Nitrotyrosine formation with endotoxin-induced kidney injury detected by immunohistochemistry

KA BIAN,1 KAREN DAVIS,1 JEFF KURET,2 LESTER BINDER,2 AND FERID MURAD1

1Department of Integrative Biology, Physiology, and Pharmacology, University of Texas-Houston Medical School, Houston, Texas 77030; and 2Department of Cell Biology, Northwestern University Medical School, Chicago, Illinois 60611

Bian, Ka, Karen Davis, Jeff Kuret, Lester Binder, and Ferid Murad. Nitrotyrosine formation with endotoxin-induced kidney injury detected by immunohistochemistry. Am. J. Physiol. 277 (Renal Physiol. 46): F33–F40, 1999.—The presence of nitrotyrosine in the kidney has been associated with several pathological conditions. In the present study, we investigated nitrotyrosine formation in rat kidney after animals received endotoxin for 24 h. With lipopolysaccharide (LPS) treatment, immunohistochemical data demonstrated intense nitrotyrosine staining throughout the kidney. In spite of marked nitrotyrosine formation, the architectural appearance of tubules, glomeruli, and capillaries remained intact when examined by reticulin staining. Our data suggested that the marked staining of nitrotyrosine in proximal tubular epithelial cells was in the subapical compartment where the endocytic lysosomal apparatus is located. Thus a large portion of nitrotyrosine may come from the hydrolysis of nitrated proteins that are reabsorbed by the proximal tubule during the LPS treatment. We also found the colocalization of nitric oxide synthase (NOS-1) and nitrotyrosine within the macula densa of LPS-treated rats by using a double fluorescence staining method. In renal arterial vessels, vascular endothelial cells were more strongly stained for nitrotyrosine than vascular smooth muscle cells. Control animals without LPS treatment showed much less renal staining for nitrotyrosine. The general distribution of nitrotyrosine staining in control rat renal cortex is in the proximal and convoluted tubules, whereas the endothelial cells of vasa recta are major areas of nitrotyrosine staining in inner medulla. The renal distribution of nitrotyrosine in control and LPS-treated animals suggests that protein nitration may participate in renal regulation and injury in ways that are yet to be defined.

NITRIC OXIDE (NO·), a free radical gas, can regulate an ever-growing list of biological processes [reviewed by Murad (25, 26)]. In the normal kidney, NO· is actively involved in the regulation of glomerular hemodynamics and filtration and the control of tubular function and sodium excretion (30, 36). However, excessive NO· production may contribute to several renal diseases, including immune-mediated glomerulonephritis, post-ischemic renal failure, radiocontrast nephropathy, obstructive nephropathy, and renal allograft rejection [reviewed by Kone (17)].

The paramagnetic NO· rapidly reacts with superoxide (O2·⁻) to form peroxynitrite anion (ONOO⁻·), a potent nitrating and oxidizing agent, resulting in oxidative damage to proteins, lipids, carbohydrates, DNA, and sulfhydryl groups. ONOO⁻· can give several reactive products, including hydroxyl radical and nitronium ion. It can nitrate aromatic amino acids, and nitration on the three position of tyrosine can form free or protein-associated nitrotyrosine (13, 32, 34). Nitrotyrosine has been used as an index of ONOO⁻· formation. The presence of nitrotyrosine in the kidney detected with immunohistochemistry has been associated with several pathological conditions, such as human renal allograft rejection (19), experimental glomerulonephritis (10), and the Goldblatt animal model for hypertension (2).

Acute renal failure due to endotoxemia is a common problem in clinical medicine. Endotoxins are released from the outer membrane of gram-negative bacteria and are composed of lipopolysaccharide (LPS). Endotoxins can activate macrophages and other cells that can release cytokines and inflammatory mediators, which increase the formation of the inducible isoform of nitric oxide synthase (NOS-2). The increased production of NO· and subsequent formation of ONOO⁻· and nitrotyrosine are thought to contribute to the inflammatory response (22, 29, 38). In the present study, we have examined the renal distribution of nitrotyrosine with immunohistochemical methods after rats have received LPS. With Western immunoblots of kidney extracts, we have also found several proteins (~39 and 70–75 kDa) that probably contributed to the immunohistochemical staining observed.

MATERIALS AND METHODS

Animal and tissue preparation. Male Sprague-Dawley rats (275–300 g) were obtained from Charles River. The experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Texas Medical School Animal Care and Use Committee. The rats were divided into control and experimental groups. LPS (serotype 0111:B4; Sigma) dissolved in sterile saline was administered intraperitoneally at a dose of 20 mg/kg body wt, and rats were killed under ether anesthesia at 6, 12, or 24 h after LPS injection. For the control group, rats were injected with the same volume of physiological saline. After death, the kidneys were collected and immediately frozen in liquid nitrogen. The tissues were stored at −135°C until further processing.

Frozen kidneys were pulverized with a pestle and mortar that contained liquid nitrogen and then homogenized at 4°C in 20 mM Tris·HCl buffer (pH 7.4) containing protease...
inhibitors (final concentration: 10 µg/ml soybean trypsin inhibitor, 10 µg/ml benzamidine, 5 milliISU inhibitor units/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 5 µg/ml antipain, 0.2 mM phenylmethane sulfonate fluoride, and 0.1 mM EDTA). Each sample was sonicated on ice with 10 pulses at 40% duty cycle and output 3 and then was centrifuged at 1,000 g for 15 min at 4°C. The supernatant fractions were used for SDS-PAGE and Western immunoblotting.

Immunohistochemistry. The animals were anesthetized with ether, and kidneys were perfusion fixed via the left ventricle with 4% paraformaldehyde. Kidneys were removed and incubated at 4°C overnight in 30% sucrose in Tris buffer (100 mM Tris base, pH 8.0). Tissues were embedded in optimum cutting temperature compound (Miles, Elkhart, IN) and were frozen in ethanol cooled on dry ice. The 3- to 8-µm frozen sections were prepared with a Minotome (Damon, MA) and were thaw mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Tissue sections were treated with 0.1% Triton X-100 for 5 min to permeabilize cell membranes. After being rinsed with 100 mM Tris buffer (pH 8.0), slides were incubated with donkey serum (at 1:20 dilution) for 1 h at room temperature. This was followed by overnight incubation with an anti-nitrotyrosine antibody (at 1:100 to 1:500 dilution). The anti-nitrotyrosine mouse monoclonal antibody was prepared against a random peptide of L-nitrotyrosine, L-alanine, and L-glycine coupled to keyhole limpet hemocyanin. Antibody purified from cell culture medium of a selected mouse clone was utilized. To assess nonspecific staining, control experiments were performed by incubating the slides without primary antibody or with primary antibody preincubated with nitrated BSA for 1 h at room temperature. Nitrotyrosine-containing BSA was prepared by treating BSA with peroxynitrite, tetranitromethane, or 3-morpholinosydnonimine as recently described (16).

Primary antibody preincubated under the same condition with either free L-3-nitrotyrosine (20 mM) or BSA that was not nitrated was also tested in our experiments. Biotin-conjugated donkey anti-mouse antibody (1:2,000) was used as a secondary antibody for avidin-biotin amplified 3,3'-diaminobenzidine (DAB) staining and was applied to sections for 1 h at room temperature. Sections were also stained with hematoxylin and silver impregnation (Gomori's method) was used for reticulin staining. In the immunofluorescence staining, a rabbit polyclonal antibody against nitric oxide synthase-1 (NOS-1; bNOS 251–270; Sigma) was used as a second primary antibody. Lissamine Rhodamine (LRSC)-conjugated affinity purified donkey anti-rabbit IgG (1:200; Jackson Immuno-chemicals, West Grove, PA) was used for detecting NOS-1. In the same section, Fluorescein (FITC)-conjugated affinity purified donkey anti-mouse IgM (1:200; Jackson Immuno-chemicals) was used for staining of nitrotyrosine. After incubation with both NOS-1 and nitrotyrosine primary antibodies overnight, the slides were incubated with the LRSC-conjugated secondary antibody for 1 h at room temperature, rinsed with 100 mM Tris buffer (pH 8.0), and further incubated with the FITC-conjugated secondary antibody under the same conditions. Sections were examined with a Zeiss Axiophot (D-7082) microscope. The fluorescence photomicrographs were taken using Kodachrome slide film, and Kodak LoyalGold film was used for color pictures.

Immunoblotting. The kidney lysates were subjected to standard SDS-PAGE and Western blotting techniques. The samples were electrophoresed using a 5% polyacrylamide stacking gel and a 7.5 or 10% polyacrylamide separating gel (gel size, 12 × 15 × 0.15 cm). Equal amounts of proteins (400 µg/well) were loaded onto the gel for each experimental sample. Separated proteins were transferred to nitrocellulose membranes, and the membranes were treated with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris-HCl and 130 mM NaCl, pH 7.6) plus 0.1% Tween 20 (TBS-T) and then were incubated at 4°C overnight with the anti-nitrotyrosine antibody. The membranes were washed with TBS-T and incubated with peroxidase-conjugated goat anti-mouse antibody. Chemiluminescence was used to identify nitrotyrosine-containing proteins according to the enhanced chemiluminescence Western blotting detection system (Amersham Life Science).

RESULTS

Immunoblotting. Western blot analysis indicated that there are two major groups of nitrotyrosine-containing proteins recognized by the anti-nitrotyrosine antibody in normal kidney. As shown in Fig. 1, the nitrated proteins of ~70–75 kDa were markedly increased in a time-dependent manner after LPS treatment. Similarly, another group of proteins of ~39 kDa were found in normal kidney extracts, and these proteins and/or

![Fig. 1. Representative Western blot analysis of nitrotyrosine-containing proteins in kidney extracts from a control rat (0 h) and rats after 6, 12, and 24 h of lipopolysaccharide (LPS, 20 mg/kg ip) treatment (n = 3 rats).](http://ajprenal.physiology.org/Downloadedfrom)
their nitrotyrosine content also increased with LPS treatment.

Immunohistochemistry. LPS treatment resulted in intense renal cortical staining of nitrotyrosine (Fig. 2, B and D). Preabsorption of the anti-nitrotyrosine antibody with nitrated BSA prevented renal staining (Fig. 2C). Similar inhibition of staining was observed when the antibody was pretreated with free nitrotyrosine (data not shown). However, preabsorption of the antibody with BSA did not alter the histochemical staining (Fig. 2D). In the LPS-treated renal cortex, intense nitrotyrosine staining was observed in the region of proximal and distal convoluted tubules, initial collecting tubules, interlobular vascular endothelium, and glomeruli. Control animals without LPS treatment showed much less renal staining for nitrotyrosine, and the general distribution of nitrotyrosine staining was in the area of proximal and convoluted tubules, but not in glomeruli.

Figure 3, A–C, shows a general distribution of nitrotyrosine staining in LPS-treated rat kidney outer medulla (A) and inner medulla (B and C). Strong nitrotyrosine staining was found in the inner medulla, which contains the thin limb of the loop of Henle, collecting tubules and ducts, and vasa recta. It is notable that nitrotyrosine staining of the endothelial cells was observed not only in the renal of LPS-treated animal but also in the vasa recta from control rats (Fig. 3D).

Despite the intense nitrotyrosine formation induced by 24 h of LPS treatment, the reticulin framework outlining renal glomeruli and tubules was not altered (Fig. 4) compared with normal kidney sections (data not show). The architectural appearance of tubules, glomeruli, and/or capillaries remained intact as demonstrated by silver staining of type III collagen, the basis of the reticulin stain (Fig. 4).

As demonstrated in Fig. 2, proximal convoluted tubules represent the major parenchymal component. A high-magnification micrograph of the pars convoluta of LPS-treated proximal tubule is shown in Fig. 5. The initial convoluted portion of the tubule is a direct continuation of parietal epithelium of Bowman’s capsule and has a characteristic tall brush border and a well-developed endocytic lysosomal apparatus system that is located at the apical cytoplasm beneath the luminal brush border. Histologically, numerous mitochondria are distributed in the basal part of the tubular cells. The nitrotyrosine staining was localized in the brush border and was greatest underneath the luminal brush border where the endocytic lysosomal apparatus is distributed. Although the staining seems much less than that of the tubular cells, the area where glomerular basement membrane and mesangial cells are located was also stained by anti-nitrotyrosine antibody (Fig. 5).
In the region of the cortical thick ascending limb adjacent to the hilus of the glomeruli of LPS-treated rats, a group of low columnar cells with the morphological appearance of macula densa cells (Fig. 6) demonstrated intense basolateral staining of nitrotyrosine. We confirmed the presence of nitrotyrosine-containing proteins in macula densa cells by co-staining the same section with anti-NOS-1 antibody (LRSC fluorescence, Fig. 7A) and anti-nitrotyrosine antibody (FITC fluorescence, Fig. 7B). Figure 7C indicated the colocalization of NOS-1 and nitrotyrosine within the macula densa. In control animals, there was no detectable nitrotyrosine formation in the macula densa.

The renal vasculature is a major component of the kidney and can be divided into three major groups: arterial vessels, glomeruli, and vasa recta (including capillaries). As described previously, the glomeruli and vasa recta demonstrated different intensity of nitrotyrosine staining. In renal arterial vessels, vascular endothelial cells were more strongly stained for nitrotyrosine than vascular smooth muscle cells (Fig. 8A). Figure 8B depicts the transverse course of an afferent (hilar) arteriole that connects a glomerulus and an interlobular artery and shows an intense endothelium staining of nitrated protein. It is notable that nitrotyrosine staining of endothelial cells was also clearly observed in renal vasa recta from control rats without LPS treatment (Fig. 3D).

**DISCUSSION**

NOS-2 expression and activity by LPS in various organs, including kidney, has been well documented (4, 8, 9, 11, 23, 28, 33, 37, 39, 41). This results in the formation of nitrotyrosine.
increased formation of NO\textsuperscript{•} and its oxidized products (7, 15, 44). Several studies have shown that enzymatically generated NO\textsuperscript{•} is the sole source of nitrogen oxides in vivo and nitration of tyrosine residues is almost certainly derived from NO\textsuperscript{•} [reviewed by Ischiropoulos (12)]. In fact, inhibition of NO\textsuperscript{•} synthesis successfully prevented the increases in nitrotyrosine content of various tissues (21, 31, 42). In the current study, the administration of LPS has markedly increased several groups of nitrated proteins of kidney lysates in a time-dependent manner (Fig. 1), which further confirmed the existence of endotoxin-triggered nitrotyrosine formation in rat kidney.

In LPS-treated rat kidney cortex, we have demonstrated that the most intense staining of nitrotyrosine was observed in the region of cortical labyrinth that consists of proximal and distal convoluted tubules, initial collecting tubules, and glomeruli. In the region of cortex, proximal tubules account for \(\sim 70\%\) of the relative tissue volume (14). The proximal tubule begins abruptly at the urinary pole of the glomerulus (Fig. 5) and consists of an initial convoluted portion (the pars convoluta) and a straight portion (the pars recta). The luminal brush border of the proximal tubule is composed of numerous microvilli that were stained by the anti-nitrotyrosine antibody. The cytoplasm immediately beneath the brush border contains many well-developed endocytic/lysosomal apparatuses that demonstrated very strong nitrotyrosine staining. However, less staining was found at the basal part of these cells where mitochondria are located (5). Further studies will be required, however, to define the specific intracellular sites of nitrotyrosine expression. Our finding suggests that the nitrotyrosine-containing proteins observed in the proximal tubule may have come from two sources. First, nitrotyrosine is formed in the proximal tubular cells as a consequence of LPS-induced NO\textsuperscript{•} production, since the tubule epithelium is the location for both endothelial nitric oxide synthase (NOS-3; see Ref. 43) and NOS-2 (23). The second source may be the reabsorption of nitrated proteins by the endocytic/lysosomal apparatus of the proximal tubule, which is responsible for the reabsorption of \(\sim 60\%\) of the glomerular ultrafiltrate, including glucose, amino acids, and minerals. It is known that ONOO\textsuperscript{−} reacts selectively and rapidly with the iron-sulfur centers of several mitochondrial respiratory proteins (35). However, in the present study, the strongest staining of nitrotyrosine in proximal tubular epithelial cells is not in the basal mitochondria region. Thus the cellular location of nitrotyrosine staining suggests that a large portion of nitrotyrosine may come from the hydrolysis of nitrated proteins during the 24 h of LPS treatment. The pathological implication of reabsorbed nitrotyrosine-containing proteins is unknown. The fate of the nitrated proteins is also unclear. They may be further metabolized in the kidney, redistributed to the blood and body, and/or be excreted.

The current study clearly colocalized NOS-1 and nitrotyrosine to the LPS-treated macular densa segment (MDS; Fig. 6 and 7). The concept that MDS is the principal site of NOS-1 gene expression in the rat kidney is generally accepted (40, 45), although NOS-1 immunoreactivity was also observed in the structures such as endothelium of efferent arterioles, single cells of the glomerular visceral epithelium, and perivascular nerves [reviewed by Kone and Baylis (18)]. Although Bachmann et al. (1) described that the NOS-1 labeling frequently extended beyond the typical location of the MDS in rat kidney, “perimacular” NOS-positive staining was sometimes located on the opposite side of the MDS or at a considerable distance upstream or downstream to the MDS when portions of the thick ascending limb were longitudinally sectioned (1). In our cross-sectional profiles of MDS, NOS-1 staining was largely restricted to the morphologically defined site of the MDS plaque adjacent to the extraglomerular mesangium. Several in vitro studies have indicated that
phosphorylation of NOS-1 by different kinases may affect the catalytic activity of NOS-1 (3, 6, 27); however, an effect of LPS on regulation of NOS-1 expression has not been described. Although our immunolocalization experiments should not be viewed as quantitative, NOS-1 immunoreactivity in the MDS appeared to be comparable between normal and LPS-treated rats. Perhaps LPS directly or indirectly increases protein kinases activity and stimulates the expression and/or activity of NOS-1 in MDS that results in increased nitrotyrosine formation. NO$^\cdot$, O$_2$^\cdot$, or ONOO$^-$ might also diffuse into the MDS from adjacent cells where abundant NO$^\cdot$ was produced after LPS stimulation. Although it has been demonstrated that NOS activity in MDS is directly linked with glomerular capillary pressure (45), the role of nitrotyrosine formation in MDS remains to be established.

Biochemical studies have indicated that the renal medulla has a greater capacity to generate NO$^\cdot$ than the renal cortex. The renal medulla has a high basal NOS activity, approximately threefold greater than that of the renal cortex (20). In the present study, our finding demonstrated an intense staining of nitrotyrosine not only in the vasa recta stimulated by LPS (Fig. 3C) but also in the vasa recta of untreated control animals (Fig. 3D). Both NOS-2 and NOS-3 expressions have been detected in the vasa recta (1, 24). Although little is known about the regulation of NOS-3 expression in kidney, endothelial cell culture studies suggest that both shear stress and oxygen tension influence the level of NOS-3 expression and activity (18). Perhaps changes of shear stress and oxygen tension in vasa recta can regulate NO$^\cdot$ production, which enhances nitrotyrosine formation.

Fig. 7. A-C: images of the same macula densa (magnification: ×1,000) from LPS-treated rat kidney of Fig. 6. Co-staining macula densa with anti-nitric oxide synthase (NOS)-B1 antibody (lissamine rhodamine fluorescence, A) and anti-nitrotyrosine antibody (FITC fluorescence, B). C: colocalization of NOS-B1 and nitrotyrosine within the macula densa.

Fig. 8. Nitrotyrosine staining in endothelium of arterial vessels of LPS-treated rat kidney. A: intense nitrotyrosine staining (arrows) in vascular endothelial cells of an arcuate artery and an interlobular artery (magnification: ×200). Note that vascular smooth muscle cells have much less staining. B: transverse course of afferent (hilar) arteriole that connects a glomerulus (G) and interlobular artery (A); intense endothelium staining of nitrated protein can also be seen (arrow; magnification: ×400).
The kidney is an intricate structure with diverse roles of excreting waste products, regulating body fluid and solute balance, regulating blood pressure, and secreting hormones. The three major NOS isoforms are broadly distributed in renal tissues, and NO plays an important role in many renal processes. Extensive protein nitration is evident in the kidneys of LPS-treated rats (present study), indicating that NO−, O2−, and ONOO− are produced in these kidney compartments. In addition to illustrating extensive protein nitration in LPS-stimulated rats, this study also demonstrates tyrosine nitration in control animals. These observations suggest both pathophysiologically and normal biological roles for nitrotyrosine in renal regulation and cellular signal transduction. The functions and effects of nitrotyrosine have yet to be defined, and many additional studies are suggested from those results.

Address for reprint requests and other correspondence: F. Murad, Dept. of Integrative Biology, Physiology, and Pharmacology, The Univ. of Texas-Houston Medical School, 6431 Fannin, Houston, TX 77030 (E-mail: fmrnad@girch1.med.uth.tmc.edu).

Received 9 November 1998; accepted in final form 1 April 1999.

REFERENCES


sion and vascular hyperactivity in the rat: tissue analysis of nitric oxide synthase mRNA and protein expression in the presence and absence of dexamethasone, N6-monomethyl-L-


