ANTIOXIDANTS & REDOX SIGNALING
Volume 3, Number 1, 2001
Mary Ann Liebert, Inc.

Original Research Communication

Antioxidant Treatment Inhibits Activation of Myocardial Nuclear Factor κB and Inhibits Nitrosylation of Myocardial Heme Protein in Cardiac Transplant Rejection

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ABSTRACT

Nitric oxide production via inducible nitric oxide synthase (iNOS) is believed to play a role in cardiac allograft rejection. Previously, we showed that antioxidants can significantly prolong cardiac graft survival, but the nature of this protection is unknown. In the present study, we examined the protective effect of another antioxidant, dimethylthiourea (DMTU), in a model of cardiac allograft rejection. Specifically, we hypothesized that DMTU would prolong graft survival and decrease activation of nuclear factor-κB (NF-κB), an important redox-sensitive transcription factor necessary for iNOS gene expression. NF-κB was activated by twofold as early as postoperative day 2 in allografts. NF-κB activation in allografts progressed to a peak of ninefold by postoperative day and remained increased until postoperative day 6. No activation of NF-κB was observed in isografts for comparable time periods. Treatment with DMTU resulted in a significant prolongation of graft survival. This beneficial effect was associated with diminished activation of myocardial NF-κB. Treatment with DMTU also resulted in decreased formation of iron-nitrosylprotein complexes as evidenced by electron paramagnetic resonance spectroscopy. These studies provide evidence that reactive oxygen plays a significant role in signal transduction for activation via the transcription factor, NF-κB, thereby modulating distal actions and consequences of iNOS-derived nitric oxide. Antioxid. Redox Signal. 3, 81–88.

INTRODUCTION

SEVERAL LINES OF EVIDENCE obtained from our laboratory and elsewhere suggest that nitric oxide (NO) production via inducible nitric oxide synthase (iNOS) plays a significant role in cardiac allograft rejection. Nonselective (17) and selective iNOS (18, 19) inhibitors have been shown to prolong cardiac allograft survival. The iNOS-selective inhibitor, aminoguanidine, has also been shown to reduce myocardial iron nitrosylprotein complex formation (10, 18), suggesting that such intervention limits targeting of myocardial proteins by excess NO. In our laboratory, we have shown that interventions that prolong cardiac graft survival also limit the formation of myocardial protein nitrosylation (4, 10, 11, 13). These interventions include such diverse agents as cyclosporine (an immunosuppressant), aminoguanidine (an iNOS in-

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hibitor), pyrrolidine dithiocarbamate (an antioxidant and metal chelator), and NOX-100 (a water-soluble NO scavenger).

The studies using pyrrolidine dithiocarbamate suggest that reactive oxygen may play a role in cardiac allograft rejection. This concept is supported by additional studies in our laboratory that show that cardiac graft survival is enhanced by treatment with vitamins in the absence or presence of concurrent administration of immunosuppressant (14). Other evidence that reactive oxygen may be increased is supported by studies showing that the antioxidant enzyme, superoxide dismutase (SOD), is decreased in cardiac allografts, but not isografts (7, 12).

The precise mechanism by which reactive oxygen contributes to graft rejection is unknown. Preliminary studies in our laboratory suggest that antioxidants such as pyrrolidine dithiocarbamate might limit iNOS expression by inhibiting the activation of the transcription factor, nuclear factor κB (NF-κB) (4). Indeed, NF-κB, is widely known to be a redox-sensitive transcription factor (5). Thus, activation of NF-κB may be a potential site of action of antioxidants.

The importance of the activation of NF-κB in cardiac rejection was strengthened by our studies showing that the increase in NF-κB precedes the peak development of plasma NO products and myocardial nitrosylprotein formation (4). This temporal relationship suggests that reactive oxygen may play a role in early stages of organ rejection as a signal transduction event by regulating activation of the redox-sensitive transcription factor, NF-κB. Activation of NF-κB is known to be important in iNOS gene expression induced by cytokines in various *in vitro* cell preparations (1). Furthermore, agents such as pyrrolidine dithiocarbamate that antagonize the hydroxyl radical ($^{\bullet}$OH) also decrease NF-κB activation, suggesting a role specifically for $^{\bullet}$OH in redox signaling for expression of NF-κB-dependent gene products such as iNOS in the transplant rejection model.

In the present study, we attempted to explore the potential role of reactive oxygen in cardiac allograft rejection. Specifically, we examined the effects of the antioxidant, dimethylthiourea (DMTU), on activation of myocardial NF-κB, formation of nitrosylprotein complexes, and graft survival in a model of heterotopic cardiac transplantation.

MATERIALS AND METHODS

Animals and surgery

Rats weighing ~210–230 g were purchased from Harlan Sprague–Dawley (Indianapolis, IN, U.S.A.). Lewis (Lew: RT1^{1}) and Wistar–Furth (WF: RT1^{U}) strain rats representing genetic disparity at both the major and minor histocompatibility loci were chosen for these studies. Surgery was performed under sterile conditions in animals anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital. Isogeneic (Lew → Lew) or allogeneic (WF → Lew) heterotopic cardiac transplantation was performed by grafting to the abdominal aorta and vena cava using established microsurgical techniques. Graft function was monitored twice daily by external palpation. Rejection was defined as a loss of palpable contractile activity, which was confirmed on direct inspection following laparotomy.

Experimental groups

Isograft or allograft experiments were terminated at various days up until postoperative day 6 (POD6) or until day of rejection. A subset of allograft recipients received daily intraperitoneal loading doses of 130 mg/kg DMTU for 3 days prior to surgery. On the day of surgery, animals received 50 mg/kg i.p. at 30 min prior to surgery and 20 mg/kg i.v. upon transplantation of the graft. Thereafter, animals received single daily intraperitoneal injections of 65 mg/kg DMTU. Animals were treated until graft rejection or grafts harvested at POD6 and then either nuclear protein was extracted for NF-κB analysis or tissue was frozen in liquid nitrogen for myocardial nitrosylprotein formation using electron paramagnetic resonance (EPR) spectroscopy.

Native and transplanted hearts were harvested from rats by metabolically and functionally arresting *in situ* with 4°C UW cardioplegic solution. A portion of the heart was minced and placed in a 4.0-mm quartz tube, frozen in liquid nitrogen, and stored at 77 K for EPR analysis. Other tissue was taken for preparation of nuclear extracts.

Nuclear protein extraction

Ventricle portions were minced and the tissue transferred to a 15-ml polypropylene tube containing 1.5 ml of buffer A: 10 m*M* HEPES, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.5 m*M* dithiothreitol (DTT), 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), 200 μM sodium orthovanadate, 10 ng/ml aprotinin, 10 ng/ml leupeptin, and 0.1% NP-40. The tissue was homogenized, transferred to 1.5-ml microfuge tubes, and centrifuged at 4°C for 10 min at 12,000 *g*. The supernatant was discarded, and the nuclear pellet was resuspended in 400 μL of buffer C: 20 m*M* HEPES, 25% (vol/vol) glycerol, 0.42 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* DTT, 0.5 m*M* PMSF, 200 μM sodium orthovanadate, 10 ng/ml aprotinin, and 10 ng/ml leupeptin and incubated at 4°C for 10 min. The samples were centrifuged at 14,000 *g* for 10 min at 4°C. The supernatant (nuclear fraction) was transferred to a new tube with 900 μl of buffer D: 20 m*M* HEPES, 20% (vol/vol) glycerol, 0.42 m*M* NaCl, 0.05 m*M* KCl, 0.2 m*M* EDTA, 0.5 m*M* DTT, 0.5 m*M* PMSF, 200 μM sodium orthovanadate, 10 ng/ml aprotinin, and 10 ng/ml leupeptin. Protein concentration was determined using a bicinchoninic acid assay.

Electrophoretic mobility gel shift assay (EMSA) for NF-κB

Double-stranded NF-κB oligonucleotide (Promega, Madison, WI, U.S.A.) was end-labeled with [γ-^{32}P]ATP and polynucleotide kinase for 10 min at 37°C. After incubation, the labeled oligonucleotide was desalted and resuspended in Tris–EDTA buffer. DNA binding reactions were performed at room temperature containing 30 μg of nuclear extract, 0.5 ng of labeled oligonucleotide, and 3 μg of poly(dI-dC) (Pharmacia–Upjohn, Kalamazoo, MI, U.S.A.). After incubation for 30 min, the reactions were electrophoresed on 4% polyacrylamide in 0.5 × Tris–borate–EDTA at 10 V/cm. Specificity for NF-κB binding activity was verified by competition with 100-fold excess of unlabeled mutant or wild-type competitor oligonucleotides. Gels were dried on Whatman 3-mm filter paper and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY, U.S.A.). Intensity of NF-κB binding activity was determined by phosphorimaging (Molecular Dynamics, Sunnyvale, CA, U.S.A.). Quantitative results are expressed as a percentage of the total amount of radioactivity that is shifted to the NF-κB binding activity.

Immunohistochemistry

In a subset of animals at POD5, hearts were harvested and fixed in formalin and embedded in paraffin. Deparaffinized sections were taken for immunolocalization of activated NF-κB within allografts and examined with or without hematoxylin and eosin counterstaining. To quench endogenous background, sections were incubated with 1% H_2O_2 in methanol for 5 min, followed by washing twice in 7.4% buffered saline. Unspecified binding was blocked at room temperature for 30 min using commercial blocking serum (Vector Elite ABC kit no. PK-6101, Vector Labs, Burlingame, CA, U.S.A.). Sections were incubated for 45 min with a 1:50 dilution of primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for p65 NF-κB, followed by washing twice in buffered saline and incubation at room temperature for 30 min with biotinylated secondary antibody (Vector Elite Kit). After washing in buffered saline, sections were incubated with Vectastain Elite ABC reagent at room temperature for 30 min. Sections were then washed twice in

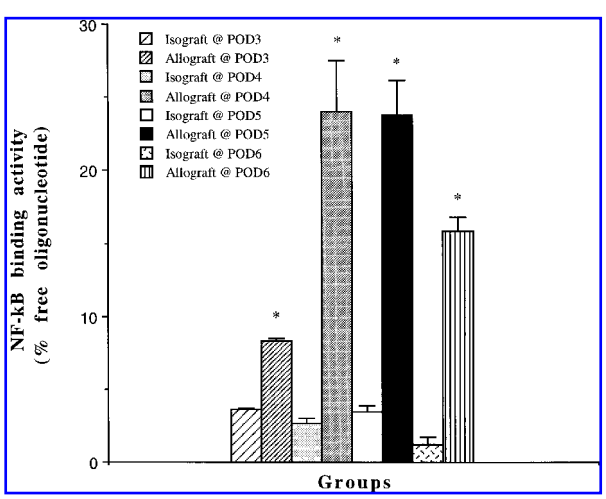

FIG. 1. Quantitation of NF-κB nuclear binding activity (determined by phosphorimaging analysis) at various days post transplant in allografts versus isografts and the decrease by DMTU treatment determined by phosphorimaging. Results are the means ± SEM for three to five samples each. $n = 3$ each isografts or $n = 5$ each allografts. *$p < 0.05$ versus isograft at respective time.

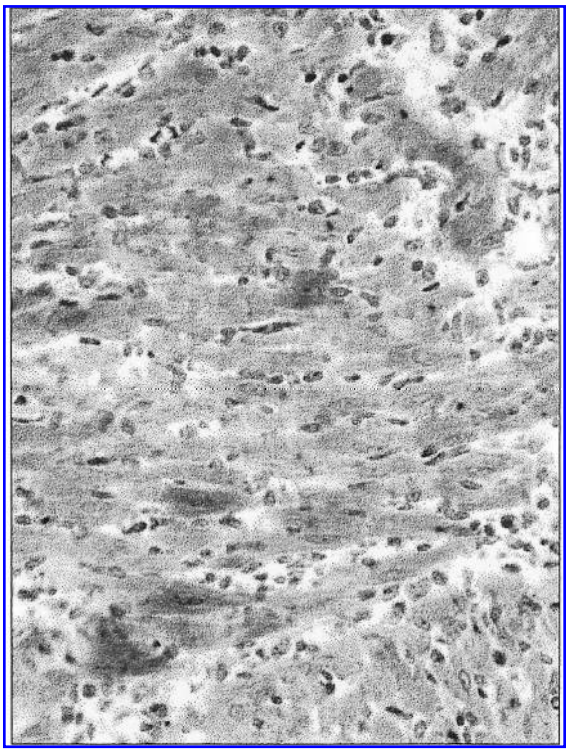

FIG. 2. Evidence of immunoreactive NF-κB (denoted by arrow) within myocardium of an untreated allograft at POD5.

buffered saline, incubated with 0.03% (wt/vol) 3,3′-diaminobenzidine with 0.003% (vol/vol) H_2O_2, rinsed, and examined with or without hematoxylin and eosin counterstaining.

Slides were visualized using a BX40 Olympus microscope (Leeds Precision Instruments, Minneapolis, MN, U.S.A.), captured with a three-chip color CCD camera (Dage MTI, Inc., Michigan City, IN, U.S.A.), and analyzed using the software programs Adobe Photoshop 4.0 (Adobe Systems, Inc., San Jose, CA, U.S.A.) and Optimas 6.0 (Optimas Corp., Bothell, WA, U.S.A.). All images were adjusted for optimal brightness and contrast using the adjust levels, sharpen and image size operations; however, these settings were uniform for a given set of analysis.

EPR spectroscopy

X-band EPR analysis was performed using a liquid nitrogen finger dewar on a Varian E-109 spectrometer (Varian Instruments, Palo Alto, CA, U.S.A.). For relative comparisons of signal intensity, samples from DMTU-treated recipient hearts were always normalized to identical instrument settings for untreated allograft recipient hearts. Experimental conditions included the following: 1,000-G scan range, 4-min scan time, 0.25-s time constant, 5.0-G modulation amplitude, 100-kHz modulation frequency, and 5-mW microwave power. The magnetic field was calibrated with Fremy's salt by using a g value of 2.0055 ± 0.0001.

Data analysis

EPR spectra were processed for presentation by using SUMSPEC and Grapher programs (Golden Software, Golden, CO, U.S.A.). Statistical analysis of data was performed by analysis of variance for multiple group means or by Student's t test for comparisons between two group means. Statistical significance was determined at the 95% confidence level (i.e., p value < 0.05).

RESULTS

Phosphorimaging analysis of EMSA revealed a twofold increase in NF-κB nuclear

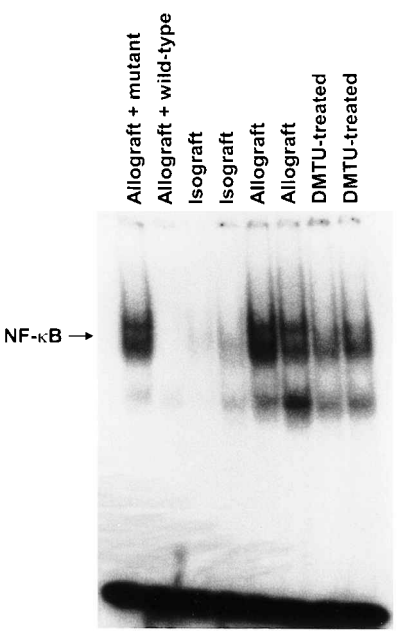

FIG. 3. Example of EMSA showing increase in NF-κB nuclear binding activity at POD6 in untreated isografts or in allografts (without or with DMTU). Specificity for NF-κB binding activity is verified by competition using excess mutant or wild-type oligonucleotides (lanes 1 and 2).

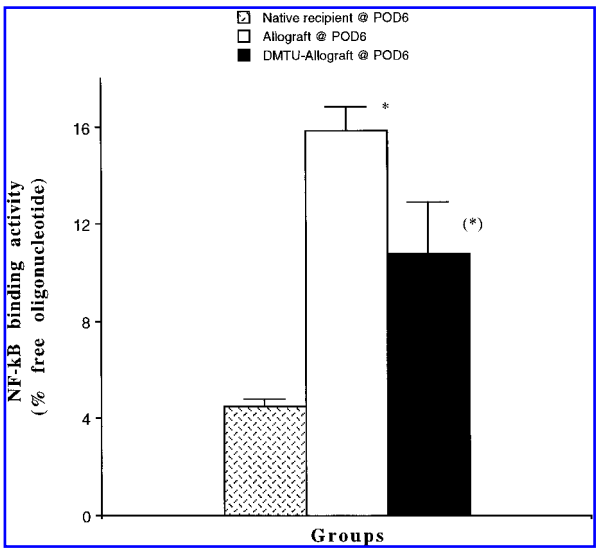

FIG. 4. Quantitation of the decrease in NF-κB nuclear binding activity by treatment of allograft recipients with DMTU. Results are the means ± SEM for native hearts ($n = 3$), untreated allografts ($n = 8$), or DMTU-treated allografts ($n = 5$). *$p < 0.05$ versus isograft; (*)$p < 0.05$ versus untreated allograft.

binding activity in allografts at POD2, but not at POD1, compared with native control hearts of allograft recipients (% NF-κB binding activity: allograft at POD2, 5.1 ± 0.9%; allograft at POD1, 2.7 ± 0.4%; native control hearts of allograft at POD2, 2.4 ± 0.2%; $p < 0.05$, $n = 3$–5 each). NF-κB nuclear binding activity increased progressively to a peak at POD4 in allografts and remained elevated relative to isografts up to POD6 (Fig. 1).

Sections of heart tissue at POD5 were used for immunohistochemical examination for cardiomyocyte localization of immunoreactive NF-κB. In allografts (Fig. 2), there were intense, concentrated areas of staining for NF-κB. Concentrated areas of immunoreactive staining for NF-κB were not obvious in isografts (data not shown). Immunoreactive NF-κB was localized not only to cellular infiltrate (data not shown), but also to cardiomyocytes *per se* (Fig. 2).

EMSA analysis of nuclear extracts from hearts at POD6 revealed that the increased level of NF-κB DNA binding activity was decreased in allograft hearts treated with DMTU compared with untreated allograft controls (Fig. 3). Specificity for NF-κB binding in this assay was verified by competition with excess cold mutant or wild-type oligonucleotide (Fig. 3, lanes 1 and 2). Quantitative analysis of NF-κB binding activity using phosphorimaging revealed a significant ($p < 0.05$) attenuation by treatment with DMTU (Fig. 4).

EPR analysis of samples at POD6 revealed marked differences between isografts and allografts. Background signals for reduced Fe-S clusters at $g = 2.02$ and $g = 1.94$ or for semiquinone at $g = 2.004$ were observed in isograft hearts (Fig. 5). In contrast for allograft hearts, additional signals characteristic of protein nitrosylation were observed that were never seen in any isograft or native hearts of allograft recipients at any time (data not shown). These additional signals consisted of a triplet signal at $g = 2.014$ with hyperfine splitting of 17.5 G (which is designated with the generic term, nitrosoheme) and a broad signal at $g = 2.08$ [attributed to nitrosylmyoglobin (NO-Mb)]. Compared with untreated allografts, the intensity of nitrosylprotein signal formation was decreased in samples obtained from allograft recipients receiving treatments with DMTU (Fig. 6).

In contrast to allografts (Fig. 7), there was no rejection of any isograft. Graft survival time was increased by 40% in allografts treated with DMTU (Fig. 7).

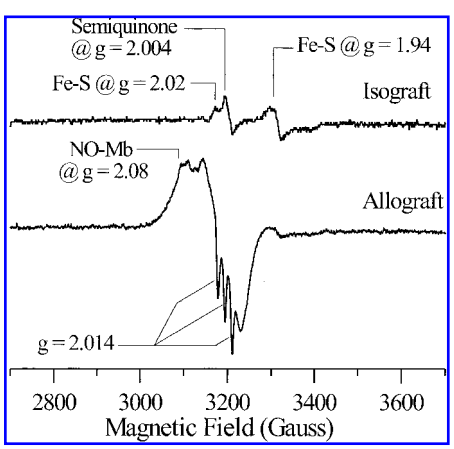

FIG. 5. Example showing the EPR spectra for isograft versus allograft at POD6. Analysis for isografts indicates background signals for semiquinone at $g = 2.004$ and reduced Fe-S complexes at $g = 2.02$ and $g = 1.94$. For allografts, additional nitrosoheme signals consisting of a triplet at $g = 2.014$ and for NO-Mb at $g = 2.08$ are shown. Examples shown are spectra collected under identical conditions including spectrometer gain settings.

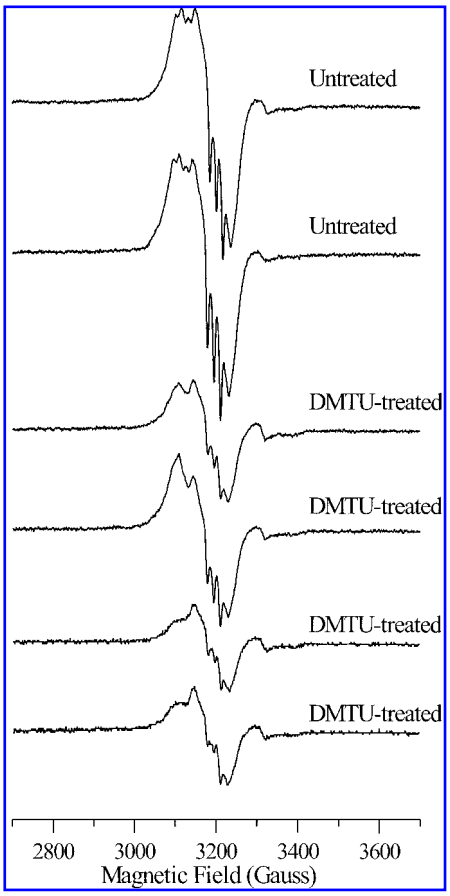

FIG. 6. Decrease by DMTU treatments of EPR signals for NO-Mb and nitrosoheme in untreated allografts. For comparative purposes, examples shown are spectra from individual hearts and were collected under identical conditions including spectrometer gain settings.

DISCUSSION

The present study demonstrated that treatment with the antioxidant, DMTU, decreased the activation of myocardial NF-κB, decreased myocardial protein nitrosylation, and prolonged graft survival in a model of cardiac allograft rejection. These actions of DMTU were achieved in the absence of any immunosuppressant therapy. These results complement previous studies from our laboratories showing that treatment with pyrrolidine dithiocarbamate limited NF-κB activation and NO-Mb formation and significantly prolonged cardiac graft survival (4, 10). Our results are analogous to the findings in another model of iNOS-induced injury in which DMTU treatment diminished endotoxin-induced activation of NF-κB and tissue injury (15). Collectively, these data suggest that antioxidants may be useful regimens to prevent iNOS-induced pathologies by interfering with transcription factor activation.

Previously, it has been speculated that antioxidants may be useful adjunct therapies in clinical organ transplantation (8). The rationale for antioxidant treatment was acknowledged to be due to counteraction of the effects of increased reactive oxygen production as a consequence of prolonged periods (i.e., hours) of ischemia/reperfusion (3). In this regard, it is known that normothermic ischemia plus reperfusion can activate myocardial NF-κB and stimulate iNOS gene expression (2, 6, 9).

In contrast in the present model, the effects of ischemia/reperfusion are minimized for at least two reasons. First, hearts in our model are not stored for prolonged periods of time prior to transplantation. Rather, hearts are rapidly arrested in cold UW cardioplegic solution and revascularized to recipient animals within 15 min. In this regard, previous studies from our laboratory (4) and the present study show that activation of myocardial NF-κB does not occur in isografts that are subjected to the same surgical protocol. Additional studies reveal no NF-κB activation within allografts even after 2 h of revascularization (unpublished observations). Collectively, these findings argue strongly against a generalized increase in ischemia/reper-

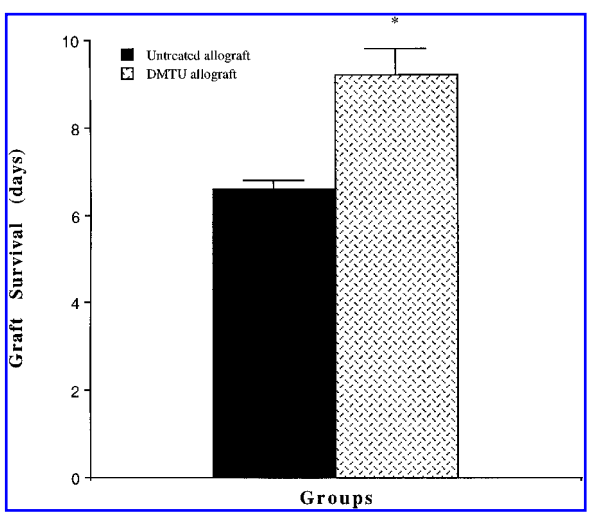

FIG. 7. Prolongation of graft survival by treatment with DMTU. Results are the means ± SE of $n = 7$ each group. *$p < 0.05$ versus untreated allografts.

fusion-mediated reactive oxygen production accounting for our findings.

In contrast to the model in which reactive oxygen is believed to be released upon reperfusion of organs following prolonged ischemic storage conditions, we believe that oxidant signals are also likely to occur at a time period of hours to days after transplantation, despite brief cardioplegic arrest prior to transplantation. Indeed, findings from our laboratory and elsewhere (7, 12) indicate that myocardial activity of SOD is significantly decreased in cardiac allografts, but not isografts, several days following transplantation, but prior to rejection. This decreased myocardial SOD activity suggests indirect evidence for increased levels of $^{\cdot}O_2^-$ following allogeneic transplantation. Because of this proposed increased in $^{\cdot}O_2^-$, it is also likely that reactive species distal to $^{\cdot}O_2^-$ production are also increased.

The increased production of reactive oxygen at time points removed from revascularization of grafted organs may have an important role to play in organ rejection. Specifically, results from the present study and elsewhere from our laboratory (4) suggest that reactive oxygen may be important for signal transduction via activation of the redox-active transcription factor, NF-κB. Limiting activation of NF-κB would be expected to limit iNOS gene expression and distal actions of iNOS-mediated nitrosylation of myocardial proteins. Indeed, our studies indicated that DMTU decreased nitrosylprotein formation as demonstrated by EPR spectroscopy.

In general, the nascent reactive oxygen species responsible for NF-κB activation by cytokines in other cell types *in vitro* is believed to be the $^{\cdot}OH$. The reactive oxygen species involved *in vivo* is believed to be similar, and our present studies using DMTU in the cardiac transplant model are consistent with this notion because DMTU is known to have higher selectivity to scavenge $^{\cdot}OH$ rather than other reactive oxygen species (16).

PERSPECTIVES

The present studies suggest that reactive oxygen may play an important role in early stages of transplant rejection. Specifically, reactive oxygen is implicated in activation of NF-κB, which leads to expression of the NF-κB-dependent gene product, iNOS. Thus, the distal actions of NO derived from iNOS in cardiac transplant rejection might be regulated by interfering with the signaling by reactive oxygen of the activation of NF-κB.

ACKNOWLEDGMENTS

We thank Karen Hyde for reproduction of EPR spectra using the SUMSPEC and Grapher programs. This work was supported, in part, by the Biomedical Research Center grant no. RR01008.

ABBREVIATIONS

DMTU, dimethylthiourea; DTT, dithiothreitol; EMSA, electrophoretic mobility gel shift assay; EPR, electron paramagnetic resonance; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor κB; NO, nitric oxide; NO-Mb, nitrosylmyoglobin; $^{\cdot}OH$, hydroxyl radical; PMSF, phenylmethylsulfonyl fluoride; POD, postoperative day; SOD, superoxide dismutase.

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Received for publication March 25, 2000; accepted July 2, 2000.

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