Role of Nitric Oxide and Superoxide in Acute Cardiac Allograft Rejection in Rats (44564)

EIJI AKIZUKI,*† TAKAAKI AKAIKE,* SINICHIROU OKAMOTO,* SHIGEMOTO FUJII,* YASUO YAMAGUCHI,† MICHIO OGAWA,† AND HIROSHI MAEDA*,[1]

Departments of Microbiology and †Surgery II, Kumamoto University School of Medicine, Kumamoto 860–0811, Japan

Abstract. **The role of NO and superoxide (O_2^-) in tissue injury during cardiac allograft rejection was investigated by using a rat *ex vivo* organ perfusion system. Excessive NO production and inducible NO synthase (iNOS) expression were observed in cardiac allografts at 5 days after cardiac transplantation, but not in cardiac isografts, as identified by electron spin resonance spectroscopy and Northern blotting. Cardiac isografts or allografts obtained on Day 5 after transplantation were perfused with Krebs bicarbonate buffer with or without various antidotes for NO or O_2^-, including N^ω-monomethyl-L-arginine (L-NMMA; 1 m*M*), 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO; 100 μ*M*), 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP; a xanthine oxidase inhibitor; 100 μ*M*), and superoxide dismutase (SOD; 100 units/ml). Treatment of the cardiac allografts with PTIO showed most remarkable improvement of the cardiac injury as revealed by significant reduction in aspartate transaminase, lactate dehydrogenase, and creatine phosphokinase concentrations in the perfusate. Similar but less potent protective effect on the allograft injury was observed by treatment with L-NMMA, AHPP, and SOD. Immunohistochemical analyses for iNOS and nitrotyrosine indicated that iNOS is mainly expressed by macrophages infiltrating the allograft tissues, and nitrotyrosine formation was demonstrated not only in macrophages but also in cardiac myocytes of the allografts, providing indirect evidence for the generation of peroxynitrite during allograft rejection. Our results suggest that tissue injury in rat cardiac allografts during acute rejection is mediated by both NO and O_2^-, possibly through peroxynitrite formation.** [P.S.E.B.M. 2000, Vol 225:151–159]

Excessive production of nitric oxide (NO) has been observed during organ transplant rejection (1–4), and several different experimental approaches strongly suggest that the rejected organ is the source of increased end products of L-arginine-NO synthesis. In a study of rat cardiac allograft rejection, nitrosyl iron hemoglobin signals were detected in grafts but not in other recipient organs (3). Activated/infiltrated phagocytes produce high levels of both superoxide and NO, with the formation of peroxynitrite (5), which may contribute critically to the NO-induced cellular damage in various diseases (6–10). In fact, it has been suggested that peroxynitrite may exert significant cellular toxicity through its potent nitrating and oxidizing properties (6–10). Specifically, peroxynitrite mediates oxidative injury of a variety of biomolecules including proteins and nonprotein thiols (11), DNA (12), and lipids (13), and it also nitrates aromatic amino acids, most typically tyrosine (14, 15). Although opposing effects of NO have been recently reported in the model of cardiac rejection (16), we envisaged that high levels of both O_2^- and NO through peroxynitrite formation may induce tissue injuries of the transplanted allografts during transplant rejection.

It is now well documented that N^ω-monomethyl-L-arginine (L-NMMA) suppresses overproduction caused by inducible NO synthase (iNOS), showing beneficial effects in various disease models, including viral infections, septic

This work is supported by a Grant-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan, and a grant from the Ministry of Health and Welfare of Japan for surveys and research on specific diseases.

[1] To whom requests for reprints should be addressed at the Department of Microbiology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan. E-mail: msmaedah@gpo.kumamoto-u.ac.jp

Received December 20, 1999. [P.S.E.B.M. 2000, Vol 225]
Accepted May 8, 2000.

0037-9727/00/2252-0151$15.00/0

shock, and bacterial meningitis (17–19). We found previously that 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) can scavenge NO and completely abolish biological activity of NO *in vitro* as well as *in vivo* (20, 21), and that PTIO exhibits a potent antihypotensive effect in endotoxin shock in animals and has a therapeutic effect in cardiac and circulatory disorders (21). Xanthine oxidase (XO) is supposed to be a major O_2^--producing enzyme in the vascular system (22) and also in inflamed tissue (17). We reported earlier that 4-amino-6-hydroxypyrazolo[3,4-*d*]pyrimidine (AHPP) specifically inhibits the generation of O_2^- and conversion of hypoxanthine and xanthine to uric acid (23).

In the present study, we investigated the roles of NO and O_2^- in tissue injury during cardiac allograft rejection in rats. An *ex vivo* organ perfusion system was employed for the analysis of NO biosynthesis and of the effect of various antidotes for NO and O_2^-, such as L-NMMA, PTIO, AHPP, Cu,Zn-superoxide dismutase (SOD), and PTIO, on cardiac injury caused by organ rejection responses. Our present results indicate that NO and O_2^- and their reaction product peroxynitrite may contribute to the pathogenesis of cardiac allograft rejection.

Materials and Methods

Animals. Male LEW(RT1 ^{l}) rats were used as recipients and ACI(RT1^a) rats as donors. These rats were obtained from the Central Institute for Experimental Animals, Kawasaki, Japan. This strain combination is fully allogeneic, and thus acute rejection of cardiac transplants results (24). The ACI-to-ACI combination was used for control isografts. All animals, weighing 225–250 g, were maintained under standard conditions and received water and rodent chow *ad libitum*.

Cardiac Transplantation. Heterotopic cardiac transplantations were performed with the cuff method. Donor ACI hearts were anastomosed to the internal carotid artery and jugular vein of LEW recipients. Cardiac function was monitored daily by palpation. A rejection reaction of the cardiac allograft was determined by a decrease in the number of beats or contractions of the hearts.

***Ex Vivo* Perfusion of Isolated Cardiac Grafts.** Cardiac grafts were obtained from transplant recipients under ether anesthesia on Day 5 after transplantation. The hearts were excised, and the aorta was cannulated with polyethylene tubing (Becton Dickinson, Sparks, MD). Retrograde perfusion of the hearts was then performed by perfusing from the aorta at a constant pressure of 80 mmHg with Krebs bicarbonate buffer (17 m*M* glucose, 120 m*M* sodium chloride, 25 m*M* sodium bicarbonate, 2.5 m*M* calcium chloride, 5.9 m*M* potassium chloride, and 1.2 m*M* magnesium chloride), which was treated with 95% O_2 and 5% CO_2 gas at 37°C, as described previously (25). After the initial 20-min perfusion period to remove the endogenous blood (plasma) components from the heart tissues, the organ perfusion was continued with 20 ml of Krebs bicarbonate buffer, and the efflux from the organ was pooled and recycled for the perfusion. The *de novo* release of NO and various enzymes from the cardiac tissues during perfusion was analyzed with use of the organ perfusate. Specifically, the perfusates that were recovered as efflux from the right atrium were obtained every 10 min during a 2-hr perfusion to measure nitrite/nitrate concentrations as well as various cardiac enzymes, such as aspartate transaminase (AST), lactate dehydrogenase (LDH), and creatine phosphokinase (CPK), as described below.

Ex vivo cardiac perfusion was performed with the perfusate containing L-NMMA (Calbiochem-Novabiochem International, San Diego, CA); PTIO, a scavenger for NO (Wako Pure Chemical, Osaka, Japan); AHPP, an XO inhibitor (Aldrich Chemical, Milwaukee, WI); and Cu,Zn-SOD (Sigma Chemical, St. Louis, MO).

Cardiac grafts were randomly divided into seven experimental groups: Group I, untreated isografts; Group II, untreated allografts; Group III, allografts treated with L-NMMA (1 m*M*); Group IV, allografts treated with PTIO (100 μ*M*); Group V, allografts treated with L-NMMA (1 m*M*) plus PTIO (100 μ*M*); Group VI, allografts treated with AHPP (100 μ*M*); and Group VII, allografts treated with Cu,Zn-SOD (100 units/ml).

Measurement of Nitrite/Nitrate and Various Enzymes in Plasma and Perfusate. Blood was obtained by cardiac puncture, and plasma from four transplant recipients was separated by centrifugation at 1, 3, 5, and 7 days after transplantation. The nitrite/nitrate concentration was determined by using high-performance liquid column chromatography combined with a Griess reagent flow reactor (26). To minimize the influence of food intake on plasma nitrite/nitrate levels, the animals received distilled water and were fasted for 24 hr before they were sacrificed. Similarly, nitrite/nitrate produced from cardiac grafts was quantitated by use of the organ perfusate. In addition, activities of AST, LDH, and CPK in plasma and perfusate were determined by using a sequential multiple autoanalyzer system (Hitachi Ltd, Tokyo, Japan).

Electron Spin Resonance (ESR) Spectroscopy for NO Detection in Cardiac Grafts. NO generation in cardiac tissues was measured directly by ESR spectroscopy with a diethyldithiocarbamate (DETC)-iron complex as a spin trap for NO (17). Briefly, DETC · $3H_2O$ (Wako Pure Chemical) and $FeSO_4$ · $7H_2O$ were administered intramuscularly at different sites in the lower limbs of the rats at 400 mg/kg and 20 mg/kg body weight, respectively. Thirty minutes after injection of DETC and $FeSO_4$, the heart was perfused with saline containing heparin (10 units/ml) for 5 min, with the rat under pentobarbital anesthesia, after which the heart was removed and then frozen immediately in a quartz sample tube. ESR spectroscopy was carried out by using an X-band ESR spectrometer (Bruker ESP 380E, Rheinstein, Germany) at 110K. The conditions for ESR

measurement were microwave 4 mW and modulation amplitude 0.5 mT.

Immunohistochemical Analyses. After cardiac transplant recipients were exsanguinated at 5 days after transplantation ($n = 4$ for each group), immunohistochemistry was performed to examine iNOS expression, nitrotyrosine formation, and macrophage infiltration in the cardiac grafts as described previously (17, 27–29). The grafted hearts were excised and fixed with 2% periodate-lysine-paraformaldehyde for 6 hr. The tissue was then embedded in tissue-embedding medium and frozen in liquid nitrogen, followed by preparation of 6-μm-thick sections by use of a cryostat. After inhibition of endogenous peroxidase activity, the specimens were incubated with an antimurine iNOS polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hr at room temperature. After the sections were washed with 0.01 *M* phosphate-buffered 0.15 *M* saline (PBS, pH7.4), they were incubated for 1hr with a sheep anti-immunoglobulin [$F(ab')_2$] conjugated with peroxidase diluted 1:100. Tissue-bound peroxidase activity was visualized by using 3,3′-diaminobenzidine as substrate; hematoxylin was used for nuclear staining. Similarly, formation of nitrotyrosine in cardiac grafts was detected with an antinitrotyrosine polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) (17). In addition, tissue sections were stained immunohistochemically with a specific monoclonal antibody against rat macrophages (RM-4) (29). As the control, sections were incubated with nonimmunized mouse serum or PBS instead of primary antibody, after which they were processed in the manner just described.

Northern Blot Analysis for iNOS Expression. Cardiac specimens were obtained from the animals at 5 days after transplantation. The specimens were then quickly frozen in liquid nitrogen and stored at −70°C before RNA extraction. iNOS expression was examined by Northern blotting as described previously (27, 28). Briefly, formaldehyde-agarose gel electrophoresis of total RNA (20 μg), which had been extracted from the tissues with Trizol reagent (Gibco BRL, Gaithersburg, MD), was performed, after which the RNA was transferred to a positively charged nylon membrane (Hybond-N^+, Amersham International plc, Little Chalfont, England) by a capillary transfer method. The membrane was then hybridized with a DNA probe for the iNOS in 5 × SSPE (0.9 *M* NaCl, 0.05 *M* sodium phosphate, and 5 m*M* ethylenediaminetetraacetic acid; pH 7.7) plus 0.5% sodium dodecyl sulfate (SDS), 5 × Denhardts solution, and 0.5 μg/ml salmon sperm DNA at 65°C. Similarly, Northern blot analysis was performed with use of a cDNA fragment for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a control housekeeping gene in the heart. The ^{32}P-labeled cDNA probe for iNOS was prepared by the random primer technique (Megaprime DNA labeling system, Amersham) using a rat iNOS cDNA fragment (−97~ +429) as a template. After the membrane was hybridized with the iNOS DNA probe, it was washed twice for 10 min in 2 × SSPE plus 0.5% SDS at room temperature and twice for 20 min in 0.1 × SSPE plus 0.5% SDS at 65°C. The radioactive band on the hybridized membrane was detected with a bioimage analyzer (BAS2000, Fuji Photo Film, Tokyo, Japan).

Statistical Analysis. Analysis of variance (ANOVA) and the *t* test of independent means were used to determine differences between multiple groups and two groups, respectively. When F ratios were significant, means were compared by using Bonferroni's test as the *posthoc* comparison. *P*-values of <0.05 were considered significant.

Results

Cardiac allografts from ACI to LEW rats survived 7.0 ± 0.9 days (means ± SD, $n = 9$), whereas control ACI-to-ACI isografts survived indefinitely (>60 days). In the allogeneic combination, cellular infiltration was prominent at early time points (Days 3 and 5) and progressed to severe diffuse tissue damage by Day 8. In cardiac isografts, the inflammatory infiltrates after transplantation were considerably less extensive than those in cardiac allografts (data not shown).

The levels of AST, LDH, and CPK in the plasma of cardiac allograft recipients were significantly higher than those observed in isograft recipients on Day 5 after transplantation (Fig. 1). The plasma nitrite/nitrate concentrations in allograft recipients were significantly elevated on Days 5 and 7 after transplantation compared with those in cardiac isograft recipients (Fig. 2). A strong ESR signal of the NO-

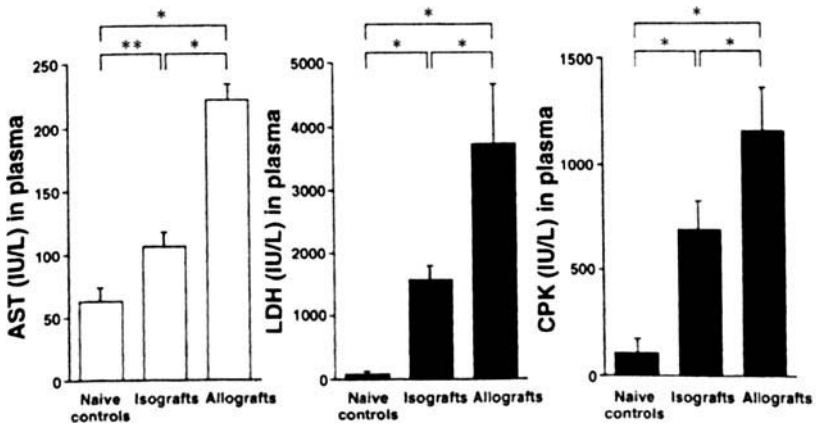


Figure 1. Plasma concentrations of AST, LDH, and CPK on Day 5 after transplantation. Each experimental group consisted of four animals. Data represent means ± SEM. *$P < 0.01$; **$P < 0.05$; AST, aspartate transaminase; LDH, lactate dehydrogenase; CPK, creatine phosphokinase.

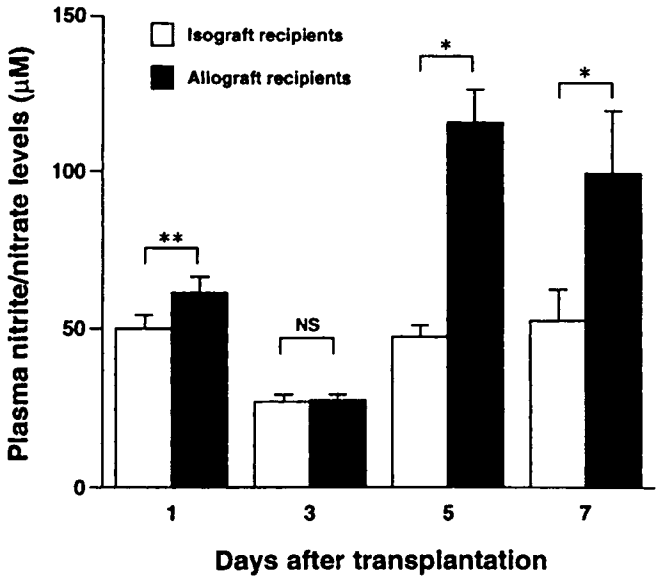


Figure 2. Time profile of change in plasma nitrite/nitrate concentrations in recipient rats after cardiac transplantation. Each experimental group consisted of four animals. Data represent means ± SEM. *P < 0.01; **P < 0.05; NS, not significant.

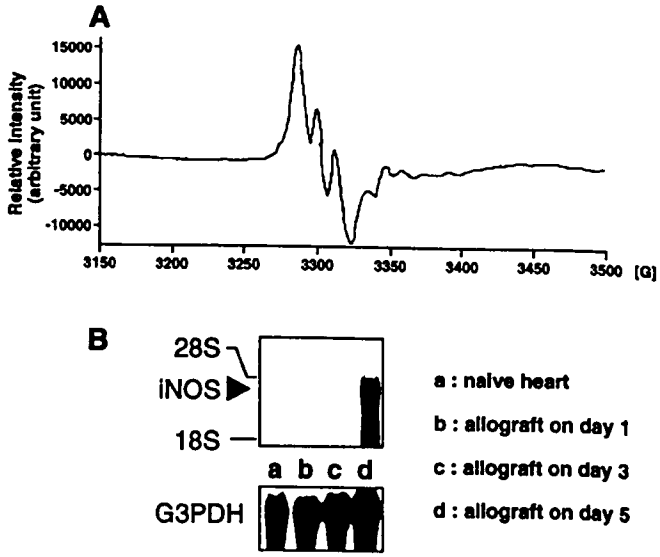


Figure 3. (A) NO production as detected by ESR spin trapping and (B) iNOS mRNA expression as identified by Northern blotting in cardiac allografts. ESR spectroscopy was performed at 110K by using a DETC-Fe complex with the grafted heart on Day 5 after transplantation. iNOS mRNA was assayed by Northern blotting with use of a cDNA probe for rat iNOS or G3PDH and naive heart or allografts obtained at 1, 3, and 5 days after transplantation. Note that (A) a characteristic three-line signal of the DETC-Fe-NO adduct was observed for the cardiac allograft, and (B) simultaneously strong iNOS mRNA expression was evident.

DETC-iron adduct as well as induction of iNOS mRNA expression was observed for cardiac allografts on Day 5 (Figs. 3A & 3B), suggesting excessive production of NO induced by iNOS in heart tissue during acute rejection.

To further identify the iNOS expression in the allograft tissues, immunohistochemical analysis was performed and iNOS localization was correlated with that of macrophages. The iNOS expression was localized mainly in macrophages infiltrating the allograft tissue (Figs. 4A vs 4B; Figs. 4D vs 4E). Interestingly, immunohistochemical analysis for nitrotyrosine showed that nitrotyrosine formation was evident not only in the macrophages infiltrating the interstitial spaces of the heart but also in cardiac myocytes (Figs. 4C & 4F). Control staining without each primary antibody showed no appreciable immunohistochemical reaction in the sections of allografts and isografts (unpublished data). Similarly, no significant immunostaining for iNOS and nitrotyrosine was observed with the transplanted isograft, although a very little staining for macrophages was observed in the same tissue specimens.

NO production from cardiac allografts was further evaluated by using an *ex vivo* organ perfusion system, and the amount of NO generated was assessed by measuring both nitrite and nitrate in the perfusate (Fig. 5). Nitrite/nitrate concentrations of the perfusate in allografts (Group II) were significantly higher than those in isografts (Group I) (P < 0.01, Group I vs Group II). Treatment of allografts with L-NMMA (Group III) significantly reduced nitrite/nitrate levels in the perfusate compared with untreated allografts (Group II) (P < 0.01, Group II vs Group III). Although allografts treated with PTIO (Group IV) showed higher perfusate nitrite/nitrate concentrations than did untreated allografts (Group II) (P < 0.01, Group II vs Group IV), a decrease in perfusate nitrite/nitrate concentrations was observed by treatment with both L-NMMA and PTIO (Group V) (P < 0.01, Group IV vs Group V). Nitrite/nitrate concentrations in allografts treated with either AHPP (Group VI) or SOD (Group VII) were lower than those in untreated allografts (Group II) (P < 0.05, Group II vs Group VI or VII) but higher than those in control isografts or allografts treated with L-NMMA (Group III) (P < 0.01, Group I vs Group VI or VII; Group III vs Group IV or VII). The combination treatment of the allograft with L-NMMA plus SOD or AHPP resulted in almost complete suppression of nitrite/nitrate production (unpublished data).

Perfusate AST, LDH, and CPK concentrations at 2 hr after *ex vivo* cardiac perfusion are shown in Figures 6 and 7. The levels of all three enzymes in the perfusate of untreated allografts (Group II) were significantly higher than those in isograft perfusate (Group I). Treatment of allografts with L-NMMA (Group III; Fig. 6) resulted in significantly lower enzyme levels compared with those of untreated allografts (Group II; Fig. 6). In addition, PTIO (Group IV; Fig. 6) produced the greatest reduction in concentrations of all enzymes in the perfusate in comparison with untreated allografts (Group II) or allografts treated with L-NMMA (Group III). However, treatment with L-NMMA and PTIO (Group V; Fig. 6) resulted in higher enzyme levels than in groups treated with each agent alone (III and IV). Treatment of allografts with either AHPP (Group VI; Fig. 7) or SOD (Group VII; Fig. 7) significantly lowered the levels of AST, LDH, and CPK compared with those in untreated allografts (Group II).

Also, we examined the effect of concentrations of PTIO

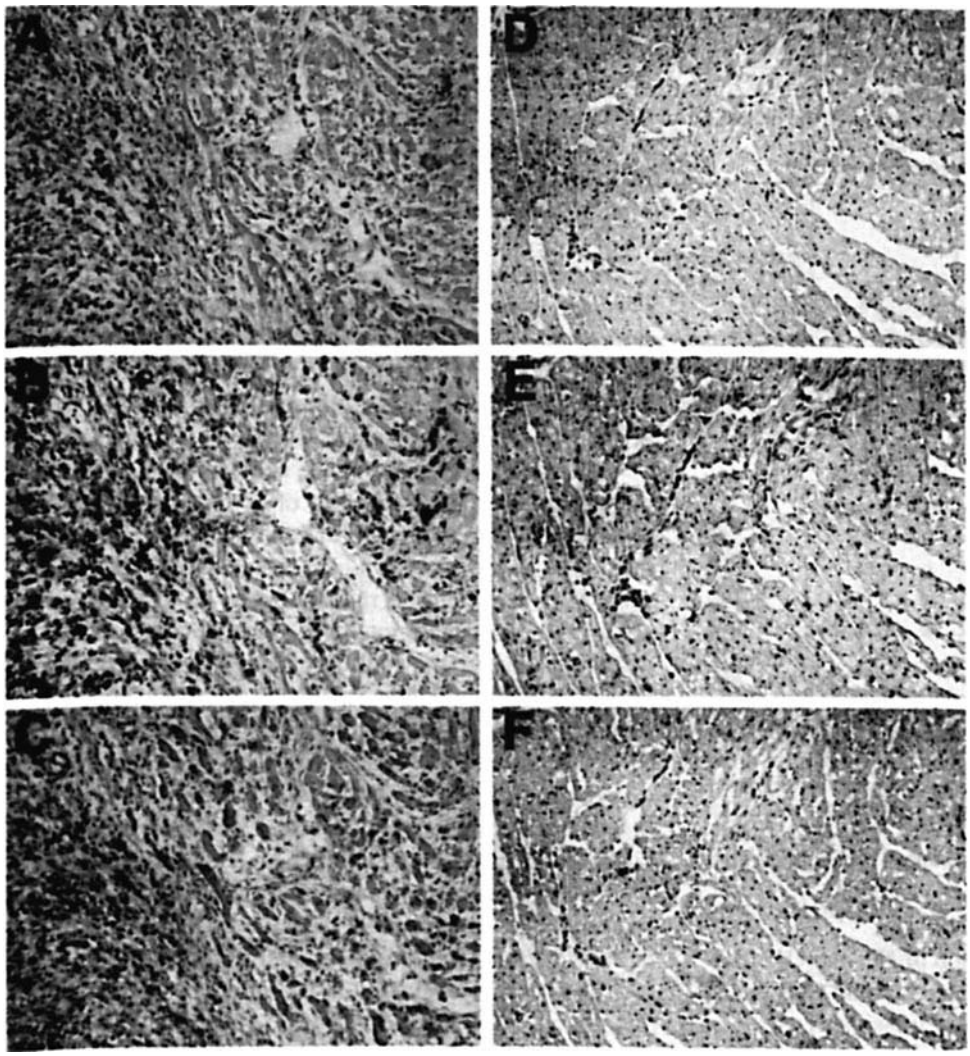


Figure 4. Immunohistochemistry for iNOS, macrophages, and nitrotyrosine in cardiac allografts. Immunostaining was performed with use of anti-iNOS antibody, antinitrotyrosine antibody, or anti-rat macrophage antibody (RM-4) with cardiac allografts obtained on Day 5 after transplantation. (A, B, and C) Serial sections (B → A → C) of allograft tissues immunostained with anti-iNOS, antimacrophage, and antinitrotyrosine antibodies, respectively. (D, E, and F) Serial sections (E → D → F) of isograft tissues immunostained with anti-iNOS, antimacrophage, and antinitrotyrosine antibodies, respectively. Magnification: ×90. Extensive infiltration of macrophages were observed mainly in the peripheral interstitial spaces of the cardiac allografts (A–C).

on the allograft rejection. As shown in Figure 8, the maximum reduction of LDH release was obtained with 100 μM PTIO; the elevated level of the enzyme released was decreased to the normal range of control cardiac tissues (normal naive hearts and isograft transplant). PTIO up to 100 μM showed a dose-dependent suppression of the cardiac enzyme release, but the suppressive effect was not so remarkable at a concentration of 200 μM compared with those of lower doses of PTIO. A similar bell-shape pattern of improvement of the cardiac enzyme release was observed with L-NMMA particularly at the concentrations more than 1 mM (unpublished data). Furthermore, to confirm that iNOS induced as a specific response to allograft transplants is responsible for the cardiac allograft rejection, we examined the effect of PTIO on the enzyme release from isograft as a control transplant. The result showed that PTIO did not appreciably affect the level of the cardiac enzymes.

Discussion

In the present study, the contribution of NO and O_2^- to the rejection response of rat cardiac allografts was investigated. By using a rat model of cardiac transplantation, we showed that a high level of NO was produced by the transplanted organ during acute rejection. Upregulation of iNOS mRNA was clearly demonstrated, and overproduction of NO was identified directly by ESR spectroscopy of the grafted heart.

Our present study using an *ex vivo* allograft perfusion system showed involvement of NO generation in myocardial damage in cardiac allografts (Figs. 5–8). Although NO inhibitors used in this study are not entirely specific to iNOS, the NOS inhibitor L-NMMA as well as the NO scavenger PTIO reduced tissue injury in the cardiac allograft as assessed by levels of various enzymes released from myocardial cells in the organ perfusate. In contrast, we did not observe any appreciable effect of PTIO on the enzyme release from isografts, indicating that the beneficial effect of the NO scavenger is brought about by its suppression of NO from iNOS specifically induced in the allografts after transplantation. In addition, because both NO antidotes (L-NMMA and PTIO) and O_2^- inhibitors (AHPP and SOD) reduced injury of the cardiac allografts, as evidenced by our *ex vivo* organ perfusion study, O_2^- (not only NO) also contributes to the allograft rejection reaction. Collectively,

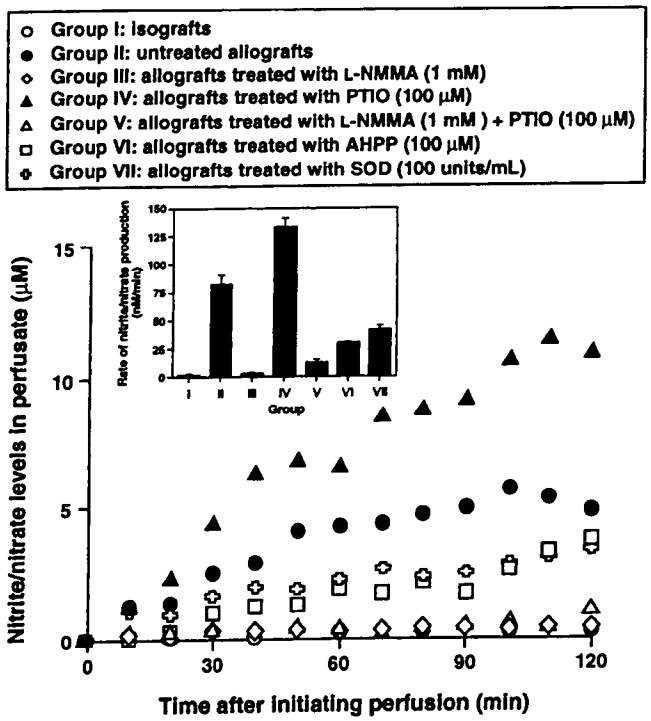


Figure 5. Time profile of release of nitrite/nitrate concentrations to the perfusate during *ex vivo* cardiac perfusion. The cardiac grafts were obtained on Day 5 after transplantation. Each experimental group consisted of four animals. Data represent mean values for each time point after initiating organ perfusion. The rate of nitrite/nitrate formation in the perfusate in each group is shown in the inset as means ± SEM.

these data indicate that overproduction of NO and O_2^- leading to peroxynitrite formation causes cytotoxic effects and pathological consequences for cardiac transplants.

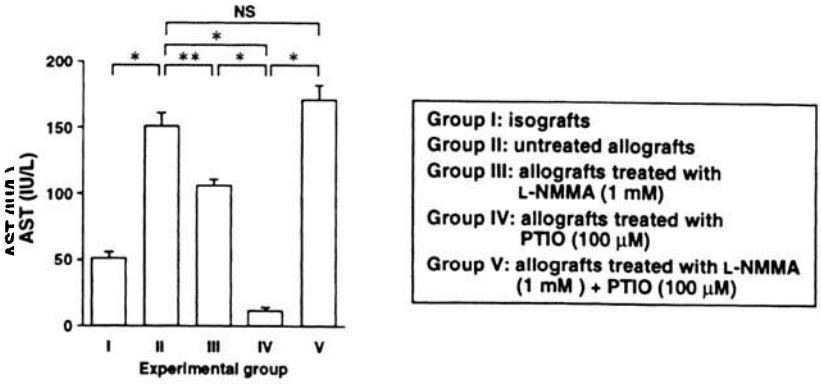
However, enzyme levels in the organ perfusate of the allografts increased after simultaneous treatment with L-NMMA and PTIO compared with those after either L-NMMA or PTIO alone. Similarly, although PTIO shows a potent cytoprotective action for the allograft rejection, the suppressive effect on the cardiac damage is not so remarkable at a high dose of PTIO (200 μ*M*). This might not be due to the direct toxic effects of PTIO *per se* or of its reaction products with NO because the same trend is observed with L-NMMA treatment of the allografts in terms of the effect of its concentrations. Therefore, complete inhibition of NO appears not to be necessarily beneficial to the allograft survival, but rather it may worsen cardiac allograft damage. Accordingly, the role of NO in organ rejection seems to be dual with deleterious and beneficial effects. Such opposing effects of NO are often documented not only for the organ rejection (16, 30–32) but also for other diseases such as mircobial infections and inflammatory disorders (17, 28, 33–35).

The diverse physiological and pathophysiological functions of NO may be brought about through formation of different redox forms of NO such as nitrosothiols and peroxynitrite (7–10, 36, 37). For example, formation of nitrosothiols such as *S*-nitrosoglutathione is suggested to produce beneficial and cytoprotective effects of NO for various oxidative stress *in vivo* (36–39). In contrast, it is now well accepted that peroxynitrite has cytotoxic potentials through oxidation and nitration of lipids, proteins, and nucleic acids (6–14). Peroxynitrite causes impairment of energy metabolism by suppressing aconitase and mitochondrial function directly, or by consuming intracellular energy stores indirectly (40–43). In addition, we reported recently that peroxynitrite activates precursors of matrix metalloproteinases (44), and it was revealed that a tissue inhibitor of metalloproteinase-1 (TIMP-1) can also be inactivated by peroxynitrite (45). It is thus anticipated that peroxynitrite will be shown to potentiate metalloproteinase-mediated tissue breakdown in the graft rejection model.

Nitration of protein tyrosine residues to give 3-nitrotyrosine can serve as a footprint of peroxynitrite formed *in vivo* (15). In fact, tyrosine nitration is found in tissue specimens obtained in various human and animal disease models, such as atherosclerosis, rheumatoid arthritis, sepsis, viral pneumonia, idiopathic interstitial pneumonia, neurodegenerative diseases, and adult respiratory distress syndrome (14, 17, 46–49). In this study, we identified, by use of immunohistochemistry, nitrotyrosine formation in cardiac allografts, particularly macrophages and cardiac myocytes occurring in the inflamed area of the allograft tissues. This may indicate the generation of biologically reactive peroxynitrite in cardiac allografts during acute rejection responses.

As was proposed recently by Eiserich *et al.* (50), nitrotyrosine could be produced *via* H_2O_2 and nitrite catalyzed by myeloperoxidase to form a putative potent nitrating species. However, our immunohistochemical analysis showed that not only iNOS expression but also nitrotyrosine formation is mainly localized in macrophages infiltrating cardiac tissue, suggesting that peroxynitrite rather than the H_2O_2/nitrite/myeloperoxidase system is more likely to contribute to the tyrosine nitration in the allograft tissues. Recently, there has been some argument about contribution of peroxynitrite to tyrosine nitration occurring *in vivo*. Specifically, Pfeiffer and Mayer (51) reported an apparent lack of tyrosine nitration by peroxynitrite formed *in situ* from NO and O_2^- generated by the hypoxanthine/xanthine oxidase system. However, our recent investigation shows that NO co-generated with O_2^- from xanthine oxidase produces a significant amount of nitrotyrosine even with nanomolar concentrations of NO/O_2^- flux (52). A similar result was observed by Beckman's group using peroxynitrite produced *in situ* from potassium superoxide and NO (53).

Addition of PTIO to the perfusion system of the allografts led to significantly raised nitrite/nitrate concentrations. Because autooxidation of physiological concentrations of NO by O_2 proceeds very slowly in solution at neutral pH under ambient condition (54), PTIO can significantly increase the generation of nitrite from NO produced biologically through its potent NO oxidation reaction as we and other groups reported earlier (55, 56). Therefore, the enhancement of nitrite/nitrate concentrations in the perfus-



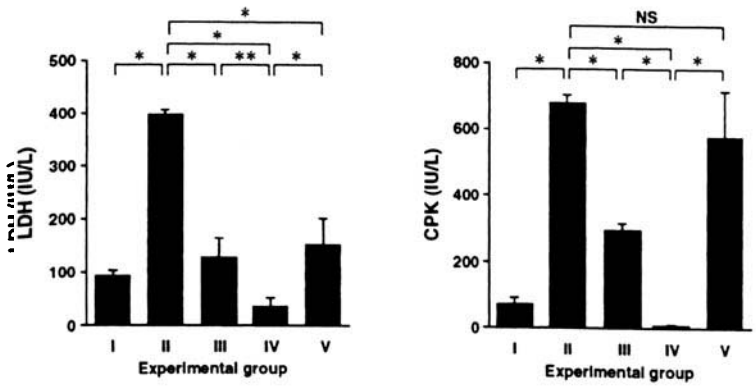
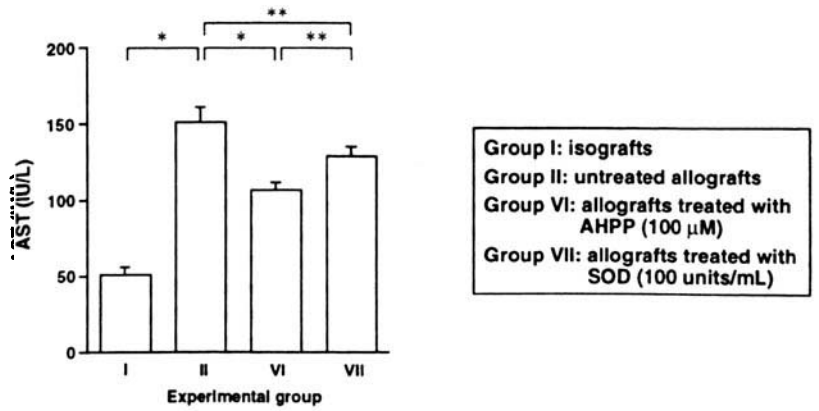


Figure 6. Concentrations of AST, LDH, and CPK in perfusate 2 hr after *ex vivo* cardiac perfusion of allografts obtained on Day 5 after transplantation, with or without L-NMMA and PTIO. Each experimental group consisted of four animals. Data represent means ± SEM. $^{*}P < 0.01$; $^{**}P < 0.05$; NS, not significant.



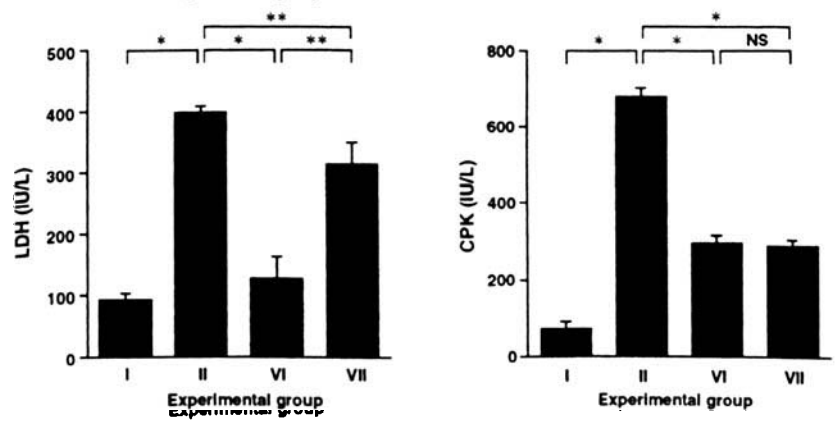


Figure 7. Concentrations of AST, LDH, and CPK perfusate 2 hr after *ex vivo* cardiac perfusion of allografts with or without AHPP or SOD. Each experiment was performed in the same manner as in Figure 6. Data represent means ± SEM. $^{*}P < 0.01$; $^{**}P < 0.05$; NS, not significant.

ate may indirectly indicate that PTIO effectively scavenged NO generated excessively in the cardiac allografts. In contrast, the level of nitrite/nitrate in the perfusate is decreased by SOD and AHPP. The mechanism of the apparent decrease of nitrite/nitrate production remains unclear. A possible and speculative explanation is that NO being trapped rapidly by O_2^- is converted effectively to nitrate (or nitrite in the presence of reducing agents abundant in the tissues); whereas NO that escapes from the reaction with O_2^-, in the presence of SOD or AHPP, may partition between perfusate

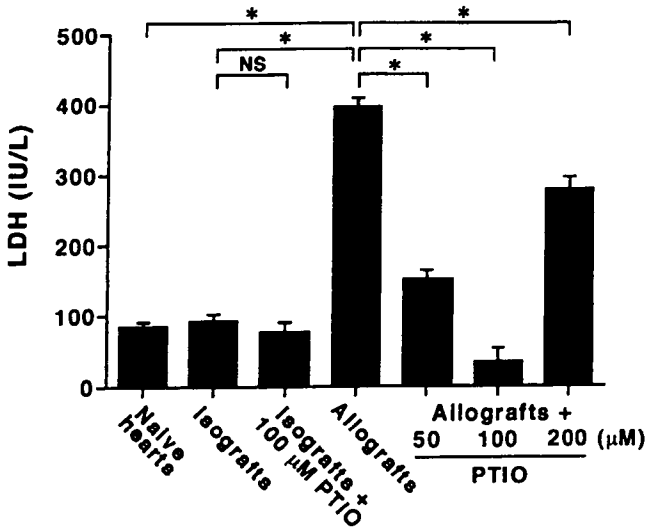


Figure 8. Effect of PTIO concentrations on the level of LDH in the perfusate obtained 2 hr after *ex vivo* cardiac perfusion of cardiac allografts. Normal levels of LDH in perfusates of the normal hearts and isografts are shown as the control. Each experiment was performed in the same manner as in Figure 6. Data represent means ± SEM. $^{*}P < 0.01$; $^{**}P < 0.05$; NS, not significant.

solution and the gas phase during the organ perfusion that is partly open to the atmosphere, resulting in loss of NO releases from the allograft tissue to the atmosphere. Partition of NO to the gas phase from aqueous milieu often occurs due to its physical nature as a gaseous hydrophobic radical. Therefore, we may observe such an apparent reduction in NO production with SOD and AHPP treatment. In any event, further study is needed to identify the mechanism of NO loss in this study.

In conclusion, NO and O_2^- appear to play an important role in acute rejection and tissue damage possibly through peroxynitrite formation. Therefore, pharmacological intervention by using appropriate inhibitors or scavengers of NO and O_2^-, or peroxynitrite *in vivo*, may have significant value in controlling acute rejection of allografted organs.

The authors thank Ms. Judith Gandy for editing our manuscript.

1. Langrehr JM, Hoffman RA, Lancaster JR, Simmons RL. Nitric oxide—a new endogenous immunomodulator. Transplantation **55**:1205–1212, 1993.
2. Langrehr JM, Murase N, Markus PM, Cai X, Neuhaus P, Schraut W, Simmons RL, Hoffman RA. Nitric oxide production in host-versus-graft and graft-versus-host reactions in the rat. J Clin Invest **90**:679–683, 1992.
3. Lancaster JR Jr., Langrehr JM, Bergonia HA, Murase N, Simmons RL, Hoffman RA. EPR detection of heme and nonheme iron-containing protein nitrosylation by nitric oxide during rejection of rat heart allograft. J Biol Chem **267**:10994–10998, 1992.
4. MacMillian-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. Proc Natl Acad Sci U S A **93**:11853–11858, 1996.
5. Ischiropoulos H, Zhu L, Beckman JS. Peroxynitrite formation from macrophage-derived nitric oxide. Arch Biochem Biophys **298**:446–451, 1992.
6. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A **87**:1620–1624, 1990.
7. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and the ugly. Am J Physiol **271**:C1424–C1437, 1996.
8. Freeman BA. Free radical chemistry of nitric oxide: Looking at the dark side. Chest **105**:79S–84S, 1994.
9. Darley-Usmar V, Wiseman H, Halliwell B. Nitric oxide and oxygen radicals: A question of balance. FEBS Lett **369**:131–135, 1995.
10. Rubbo H, Darley-Usmar V, Freeman BA. Nitric oxide regulation of tissue free radial injury. Chem Res Toxicol **9**:809–820, 1996.
11. Radi R, Beckman JS, Bush K, Freeman BA. Peroxynitrite oxidation of sulfhydryls: The cytotoxic potential of superoxide and nitric oxide. J Biol Chem **266**:4244–4250, 1991.
12. Szabó C, Ohshima H. DNA damage induced by peroxynitrite: Subsequent biological effects. Nitric Oxide **1**:373–385, 1997.
13. Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys **288**:481–487, 1991.
14. Ischiropoulos H. Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. Arch Biochem Biophys **356**:1–11, 1998.
15. Crow JP, Beckman JS. Reactions between nitric oxide, superoxide, and peroxynitrite: Footprints of peroxynitrite *in vivo*. Adv Pharmacol **34**:17–43, 1995.
16. Koglin J, Glysing-Jensen T, Mudgett JS, Russel ME. NOS2 mediates opposing effects in models of acute and chronic cardiac rejection: Insight from NOS2-knockout mice. Am J Pathol **153**:1371–1376, 1998.
17. Akaike T, Noguchi Y, Ijiri S, Setoguchi K, Suga M, Zheng YM, Dietzschold B, Maeda H. Pathogenesis of influenza virus–induced pneumonia: Involvement of both nitric oxide and oxygen radicals. Proc Natl Acad Sci U S A **93**:2448–2453, 1996.
18. Wray G, Thiemermann C. NO in sepsis. In: Fang FC, Ed. Nitric Oxide and Infection. New York: Kluwer Academic/ Plenum Publishers, pp265–280, 1999.
19. Townsend G, Scheld M. Nitric oxide in bacterial meningitis. In: Fang FC, Ed. Nitric Oxide and Infection. New York: Kluwer Academic/ Plenum Publishers, pp417–428, 1999.
20. Akaike T, Yoshida M, Miyamoto Y, Sato K, Kohno M, Sasamoto K, Miyazaki K, Ueda S, Maeda H. Antagonistic action of imidazolineoxyl *N*-oxides against endothelium-derived relaxing factor/·NO through a radical reaction. Biochemistry **32**:827–832, 1993.
21. Yoshida M, Akaike T, Wada Y, Sato K, Ikeda K, Ueda S, Maeda H. Therapeutic effects of imidazolineoxyl *N*-oxide against endotoxin shock through its direct nitric oxide-scavenging activity. Biochem Biophys Res Commun **202**:923–930, 1994.
22. White CR, Darley-Usmar V, Berrington WR, McAdams M, Gore JZ, Thompson JA, Parks DA, Tarpey MM, Freeman BA. Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. Proc Natl Acad Sci U S A **93**:8745–8749, 1996.
23. Miyamoto Y, Akaike T, Yoshida M, Goto S, Horie H, Maeda H. Potentiation of nitric oxide-mediated vasorelaxation by xanthine oxidase inhibitors. Proc Soc Exp Biol Med **211**:366–373, 1996.
24. Yamaguchi Y, Harland RC, Wyble C, Mori K, Bollinger RR. Variable allograft responses to pretreatment with donor splenocytes treated with mitomycin C in the rat. Transplantation **47**:360–363, 1989.
25. Zweier JL. Measurement of superoxide-derived free radicals in the reperfused heart: Evidence for a free radical mechanism of reperfusion injury. J Biol Chem **263**:1353–1357, 1988.
26. Akaike T, Inoue K, Okamoto T, Nishino H, Otagiri M, Fujii S, Maeda H. Nanomolar quantification and identification of various nitrosothiols

by high performance liquid chromatography coupled with flow reactors of metals and Griess reagent. J Biochem **122**:459–466, 1997.
27. Setoguchi K, Takeya M, Akaike T, Suga M, Hattori R, Maeda H, Ando M, Takahashi K. Expression of inducible nitric oxide synthase and its involvement in pulmonary granulomatous inflammation in rats. Am J Pathol **149**:2005–2022, 1996.
28. Fujii S, Akaike T, Maeda H. Role of nitric oxide in pathogenesis of herpes simplex virus encephalitis. Virology **256**:203–212, 1999.
29. Iyonaga K, Takeya M, Yamamoto T, Ando M, Takahashi K. A novel monoclonal antibody, RM-4, specifically recognizes rat macrophages and dendritic cells in formalin-fixed, paraffin-embedded tissues. Histochem J **29**:105–116, 1997.
30. Worrall NK, Misko TP, Sullivan PM, Hui JJ, Ferguson TB Jr. Inhibition of inducible nitric oxide synthase attenuates established acute cardiac allograft rejection. Ann Thorac Surg **62**:378–385, 1996.
31. Roza AM, Cooper M, Pieper G, Hilton G, Dembny K, Lai CS, Lindholm P, Komorowski R, Felix C, Johnson C, Adams M. NOX 100, a nitric oxide scavenger, enhances cardiac allograft survival and promotes long-term graft acceptance. Transplantation **69**:227–231, 2000.
32. Pieper GM, Cooper M, Johnson CP, Adams MB, Felix CC, Roza AM. Reduction of myocardial nitrosyl complex formation by a nitric oxide scavenger prolongs cardiac allograft survival. J Cardiovasc Pharmacol **35**:114–120, 2000.
33. Nathan C. Inducible nitric oxide synthase: What difference does it make? J Clin Invest **100**: 2417–2423, 1997.
34. Akaike T, Maeda H. Pathophysiological actions of high-output production of nitric oxide. In: Ignarro L, Ed. Nitric Oxide: Biology and Pathobiology. San Diego: Academic Press, pp733–745, 2000.
35. Hickey MJ, Sharkey KA, Sihota EG, Reinhardt PH, Macmicking JD, Nathan C, Kubes P. Inducible nitric oxide synthase–deficient mice have enhanced leukocyte-endothelium interactions in endotoxemia. FASEB J **11**:955–964, 1997.
36. Akaike T. Mechanism of biological *S*-nitrosation and its measurement. Free Radic Res (in press) 2000.
37. Inoue K, Akaike T, Miyamoto Y, Okamoto T, Sawa T, Otagiri M, Suzuki S, Yoshimura T, Maeda H. Nitrosothiol formation catalyzed by ceruloplasmin: Implication for cytoprotective mechanism *in vivo*. J Biol Chem **274**:27069–27075, 1999.
38. Rauhala P, Lin AM, Chiueh CC. Neuroprotection by *S*-nitrosoglutathione of brain dopamine neurons from oxidative stress. FASEB J **12**:165–173, 1998.
39. Konorev EA, Tarpey MM, Joseph J, Baker JE, Kalyanaraman B. *S*-nitrosoglutathione improves functional recovery in the isolated rat heart after cardioplegic ischemic arrest: Evidence for a cardioprotective effect of nitric oxide. J Pharmacol Exp Ther **274**:200–206, 1995.
40. Castro L, Rodriguez M, Radi R. Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. J Biol Chem **269**:29409–29415, 1994.
41. Hausladen A, Fridovich I. Superoxide and peroxynitrite inactivate aconitase, but nitric oxide does not. J Biol Chem **269**:29405–29408, 1994.
42. Szabó C, Cuzzocrea S, Zingarelli B, O'Connor M, Salzman AL. Endothelial dysfunction in a rat model of endotoxic shock: Importance of the activation of poly (ADP-ribose) synthetase by peroxynitrite. J Clin Invest **100**:723–735, 1997.
43. Szabó C, Zingarelli B, O'Connor M, Salzman AL. DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. Proc Natl Acad Sci U S A **93**:1753–1758, 1996.
44. Okamoto T, Akaike T, Nagano T, Miyajima S, Suga M, Ando M, Ichimori K, Maeda H. Activation of human neutrophil procollagenase by nitrogen dioxides and peroxynitrite: A novel mechanism for procollagenase activation involving nitric oxide. Arch Biochem Biophys **342**:261–274, 1997.
45. Frears ER, Zhang Z, Blake DR, O'Connell JP, Winyard PG. Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. FEBS Lett **381**:21–24, 1996.
46. Beckman JS, Ye YZ, Anderson P, Chen J, Accavetti MA, Tarpey MM, White CR. Extensive nitration of protein tyrosines observed in human atherosclerosis detected by immunohistochemistry. Biol Chem Hoppe Seyler **375**:81–88, 1994.
47. Miller MJS, Thompson JH, Zhang X-J, Sadowska-Krowicka H, Kakkis JL, Munshi UK, Sandoval M, Rossi JL, Eloby-Childress S, Beckman JS, Ye YZ, Rodi CP, Manning PT, Currie MG, Clark DA. Role of inducible nitric oxide synthase expression and peroxynitrite formation in guinea pig ileitis. Gastroenterology **109**:1475–1483, 1995.
48. Kooy NW, Royall JA, Ye YZ, Kelley DR, Beckman JS. Evidence for *in vivo* peroxynitrite production in human acute lung injury. Am Rev Respir Dis **151**:1250–1254, 1995.
49. Estévez AG, Crow JP, Sampson JB, Reiter C, Zhuang Y, Richardson GJ, Tarpey MM, Barbeito L, Beckman J. Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. Science **286**:2498–2500, 1999.
50. Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A. Formation of nitric oxide–derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature **391**:393–397, 1998.
51. Pfeiffer S, Mayer B. Lack of tyrosine nitration by peroxynitrite generated at physiological pH. J Biol Chem **273**:27280–27285, 1998.
52. Sawa T, Akaike T, Maeda H. Tyrosine nitration by peroxynitrite formed from nitric oxide and superoxide generated by xanthine oxidase. J Biol Chem **275** (in press) 2000.
53. Reiter CD, Teng RJ, Beckman JS. Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. J Biol Chem **275** (in press) 2000.
54. Goldstein S, Czapski G. Kinetics of nitric oxide autooxidation in aqueous solution in the absence and presence of various reductants: The nature of the oxidizing intermediates. J Am Chem Soc **117**:12078–12084, 1995.
55. Amano F, Noda T. Improved detection of nitric oxide radical (NO) production in an activated macrophage culture with a radical scavenger, carboxy PTIO, and Griess reagent. FEBS Lett **368**:425–428, 1995.
56. Akaike T, Maeda H. Quantitation of nitric oxide using 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO). Meth Enzymol **268**:211–221, 1996.