Visualizing Tubular Lipid Peroxidation in Intact Renal Tissue in Hypertensive Rats

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Abstract. An imbalance between production of reactive oxygen species (ROS) and antioxidant defense is involved in the pathogenesis of diverse chronic parenchymatous diseases. To identify the primary site of such increased oxidative stress, a lipophilic ROS-sensitive probe (C11-Bodipy 581/591) is introduced, which allows the visualization and quantification of oxidative injury using confocal fluorescence microscopy in living cells. The properties of this probe are such that its emission wavelength irreversibly shifts from red to green upon oxidation. This probe was used to identify the spatiotemporal distribution of lipid peroxidation in the rat kidney during chronic NOS inhibition, a model associated with hypertension and proteinuria. Chronic NOS inhibition resulted in increased lipid peroxidation in renal tubules but hardly any in glomeruli or blood vessels. This peroxidation preceded the loss of renal function characteristic of the model and was accompanied by parallel changes in thiobarbituric acid reactive substances in the renal cortex. Furthermore, the increase in oxidation was dependent on angiotensin II and NADPH oxidase and prevented by vitamin E. Induction of cytoprotective heat-shock protein 70 preceded lipid peroxidation, rise in BP, or proteinuria. These findings challenge the paradigm that the vascular wall is the source and target of oxidative stress in chronic parenchymatous renal disease associated with hypertension.

An imbalance between production of reactive oxygen species (ROS) and antioxidant defense appears to be involved in the pathogenesis of diverse chronic parenchymatous diseases that occur as a sequel to diabetes, hypertension, ischemia-reperfusion, inflammatory diseases, exposure to cytostatic drugs, as well as neurodegenerative diseases and other manifestations of aging (1). This imbalance results in oxidative damage of biomolecules, including the unsaturated fatty acid moieties of phospholipids. Because vascular changes that ultimately result in atherosclerosis are involved in many of these conditions, it is generally assumed that the vascular wall is frequently the source and target of oxidative stress (2). Nevertheless, this assumption has not been addressed in most chronic parenchymatous disease states. Testing the paradigm that the vascular wall is the primary source of oxidative stress, thereby identifying the primary site of increased oxidative stress, would greatly enhance our insight into the mechanisms of chronic parenchymatous disease. We recently introduced a new, lipophilic ROS-sensitive probe (C11-Bodipy 581/591), which allows the visualization and quantification of oxidative injury in cellular membranes using confocal fluorescence microscopy in living cells. The fluorescence properties of this probe are such that its emission wavelength irreversibly shifts from red to green upon oxidation (3). Until now, this probe has been applied in cultured cells, artificial lipid vesicles, and blood plasma (3–5). The application of this probe to study the spatial and temporal distribution of lipid peroxidation in intact tissues will provide novel insights into oxidative processes underlying organ damage.

Many of the effects of oxidative stress on the vascular wall, irrespective of pathogenesis, are ascribed to inactivation of endothelial NO synthase (NOS)-derived NO, by ROS (2). The kidney is rich in NOS, and constitutive expression of all three isoforms has been documented (6). Chronic NOS inhibition in rodents results in hypertension, renal injury and proteinuria (7). Oxidative stress was elevated during NOS inhibition in the systemic circulation (8–10) and in the kidney (11). Moreover, the increased superoxide activity was reduced by vitamin E treatment in the kidney (11). We recently observed that apocynin, a compound that specifically inhibits the assembly and thus the activity of NAD(P)H oxidase complex (12), completely inhibits superoxide activity in the intact renal cortex (11). Thus, in the chronic NOS inhibition model, NAD(P)H oxidase may well be an important source of superoxide production. The effects of chronic NOS inhibition can be prevented by AT1-receptor blockade, suggesting an important role for angiotensin II (AngII), both in the circulation (10) and in
the kidney (13). It is well known that AngII can stimulate vascular superoxide production via NADPH oxidase (14). However, within the kidney, tubular epithelial cells also appear to be a source of superoxide via AngII stimulation of NAD(P)H oxidase (15).

Stress can induce the expression of molecular chaperones such as heat-shock protein 70 (HSP70i) that extend the interval before irreversible cell damage can occur (16,17). After only 4 d in the chronic NOS inhibition model, we previously reported a marked reduction in endothelial-dependent relaxation in aortic rings, despite the absence of superoxide activity, hypertension, or proteinuria (11). This indicates that NO synthesis is already markedly inhibited at this stage. Thus, it is readily conceivable that cytoprotective proteins such as HSP70i are upregulated at a very early stage in this rapid and aggressive model of vascular and renal injury.

Whether lipid peroxidation occurs in the chronic NOS inhibition model, and whether the cellular site of lipid peroxidation is also vascular, as has been found outside the kidney, has not been investigated. Thus the present investigation was conducted, (1) to identify the spatiotemporal distribution of lipid peroxidation in the kidney during chronic NOS inhibition, (2) to determine whether NADPH oxidase is the primary source of superoxide at the site of lipid peroxidation, (3) to investigate the role of AngII in the generation of oxidative stress in the kidney during chronic NOS inhibition; and (4) to assess the time-course of increased oxidative stress in relation to HSP70i expression in the kidney during chronic NOS inhibition.

Materials and Methods

Animal Treatments

Male Sprague-Dawley rats (8 to 12 wk, 180 to 210 g; Harlan-Olac, Blackthorn, Bicester, Oxon, UK) were used. The rats received a standard diet (RMH-TM; Hope Farms, Woerden, the Netherlands) containing 100 mmol/kg sodium and tap water ad libitum. Animals were housed in cages in a room maintained at 22°C, 60% humidity with a 12-h light/dark cycle. Sentinel animals, which were monitored regularly for infection by nematodes and pathogenic bacteria, as well as antibodies to rodent viral pathogens (ICLAS, Nijmegen, the Netherlands) consistently tested negative for infection throughout the course of experiment. The Utrecht University board for studies on animals approved the protocol.

Inhibition of NOS in the animals was achieved using NO-nitro-l-arginine (l-NNA; Sigma Chemical Co., St. Louis, MO). l-NNA was dissolved in the drinking water of the animals (500 mg/L) and administered for 4, 14, or 21 d.

Protective measures were studied in two additional groups of rats. One group received the AngII type-1 (AT1) receptor antagonist L-158809 (kindly provided by Merck, Rahway, NJ) in addition to l-NNA. L-158809 (80 mg/kg chow) was mixed with finely ground chow and administered for 21 d. The other group received (+)-α-tocopherol acetate (vitamin E, Sigma) in chow at a dose of 0.7 g/kg body wt per day in addition to l-NNA (11). Systolic BP was measured in awake rats by the tail-cuff method (ITTC, San Diego, CA). During the final 24 h of treatment, the animals were placed in metabolism cages for determination of urinary protein and creatinine excretion.

Antibodies

Antibodies against inducible HSP70i (HSP70i) were purchased from Stressgen Biotech. Corp., Victoria, BC, Canada. ERK2 monoclonal antibody was purchased from Upstate Biotechnologies, Lake Placid, NY. Horseradish peroxidase–conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc, West Grove PA.

Measurement of Oxidative Stress

Following treatment with l-NNA, l-NNA and L-158809, or l-NNA and vitamin E, rats were anesthetized using sodium pentobarbital (60 mg/kg intraperitoneally) and the aorta was cannulated to collect blood samples for determination of plasma creatinine. One kidney was rapidly excised, weighed, and sliced into 1-mm slices. The slices were placed in wells of a 24-well plate containing Krebs-HEPES buffer. The other kidney was plunged in liquid nitrogen and stored at −80°C.

Oxidative stress in the kidney was measured using the C14'-BODIPY®387/01 (BoC11; 4,4-difluoro-5-[4-phenyl-1,3-butadienyl]-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) probe. The probe (2 mM stock in ethanol) was added to the buffer at a final concentration of 10 μM. In some instances, Apocynin (100 μM) was added to the slices as well to inhibit NADPH oxidase. After a 30-min incubation period at room temperature, free probe and apocynin were washed off with Krebs-HEPES buffer and fresh buffer was added to the slices. The incorporation and oxidation of BoC11 was observed using confocal laser scanning microscopy (CLSM; Bio-Rad Laboratories, Hercules, CA), equipped with a Krypton/Argon laser. Randomly selected areas of the slices were imaged in the red and green (nonoxidized and oxidized probe) spectrum every 30 min for up to a total of 90 min from the end of BoC11 labeling. Green and red fluorescence excitation wavelengths are 488 and 568 nm, respectively, and green and red fluorescence emissions are 530 and 590 nm, respectively. Images were analyzed using Scion imaging software (Scion Corp., Frederick, MD). The mean fluorescence intensity of the glomerular areas and three randomly chosen non-glomerular areas in each slice were determined in each image. At least six rats per group and three slices per kidney were used for detection of BoC11 oxidation. Furthermore, to one slice from every rat no probe was added, and the green and red autofluorescence signal was obtained and subtracted.

Renal TBARS

Lipid peroxidation was also assessed by measurement of renal cortex thiobarbituric acid reactive substances (TBARS). Tissue samples were stored at −80°C until determination. From the tissues, 0.3 g was homogenized in 3.0 ml of PBS (pH 7.2) and centrifuged for 10 min at 3000 rpm at 4°C. From the supernatants, 250-μl aliquots were mixed with 50 μl of 1% thiobarbituric acid (pH 1.5) and 10 μl of 1% butylated hydroxytoluene and boiled for 15 min. After cooling down and centrifugation, the absorbance was measured at 540 nm using a microplate reader (18). The results were expressed as pmol of malondialdehyde per mg of protein.

Preparation of Samples and Western Blotting

Kidneys frozen and stored at −80°C as described above were homogenized in buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.25% sodium deoxycholate, 1 mM EGTA, 1% Nonidet P-40, and protease inhibitors). The homogenates were spun at 1000 rpm for 5 min and the supernatant was collected. Proteins were estimated using the BCA protein assay kit (Pierce, Rockford, IL) as per manufacturer’s instructions, and samples were taken up in sample buffer (8.3%...
Figure 1. Oxidative stress after NO synthase (NOS) inhibition occurs in renal tubules. Autofluorescence of kidney slices in the green (A) and red (B) channel. Oxidized (green fluorescence) and non-oxidized (red fluorescence) BoC11 probe in kidney slices of control rats (C and D) or rats treated with Nω-nitro-l-arginine (l-NNA) for 21 d (E and F). Panel G is an overlay of the green and red fluorescence of the l-NNA–treated kidney from panels E and F clearly showing the spatial distribution of oxidation within the kidney.
images of the oxidized and non-oxidized probe as well as the autofluorescence signal in kidney slices obtained from untreated rats or those treated with l-NNA for 21 d. A substantial increase in green signal, representing oxidized BoC11, occurred in kidneys of rats that received l-NNA for 21 d compared with control rats. Furthermore, the oxidation was mainly localized in the tubules. Figure 1G was generated by an overlay of red and green images of the 21-d l-NNA–treated group. Green structures appear to be mainly proximal tubules judged by the tall cylindrical appearance of the cells. The areas representing glomeruli were devoid of green signal. However, in the glomeruli and blood vessels red signal is present, indicating that the probe was present but not oxidized in these regions. Quantification of images obtained at different time points after labeling showed that a significant difference was observed in the 14- and 21-d groups immediately after removal of free probe (Figure 2). The oxidation of BoC11 in these groups continued for up to 60 min after incorporation into the tissue, beyond which oxidation appeared to plateau. In subsequent experiments, we quantified oxidation at 60 min after removal of excess probe.

Kidneys of control animals showed minimal oxidation, as was also found in the 4-d group. In contrast, significant oxidative stress was observed in kidneys of rats that had been treated with l-NNA for 14 or 21 d. Furthermore, BoC11 oxidation in the kidney at 14 d of l-NNA was similar to that of the 21-d l-NNA group (Figure 2). Renal cortical TBARS were also increased after 21 d of l-NNA (1897 ± 103 versus 1394 ± 107 pmol/mg protein; P < 0.05). l-NNA treatment resulted in a significant increase in BP from day 14 (P < 0.01) to day 21 (P < 0.01) compared with control animals (Table 1). Protein excretion was also significantly increased in rats treated with l-NNA for 14 to 21 d (P < 0.01). However, plasma creatinine levels were increased after 21 d (P < 0.01), but not yet at 14 d. These results clearly indicate that inhibition of NOS using l-NNA resulted in hypertension, proteinuria, tubular lipid peroxidation, and ultimately loss of glomerular function in rats.

Oxidative Stress in Kidneys is Dependent on NADPH Oxidase

A greater than fourfold rise in BoC11 oxidation was found in the 21-d l-NNA–treated rats (Figure 3). Apocynin was
added to the kidney slices at the same time as BoC11 for 30 min and washed off subsequently. Apocynin had little effect on the minimal oxidation that was observed in control kidneys or in those of rats treated with L-NNA plus L-158809, or L-NNA + vitamin E, with apocynin (buffer) or with apocynin (mean + SEM of six rats using the average signal from three slices in each rat. ** P < 0.01 versus control; *** P < 0.01 versus L-NNA 21 d.s.

**Figure 3.** L-NNA induced oxidative stress in kidneys is dependent on NADPH oxidase and on angiotensin II (AngII). BoC11 oxidation in kidney slices of control rats and rats treated for 21 d with L-NNA, L-NNA + the AT1 antagonist L-158809, and L-NNA + vitamin E were incubated with BoC11 without apocynin (buffer) or with apocynin (mean + SEM of six rats using the average signal from three slices in each rat. ** P < 0.01 versus control; *** P < 0.01 versus L-NNA 21 d.s.

Oxidative Stress in Kidneys Is Dependent on AngII

The AngII type 1 receptor antagonist, L-158809, reversed the effect of L-NNA on BP, proteinuria, and plasma creatinine levels (Table 1). In the group that received L-158809 in addition to the L-NNA, BoC11 oxidation did not increase above control levels, suggesting that oxidative stress in the kidneys observed in terms of BoC11 oxidation occurred mainly via a mechanism involving NADPH oxidase. However, the levels were numerically higher in the L-NNA–treated kidneys, suggesting a minor NADPH oxidase-independent component.

Oxidative Stress in Kidneys Is Prevented by Vitamin E

Vitamin E had no effect on BP but somewhat blunted the increase in proteinuria (Table 1). Vitamin E completely prevented the increase in BoC11 oxidation, and renal cortical TBARS were also markedly reduced as compared with L-NNA alone (932 ± 91 versus 1897 ± 103 pmol/mg protein; P < 0.01).

HSP70i Is Induced in L-NNA Treated Rat Kidneys

Stress is known to induce chaperone molecules; we therefore examined the effect of NOS inhibition on expression of HSP70i in kidney homogenates of rats treated with L-NNA for 4, 14, or 21 d. Substantial expression of HSP70i was detected in rat kidney homogenates after 4 d of L-NNA supplementation, suggesting stimulation by an effect of NO deficiency that precedes oxidative stress. HSP70i continued to be expressed at a high level after 14 d of treatment with L-NNA. However, by 21 d, HSP70i expression was no longer significantly increased, although still numerically higher than in controls.

Discussion

Chronic NOS inhibition resulted in increased lipid peroxidation in the renal tubules but practically not in glomeruli or blood vessels. This effect preceded the loss of renal function that is characteristic of this model. Furthermore, the increase in oxidation was dependent on AngII and NADPH oxidase and prevented by vitamin E. We also observed expression of HSP70i after NOS inhibition before any detectable lipid peroxidation or symptoms of NO deficiency such as a rise in BP or proteinuria.

We have used the BoC11 probe in fresh tissue sections to identify ex vivo the localization in the kidney where oxidative stress occurs in response to NOS inhibition. In cell-free systems and in vitro, BoC11 has been demonstrated to be a marker of oxidative stress with a sensitivity toward oxidizing species comparable to endogenous unsaturated fatty acids (3,5,19). Our results show that BoC11 can also be used effectively to measure oxidative stress in intact kidney tissue slices. As such, it provides a valuable tool for localization and visualization of areas of elevated oxidative stress versus those where the probe was not oxidized. Superoxide activity in the kidneys was increased after 21 d of NOS inhibition (11), and increased TBARS indicates that this results in increased lipid peroxidation of renal cortical tissue. Vitamin E decreased superoxider activity in this model without affecting BP and with only a small reduction of proteinuria (11). The protective effects of vitamin E in this model were confirmed in the present series of experiments, and the antioxidative capacity of vitamin E was supported by the marked reduction of renal cortical TBARS. However, these measurements cannot identify the site of oxidation. Application of BoC11 revealed that in this model the extravascular compartment was the primary site of lipid peroxidation, suggesting that nonvascular cells can act as a major source of oxidative stress in the kidney. It can be hypothesized therefore, that the main site of increased superoxide production in the kidney was the tubular epithelium. The finding that during L-NNA treatment an increase in lipid peroxidation in tubular structures preceded an increase in plasma creatinine supports the idea that tubulointerstitial injury is an important contributor to the progressive loss of nephrons, and thus is causally related to loss of glomerular function (20).

The tubular epithelium is clearly accessible to vitamin E because both the increases in BoC11 oxidation and in renal TBARS were completely prevented by co-treatment with vitamin E. In-
terestingly, this protection occurred in the presence of the same level of BP as L-NNa alone, indicating the BP per se is not directly responsible for increased renal lipid peroxidation. Furthermore, proteinuria was decreased but not absent, which supports the notion that tubular lipid peroxidation is an event down-stream of proteinuria. However, vitamin E treatment could not prevent loss of glomerular filtration. Presumably, a longer period of un unabated severe hypertension does result in glomerular damage despite a reduction of tubular lipid peroxidation. We previously observed a marked increase in renal interstitial monocyte/macrophage influx but no increase in renal cortical superoxide activity after only 4 d in this model (11). These observations, in combination with the absence of an increase in lipid peroxidation after the same period in the present study, do not support the notion that invading inflammatory cells are an important source of superox-idation in the present model.

Endogenous unsaturated fatty acids and BoC11 cannot be directly oxidized by superoxide (19), hence oxidation of the probe suggests that superoxide is converted to reactive species such as hydrogen peroxide and subsequent hydroxyl radical, which in turn cause BoC11 oxidation. Nevertheless, apocynin, a specific inhibitor of NAD(P)H oxidase, strongly attenuated BoC11 oxidation; superoxide must therefore be the ROS primarily responsible for the oxidative stress. The data in Figure 3 show that although apocynin largely abolished the oxidation of BoC11, a minor NADPH oxidase-independent component of lipid peroxidation might be present.

Our results show that the elevated oxidation in tubules after L-NNa administration was completely abolished in rats that received the AngII antagonist L-158809. Furthermore, we observed that L-158809 also protected the rats from hypertension, renal injury, and proteinuria. Verhagen et al. (13) demonstrated that inhibition of the AT1 receptor can prevent renal damage caused by NOS inhibition. Similarly, Kitamato et al. (10) showed that AT1 receptor blockade also prevents the effects of NOS inhibition in the circulation, indicating that AngII plays an important role in protection from the deleterious effects of NOS inhibition. AngII stimulates O2− production via NAD(P)H oxidase in renal epithelial cells (15). Indeed, incubation with the specific NAD(P)H oxidase inhibitor apocynin completely prevented superoxide activity (11) and oxidative stress in renal tubules resulting from NOS inhibition. These data support the hypothesis that superoxide is generated in the renal tubules during chronic NOS inhibition via AngII and NAD(P)H oxidase.

The kidney appeared to be able to mount an early defense against the oxidative stress induced by NO deficiency. Prior investigations using chronic NOS inhibition have demonstrated a substantial reduction of endothelial-dependent relaxation in aortic rings after 4 d, indicating marked inhibition of NO synthesis at this early time point (11). Absence of an increase in superoxide activity (11) or lipid peroxidation after 4–d L-NNa treatment implies that either increased superoxide was secondary to renal injury or antioxidant defense mechanisms were adequate at this early stage. In response to stress factors such as inhibition of NO synthesis, cells can generate cytoprotective proteins. Indeed, after 4 d of L-NNa treatment a greater than twofold increase in expression of HSP70i was observed in rat kidneys. HSP70 is induced in response to several stresses, including ischemia/reperfusion and oxidative stress (21). Specifically, in the kidney, HSP70 has found to be induced during stress conditions such as AngII infusion (22), ischemia (23,24), and ureter obstruction (25). The detection of HSP70i in the kidney homogenates of rats administered L-NNa indicates that stress conditions occur in the kidneys much earlier than any indication of lipid peroxidation or renal damage occurs. Interestingly, increased tubular expression of HSP70 was observed in the kidney of inducible NOS knockout mice that were free of injury (26). This suggests that upregulation of HSP70 may be the first line of defense when NO availability is impaired. AngII infusion increased HSP70 expression after only 3 d, and this effect was not diminished by normalization of BP with the nonspecific vasodilator hydralazine, suggesting a specific effect of AngII (22). Moreover, a similar increase in BP induced by norepinephrine had no effect on HSP70 expression (22). In conjunction with our data, this suggests that an imbalance between AngII and NO may be initially countered by induction of HSP70. The induction of HSP70 precedes conventional markers of renal injury and might be useful in the early detection of renal disease at a stage when injury is still reversible.

In summary, utilization of a novel lipophilic ROS-sensitive probe has shown that chronic NOS inhibition in the kidney results in increased lipid peroxidation in the epithelial and not in the vascular compartment. We also demonstrate that induction of HSP70 in the kidney in this model occurs much earlier than proteinuria, lipid peroxidation, or loss of renal function can be detected. Furthermore, the increase in renal lipid peroxidation occurs via AngII and NAD(P)H oxidase and precedes loss of renal function. The increase in lipid peroxidation was confirmed by renal cortical TBARS and prevented by vitamin E, a lipophilic antioxidant. Application of this technique has important consequences for the paradigm that oxidative stress in renal failure is largely confined to the vascular compartment. The extravascular compartment may also be an interesting focus for interventions.

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