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Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts

(peroxynitrite/tyrosine nitration/mitochondria/transplantation/inflammation)

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ABSTRACT **Inflammatory processes in chronic rejection remain a serious clinical problem in organ transplantation. Activated cellular infiltrate produces high levels of both superoxide and nitric oxide. These reactive oxygen species interact to form peroxynitrite, a potent oxidant that can modify proteins to form 3-nitrotyrosine. We identified enhanced immunostaining for nitrotyrosine localized to tubular epithelium of chronically rejected human renal allografts. Western blot analysis of rejected tissue demonstrated that tyrosine nitration was restricted to a few specific polypeptides. Immunoprecipitation and amino acid sequencing techniques identified manganese superoxide dismutase, the major antioxidant enzyme in mitochondria, as one of the targets of tyrosine nitration. Total manganese superoxide dismutase protein was increased in rejected kidney, particularly in the tubular epithelium; however, enzymatic activity was significantly decreased. Exposure of recombinant human manganese superoxide dismutase to peroxynitrite resulted in a dose-dependent (IC_{50} = 10 μM) decrease in enzymatic activity and concomitant increase in tyrosine nitration. Collectively, these observations suggest a role for peroxynitrite during development and progression of chronic rejection in human renal allografts. In addition, inactivation of manganese superoxide dismutase by peroxynitrite may represent a general mechanism that progressively increases the production of peroxynitrite, leading to irreversible oxidative injury to mitochondria.**

Even though renal transplantation has been a clinical reality for nearly 40 years (1, 2), chronic rejection remains a primary cause of late graft loss. Whereas advances in immunosuppressive therapy have increased 1-year graft survival, the percentage of allografts developing chronic rejection has not changed (3, 4). Histopathologically, chronic rejection in renal allografts manifests itself as progressive vascular occlusion, characteristic glomerulopathy, and processes of interstitial fibrosis accompanied by tubular atrophy, ultimately resulting in organ failure (5). Despite recognition of these pathophysiologic features, the molecular events underlying development of chronic rejection remain elusive.

The limited utility of long-term immunosuppression suggests that, in addition to antigen-dependent factors, normal inflammatory-mediated repair mechanisms may contribute to pathophysiologic processes associated with chronic rejection. Inflammatory processes in chronic rejection are important (6, 7) and provide a mechanism for targeted delivery of factors capable of inducing, sustaining, and coordinating a wound-healing process. Reactive oxygen species have been established as key participants of inflammation; wherein activated cellular infiltrate can produce high levels of both superoxide ($O_2^{\cdot-}$) and nitric oxide ($NO^{\cdot}$) (8). Increased levels of $O_2^{\cdot-}$ (9) and $NO^{\cdot}$ (10) have been reported during chronic rejection of renal allografts, observations that predict a role for oxidant stress during transplant dysfunction. Cellular protection against these reactive oxygen species involves an elaborate antioxidant defense system, including that associated with manganese superoxide dismutase (MnSOD). Human MnSOD is a tetrameric protein consisting of identical 24-kDa subunits, which are translated in the cytosol as a precursor form containing an N-terminal 24 amino acid sequence signaling mitochondrial compartmentalization (11). The signal peptide sequence is cleaved to generate the mature protein, which protects cells against the large (up to 1% of total oxygen consumption) amounts of cytotoxic $O_2^{\cdot-}$ leaking from mitochondrial respiratory chains (12). The overall importance of this enzyme is evident from transgenic animal studies, wherein MnSOD "knockout" mice suffer from a loss of mitochondrial iron–sulfur centers, a modification proving lethal to newborns (13).

Submicromolar concentrations of $NO^{\cdot}$ can compete with endogenous SOD for $O_2^{\cdot-}$ (14) by a rapid reaction (15) that generates the potent oxidizing agent peroxynitrite, which can directly attack many biological targets (16). In addition, peroxynitrite readily nitrates tyrosine residues in proteins producing a permanent modification that can be detected immunologically. Nitrotyrosine immunoreactivity has been reported in several pathological conditions, including atherosclerosis, reperfusion injury, amyotrophic lateral sclerosis, septic lung, rheumatoid arthritis, and MPTP toxicity (17–22). Nitration of tyrosine residues is a stable modification and mechanisms for eliminating this species other than proteolysis and urinary excretion have yet to be identified (23). Despite the number of diseases in which tyrosine nitration has been observed, few specific protein targets containing modified tyrosine residues have been identified. We now report that MnSOD extracted from chronically rejected human renal allografts demonstrates both a significant increase in tyrosine nitration and a dramatic decrease in enzymatic activity. In addition, exposure of recombinant human MnSOD to peroxynitrite resulted in a dose-dependent increase in tyrosine nitration that correlated with a decrease in enzymatic activity. Collectively, these observations suggest a role for peroxynitrite during development and progression of lesions associated with chronic rejection of human renal allografts.

EXPERIMENTAL METHODS

Tissue Specimens. Renal tissue from three patients who underwent nephrectomy after graft loss secondary to chronic rejection were examined in this study. According to the Banff Classification criteria (24), all tissues were found to have either grade II or III chronic allograft nephropathy. A 17-year-old heart-beating organ donor, whose kidney was determined

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Abbreviation: MnSOD, manganese superoxide dismutase.

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unsuitable for transplantation, was utilized as a nontransplant control.

Immunohistochemistry. Renal tissue was formalin-fixed, paraffin-embedded, and processed as described (25). Thin (5 μm) sections were incubated (1 hr, 20°C) with rabbit polyclonal anti-nitrotyrosine antibody (1:300 dilution, Upstate Biotechnology) or sheep polyclonal anti-human MnSOD (1:50 dilution, Calbiochem). Antibody binding was detected with a Quick Staining kit (Dako) using the swine anti-rabbit horseradish peroxidase-conjugated secondary antibody or a rabbit anti-sheep horseradish peroxidase-conjugated secondary antibody (1:250 dilution).

Protein Extraction. Human renal tissue (1 g) was homogenized (Tissue Tearer, Biospec Products, Bartlesville, OK) in 5.0 ml of extraction buffer (4°C) consisting of phosphate buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride and aprotinin at 10 mg/ml. Solubilized extracts were sonicated (two 10-sec bursts at 30% power) using a Sonifier cell disruptor (Ultrasonics, Farmingdale, NY) and centrifuged ($325 \times g$) to remove cellular debris. Protein concentration was determined using the Bradford assay (Pierce).

Nitrotyrosine Immunoprecipitation. Solubilized proteins (2 mg) were precleared (45 min, 4°C) with 15 μl of Gamma Bind Plus Sepharose (Pharmacia) and the supernatant was incubated (16 hr, 4°C) with 10 μg of monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology). Immune complexes were precipitated (1.5 hr, 4°C) with 50 μl of Gamma Bind Plus Sepharose, washed in extraction buffer, resuspended in sample loading buffer (16 mM Tris·HCl, pH 6.8/2.5% glycerol/0.5% SDS/200 mM 2-mercaptoethanol/0.001% bromophenol blue), heated (95°C, 5 min), and immediately fractionated by reducing SDS/PAGE in 12% gels.

Western Blot Analysis. After separation by SDS/PAGE, proteins were transferred electrophoretically (100 V, 1 hr) to nitrocellulose membranes (Bio-Rad), which were blocked (1 hr, 20°C) with 5% nonfat milk in 50 mM Tris·HCl, pH 7.4/150 mM NaCl/0.05% Tween 20 (TBS/T). For detection of Mn-SOD, blots were incubated (2 hr, 20°C) with sheep polyclonal anti-MnSOD antibody (1:800 dilution, Calbiochem). This commercial antibody does not demonstrate cross reactivity with recombinant human Cu,ZnSOD (data not shown). Nitrotyrosine-containing proteins were detected by incubating (2 hr, 20°C) blots with a mouse monoclonal anti-nitrotyrosine antibody (1:1000 dilution, Upstate Biotechnology). After three 15-min washes in TBS/T, the immunocomplexed membranes were probed (45 min, 20°C) with either a rabbit anti-sheep (1:20,000 dilution, Calbiochem) or goat anti-mouse (1:25,000, Kirkegaard & Perry Laboratories) horseradish peroxidase-conjugated secondary antibody. Probed membranes were washed (20 min, TBS/T) three times and immunoreactive proteins were detected using enhanced chemiluminescence (LumiGLO, Kirkegaard & Perry Laboratories).

Microsequencing. After immunoprecipitation and SDS/PAGE separation, tyrosine-nitrated proteins were transferred electrophoretically (2 hr, 70 V) to Immobilon-PVDF^{SQ} membranes (Pharmacia) using 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11) containing 10% methanol. Proteins were stained (15 sec) with 0.1% Coomassie blue in 60% methanol and destained (15 min) with 50% methanol. Identified bands were excised and sent to the Biotechnology Instrumentation Facility at the University of California at Riverside for microsequencing analysis. Proteins were analyzed at the 0.3- to 1.6-pmol level and identified amino acid sequences were confirmed by two independent integrators. Sequence identity was ascertained by using the BLAST network service at the National Center for Biotechnology Information by searching the Swiss Protein database.

MnSOD Activity Assays. MnSOD activities in total solubilized renal extracts were measured by the cytochrome *c* reduction method (26) in the presence of 1 mM potassium cyanide to inhibit both Cu,ZnSOD and extracellular SOD. Assays were performed in triplicate and each experiment was done three times. Data are reported as the mean ± SD and statistical significance assessed by factorial analysis of variance (ANOVA). A difference of $P < 0.01$ was considered significant.

Nitration of MnSOD *in Vitro*. Peroxynitrite was prepared and quantitated as described (16). Reactions (0.192 ml) contained recombinant human MnSOD (0.5 μM) in 100 mM potassium phosphate (pH 7.8) containing 0.2 mM EDTA. Peroxynitrite was added (20°C) while vortex mixing at the final indicated concentrations. Working solutions of peroxynitrite were prepared by diluting stocks in 100 mM NaOH prior to use. Immediately following peroxynitrite addition, catalase (8000 units, Sigma) was added to scavenge any residual hydrogen peroxide (H_2O_2). Aliquots of each reaction were assayed for MnSOD activity and examined by Western blot analysis as described above.

RESULTS

Immunohistochemical Analysis of Nitrotyrosine in Human Kidneys. Routine histological analysis of the control kidney was unremarkable. In contrast, each of the nephrectomized tissues demonstrated pathologic evidence of chronic rejection characterized by vasculopathy, typical glomerulopathy, interstitial cellular infiltration and fibrosis, and tubular atrophy. A clinical diagnosis of chronic rejection was supported by elevated serum creatinine, proteinuria, and hypertension. Compared with the control kidney (Fig. 1*A*), extensive immunostaining for nitrotyrosine was observed primarily in tubular epithelial cells associated with chronic rejection (Fig. 1*B*). Minimal nitrotyrosine immunoreactivity was noted in the glomeruli of either control or chronically rejecting tissues. Preincubation (30 min, 20°C) of the antibody with 10 mM 3-nitrotyrosine (Sigma) completely blocked immunostaining (Fig. 1*C*).

Identification of Nitrotyrosine-Containing Proteins in Human Kidneys. The presence of elevated nitrotyrosine was confirmed by subjecting proteolyzed rejecting kidney extracts to an HPLC-electrochemical detection system (22). When compared with the control tissue, a 5-fold increase in total polypeptide-derived nitrotyrosine was detected in chronically rejected kidney extracts. In addition, Western blot analysis of total tissue extracts from chronically rejected kidneys demonstrated that increased tyrosine nitration was limited to a few specific tyrosine-nitrated proteins. As a control for nitrotyrosine monospecificity, the monoclonal antibody was preincubated with 10 mM nitrotyrosine, which completely blocked immunoblot staining (data not shown).

To concentrate, purify, and identify specific polypeptides containing nitrated tyrosine residues, a novel immunoprecipitation technique was developed using the monoclonal anti-nitrotyrosine antibody. Western blot analysis of immunoprecipitated proteins fractionated by SDS/PAGE was achieved using the same nitrotyrosine antibody to permit detection of tyrosine-nitrated proteins that were well-resolved from the dominant heavy and light chain immunoglobulins (Fig. 2*A*). Nitration of tyrosine residues in a polypeptide with an apparent molecular mass of 24 kDa (Fig. 2*A*, arrow) was consistent with this strategy. Microsequence analysis provided a sequence (KHDLPDLPYDY) of the first 11 N-terminal residues of this polypeptide. A computerized data base search identified that 10 of these 11 amino acids were identical to the peptide KHSLPDLPYDY, which corresponds to residues 25–35 in the precursor form of human MnSOD as well as residues 1–11 in the mature mitochondrial form of this antioxidant enzyme (nonidentical residue is underlined).

Nitration of tyrosine residues in MnSOD was confirmed further by Western blot analysis of SDS/PAGE-fractionated

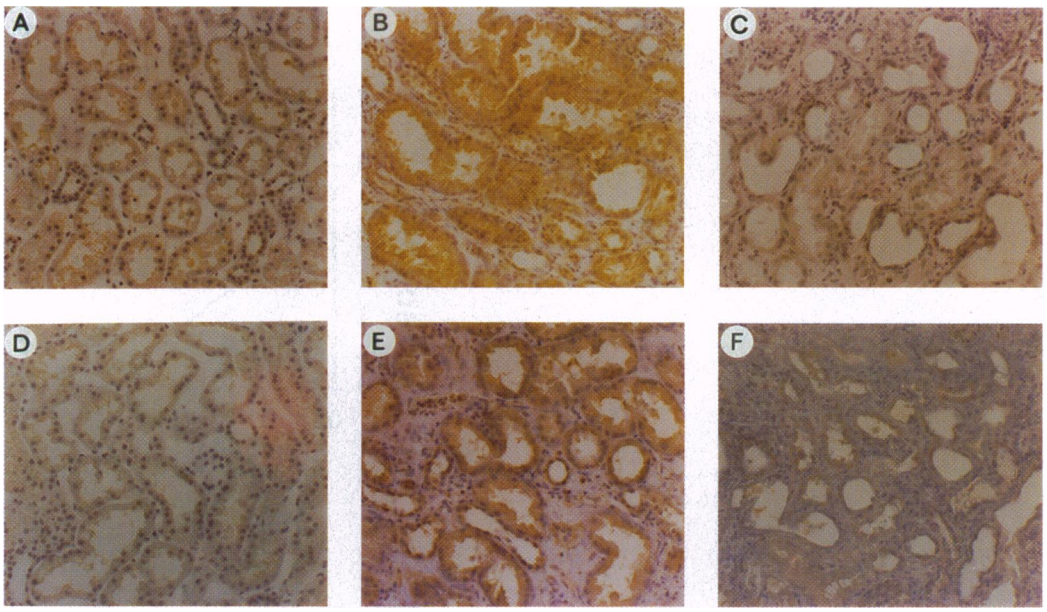

FIG. 1. Thin sections of formalin-fixed paraffin-embedded renal tissue obtained from either a control (*A* and *D*) or a chronically rejected allograft (*B*, *C*, *E*, and *F*) were prepared and examined by light microscopy after staining with either polyclonal anti-nitrotyrosine antibody (*A* and *B*) or polyclonal anti-MnSOD antibody (*D* and *E*) followed by secondary antibodies conjugated to horseradish peroxidase. Peroxidase-labeled sections were developed with 3,3′-diaminobenzidine (brown) and counterstained with Mayer's hematoxylin (blue). Pretreatment of the nitrotyrosine antibody with 10 mM 3-nitrotyrosine prevented staining (*C*), and pretreatment of the MnSOD primary antibody with a 10-fold molar excess of human MnSOD resulted in negative staining (*F*). (original magnification ×200.)

tyrosine-nitrated proteins, which had been immunoprecipitated from total renal extracts using the monoclonal anti-nitrotyrosine. Immunodetection with polyclonal anti-MnSOD demonstrated low but detectable levels of tyrosine-nitrated MnSOD in control tissue (Fig. 2*B*). In contrast, each of the chronically rejected renal allografts exhibited an increased level of immunoreactive tyrosine-nitrated MnSOD. Anti-nitrotyrosine staining of proteins immunoprecipitated with the anti-MnSOD antibody produced similar results. In addition, peroxynitrite (1 mM) treatment of total proteins extracted from the control renal tissue resulted in tyrosine nitration of similar polypeptide targets, including increased tyrosine nitration of MnSOD (data not shown).

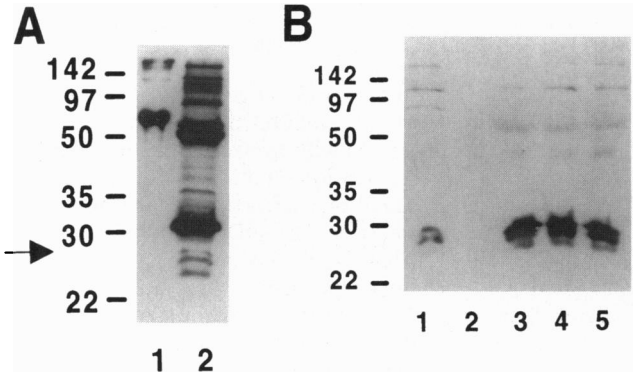

FIG. 2. (*A*) Immunoprecipitated tyrosine-nitrated proteins extracted from chronically rejected human renal allografts were resolved in 12% gels by reducing SDS/PAGE and examined by Western blot analysis with the monoclonal antibody against nitrotyrosine (lane 2). Tyrosine-nitrated bovine serum albumin (lane 1) served as a positive control. Arrowhead identifies the specific tyrosine-nitrated polypeptide subjected to microsequence analysis. (*B*) Immunoprecipitated tyrosine-nitrated proteins extracted from control (lane 1) and three separate chronically rejected (lanes 3–5) renal tissue were resolved in 12% gels by reducing SDS/PAGE and examined by Western blot analysis with the polyclonal antibody against human MnSOD. Lane 2, blank. Approximate sizes (kDa) were estimated using prestained molecular mass markers to the left.

Immunoblot analysis of total tissue extracts in both control and chronically rejecting kidneys using polyclonal anti-MnSOD demonstrated the presence of a band corresponding to the molecular mass (24 kDa) of monomeric MnSOD. However, chronically rejected tissue also contained increased levels of MnSOD migrating primarily as higher molecular mass bands (Fig. 3*A*) that were not dissociated by routine conditions of reducing SDS/PAGE. Preincubation (30 min, 20°C) of the primary MnSOD antibody with a 10-fold molar excess of recombinant human MnSOD blocked immunostaining of these higher molecular mass aggregates. Although higher molecular mass MnSOD immunoreactive bands have been reported in hamster proximal tubular epithelium (27), the significance and exact identity of the modifications leading to these species is unknown. Immunohistochemical analysis of MnSOD in renal tissue complemented this effort. Compared with control tissue (Fig. 1*D*), increased staining for MnSOD was observed in chronically rejecting kidneys (Fig. 1*E*). Preincubation (30 min, 20°C) of the primary MnSOD antibody with a 10-fold molar excess of human recombinant MnSOD eliminated immunostaining (Fig. 1*F*). Interestingly, immunoreactive MnSOD was localized to tubular epithelial cells, a pattern similar to that observed for nitrotyrosine immunoreactivity (Fig. 1*B*).

MnSOD Activity in Human Kidneys. Compared with the total MnSOD activity in the control kidney (39 ± 5 units/mg), activity in protein extracts recovered from each ($n = 3$) of the chronically rejected renal allografts (12 ± 0.4, 9.7 ± 0.3, and 6.4 ± 0.2 units/mg) was significantly ($P < 0.001$) reduced (Fig. 3*B*). The decrease in MnSOD activity in rejecting extracts was verified further (data not shown) using SOD activity gels (28). However, the total percentage of inactivated MnSOD enzyme within chronically rejected renal allografts is probably much greater, considering that these tissues demonstrated increased levels of immunoreactive MnSOD protein (Figs. 1*E* and 3*A*).

Peroxynitrite Inhibits MnSOD Activity *in Vitro*. Exposure of recombinant MnSOD (0.5 μM) to peroxynitrite (0–50 μM) resulted in a dose-dependent (IC_{50} = 10 μM) decrease in enzymatic activity (Fig. 4*A*). This decrease in MnSOD activity was related directly to peroxynitrite and not other trace

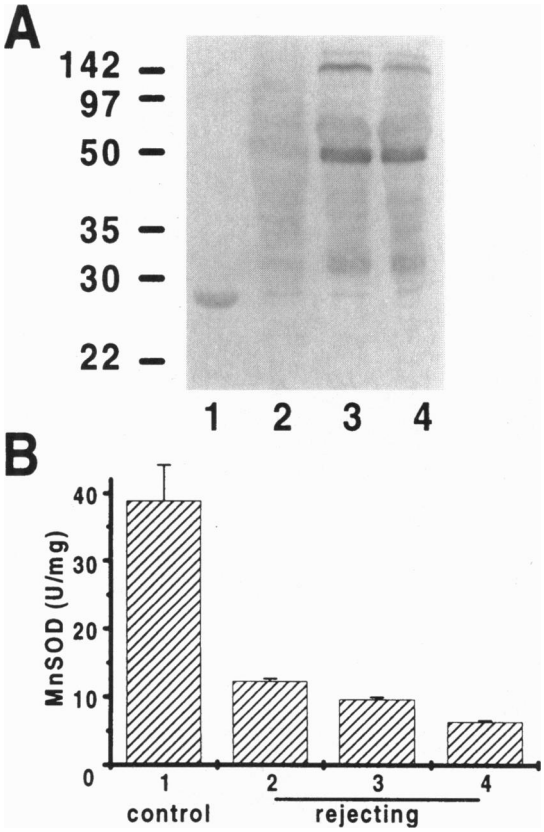

FIG. 3. (*A*) Total proteins extracted from control (lane 2) and two separate chronically rejected (lanes 3 and 4) renal tissue were resolved in 12% gels by reducing SDS/PAGE and examined by Western blot analysis with the polyclonal antibody against human MnSOD. Recombinant human MnSOD served as a positive control (lane 1). Approximate sizes (kDa) were estimated using prestained molecular mass markers to the left. (*B*) The specific activity (units /mg) of MnSOD was determined in total protein extracts recovered from control (bar 1) and three separate chronically rejected renal tissue (bars 2–4). The MnSOD activity in rejecting extracts (bars 2–4) were 32, 25, and 16% of control levels, respectively ($n = 3$; $P < 0.001$).

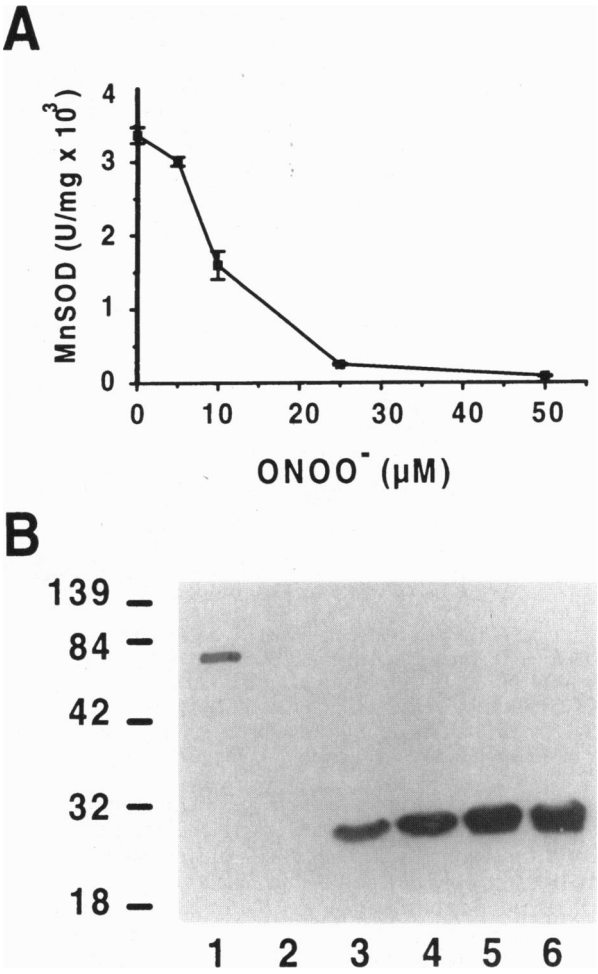

FIG. 4. (*A*) Specific activity (units/mg) of recombinant human MnSOD was determined after exposure to increasing concentrations of peroxynitrite (0–50 μM). (*B*) Recombinant human MnSOD was exposed to specific doses of peroxynitrite (0, 5, 10, 25, and 50 μM; lanes 2–6), resolved in 12% gels by reducing SDS/PAGE, and examined by Western blot analysis with the monoclonal antibody against nitrotyrosine. Tyrosine-nitrated bovine serum albumin (lane 1) served as a positive control. Approximate sizes (kDa) were estimated using prestained molecular mass markers to the left.

contaminants (such as nitrate, nitrite, or H_2O_2), since (*i*) exposure to decomposed peroxynitrite did not affect activity and (*ii*) catalase was included in each reaction to scavenge any traces of H_2O_2. Immunoblot analysis (Fig. 4*B*) of identical aliquots generated by the peroxynitrite dose-response reaction demonstrated an inverse relationship, wherein increased levels of tyrosine nitration accompanied the decrease in MnSOD activity.

DISCUSSION

The activity of the mitochondrial antioxidant enzyme MnSOD is dramatically decreased in chronically rejecting human renal allografts despite an overall increase in translated MnSOD protein. This paradoxical finding may be a consequence of enzyme inactivation after endogenous exposure to the short-lived potent oxidant and nitrating agent peroxynitrite. Increased levels of tyrosine-nitrated MnSOD were present in chronically rejected renal tissue and peroxynitrite was found to nitrate and inactivate recombinant human MnSOD *in vitro*. Enhanced immunostaining for both MnSOD and nitrotyrosine was colocalized to tubular epithelial cells in chronically rejected renal allografts, suggesting a cytotoxic role for peroxynitrite during renal transplant dysfunction. Tubular damage and atrophy is a major component of chronic renal allograft rejection and may be the most important determinant of eventual loss of renal function in these transplants (29–31).

Eukaryotic regulation of MnSOD gene expression remains unclear. Decreased levels of MnSOD mRNA and activity have been found to be associated with hypoxia, human immunodeficiency virus 1 TAT expression, and several tumor cell lines (27, 32–34); whereas additional efforts have demonstrated a hypoxia-mediated increase in MnSOD activity (35). In contrast, induction of MnSOD mRNA and protein has been correlated with ischemia-reperfusion, irradiation, leukocyte invasion, hyperoxia, glomerular inflammation, and colitis (36–42). Increased MnSOD expression represents an appropriate response to oxidative injury as a mechanism to protect against increasing levels of reactive oxygen species. This rationale is consistent with transfection experiments wherein overexpression of MnSOD protected endothelial cells from hyperoxic conditions (43). The molecular mechanisms involved in induction of MnSOD during oxidative stress have not been completely elucidated; however, the proinflammatory cytokines tumor necrosis factor α and interleukin 1 have been implicated (44, 45).

Results described herein provide additional insights into regulation of MnSOD activity beyond that previously reported. Compared with control kidney, tissue homogenates from chronically rejected human renal allografts demon-

strated both decreased levels of MnSOD activity and increased expression of MnSOD protein. Similar results have been demonstrated in type II alveolar epithelial cells exposed to hyperoxic conditions *in vivo* (39). The observed increase in MnSOD protein may occur in response to decreased MnSOD activity in an attempt to compensate for the loss of protection against oxidative stress. However, during chronic rejection this compensatory response appears to be inadequate, since the rate of MnSOD inactivation by peroxynitrite may exceed that associated with *de novo* synthesis.

Chronically rejecting renal extracts also contained a few specific tyrosine-nitrated polypeptides, including the mature mitochondrial form of MnSOD. *In vitro* studies demonstrated that peroxynitrite readily tyrosine-nitrates MnSOD and inhibits its enzymatic activity, a process which would increase the steady-state concentration of mitochondrial $O_2^{\cdot-}$ *in vivo*. Enzymatically generated $NO^{\cdot}$ is the sole source of nitrogen oxides *in vivo*, yet $NO^{\cdot}$ is incapable of both nitrating tyrosine (46) and inactivating MnSOD (47). Both $NO^{\cdot}$ and peroxynitrite have been shown to have adverse affects on mitochondrial respiration (48–56); however, $NO^{\cdot}$ most likely exerts its irreversible cytotoxic actions through peroxynitrite formation (57). Tyrosine nitration and inactivation of MnSOD during chronic rejection may represent a more general mechanism for progressively enhancing mitochondrial damage during oxidant stress (Fig. 5).

$NO^{\cdot}$ inhibits oxygen consumption in isolated mitochondria (52) by competing with oxygen for the binding site of cytochrome *c* oxidase (complex IV). Inhibition of complex IV by $NO^{\cdot}$ is rapidly reversible and, therefore, unlikely to be lethal to the cell (Fig. 5, solid lines). However, this process will decrease electron transport and potentially increase the concentration of $O_2^{\cdot-}$ as electrons (e^-) leak from reduced electron transport centers (e.g., complex I). Moreover, the concentrations of $NO^{\cdot}$ required to inhibit complex IV are sufficient to compete effectively with MnSOD for $O_2^{\cdot-}$ (14) by a rapid reaction (15) generating peroxynitrite ($ONOO^-$), which can nitrate tyrosine residues (Y-NO_2) and inactivate MnSOD to increase further the intramitochondrial level of $O_2^{\cdot-}$. Diffusion of $NO^{\cdot}$ into this

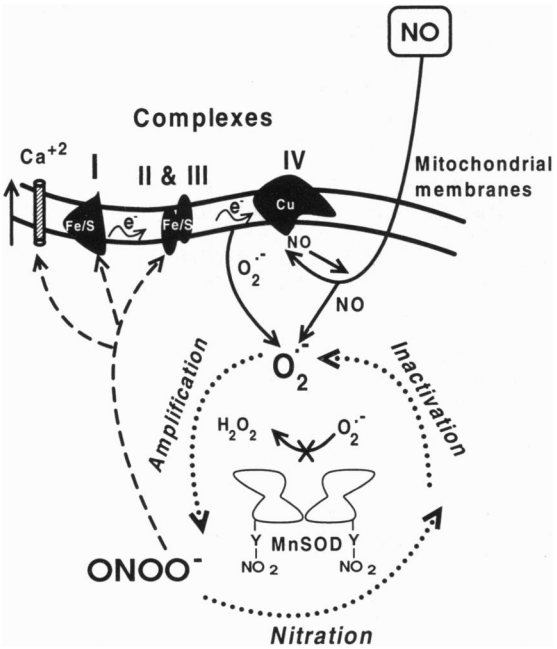

FIG. 5. Schematic diagram demonstrating the peroxynitrite-mediated amplification cycle, a general mechanism whereby tyrosine nitration and inactivation of MnSOD progressively enhances peroxynitrite production and subsequent mitochondrial damage during oxidant stress (see *Discussion* for description).

$O_2^{\cdot-}$-enriched environment may initiate a futile biological cascade resulting in additional generation of $ONOO^-$ (Fig. 5, dotted lines). Consequently, this peroxynitrite-mediated amplification cycle would result in a progressive increase of mitochondrial levels of $ONOO^-$, which can induce additional cytotoxic effects.

Peroxynitrite can inactivate aconitase, a Krebs cycle enzyme, after a direct attack of the iron–sulfur (Fe–S) centers (53). Peroxynitrite has been shown to inactivate irreversibly both NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II and III) in the electron transport chain (54). Peroxynitrite exposure also opens a cyclosporin sensitive Ca^{2+} pore in mitochondria (58), releasing mitochondrial calcium stores into the cytoplasm to induce further cell injury (Fig. 5, dashed lines). Carbon dioxide, which would be elevated in this microenvironment, has been shown to enhance the efficiency of tyrosine nitration by peroxynitrite (59). In addition to the adverse effects on mitochondrial enzymes, high steady-state concentrations of peroxynitrite generated by this amplification cycle may induce (*i*) the loss of intracellular energy stores (56), (*ii*) the initiation of lipid peroxidation (60), (*iii*) DNA strand breakage (56), and (*iv*) cellular apoptosis (61, 62). This proposed cytotoxic scheme involving peroxynitrite-induced modification of MnSOD is consistent with the lethal effects observed in MnSOD "knockout" transgenic mice (13).

Tyrosine nitration and inactivation of MnSOD demonstrated a similar dose-dependent response to peroxynitrite, suggesting that enzymatic activity is sensitive to tyrosine modification. Analysis of crystallographic data obtained from homodimeric human MnSOD (63) indicates that Tyr_{35} and Tyr_{166} are oriented toward the manganese atom within the active site. Nitration of critical tyrosine residues would introduce a negative charge and potentially block $O_2^{\cdot-}$ from gaining access to the active site. Preliminary data suggests further that MnSOD may catalyze its own tyrosine nitration similar to what has been described for Cu,ZnSOD (64). Even though results presented herein provide a compelling argument that peroxynitrite may effect MnSOD activity by nitration of tyrosine residues, inhibition also could occur after oxidation of either the manganese atom or other amino acid residues, including cysteine (65), methionine (66), and tryptophan (67). However, regardless of whether MnSOD inactivation results from nitration or oxidation, once this crucial antioxidant enzyme becomes tyrosine nitrated, it is inactivated.

Results from this study demonstrate that endogenous tyrosine nitration is not promiscuous since only a few specific proteins appear to be targeted during chronic rejection. Exposure of control kidney extracts to peroxynitrite *in vitro* results in tyrosine nitration of similar polypeptides including MnSOD, providing further evidence that (*i*) tyrosine nitration detected in rejecting kidney extracts is probably a consequence of peroxynitrite rather than other putative nitrating agents like nitrogen dioxide (68) and (*ii*) peroxynitrite is able to target tyrosine nitration of MnSOD within the internal cellular milieu. Regardless of the exact reactive nitrogen species, MnSOD is tyrosine-nitrated and inactivated during chronic rejection of human renal allografts. The low level of nitrated MnSOD observed in the control kidney may be reflective of either trauma prior to death, post-mortem tissue ischemia, or basal tyrosine nitration levels under normal physiologic conditions. Because nitration of tyrosine occurs in many other pathophysiologic conditions (17–22) and is not unique to chronic rejection, current efforts include determining the involvement of modified MnSOD in other disease processes. Early preliminary results suggest that tyrosine nitration of MnSOD is associated with rheumatoid arthritis, sepsis, and myocarditis. These observations predict that inactivation of MnSOD by the peroxynitrite-mediated amplification cycle

may be a general cytotoxic mechanism in many different pathophysiologic conditions.

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