Oxidative and nitrosative stress in acute renal ischemia

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Departments of 1Nephrology and Endocrinology and 5Cardiovascular Disease, The University of Tokyo, Tokyo 113–8655; 2Laboratory of Food and Biodynamics, Nagoya University, Nagoya 464-8601; 4Department of Pediatrics, Fukui Medical University, Fukui, Japan 910-1193; and 2Departments of Medicine and Physiology, State University of New York at Stony Brook, Stony Brook, New York 11794-8152

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Noiri, Eisei, Akihide Nakao, Koji Uchida, Hirokazu Tsukahara, Minoru Ohno, Toshiro Fujita, Sergey Brodsky, and Michael S. Goligorsky. Oxidative and nitrosative stress in acute renal ischemia. Am J Physiol Renal Physiol 281: F948–F957, 2001.—Generation of reactive oxygen species and nitric oxide in hypoxia-reperfusion injury may form a cytotoxic metabolite, peroxynitrite, which is capable of causing lipid peroxidation and DNA damage. This study was designed to examine the contribution of oxidative and nitrosative stress to the renal damage in ischemic acute renal failure (iARF). iARF was initiated in rats by 45-min renal artery clamping. This resulted in lipid peroxidation, DNA damage, and nitrotyrosine modification confirmed both by Western and immunohistochemical analyses. Three groups of animals were randomly treated with an inhibitor of inducible nitric oxide synthase (NOS), L-NAME-(1-iminoethyl)lysine (l-Nil), cell-permeable lecithinized superoxide dismutase (SOD), or both. Each treatment resulted in amelioration of renal dysfunction, as well as reduced nitrotyrosine formation, lipid peroxidation, and DNA damage, thus suggesting that peroxynitrite rather than superoxide anion is responsible for lipid peroxidation and DNA damage. Therefore, in a separate series of experiments, a scavenger of peroxynitrite, ebselen, was administered before the reperfusion period. This treatment resulted in a comparable degree of amelioration of iARF. In conclusion, the present study provides the first attempt to elucidate the role of peroxynitrite in initiation of the cascade of lipid peroxidation and DNA damage to ischemic kidneys. The results demonstrate that l-Nil, lecithinized SOD, and ebselen treatments improve renal function due to their suppression of peroxynitrite production or its scavenging, consequently preventing lipid peroxidation and oxidative DNA damage.

4-hydroxy-2-nonenal; 8-hydroxy-2′-deoxyguanosine; L-NAME-(1-iminoethyl)lysine; lecithinized superoxide dismutase; ebselen

THE CONSEQUENCES OF OXIDATIVE stress are multiple and invariably ominous. They include lipid peroxidation, resulting in the destruction of membrane lipids (38), and oxidative DNA damage (30), collectively leading to the loss of cell viability, either via necrotic or apoptotic pathways (7). Therefore the processes of lipid peroxidation and oxidative damage of DNA in ischemia-reperfusion injury in general and renal injury in particular warrant further in-depth investigation.

Both processes are characterized by the multiplicity of chemical reaction products. Specifically, oxidative stress has been associated with more than 20 different modifications of DNA, such as oxidation of sugars, bases, and strand breaks, to name a few (34). Similarly, oxidative damage of lipids, involving abstraction of a hydrogen atom, leads to the formation and propagation of lipid radicals with generation of a broad range of breakdown products, like aldehydes, ketones, alcohols, and ethers (9). This panoply of oxidative products poses significant barriers to their use as potential diagnostic tools. Notwithstanding this difficulty, important markers of lipid peroxidation and DNA oxidation have recently been identified. They include formation of an α,β-unsaturated aldehyde, 4-hydroxy-2-nonenal (HNE), produced in the process of peroxidative metabolism of arachidonic or linoleic acids (47). HNE rapidly derivatizes different proteins on lysine, cysteine, and histidine residues, leading to the loss of protein functions (48, 49). Antibodies raised against HNE (50) have been successfully used for the immunodetection of lipid peroxidation, both in kidney sections and lysates (47). On the other hand, a highly reliable tool for detection of oxidative DNA damage has been established by developing monoclonal antibodies specific for the 8-hydroxy-2′-deoxyguanosine (8-OHdG) moiety in DNA, one of the major products of oxidative modification of DNA (34).

Development of oxidative stress in acute renal ischemia-reperfusion has long been suggested (26, 38, 39). No agreement has been reached as to the cellular sources of radicals: oxidation of hypoxanthine (37), mitochondrial production of free radicals, and lipoxygenase- or prostaglandin H-dependent production during arachidonic acid metabolism (8). It has been appreciated that hydroxyl radical-like activities are

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generated from peroxynitrite (1). This latter compound is emerging as one of the important sequelae of oxidative and nitrosative stress: the reaction between superoxide ion and nitric oxide (NO) proceeds at a near-diffusion-limited rate, thus resulting in an almost instantaneous generation of peroxynitrite in preference to nitrites, nitrates, or hydrogen peroxide (1). It has been recently demonstrated that nitrosative stress accompanies acute renal ischemia and contributes to the pathophysiology of renal damage (28, 36, 57). Specifically, it has been shown that chemical inhibitors of the inducible nitric oxide synthase (iNOS), antisense oligonucleotides targeting mRNA encoding the enzyme or iNOS gene knockout, all result in the alleviation of renal tubular injury and improved structural and functional outcome (27, 36). Cytotoxicity of NO and its metabolite peroxynitrite has been suggested to play a role in mitochondrial membrane lipid peroxidation (16) and in the inhibition of DNA synthesis (25).

To assign to peroxynitrite the role of the instigator molecule in the observed lipid peroxidation and DNA damage, it is necessary, however, to demonstrate that inhibition of either nitrosative or oxidative stress is equally capable of preventing these processes. The present study was designed to evaluate the possibility that peroxynitrite formation in the course of renal ischemia-reperfusion is responsible, at least in part, for lipid peroxidation and oxidative DNA damage. We utilized recently developed antibodies against HNE and 8-OHdG for immunodetection of products of protein and DNA modification. Furthermore, by inhibiting iNOS activity using a selective inhibitor of the enzyme or by accelerating the dismutation of superoxide anions with a cell-permeable superoxide dismutase (SOD), we were able to demonstrate that HNE and 8-OHdG, which accumulate in the kidney after renal artery cross-clamping, could be dramatically reduced by suppressing nitrosative and/or oxidative stress. On the basis of these findings, we next explored the effect of a peroxynitrite scavenger, a seleno-organic compound [(2-phenyl-1,2-benzisoselenazol-3(2H)-one); ebselen], which has no direct effect on NO or superoxide anions. Amelioration of renal dysfunction and HNE and 8-OHdG formation in ischemic kidneys provides not only a strong argument in favor of the proposed peroxynitrite-driven mechanism of lipid peroxidation and DNA damage but also introduces ebselen as a potential therapeutic tool in ameliorating reperfusion injury.

MATERIALS AND METHODS

Materials. L-N\(^{6}\)-\((1\)-iminoethyl\)lysine (L-Nil) was purchased from Alexis (San Diego, CA), and 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol) was purchased from Sigma (St. Louis, MO). Lecithinized SOD was generously provided by Seikagaku-Kogyo (Tokyo, Japan). Ebselen was also generously provided by Daiichi Pharmaceutical (Tokyo, Japan). All the chemicals were purchased from Wako Pure Chemicals (Osaka, Japan) unless otherwise specified.

Cell culture. The murine macrophage-like cell line RAW264 was obtained from Riken Cell Bank (Wako, Saitama, Japan) and cultured in minimum essential medium (MEM; GIBCO, Gaithersburg, MD) supplemented with 10% fetal bovine serum (GIBCO), nonessential amino acid (GIBCO), 100 U/ml penicillin, and 100 g/ml streptomycin (GIBCO). African green monkey kidney epithelial cells, BSC-1, were obtained from ATCC (Manassas, VA) and cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum and 100 U/ml penicillin and 100 g/ml streptomycin.

Nitrite analysis. Nitrite production by RAW264 cells was measured using a colorimetric assay on the basis of the method of Griess (39). Cells were grown in 24-well plates in MEM without phenol red, supplemented with 0.1% bovine serum albumin (Sigma). At confluence, different concentrations and combinations of agents were added for 24 h, and 100 \(\mu\)l of the incubation medium were withdrawn for determination of nitrite concentration. The Griess reagent (100 \(\mu\)l) of the following composition [1 part 1% sulfanilamide and 1 part 0.1% naphthyleldiamine dihydrochloride (Nakarai, Kyoto, Japan) in 5% phosphoric acid] was used. The absorbance was measured at 540 nm by a microtiter plate reader (Techne, Tokyo, Japan), and the nitrite concentration was determined using a calibration curve with NaNO\(_2\) as a standard. Results were expressed per 1,000 cells. Cell numbers are subsequently counted after detachment by trypsinization.

Cell detachment assay. Electrode fabrication and the design of an electric cell-substrate impedance sensor (ECIS) have been reported previously (18). Electrodes were precoated with 10 \(\mu\)g/ml fibronectin (Collaborative Biomedical Products). BSC-1 cells, at a density of 2 \(\times\) 10\(^5\), were seeded on the electrodes after 12 h incubation. To study cell detachment from the electrodes, confluent epithelial monolayers grown on electrodes were exposed to hydrogen peroxide at a concentration of 0.5 mM in combination with different agents.

Preparation of an HNE antibody. A polyclonal antibody purified by affinity chromatography, using an HNE-histidyl peptide column, was raised by immunization of New Zealand White rabbits with an HNE-modified histidyl peptide (Gly3-His-Gly3) conjugated with keyhole limpet hemocyanin, as previously reported (50).

Surgical procedure. All experiments were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” [DHEW Publication No. (NIH) 86–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. Male Sprague-Dawley rats, weighing 120–150 g, were allowed food and water ad libitum. Twelve hours before the experiment, L-Nil (0.36 mg/kg) was injected intravenously (L-Nil-treated group). After an overnight fast, animals were anesthetized with a combination of ketamine hydrochloride (11.6 mg/100 g) and xylazine hydrochloride (0.77 mg/100 g). The animals were placed on a heated surgical pad, and rectal temperature was maintained at 37°C. An intramuscular injection of 250 U/kg heparin was given 15 min before the operation. A 2.5-cm anterior midline incision was made, the right kidney was exposed, and two 3-0 sutures were passed under the renal pedicle. The left kidney was exposed, and the renal artery was separated from the renal vein and also underpadded with a 3-0 suture. Both ends of the suture were passed through a 1-cm polyethylene cannula. Renal ischemia was initiated by clamping the left renal artery. After 45 min, the right renal pedicle and the right ureter were ligated, and a right nephrectomy was performed. The left renal artery was subsequently released. In the L-Nil-treated group, L-Nil (0.36 mg/kg) was again injected intravenously before clamp release of the left renal artery. SOD (10,000 U/kg) was administered similarly in a SOD-treated group.
group of animals; a combination therapy (L-Nil+SOD) was administered using the same doses of individual pharmacueticals. In a separate group of animals, ebselen (10 mg/kg) dissolved in DMSO was intraperitoneally injected 5 min after clamp release (ebselen-treated group). The incision was closed with 3-0 sutures and surgical staples. Blood was drawn for blood urea nitrogen (BUN) and serum creatinine (Cr) analysis 24 h after the surgery. Sham-operated rats served as a control. Kidney specimens were collected 3 and 24 h after clamp release for 8-OHdG immunohistochemistry and 24 h after clamp release for other analytic procedures, as detailed below.

Western analysis. Harvested kidneys were divided into cortex and medulla and homogenized on ice in an extraction buffer of the following composition: 0.1% SDS, 0.5 mM dithiothreitol, 0.02% bromophenol blue, 20% glycerol, 60 mM Tris, pH 6.8. The lysates were electrophoretically separated on a 10% polyacrylamide gel. After the membrane transfer and blocking by a blocking reagent (Bio-Rad) following the manufacturer's protocol. Control sections were subjected to the secondary antibody only (blank). Mounted preparations were examined under an Olympus light microscope.

Statistical analysis. The differences among experimental groups were detected by ANOVA using Bonferroni’s post hoc analysis. P < 0.05 was considered as significant.

RESULTS

In vivo analysis of lipid peroxidation and DNA damage in renal ischemia. BUN and Cr were significantly elevated 24 h after renal artery cross-clamping. In the ischemia group, BUN averaged 101.3 ± 15.4 (SE) mg/dl and serum Cr was 2.89 ± 0.12 mg/dl (n = 7) compared with sham-operated animals (BUN 11.7 ± 1.0 mg/dl, Cr 0.33 ± 0.02 mg/dl, n = 7; P < 0.05) (Fig. 1). HNE-modified proteins were prominent in cortical kidney homogenates obtained from the ischemic rats (Fig. 2A), and immunohistochemical analyses revealed intense cytoplasmic staining of some tubular cells, adjacent tubular basement membranes, and exfoliated cells (see below and Fig. 3A). Immunocytochemical localization of 8-OHdG was predominantly detected at

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**Fig. 1.** Serum creatine (Cr; A) and blood urea nitrogen (BUN; B) in rats (n) subjected to 45 min of renal ischemia. Sham, sham-operated group; Cont Isch, control ischemia group; L-Nil (L-Nil), superoxide dismutase (SOD), and L-Nil+SOD groups were administered either L-Nil, SOD, or L-Nil+SOD before experiments (detailed in MATERIALS AND METHODS). *P < 0.05 compared with Sham animals. **P < 0.05 compared with Sham and L-Nil animals.
the nuclear region of desquamated tubular epithelial cells in the ischemic kidneys (see below and Fig. 3B). Peroxynitrite was monitored in kidney homogenates by Western analysis using an anti-nitrotyrosine antibody (Fig. 2B). Ischemic kidney homogenates demonstrated a higher intensity of immunoreactive bands of tyrosine-nitrated proteins compared with those from sham-operated animals. Immunohistochemical analysis showed staining of desquamated tubular epithelial cells in ischemic kidneys (see below and Fig. 3C). Collectively, these findings reaffirmed previous observations of lipid peroxidation and DNA damage and the concomitant occurrence of oxidative and nitrosative stress in renal ischemia (reaction 1).

**In vitro effect of L-Nil and cell-permeable SOD.** In our previous studies, we employed antisense oligonucleotides hybridizing to the open reading frame of iNOS mRNA (47). Recently, a selective iNOS inhibitor has been established, L-Nil, with an IC$_{50}$ for mice iNOS 6 times more potent than N$^{G}$-monomethyl-L-arginine (L-NMA) and an IC$_{50}$ for rat brain constitutive (c)NOS 10 times less potent (33). To ascertain the optimal therapeutic range of this inhibitor, initial studies were performed in cultured cells. The RAW264 cell line has been established as a model for macrophage function, including induction of iNOS and NO production by lipopolysaccharide (LPS) and cytokines (44). The minimal effective concentration of L-Nil inhibiting either LPS-induced or hydrogen peroxide-induced iNOS was examined by measuring nitrite production using the Griess assay (39). As shown in Fig. 4, the IC$_{50}$ for L-Nil suppression of nitrites by RAW264 cells activated by either LPS or hydrogen peroxide was ~20 μM. These findings are in agreement with the previously reported observations that L-Nil inhibits inducible NOS but not constitutive isoforms of NOS, within the similar concentration range (42, 33).

We and others have previously demonstrated that oxidative and nitrosative stress to renal tubular epithelial cells result in the loss of cell-matrix adhesion and eventual desquamation (17, 28, 36). This process was monitored in real time using ECIS, which provides a second-to-second readout of the electrical resistance of epithelial monolayers. As shown in Fig. 5A, application of hydrogen peroxide resulted in a gradual decline of the electrical resistance, consistent with the loss of cell-matrix adhesion and integrity of the monolayers. Coadministration of L-Nil resulted in a partial protection of epithelial integrity, especially 2 h after the oxidative stress. Neither SOD nor tempol, a scavenger of free radicals (54), prevented the hydrogen peroxide-induced decline in the electrical resistance. Much more complete protection against oxidative stress could be achieved by the combination of antioxidants and iNOS inhibition. Specifically, L-Nil in combination with either SOD, or tempol virtually abolished the loss of epithelial integrity, as judged by the maintained resting levels of the electrical resistance (Fig. 5B).

**In vivo effects of L-Nil and cell-permeable SOD in renal ischemia.** In view of the observed in vitro efficacy of L-Nil in suppressing iNOS and preventing the loss of epithelial integrity, this inhibitor was next utilized in vivo. L-Nil was administered at the dose of 0.36 mg/kg 12 h before the surgery and immediately before the renal artery clamp was released (reaching a final concentration of ~20 μM, considering extracellular and intracellular distribution of this agent). SOD was infused using the same timetable at a concentration of 10,000 U/kg, following a previous report (45). When L-Nil and SOD were administered separately, renal function improved significantly: in the L-Nil-treated group, BUN averaged 83.7 ± 3.7 mg/dl and Cr 1.46 ± 0.11 mg/dl (n = 7, P < 0.05 vs. ischemia group), and in SOD-treated group BUN averaged 52.9 ± 4.3 mg/dl and Cr 0.99 ± 0.07 mg/dl (n = 7, P < 0.05 vs. ischemia group) (Fig. 1). These parameters improved even more in the L-Nil+SOD-treated group (BUN 52.9 ± 4.3 mg/dl and Cr 0.99 ± 0.07 mg/dl, n = 7, P < 0.05) (Fig. 1). These data suggested that ischemia-induced oxidative and nitrosative stress are responsible for the functional sequelae of renal artery cross-clamping.

To further elucidate the efficacy of the suppression of both oxidative and nitrosative stress in ischemic ARF, Western blot analysis and immunocytochemical detection with an anti-nitrotyrosine antibody were performed. Figure 2B depicts a typical electrophoretic profile of nitrotyrosine-modified proteins in the ischemic cortical kidney lysates. The extent of nitrotyrosine formation was decreased in L-Nil+SOD-treated kidneys. In accordance with this finding, immunohistochemical staining with anti-nitrotyrosine antibodies showed diminished intensity of staining in L-Nil+SOD-treated kidneys (Fig. 3C).

Because peroxynitrite is one of the presumed sources of hydroxyl radicals, which initiate lipid peroxidation, we next investigated the effect of renal ischemia-reperfusion on lipid peroxidation using specific antibodies to HNE-modified proteins. HNE-modified proteins were prominent in kidney homogenates obtained from the ischemic rats, as discussed above. Immunohistochemical analysis with an anti-HNE antibody revealed intense staining of exfoliated cells and cytoplasmic staining of some tubular epithelial cells and adjacent tubular epithelial cells.
tubular basement membranes (Fig. 3A). In L-Nil+SOD-treated kidneys, immunodetectable HNE-modified proteins were dramatically reduced on both Western and immunohistochemical analyses (Figs. 2A and 3A).

A possible role for ischemia-reperfusion-induced oxidative and nitrosative stress in DNA damage was tested using immunodetectable 8-OHdG. A monoclonal antibody was raised against 8-OHdG (46) and proved to have an improved specificity over other reported antibodies against 8-OHdG (16, 34). With the use of this antibody, 8-OHdG was predominantly detected at the nuclear region of desquamated tubular epithelial cells in the ischemic kidneys (Fig. 3B). Immunocytochemical staining was minimal in kidney sections obtained from L-Nil+SOD-treated animals. Moreover, immunohistochemical staining with antibodies against nitrotyrosine, HNE, or 8-OHdG was found to be reduced in either L-Nil or SOD-treated kidney (data not shown).

In vivo studies of the peroxynitrite scavenger ebselen. The above in vivo analysis of the comparable efficacy of L-Nil and lecithinized SOD in ameliorating posts ischemic renal dysfunction invokes two alternative explanations: either nitrosative and oxidative stress are separate contributors to the ensuing injury, or these two pathways cooperate in generating a common pathway product, peroxynitrite. In an attempt to resolve this dichotomy, we next turned to ebselen, the recently
identified scavenger of peroxynitrite, in a separate series of experiments. As shown in Fig. 6, the ebselen-treated ischemia-reperfusion group showed a significantly lesser retention of Cr (1.10 ± 0.05, n = 8, P < 0.01) and BUN 64.9 ± 5.0 (P < 0.05) on postoperative day 1, compared with both positive (Cr 2.44 ± 0.21, BUN 105.4 ± 4.7, n = 11) and negative control groups [animals treated with either the vehicle (Cr 0.42 ± 0.05, BUN 14.3 ± 0.92, n = 10) or with ebselen (Cr 0.63 ± 0.10, BUN 17.7 ± 3.3, n = 4)]. This was associated with the decrease in the detectable lipid peroxidation and DNA damage (Figs. 2 and 3).

DISCUSSION

The data presented herein demonstrate the role of oxidative and nitrosative stress, leading to peroxynitrite formation, in renal damage and confirm the utility of two recently developed antibodies for immunodetection of nonenzymatic chemical protein and DNA modification by oxidative and nitrosative stress to the rat kidney. Lipid peroxidation is a crucial factor in the propagation of cellular damage from ischemia-reperfusion injury, leading to the increased permeability of the plasma membrane (38) as well as of mitochondrial and lysosomal membranes (24). It is well accepted that superoxide anions participate in cellular damage in a ischemia-reperfusion model, but the precise molecular species responsible for cellular damage remain uncertain. Superoxide reacts with Fe^{3+} at the rate of 10^8 M/s (52) and with ascorbate at the rate of 10^5 M/s (35). Whereas polyunsaturated fatty acid residues of phospholipids in all cell membranes are critical targets for reactive oxygen species (9), Bielski et al. (6) calculated the second-order rate constants for the

Fig. 4. Effective inhibitory concentration of L-Nil on release of nitrates using Griess reaction. Inducible NOS was stimulated in RAW264 by either lipopolysaccharide (LPS; 50 μg/ml) or 0.3 mM hydrogen peroxide (HP). Arrow, IC_{50}; C, control group.

Fig. 5. Cell detachment assay. A: the resistance of BSC-1 cell monolayers was monitored after the addition of 0.5 mM hydrogen peroxide, and efficacy of L-Nil (LN), 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol; TEMP), and SOD were simultaneously examined. B: additive effect of L-Nil with either SOD or tempol.

Fig. 6. Serum Cr and BUN after 24 h of renal ischemia and efficacy of ebselen in ameliorating renal dysfunction in rats (n).
reaction of superoxide with unsaturated fatty acids such as linoleic acid, linolenic acid, arachidonic acid, oleic acid, 9,11-octadecadienoic acid, and 10,12-octadecadienoic acid; they found that measured superoxide decay was similar in the presence or absence of unsaturated fatty acids, thus questioning the role of superoxide in lipid peroxidation. The relative lack of a detectable effect of lecithinized SOD on hydrogen peroxide-treated epithelial cells in culture is in contrast to the substantial effect of this compound in vivo, suggesting that polymorphonuclear leukocytes, rather than the epithelium, provide the major source of superoxide radicals.

Studies from Schrier’s laboratory (57) first suggested that inhibition of NOS in hypoxic proximal tubules results in the improved cell survival. We have previously demonstrated the deleterious effect of iNOS in ischemic acute renal failure using antisense oligonucleotides targeting iNOS (36). These findings were furthered by the establishment of improved tubular cell viability after hypoxic insult in iNOS-deficient mice (28). It was proposed that cellular damage is attributable to a powerful and cytotoxic oxidant peroxynitrite, which is generated by the diffusion-limited interaction of NO with superoxide. The reactivity of peroxynitrite is reported as pH dependent (12). It is readily isomerized from stable cis to reactive trans peroxynitrous acid in acidic conditions like the reperfusion phase in an ischemic kidney. Among oxidative reactions of peroxynitrite, its hydroxyl radical-like reactivity is extremely potent (1) and may lead to the propagated lipid peroxidation. Because hydroxyl radicals convert virtually any organic molecule to the corresponding free radicals, and because they can be particularly damaging to cell membranes rich in polyunsaturated fatty acids, the initiation of free radical chain reactions based on abstraction of the allylic hydrogen is highly plausible (15). These reactions should amplify cell damage (40). One of the oxidative reactions initiated by peroxynitrite is the nitration of phenolic rings (2). In the present study, we used the antibody recognizing nitrotyrosine-containing proteins (4) for both Western and histochemical analyses and found that it was specifically localized on the surface of exfoliated epithelial cells inside the tubular lumen. Both NO and superoxide are required for the generation of peroxynitrite. Inhibition of either molecular species with \( L\)-Nil or SOD was associated with improved renal function and decreased oxidative damage to lipids and DNA. An additive effect was achieved by combining \( L\)-Nil and SOD, further supporting the notion that the observed cytotoxicity was due to the generation of peroxynitrite in ischemic-reperfused kidneys. Apart from scavenging peroxynitrite, ebselen has been proposed to inhibit the peroxidation of membrane phospholipid, inhibit lipoxygenase in the arachidonate cascade, block the production of superoxide anions from activated leukocytes, inhibit iNOS, and thus exhibit a sustained protective effect against peroxynitrite (55). Our observation that ebselen, the scavenger of peroxynitrite, is comparable to \( L\)-Nil and SOD in ameliorating lipid peroxidation, DNA damage, and renal dysfunction after ischemia provides the ultimate proof of peroxynitrite’s participation in the reperfusion injury.

There is accumulating evidence that peroxynitrite propagates lipid peroxidation (21, 41). Radi et al. (41) measured the extent of lipid peroxidation, as a function of peroxynitrite concentration, in soybean phosphatidylcholine liposomes, using both malondialdehyde and conjugated diene. Therefore, we addressed the issues of whether lipid peroxidation is involved in ischemic acute renal failure and whether peroxynitrite contributes to lipid peroxidation in an in vivo ischemia-reperfusion model. Because lipid peroxidation results in the highly reactive aldehydes, we have focused on HNE, a major aldehyde product of lipid peroxidation (14). Although chemical protein modifications by products of lipid peroxidation are numerous, HNE-modified proteins are comparatively stable, being resistant to digestion by proteases due to the chemically stable hemiacetal adducts formed in the process of reactions with lysine, histidine, or cysteine residues (48, 49). We have recently raised a specific antibody to HNE-modified proteins (50). HNE-modified proteins were conspicuously detected in the ischemic kidney. Expression of HNE-modified proteins was significantly decreased when production of both inducible NO and superoxide was inhibited by \( L\)-Nil+SOD pretreatment. Immunohistochemical analysis revealed the localization of HNE-modified proteins to the cytoplasm of epithelial cells and cellular debris within the tubular lumen. This phenomenon was reduced in \( L\)-Nil+SOD-treated and in ebselen-treated ischemic kidneys. On the basis of the above observations, it is conceivable that lipid peroxidation injury in ischemic acute renal failure originates from iNOS via peroxynitrite formation and that production of NO and superoxide promotes tissue injury dependent on lipid peroxidation.

The possible mechanism of modification of deoxyribonucleotides and DNA strand breaks by NO and NO-generating compounds has been suggested, and NO-dependent mutagenic properties have been established to play a pivotal role in tissue carcinogenesis and inflammation. Hydroxyl radical-like activity of peroxynitrite could modify guanine of intact DNA to 8-OHdG and 8-nitrosoguanosine (56). This scenario was also suggested by finding of NO-induced base deamination in DNA that propagated the genetic alteration in living cells (51) and hydroxylation at the C-8 position of deoxyguanosine in DNA of activated macrophages (43). Recently, DNA damage was induced in A549 epithelial cells by crocidolite, the most carcinogenic form of asbestos, with the induction of NO and was detected as 8-OHdG formation, which was significantly inhibited by 1 mM aminoguanidine (11). In addition, peroxynitrite-induced DNA damage and lipoprotein modification were recently reported in a cell-free system (10). In the present study, 8-OHdG was used as a marker of DNA damage. Immunohistochemical studies revealed that 8-OHdG was predominantly detected at the nuclear region of ischemic tubular epithelial cells. The staining was minimal in \( L\)-Nil+SOD-
trated and ebselen-treated ischemic kidneys. Thus data presented herein support the role of peroxynitrite in DNA damage in ischemia-reperfusion injury to the kidney. Having demonstrated the role of antioxidative and antinitrosative pharmaceuticals (lecithinized SOD and l-Nil, respectively) in ameliorating ischemia-induced renal dysfunction, we have designed experiments to directly challenge the identity of the noxious product, either superoxide, NO, or peroxynitrite, by utilizing a bona fide peroxynitrite scavenger, ebselen. Peroxynitrite scavenging by ebselen shows the second-order rate constant of $2 \times 10^6$ M/s at pH 7.4 and 37°C, exceeding the rate of peroxynitrite reaction with natural antioxidants like ascorbate or glutathione by approximately three orders of magnitude (31). On the other hand, the rate of peroxynitrite decomposition, after protonation to form ONOOH, which can potentially yield $\sim 30\%$ of hydroxyl radicals, proceeds with the rate constant about three orders of magnitude faster than its scavenging (32). Therefore, efficient scavenging of peroxynitrite can be achieved only by creating an access of ebselen.

It has been demonstrated that ischemia- and endotoxin-induced renal injury are accompanied by nitrotyrosine formation (5, 36). Apart from inhibiting the functions of several highly susceptible enzymes, i.e., prostacyclin synthase (58), prostaglandin endoperoxide synthase (19), and Mn-SOD (29), detection of nitrotyrosine-modified proteins heralds the concomitant occurrence of oxidative and nitrosative stress resulting in peroxynitrite formation (1). Recently, a caution has been exercised as to the uniqueness of peroxynitrite in generating nitrotyrosine residues (22). Therefore, the finding that the peroxynitrite scavenger ebselen reduces nitrotyrosine formation in ischemic kidneys and improves the functional outcome lends additional support to the notion that oxidative and nitrosative stress occur in this condition in vivo and are mechanistically involved in the ensuing loss of kidney function. These findings establish that reactions 1 and 2

\[
\text{NO} + \text{O}_2 \rightarrow \text{ONOO}^- \xrightarrow{H^+} \text{ONOOH} \rightarrow \cdot \text{NO}_2 + \cdot \text{OH}
\]

(1)

Nitrotyrosine formation (3)

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{N} & \quad \text{N} \\
\text{H} & \quad \text{H} \\
\text{NO}_2 & \quad \text{O} \\
\end{align*}
\]

3-Nitrotyrosine C-Nitrosoctysine O-Nitrosoctysine

(2)

do take place in renal ischemia-reperfusion. Interestingly, iNOS per se can be responsible for generation of products depicted in reaction 1, especially, when L-arginine becomes depleted in macrophages (53). Furthermore, the presence of SOD may even catalyze the peroxynitrite-induced nitrotyrosine modification of target proteins, thus making this particular therapeutic choice somewhat less desirable (29). Hence, the theoretically preferred pathway for limiting oxidative and nitrosative stress could be found in a highly selective inhibition of iNOS or scavenging of peroxynitrite. Although in our experiments all three pharmaceuticals ameliorated ischemia-induced renal failure, it is conceivable that ebselen is the most promising of them. The efficacy of ebselen in preventing brain ischemic insult has been proposed, based on findings that recirculation-induced edema as well as postischemic hypoperfusion were markedly improved in a cat model of prolonged middle cerebral artery occlusion and that the infarct size was reduced in a rat transient middle cerebral artery occlusion model (13). Recently, the efficacy of ebselen in acute ischemic stroke was demonstrated in a placebo-controlled, double-blind study and is in the preregistration stage for subarachnoid hemorrhage and stroke in Japan (55). On the other hand, ebselen was used in the storage solution for harvested kidneys and was found to lack a protective effect on renal function, histological outcome, and long-term survival of rabbits after autotransplantation (20).

In conclusion, the present study provides the first attempt to elucidate the role of peroxynitrite in the initiation of the cascade of lipid peroxidation and DNA damage to ischemic kidneys and demonstrates that l-Nil, lecithinized SOD, and ebselen treatments improve renal function due to their suppression of peroxynitrite production or its scavenging (as detected by nitrotyrosine), consequently preventing lipid peroxidation and oxidative DNA damage.

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REFERENCES

1. Beckman JS, Beckman TW, Chen J, Marshall PA, and Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87: 1620–1624, 1990.


4. Beckman JS, Ye YZ, Anderson PG, Chen J, Accavitti MA, Tarpey MM, and White CR. Extensive nitration of protein...
46. Toyokuni S, Tanaka T, Hattori Y, Nishiyama Y, Yoshida A, Uchida K, Hiai H, Ochi H, and Osawa T. Quantitative im-


