Quantitative assessment of tyrosine nitration of manganese superoxide dismutase in angiotensin II-infused rat kidney


Manganese superoxide dismutase (MnSOD) is a key mitochondrial antioxidant enzyme that is inactivated in conditions of oxidant stress by reacting with peroxynitrite to form 3-nitrotyrosine in its active site. The increase in 3-nitrotyrosine content in MnSOD in the kidney of angiotensin II-infused rats was assessed in this study by immunohistochemistry, Western blotting, immunoprecipitation, and HPLC with UV detection (HPLC-UV). MnSOD activity decreased ~50% in angiotensin II-infused rat kidneys (24 ± 4.6 vs. 11 ± 5.2 U/mg) without a change in protein expression. Immunohistochemical staining showed 3-nitrotyrosine predominantly in distal tubules and collecting duct cells in the angiotensin II-infused rat kidneys. By two-photon microscopy, 3-nitrotyrosine colocalized with MnSOD. Total 3-nitrotyrosine content in kidney homogenates was increased in angiotensin II-infused rat kidney [3.2 ± 1.9 (sham treated) vs. 9.5 ± 2.3 ng/mg protein by HPLC-UV detection]. With tracer amounts of tyrosine-nitrated recombinant MnSOD, the most sensitive technique to detect tyrosine nitration of MnSOD was immunoprecipitation from tissue with anti-MnSOD antibody, followed by detection of 3-nitrotyrosine by Western blotting or HPLC. By HPLC, 3-nitrotyrosine content of kidney MnSOD increased 13-fold after angiotensin II infusion, representing an increase from approximately one-twentieth to one-fifth of the total 3-nitrotyrosine content in sham-treated and angiotensin II-infused rat kidneys, respectively. Angiotensin II-induced hypertension is accompanied by increased tyrosine nitration of MnSOD, which, because it inactivates the enzyme, may contribute to increased oxidant stress in the kidney.

nitrotyrosine; oxidant stress

ANGIOTENSIN II-INDUCED HYPERTENSION is associated with an increase in superoxide production by the activation of NADPH oxidase (23, 24, 30). Superoxide anion readily reacts with nitric oxide (NO) to generate peroxynitrite (ONOO−), which can nitrate the 3-position of tyrosine residues on proteins (5). Therefore, 3-nitrotyrosine is a marker of oxidative stress, which is evoked in various diseases including human atherosclerosis, pulmonary and heart disease, acute and chronic kidney rejection, Alzheimer disease, Parkinson disease, and amyotrophic lateral sclerosis (2, 6, 15–19, 21, 25). Moreover, 3-nitrotyrosine formation itself may change protein function. For example, manganese superoxide dismutase (MnSOD; Refs. 18, 19, 31), neurofilament L (10, 26), the type II isoform of the sarcoplasmic reticulum calcium ATPase (1), and prostacyclin synthase (32) have been reported to be inactivated by 3-nitrotyrosine formation in vivo.

MnSOD, which is the major antioxidant defense within mitochondria, is susceptible to rapid inactivation by relatively low concentrations of ONOO− (19). The overall importance of this enzyme is evident from studies in which mice genetically deficient in MnSOD suffer from a loss of mitochondrial iron-sulfur centers, a modification proving lethal to newborns (18). MnSOD in the kidney is reported to be tyrosine nitrated during aging (28) and transplant graft rejection (18). Therefore, tyrosine nitration of MnSOD may decrease its function and enhance the oxidative damage of cells in disease states.

Here we report that MnSOD from angiotensin II-infused rat kidney demonstrates both significant increase in tyrosine nitration and decrease in its enzymatic activity. We used immunohistochemistry, Western blot analysis, and HPLC-UV detection with immunoprecipitation techniques to demonstrate 3-nitrotyrosine in the protein. Recombinant MnSOD was tyrosine nitrated with peroxynitrite to track the recovery and sensitivity of detection of the modified native protein.

MATERIALS AND METHODS

Surgical procedure. The study was approved by the Boston University Medical Center Institutional Animal Care and Use Committee.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Use Committee. Male Wistar rats (Charles River Laboratories, Wilmington, MA) were acclimatized in a temperature- and light-controlled room and allowed access to standard rat chow and water. The rats were randomly divided into sham-treated and angiotensin II-infused groups. Osmotic mini-pumps (model 2001; Alza, Palo Alto, CA) were implanted under sterile conditions in the midscapular region of rats (250–275 g) after removal of paraffin and embedded in paraffin and cut in half, placed in 4% formalin, processed, dehydrated, tissue sections were treated with 10 mmol/l citric acid (pH 6). Sections were microwave heated (2 min, 3 times at 700 W) to recover antigenicity. Nonspecific binding was blocked with 10% normal goat serum in PBS (pH 7.4) for 30 min before incubation with polyclonal anti-nitrotyrosine antibody (1 μg/ml; Upstate Biotechnology) in PBS with 1% BSA overnight at 4°C. Tissue sections were then incubated for 30 min at room temperature with a biotinylated anti-rabbit IgG (1:800) secondary antibody by using the Vectastain ABC kit (Vector). Vector Red alkaline phosphatase substrate (Vector) was used to visualize 3-nitrotyrosine. Specificity of anti-3-nitrotyrosine antibodies was confirmed by preincubation of the antibody with free 3-nitrotyrosine (10 mmol/l) or by using a nonimmune rabbit IgG (Vector) isotypic control.

Fluorescent secondary antibodies were imaged on sections of rat kidney with a two-photon scanning confocal microscope designed and constructed in house in collaboration with the laboratory of Dr. Peter So (Massachusetts Institute of Technology, Cambridge, MA). The light source was a diode laser-pumped titanium-sapphire laser (Coherent, Santa Clara, CA). The excitation pulses were centered at a wavelength of 800 nm, and the power was reduced using polarizing optics. Fluorescence emission wavelengths were separated by a 565-nm dichroic mirror, and the emission filters were centered at 605 nm with a 55-nm bandwidth and at 535 nm with a 50-nm bandwidth for the long and short emitted wavelengths, respectively (Chroma Technology, Brattleboro, VT). Images were 512 × 512 pixels and were analyzed with NIH Image J and Adobe Photoshop.

**Nitrination of MnSOD in vitro.** Reactions between ONOO− and recombinant human MnSOD (kindly provided by Dr. Lee Ann MacMillan-Crow, Department of Surgery, School of Medicine, University of Alabama at Birmingham, Birmingham, AL) were carried out at room temperature in 0.1 mol/l phosphate buffer at pH 7.4. ONOO− was added to MnSOD while vortex mixing at the final indicated concentrations. Working solutions of peroxynitrite were prepared by diluting stocks in 0.1 mol/l NaOH before use, and the concentration of peroxynitrite was determined by spectrophotometry (ε = 1,670 M−1 cm−1; λ = 502 nm in 0.1 mol/l NaOH). Five micromoles per liter was the minimal concentration of ONOO− to obtain detectable nitrated MnSOD as detected either by Western blot with monoclonal anti-nitrotyrosine antibody or by HPLC. Adding 12.6 μM of ONOO− to 10 μg of MnSOD in a 87.4-μl final volume (50 μmol/l final concentration of ONOO−) resulted in tyrosine nitration of approximately one-half of the MnSOD, as indicated by the nitrotyrosine content determined by HPLC. Thereafter, 50 μmol/l ONOO− was used to nitrate recombinant MnSOD.

**Kidney homogenization.** Animals were anesthetized with pentobarbital sodium, and both kidneys were collected and immediately frozen in liquid N2 and stored at −80°C until further processing. Frozen tissues were homogenized manually in a buffer containing 50 mmol/l Tris·HCl (pH 7.4), 150 mmol/l NaCl, 0.1 mmol/l diethylenetriamine pentaacetic acid (DTPA), 1 mmol/l NaF, 1% NP-40, and 0.25% deoxycholic acid, with the addition of 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 μg/ml aprolin, and 10 mg/ml PMSF. Each sample was sonicated on ice with 10 pulses at 40% duty cycle and output 3 in a Branson sonifier (model 450) and then centrifuged at 16,000 g for 30 min at 4°C. The protein concentration of the supernatant was determined with the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

**MnSOD activity.** Horse heart cytochrome c (type III), xanthine, and xanthine oxidase were purchased from Sigma (St. Louis, MO). Synthetic angiotensin II (Calbiochem, La Jolla, CA) dissolved in 0.15 mol/l NaCl and 1 mmol/l acetic acid was implanted.

Under these conditions, one unit of SOD activity is defined as the amount that causes 50% inhibition of the initial rate of reduction of cytochrome c by xanthine and xanthine oxidase as described previously (20). Ten microliters of the supernatant was used in the assay mixture consisting of 50 mmol/l Tris·HCl, 0.1 mmol/l EDTA, 50 μmol/l xanthine, 10 μmol/l cytochrome c, and enough xanthine oxidase (~6 mmol/l) to cause ΔA550 = 0.025 min−1 at pH 7.0, in a total reaction volume of 300 μl. Under these conditions, one unit of SOD activity was defined as the amount that causes 50% inhibition of the initial rate of reduction of cytochrome c. MnSOD activity was measured by inhibiting extracellular and cytosolic Cu/Zn SOD activity with KCN (1 mmol/l). In a separate series of angiotensin II- or sham-infused rat kidneys we found no substantial difference in the results reported here when we made measurements at pH 7.8 and with 5 mmol/l NaCN, as suggested by Okado-Matsumoto and Friedman (22) to slow the reaction of cyanide with cytochrome c.

**Western blots.** Kidney protein samples, 50 μg/ lane, were electrophoretically size fractionated on 15% SDS-polyacrylamide gels at 120 V for 90–120 min and transferred to Immob-Blot cellulose membranes (Bio-Rad) at 200 mA for 90 min. Membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk for 1 h. For detection of MnSOD, membranes were incubated with the primary antibody (polyclonal rabbit anti-MnSOD IgG, dilution 1:2,000; Upstate Biotechnology) at 4°C overnight. Nitrotyrosine in MnSOD was detected by incubating membranes at 4°C overnight with a mouse monoclonal IgG anti-nitrotyrosine antibody (dilution 1:1,000; Upstate Biotechnology). The membranes were washed for 30 min, with the wash buffer changed every 10 min before incubation with the Fc fragment-specific, horseradish peroxidase-labeled goat anti-rabbit IgG (1:10,000; Pierce, Rockford, IL) in Tris-buffered saline with 5% nonfat milk for 1 h. For detection of MnSOD, membranes were incubated with the primary antibody (polyclonal rabbit anti-MnSOD IgG, dilution 1:2,000; Upstate Biotechnology) at 4°C overnight. Nitrotyrosine in MnSOD was detected by incubating membranes with a mouse monoclonal IgG anti-nitrotyrosine antibody (dilution 1:1,000; Upstate Biotechnology). The membranes were washed for 30 min with the wash buffer changed every 10 min before incubation with the Fc fragment-specific, horseradish peroxidase-labeled goat anti-mouse IgG (1:10,000; Pierce, Rockford, IL) for nitrotyrosine in MnSOD detection. The washes were again repeated for 30 min before the membrane was scanned for bound antibody by chemiluminescence with enhanced chemiuninescence detection reagents (Super Signal; Pierce). The staining of blots by anti-nitroty-
MnSOD activity decreased significantly by ~50% in the angiotensin II-infused group compared with the sham-treated group (24 ± 4.6 vs. 11 ± 5.1 U/mg, P < 0.01; Fig. 2C).

Detection of 3-nitrotyrosine containing recombinant MnSOD added to homogenates of rat kidney with monoclonal anti-nitrotyrosine antibody and HPLC. We first determined the sensitivity of Western blotting and HPLC to detect tyrosine-nitrated recombinant MnSOD added to kidney homogenate. When increasing amounts of tyrosine-nitrated MnSOD were added to 50 μg of normal rat kidney homogenate and then subjected to SDS-PAGE and Western blotting, the detection limit with monoclonal anti-nitrotyrosine antibody was 0.3 μg of nitrated MnSOD (containing 2.8 ng of nitrotyrosine, data not shown). The detection limit by HPLC was reached by adding 0.5 μg tyrosine-nitrated MnSOD (containing 4.7 ng nitrotyrosine) to 1 mg of kidney homogenate (data not shown).

Sensitivity of detection of tyrosine-nitrated MnSOD by immunoprecipitation from rat kidney homogenate. Different amounts of tyrosine-nitrated MnSOD were added to normal rat kidney homogenate. MnSOD was then immunoprecipitated from the mixture with polyclonal anti-MnSOD antibody. The immunoprecipitated proteins were separated by SDS-PAGE and subsequently blotted and stained with monoclonal anti-nitrotyrosine antibody. The threshold for detection of tyrosine-nitrated MnSOD was reached by adding 0.1 μg of tyrosine-nitrated MnSOD (containing 0.9 ng of nitrotyrosine) to 1 mg of kidney homogenate (data not shown).

RESULTS

Immunohistochemistry. Immunohistochemical staining performed with a polyclonal anti-nitrotyrosine antibody was far more intense in cortex, outer medulla, and papilla from kidneys of angiotensin II-infused rats compared with that in kidneys from sham-operated animals (Fig. 1; A–D). Nitrotyrosine was localized predominantly to collecting ducts of cortex (Fig. 1, A and B), outer medulla (Fig. 1E) and tubules within the inner medulla (papilla) of angiotensin II-infused rats (Fig. 1, B and C). When cortex of kidney from angiotensin II-treated rats was immunostained for nitrotyrosine and MnSOD and examined by two-photon microscopy, substantial colocalization of MnSOD and nitrotyrosine was evident within cells of the collecting ducts (Fig. 1F).

MnSOD expression and activity in angiotensin II-infused rat kidney. MnSOD protein expression was not significantly different between sham-treated and angiotensin II-infused groups (Fig. 2, A and B). The activity of total SOD in kidney was also unchanged between these two groups [901 ± 69 U/mg (n = 4) in sham-treated group vs. 833 ± 43 U/mg (n = 4) in angiotensin-II infused group; Fig. 2C]. However, MnSOD activity decreased significantly by ~50% in the angiotensin II-infused group compared with the sham-treated group (24 ± 4.6 vs. 11 ± 5.1 U/mg, P < 0.01; Fig. 2C).
nate, the nitrotyrosine staining of the MnSOD immunoprecipitate was increased (Fig. 4A).

To confirm the increase in nitrotyrosine in MnSOD caused by angiotensin II infusion, the immunoprecipitated protein from 1 mg of sham-treated or angiotensin II-infused rat kidney homogenate, with or without the addition of recombinant tyrosine-nitrated MnSOD containing 1.5 ng of nitrotyrosine, was hydrolyzed and nitrotyrosine was measured by HPLC. In sham-treated rat kidney samples, there was 0.15 ± 0.08 ng nitrotyrosine/mg tissue protein. The 3-nitrotyrosine content in MnSOD immunoprecipitated from angiotensin II-infused rat kidneys was increased 13-fold to 2 ± 0.4 ng nitrotyrosine/mg tissue protein (P < 0.01). The recovery of the added MnSOD containing 1.5 ng of nitrotyrosine was nearly quantitative. The amount of 3-nitrotyrosine in the total homogenate from angiotensin II-infused rat kidneys was approximately three times more than that of sham-treated rats (Fig. 4B). Thus 3-nitrotyrosine-containing MnSOD increased from one-twentieth to one-fifth of the total tissue 3-nitrotyrosine content after angiotensin II infusion.

DISCUSSION

Protein tyrosine nitration in the kidney has been detected in a number of pathological conditions, such as human renal allograft rejection (18), experimental glomerulonephritis (11), diabetic nephropathy (23, 27), endotoxin-induced kidney injury (7), transgenic sickle
Fig. 2. MnSOD expression and activity in angiotensin II infused rat kidney. A: Western blots of samples of sham-treated (n = 6) and angiotensin II-infused (n = 6) rat kidney homogenates, stained with polyclonal anti-MnSOD antibody. There was no change in MnSOD expression between sham-treated and angiotensin II-infused rat samples. B: summary of MnSOD expression as determined by densitometry of the blot shown in A. C: total SOD and MnSOD activity were measured with the cytochrome c method by spectrophotometry and expressed as units per milligram of protein. Angiotensin II had no effect on total SOD activity but significantly decreased MnSOD activity in rat kidney. **P < 0.01 vs. sham treated. S1–S4, sham-treated rats 1–4; A1–A4, angiotensin II-treated rats 1–4.

Fig. 3. Sensitivity of detecting 3-nitrotyrosine in MnSOD in kidney homogenate. A: different amounts of tyrosine-nitrated recombinant MnSOD were added to 1 mg of protein homogenate of normal rat kidney. After immunoprecipitation (IP) with polyclonal anti-MnSOD antibody (p-MnSOD), the protein was separated by SDS-PAGE and Western blotted (WB) for nitrotyrosine with monoclonal antibody (m-NY). The detection limit was 0.1 μg of tyrosine-nitrated MnSOD (NY-MnSOD). Note the detection of the rat IgG light chain with the anti-mouse secondary antibody at a slightly higher molecular weight than MnSOD. B: different amounts of tyrosine-nitrated MnSOD were added to 1 mg of protein homogenate of normal rat kidney. After immunoprecipitation with monoclonal anti-nitrotyrosine antibody, the protein was blotted for MnSOD with polyclonal antibody. The detection limit was 0.6 μg of tyrosine-nitrated MnSOD.

There are several methods to detect 3-nitrotyrosine in tissues including immunohistochemistry, Western blot, HPLC-UV, electrochemical detection, and GC-MS analysis. Immunodetection is sensitive and conventional; however, it is not quantitative and requires the careful consideration of the specificity of the antibodies used. HPLC-UV detection is quantitative; however, artifacts have also been reported with this method (13). There are many reports of tyrosine nitration that use only one method, at the risk of one or more of these shortcomings. Therefore, we used both immunochromatography and HPLC methods to detect tyrosine nitration on a specific protein in an animal model of disease. This study benefited from the fact that we had specific antibodies to MnSOD, enabling immunoprecipitation of the protein and increasing sensitivity of its detection. We also were able to tyrosine nitrate the recombinant MnSOD to test sensitivity and specificity of the methods used. Our data indicate that Western blot of whole kidney protein with anti-nitrotyrosine antibody has nearly twice the sensitivity of HPLC-UV when detecting tyrosine-nitrated recombinant MnSOD added to kidney homogenate (2.8 compared with 4.7 ng nitrotyrosine/mg protein). However, if the goal is to
detect low amounts of nitrotyrosine on unknown proteins in tissue homogenates, Western blot of tissue homogenates with anti-nitrotyrosine antibody is not as sensitive as HPLC. In the case of whole tissue homogenates of angiotensin II-infused rat kidney, we could not detect any protein bands by Western blot with either polyclonal or monoclonal anti-nitrotyrosine antibodies. In contrast, with HPLC of hydrolyzed MnSOD immunoprecipitate, we detected 0.15 ng nitrotyrosine/mg tissue in sham-treated rat kidney homogenates, which was increased 13-fold by angiotensin II infusion. Finally, we estimate that 5% of nitrotyrosine in sham-treated rat kidneys is accounted for by tyrosine-nitrated MnSOD, which increased to 20% of the total nitrotyrosine content in angiotensin II-infused rat kidney.

Immunoprecipitation with an anti-nitrotyrosine antibody is the preferred technique for detecting unknown tyrosine-nitrated proteins in tissues. This technique has been used to identify tyrosine-nitrated proteins including MnSOD in lung and liver of endotoxemic rats (3). However, immunoprecipitation with anti-nitrotyrosine antibody followed by Western blotting for nitrotyrosine failed to show any detectable proteins in our samples, except for the IgG present in the immunoprecipitate (data not shown). Comparing "nonspecific" immunoprecipitation with anti-nitrotyrosine antibody followed by staining with anti-MnSOD antibody with "specific" immunoprecipitation of MnSOD followed by staining for nitrotyrosine, we found the latter to be sixfold more sensitive.

Nitrotyrosine, as detected by immunohistochemistry, was more intense in kidneys of angiotensin II-treated rats compared with sham-treated rats. These findings are consistent with the quantitative data obtained from kidney homogenate. In addition, we were able to use two-photon microscopy to substantiate the colocalization of 3-nitrotyrosine and MnSOD in renal tubular cells. Interestingly, nitrotyrosine staining was most intense in distal tubules (predominantly collecting ducts) within the cortex and outer medulla and in the tubules within the renal papilla. These cells are reported to have increased expression of MnSOD and, presumably for that reason, to resist damage during ischemia-reperfusion (14). In addition, distal tubular cells have recently been reported to be those in which NADPH oxidase is induced in response to angiotensin

Fig. 4. 3-Nitrotyrosine in MnSOD from angiotensin II-infused rat kidney. A: proteins were immunoprecipitated with polyclonal anti-MnSOD antibody from 1 mg of protein homogenate of both sham-treated (S) and angiotensin II-infused (A) rat kidneys and blotted for nitrotyrosine with monoclonal antibody. Staining was observed in 2 of the 3 S animals, in all 6 of the A animals, and in all samples to which tyrosine-nitrated MnSOD (0.15 μg containing 1.5 ng nitrotyrosine) was added to enhance the signal (+NY). Note that the rat IgG was not detected as it was in Fig. 3A, because an anti-mouse Fc fragment-specific secondary antibody was used. No staining was observed if antibody (no anti) or homogenate (no hom) was omitted from the immunoprecipitation. B: proteins were first immunoprecipitated with polyclonal anti-MnSOD antibody from 1 mg of protein homogenates of both sham-treated (n = 4) and angiotensin II-infused (n = 6) rat kidneys, and the immunoprecipitate was then hydrolyzed in 6 N HCl, and nitrotyrosine was detected with HPLC-UV (left). Hatched bars indicate the values obtained in samples to which tyrosine-nitrated MnSOD (0.15 μg containing 1.5 ng nitrotyrosine) was added to enhance the signal. Total protein homogenates (1 mg) of both sham-treated (n = 8) and angiotensin II-infused (n = 10) rat kidneys were hydrolyzed by 6 N HCl, and then total homogenate 3-nitrotyrosine was detected by HPLC-UV (right). Note the different ordinate values for right and left sides of B. *P < 0.01 vs. sham-treated group. **P < 0.01 vs. with nitrotyrosine added.
II (9), and in preliminary studies (S. Xu and B. Jiang, unpublished observations) we have confirmed the presence in these cells of p47phox. It is possible that oxidants generated by this enzyme are the cause of tyrosine nitration of MnSOD, although this conclusion awaits further studies.

The study of the role of NO and oxidants in vivo is hindered by technical difficulties inherent in the non-invasive measurement and short half-life of NO, superoxide anion, peroxynitrite, and related species. In an effort to circumvent this difficulty, biomarkers that specifically indicate tissue nitration have been sought. 3-Nitrotyrosine was the first candidate for such a biomarker (5), and early attempts to detect nitrotyrosine used antibodies to provide some qualitative indication of protein nitration (29). Unfortunately, quantitative immunochemical analysis is subject to variability in both antibody specificity and affinity. We therefore used different methods to quantify nitrotyrosine in vivo, and the sensitivity of each method was compared. This approach suggests appropriate methods for identifying and quantifying novel proteins that are tyrosine nitratetd in tissues.

DISCLOSURES

These studies were supported by National Institutes of Health (NIH) Grants R01-HL-55620-07 and R01-HL-31607-20 (R. A. Cohen) and by the NIH Boston University Cardiovascular Proteomics Center (R01-HV-28178; T. Adachi, M. Kirber, and R. A. Cohen).

REFERENCES


