 0.01$ allografts versus isografts at respective time points; $\S P < 0.01$ allografts at POD6 versus corresponding allografts at POD4.

As shown in Figure 3, total tissue nitrate + nitrite levels measured in allografts at POD4 was 0.68 ± 0.04 μ mol/mg of protein which was 4-fold higher than the levels measured in isografts at the same post-transplant day. The increase in NO metabolites in allografts was not sustained but rather significantly ($P < 0.001$) decreased in allografts at POD6 (i.e. 0.24 ± 0.05 μ mol/mg of protein) from the peak observed at POD4. This finding contrasts with the results obtained for iNOS expression, which showed high levels of protein at both POD4 and POD6 in allografts.

Figure 4 shows the results for iNOS activity in heart tissue, determined *ex vivo* by following the formation of [14 C]-citrulline in incubations supplemented with [14 C]-L-arginine and BH₄ in the presence and absence of calcium. These measurements show an increase in NOS activity in allografts compared with low or nearly undetectable activity in isograft controls (Figure 4). As shown in Figure 4, the total NOS activity (determined in the presence of calcium) was not different from calcium-free NOS activity, strongly suggesting that it is mostly due to iNOS activity as previously found [16,17]. The measurements show that iNOS activity was increased in allografts at both POD4 and POD6 (i.e. 4.4 ± 0.1 and 10.0 ± 1.7 pmol citrulline $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹ respectively) with respect to iNOS activity detected in cardiac isografts at the same time points (Figure 4). In addition, an

Figure 5 Increases in biopterin levels in cardiac grafts

Tissues frozen with liquid nitrogen from isografts and allografts from POD4 and POD6 were homogenized under acidic conditions and total biopterin determined by HPLC as described in the Experimental section. Results represent the means $\pm$ S.E.M. for $n = 3$ experiments. $*P < 0.05$ allografts versus isograft controls; $(*)P < 0.05$ allografts at POD6 versus allografts at POD4.

insignificant increase in enzyme activity was seen in native hearts from allograft recipients (Figure 4). The discrepancies between NO levels measured in the tissues versus *ex vivo* iNOS activity suggest that iNOS enzyme activity is regulated by factors other than protein expression in the rejecting grafts. Thus whether or not biopterin availability is a factor determining this disparity was examined next.

Temporal changes in biopterin levels in heart grafts

HPLC measurements of tissue total biopterin (BH₄ + BH₂ + biopterin) showed an increased level in allografts versus isograft at POD4 (Figure 5). While a 2-fold increase in biopterin levels was measured in isografts at POD6 versus POD4 (i.e. 265.5 ± 63.7 versus 541.3 ± 45.0 pmol/g of wet tissue respectively), in allografts biopterin levels at POD6 decrease by approx. 40% with respect to POD4 (Figure 5). Of note, biopterin content was not different between native hearts of both isograft and allograft recipients at any time period (Figure 5) indicating that changes in biopterin are specific to the alloimmune response. To evaluate whether the decline from peak biopterin levels in allografts was related to changes in GTP cyclohydrolase I activity, the rates of neopterin formation in homogenates incubated with exogenous GTP was measured. This analysis revealed that GTP cyclohydrolase I enzyme activity was also decreased in allografts at POD6 versus POD4 (i.e. 1.489 ± 0.114 pmol $\cdot$ h⁻¹ $\cdot$ mg of protein⁻¹ versus 2.236 ± 0.163 pmol $\cdot$ h⁻¹ $\cdot$ mg of protein⁻¹, $P < 0.05$, $n = 3$ each).

The lower GTP cyclohydrolase I activity detected at POD6 versus POD4 directly correlated with the changes in GTP cyclohydrolase I protein levels (Figure 6), as determined by Western blot. The protein levels were significantly decreased ($P < 0.05$) in cardiac allografts at POD6 (Figure 6). The last effect, however, seemed unrelated to changes in gene expression since GTP cyclohydrolase I mRNA levels remained steady at POD6 versus allografts at POD4 (see Figure 2). Thus the changes in GTP cyclohydrolase I may be related to increased protein degradation occurring in allografts. In contrast, GTP cyclohydrolase I protein levels in native hearts of either isograft or allograft recipients remain constant throughout the time course of these studies

Figure 6 GTP cyclohydrolase I and β -actin protein in cardiac grafts and native hearts of graft recipients

Tissues frozen with liquid nitrogen from isografts and allografts from POD4 and POD6 were homogenized and proteins were determined by Western blot (upper panels) followed by densitometry (lower panels). Densitometry represents the means $\pm$ S.E.M. for three in each group normalized to the isograft at POD4 used for each blot. * $P < 0.05$ allografts at POD6 versus allografts at POD4 or versus isografts at POD6.

Figure 7 Time-dependent changes in ISHLT histological rejection scores in cardiac allografts

Paraffin-embedded sections were stained with haematoxylin and eosin and scored using ISHLT criteria. The findings show two stages of rejection in allografts (early: POD2 through POD4, solid bars; late: POD5 and POD6, open bars). Isograft (Iso) controls at POD6 are shown for comparison. Results are the means $\pm$ S.E.M. for $n = 5$ or more. ¶ $P < 0.001$ allografts at respective day versus allografts at POD1, (¶) $P < 0.001$ allografts at respective day versus allografts at POD4.

(Figure 6). Thus these results show that down-regulation of GTP cyclohydrolase I protein levels are time-dependent and specific to allografts and that these changes correlated with the low iNOS bioactivity at POD6.

Rejection score and *in situ* graft function

Histological evidence for rejection was not apparent at any time in either isografts (results not shown) or in allografts at POD1 (Figure 7). Rejection scores were increased in allografts on POD2 at a level that continued through POD4 (Figure 7). A secondary stage of rejection was revealed by significantly ($P < 0.001$) higher rejection scores on POD5 and POD6 relative to allografts at POD2 through POD4 and relative to isograft controls shown here for POD6 (Figure 7). As an independent assessment of graft function, we also performed *in situ* sonomicrometry on isografts and allografts. No change in any parameter was shown in either isografts or allografts from POD2 through POD4 (results

Figure 8 *In situ* analysis of cardiac function in isografts versus allografts

Function was determined after placing ultrasonic crystals on the exterior of the heart in pentobarbital-anaesthetized animals at POD4 (solid bars) and POD6 (open bars) in isografts (Iso4 and Iso6 respectively) versus allografts (Allo4 and Allo6 respectively). Analysis shows decreases in diastolic and systolic diameter, heart rate and percentage of fractional shortening in allografts at POD6. Results are the means $\pm$ S.E.M. for $n = 8$. ¶ $P < 0.001$ allografts at POD6 versus allografts at POD4 and corresponding isografts at POD6.

not shown). Therefore we compared the function at the two time periods of POD4 and POD6. Heart rate was significantly ($P < 0.01$) lower in allografts versus isografts but only on POD6 (Figure 8). Both end diastolic and end systolic diameters were significantly ($P < 0.01$) elevated in allografts versus isografts at POD6 and versus allografts earlier at POD4 (Figure 8). Furthermore, short-axis fractional segment shortening (% SS) was decreased in allografts versus isografts at POD6 and versus allografts at POD4 (Figure 8).

DISCUSSION

The major observations are: (i) NO production does not correlate with iNOS expression levels at the late stages of graft rejection; (ii) the lack of intra-graft NO production coincided with a late secondary decline in biopterin levels. This late decline arose from decreased GTP cyclohydrolase I protein, a key enzyme responsible for BH₄ synthesis.

iNOS in cardiac transplants

Up-regulation of iNOS has been believed to play a role in acute cardiac allograft rejection. Many studies have used iNOS gene expression (protein and mRNA) and peripheral measurements of NO metabolites in plasma and urine compartments to assess the role of NO from iNOS. However, despite the marked up-regulation in iNOS expression, secondary declines in plasma or urine NO metabolites have been demonstrated in models of acute cardiac rejection [19–21]. The secondary decline in NO metabolites in the periphery of acutely rejecting cardiac allografts has been hypothesized to be related to decreased NO release into the circulation due to destruction of graft microcirculation or to decreased substrate or cofactors for iNOS in the graft [21] although this has never been directly established. Furthermore, the peripheral measures of NO metabolites are limited in that they do not give information specific to the allograft. This may be a potential explanation for the lack of correlation of plasma

nitrate levels and systolic and diastolic myocardial dysfunction in human cardiac transplantation [22].

In the present study, we show that iNOS expression was distinctively up-regulated in allografts versus isografts with comparable levels at early (i.e. POD4) versus late (i.e. POD6) stages of graft rejection. Although we found no difference in iNOS mRNA at these two time points, the NO bioactivity in allografts was profoundly increased relative to isografts at POD4. However, this increase was not sustained decreasing to isograft levels by POD6. Furthermore, this could not be explained by any secondary decreases in intrinsic iNOS enzyme activity based upon the arginine-to-citrulline assays. Rather, an unexpected increase in intrinsic enzyme activity was seen in allograft homogenates at POD6 versus POD4 despite the fact that the increase in iNOS protein levels in these same homogenates was similar at both time periods. Since these enzyme activities measured *ex vivo* are determined with optimal substrate and cofactors, they do not give information about NO bioactivity derived from iNOS under *in vivo* conditions. For this reason, we determined endogenous intragraft NO content by the Griess reaction assay. We found that the increase in NO levels was transiently increased at POD4 but not at POD6 suggesting post-translational modification of iNOS activity *in vivo* at later stages of rejection.

Traditionally, calcium-independent activity has been considered to represent iNOS activity [16]. More recently, it is recognized that other NOS isoforms such as cNOS are able to enhance NO production via calcium-independent pathways involving kinase phosphorylation and activation (e.g. Atk, PI3K and PKA). In contrast, calmodulin kinase-dependent phosphorylation of nNOS is considered to be inhibitory. In our model, cNOS in allografts by day 6 is unchanged relative to isografts (G. M. Pieper, V. Nilakantan and N. L. N. Halligan, unpublished work). Even considering any putative increase in NO via calcium-independent activity of non-iNOS isoforms, the amount produced would be so overwhelmed compared with the high output by iNOS that it could not be argued to contribute significantly to the increase in NO signal produced in the analysis.

Support for this concept include previous studies showing that the iNOS-specific inhibitor, L-NIL [N^6 -(1-iminoethyl)-L-lysine], blocked in a concentration-dependent manner the increase in plasma NO metabolite levels seen in allografts at both POD4 and POD6 back to isograft control levels [2]. Furthermore, specificity for iNOS for calcium-independent NOS activity based on assays of citrulline production has been previously confirmed in rat allografts by the blockade of citrulline production in the presence of the iNOS inhibitor, aminoguanidine [17].

GTP cyclohydrolase I in cardiac transplants

To date concurrent measurements of both GTP cyclohydrolase I gene expression and biopterin measurements in any model of solid organ graft rejection are lacking. This has precluded any information in the understanding of the potential role of the BH₄ pathway in regulating iNOS bioactivity in solid organ rejection. Here it is shown that total biopterin levels are reduced at later versus early stages of rejection. The finding that protein levels for GTP cyclohydrolase I were also decreased suggest that the decline from the peak increase in BH₄ levels at later stages of rejection in allografts is related, at least in part, to decreased synthesis via this enzymatic pathway. Interestingly, this decrease cannot be explained by changes in mRNA for GTP cyclohydrolase I. The latter suggests that the secondary decrease in GTP cyclohydrolase I protein is related to some unknown post-transcriptional event. Possible factors could be via decreased stability in GTP cyclohydrolase I mRNA or increased protein turnover.

Differential profile of iNOS and GTP cyclohydrolase expression

In a possibly related context, it is often found that GTP cyclohydrolase I and iNOS are up-regulated in response to cytokines or other inflammatory stimuli; however, more recent evidence suggests that concurrent up-regulation of BH₄ and NO synthesis in response to cytokines may be regulated by divergent pathways [23,24]. Here we show a dissimilar expression of GTP cyclohydrolase I and iNOS at POD4 versus POD6. Consequently, functional activity of GTP cyclohydrolase I was decreased at POD6 from the peak increase at POD4 in allografts. These findings are reminiscent of previous findings in a hepatocyte model in which lipopolysaccharides induced an increase in GTP cyclohydrolase I protein and activity that reached a peak followed by a decrease to baseline some 24 h later [25]. At present the exact mechanism responsible for the decrease in GTP cyclohydrolase protein levels and activity is not known. However, one possibility is that it may involve activation of the proteasome as a direct consequence of increased NF- κ B S-nitrosylation by NO [26] secondary to conditions of high levels of stimulated NO generation.

Implications of suboptimal biopterin production and NO formation

Previous studies have shown that inhibition of NO levels hinders the normal course of graft rejection [1–5]. Since biopterin levels closely follow NO production by iNOS, it seemed plausible to speculate that a decrease in biopterin production could be beneficial for the graft survival. However, as shown in the present study, biopterin depletion appears to be an equally important component in the event leading to graft rejection. It has been shown that low levels of BH₄ promote superoxide anion radical production from purified endothelial NOS [27], which has been considered to be a mechanism contributing to the loss of endothelial-dependent relaxation by atherosclerotic rabbit aortas [28]. Also, the biopterin-free form of recombinant iNOS protein has been shown to produce superoxide anion radicals [29]. Thus it is possible under *in vivo* conditions that suboptimal conditions may uncouple iNOS causing it to produce superoxide.

It is possible that increases in superoxide production in the setting of NO production may provide a source for peroxynitrite formation and subsequent nitration of myocardial protein. In this context, we have shown increased nitration at POD6 in allografts that is prevented using an NO neutralizing agent suggesting a role of NOS [30]. Furthermore, we have also recently shown that nitration was increased in allografts only at POD6 (and not POD4) and this nitration was preventing the use of an iNOS enzyme inhibitor indicating that nitration arises primarily from an iNOS-dependent pathway [31].

We observed a 3-fold increase in total biopterin in our cardiac allografts similar in magnitude to the levels achieved in cardiac myocytes compared with endothelial cells stimulated with inflammatory stimuli [32]. Collectively, this indicates that biopterin may be inadequate to sustain optimal NO production in cardiac myocytes. In support of this notion, we have recently reported little or no increase in NO released into the media of cardiac myocytes stimulated *ex vivo* with inflammatory stimuli such as lipopolysaccharide or various cytokines despite up-regulation of iNOS and despite increased biopterin synthesis [33]. Thus biopterin levels are unable to sustain adequate NO production in cardiac myocytes *ex vivo*. It is likely that a similar response occurs in later stages of cardiac alloimmune activation *in vivo*. The implications of our studies are that low biopterin supply not only will decrease NO formation from iNOS but may also be a potential source of increased reactive oxygen species.

This would consequently increase the level of oxidative stress and nitrosative stress.

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