Enhanced Nitric Oxide Inactivation and Protein Nitration by Reactive Oxygen Species in Renal Insufficiency

Nosratola D. Vaziri, Zhenmin Ni, Fariba Oveisi, Kaihui Liang, Raj Pandian

Abstract—Chronic renal failure (CRF) is associated with oxidative stress which promotes production of reactive carbonyl compounds and lipoperoxides leading to the accumulation of advanced glycation and lipoxidation end products. Reactive oxygen species (ROS) avidly reacts with nitric oxide (NO) producing cytotoxic reactive nitrogen species capable of nitrating proteins and damaging other molecules. This study tested the hypothesis that CRF results in enhanced ROS-mediated NO inactivation and protein nitration which can be ameliorated with antioxidant therapy. Male Sprague Dawley rats were randomized to CRF (5/6 nephrectomy) and sham-operated controls and fed either a regular diet (vitamin E, 40 U/Kg food) or an antioxidant-fortified diet (vitamin E, 5000 U/Kg food) for 6 weeks. Blood pressure, plasma malondialdehyde (MDA), tissue NO synthase (NOS) isoforms, tissue nitrotyrosine (the footprint of NO interaction with ROS), and vascular tissue NO production were determined. CRF resulted in marked elevations of blood pressure, plasma MDA, and tissue nitrotyrosine abundance, but did not change plasma L-arginine level. This was coupled with depressed vascular tissue NO production and reduced immunodetectable NOS proteins in the vascular, renal, and cardiac tissues. Antioxidant therapy ameliorated the CRF-induced hypertension, improved vascular tissue NO production, lowered tissue nitrotyrosine burden, and reversed downregulations of NOS isoforms. In contrast, antioxidant therapy had no effects in the controls. CRF is associated with oxidative stress which promotes NO inactivation by ROS leading to functional NO deficiency, hypertension, and widespread accumulation of protein nitration products. Amelioration of oxidative stress by high-dose vitamin E enhances NO availability, improves hypertension, lowers protein nitration products, and increases NOS expression and vascular NO production in CRF animals. (Hypertension. 2002;39:135-141.)

Key Words: uremia ■ nitric oxide synthase ■ hypertension, renal ■ oxidative stress ■ oxygen

Oxidative stress occurs when generation of the reactive oxygen species (ROS) exceeds the natural antioxidant capacity of the organism. In the presence of oxidative stress, uncontained ROS attack, denature, or modify lipids, proteins, carbohydrates, DNA, and other molecules. Oxidative stress is a known feature of chronic renal failure (CRF), and its presence is evidenced by the reported elevation of lipid peroxidation product, malondialdehyde, depressed antioxidant capacity, and impaired antioxidant enzyme activities in the plasma and erythrocytes of CRF patients and animals.1–7 Increased ROS activity results in the nonenzymatic production of reactive carbonyl compounds and lipoperoxides which, in turn, react with and modify structural and functional proteins leading to formation of advanced glycation end products (AGE) and advanced lipoxidation end products (ALE).8,9 Accumulation of these products is thought to contribute to the cardiovascular and other complications of CRF. The present study extends the previous findings of oxidative stress-mediated production of AGE and ALE by demonstrating enhanced protein modification by reactive nitrogen species leading to the accumulation of protein nitration products in CRF.

ROS avidly react with and inactivate NO and, in the process, produce highly reactive and cytotoxic products, such as peroxynitrite (ONOO−) and peroxynitrous acid (ONOOH). Peroxynitrite in turn reacts with and modifies various molecules, such as lipids, DNA, and proteins. For instance, peroxynitrite reacts with the tyrosine and cysteine residues in protein molecules to produce nitrotyrosine and nitrocystein, which are considered to be footprints of NO-ROS-protein interaction.10,11 In addition to these and other harmful biochemical reactions, the oxidation of NO by ROS inevitably results in functional NO deficiency, which can contribute to the pathogenesis and maintenance of hypertension and its long-term consequences. In fact, Vaziri et al have demonstrated the role of oxidative stress in the pathogenesis of various forms of hypertension including uremic hypertension,1,12 lead-induced hypertension,13–15 diet-induced hypertension,16,17 aorta coarctation–induced hypertension,18 and genetic hypertension.19 In several models, depressed NO
availability was coupled with widespread accumulation of nitrotyrosine in all tested tissues, denoting NO inactivation and sequestration by ROS. Moreover, NO sequestration, functional NO deficiency, and hypertension improved with antioxidant therapy or elimination of the source of oxidative stress in these models. The role of oxidative stress per se in the pathogenesis of hypertension was recently proven by the demonstration that glutathione depletion can cause severe antioxidant-remediable hypertension in genetically normal, otherwise intact animals.20 The present study was designed to test the hypothesis that CRF results in enhanced ROS-mediated NO inactivation and protein nitration that can be ameliorated by antioxidant therapy.

Methods

Study Groups
Male Sprague Dawley rats weighing 230 to 250 g (Harlan Sprague Dawley Inc, Indianapolis, Ind) were used in this study. Animals were housed in a climate-controlled space with 12-hour day and night cycles. The animals were randomly assigned to either the CRF group or the sham-operated control group. Each animal in the CRF group underwent 5/6 nephrectomy by surgical resection of the upper and lower thirds of the left kidney followed by right nephrectomy 4 days later. Those in the control group underwent sham operations. The procedures were carried out under general anesthesia (pentobarbital 50 mg/kg, IP) using strict hemostrict and aseptic techniques. The nephrectomy procedures were accomplished via dorsal incisions as described in our earlier studies.21

The CRF and control animals were divided into subgroups receiving either antioxidant-fortified or regular diets. A minimum of six animals was included in each subgroup. The foods used in the study were prepared by Harlan Teklad Inc. The control diet was a regular rat chow which contained 320 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L DTT, 10 µg/mL leupeptin, and 2 µg/mL aprotinin at 0 to 4°C with the aid of a tissue grinder fitted with a motor-driven ground-glass pestle. Homogenates were centrifuged at 12 000g for 5 minutes at 4°C to remove nuclear fragments and tissue debris without precipitating plasma membrane fragments. The supernatant was used for determination of NOS isoform proteins. Total protein concentration was determined by using a Bio-Rad kit (Bio-Rad Laboratories). Endothelial, neuronal, and inducible NOS proteins were measured by Western analysis using anti-eNOS, anti-nNOS, and anti-iNOS monoclonal antibodies (Transduction Laboratories), respectively, in the same manner as that previously described by Vaziri et al.22

Measurement of Tissue Nitrotyrosine
Nitrotyrosine abundance in the plasma and tissue preparations was determined by Western blot analysis in a manner which was similar to that described in our earlier studies.13 The antinitrotyrosine antibody employed in these measurements was purchased from Upstate Biotechnology Inc. In an attempt to exclude nonspecific reactivity, the Western blot procedures for both nitrotyrosine and NOS isoforms were repeated with the omission of the respective primary antibodies. NO reactivity was observed with the secondary antibody when primary antibody was omitted.

Quantitation of Vitamin E and L-Arginine
Vitamin E (α, β, and γ tocopherols) was determined by using a high-performance liquid chromatography (HPLC) procedure. Briefly, 0.5 mL of plasma or standard tocopherols (α or γ) were extracted with 1 mL of ethanol. The protein-free extract was further extracted using hexane. The hexane extract was dried under nitrogen, reconstituted with mobile phase (acetonitrile, methanol, methylene chloride), and injected into HPLC. The eluates were detected at 290 nm using an ultra violet (UV) detector. The intra-assay and inter-assay variations of this assay are less than 12% and 16%, respectively. Plasma arginine was determined using HPLC as described by Goldsmith et al.24

Data Analysis
Analysis of variance (ANOVA), multiple range test, and regression analysis were used in statistical analysis of the data. Data are presented as mean±SEM. P values less than 0.05 were considered significant.

Results

General Data
As expected, the CRF group showed a significant reduction in creatinine clearance (1.25±0.20 mL/min) compared with that found in the sham-operated controls (3.66±0.24 mL/min, P<0.01). Antioxidant therapy did not significantly affect creatinine clearance in CRF animals fed the vitamin E–supplemented diet (1.34±0.16 mL/min). The CRF animals exhibited a marked elevation of arterial pressure during the observation period. Antioxidant therapy significantly, but partially, attenuated the CRF-induced hypertension (197±5 mm Hg in the untreated CRF, 162±4 in vitamin E–treated CRF, and 110±4 in the control animals, P<0.01). The observation that amelioration of oxidative stress with antioxidant therapy resulted in partial, as opposed to complete, correction of hypertension in the vitamin E–treated CRF animals points to the role of numerous other factors that also contribute to the pathogenesis of CRF-induced hypertension. The rise in arterial blood pressure in the CRF animals...
was coupled with a significant increase in plasma lipid peroxidation product, malondialdehyde (MDA) (0.98±0.13 versus 1.83±0.09 μmol/L, P<0.05). Amelioration of hypertension with antioxidant therapy was accompanied by a parallel decline in plasma MDA concentration (1.28±0.15). A significant positive correlation was found between plasma MDA concentration and arterial pressure in the study population (r=0.88, P<0.01). In contrast to the CRF group, the sham-operated control group showed no significant change in blood pressure (115±5 versus 120±6 mm Hg) in response to antioxidant therapy.

Plasma Arginine and Tocopherol

No significant difference was found in plasma arginine concentration (given as μmol/L) between the control (140±23), untreated CRF (144±22), or vitamin E–treated CRF (207±41) groups. Likewise, plasma concentration of combined beta and gamma tocopherol was similar in the 3 groups (data not shown). However, plasma concentration of α-tocopherol (given as μmol/L) was greatly increased in the CRF animals fed the α-tocopherol–supplemented diet (14.6±5.9, P<0.001) compared with the untreated CRF (0.2±0.001) and the normal control (0.45±0.25) groups.

Plasma and Tissue Nitrotyrosine Data

Data are depicted in Figures 1 and 2. The CRF group exhibited a significant increase in plasma nitrotyrosine abundance. Likewise, nitrotyrosine abundance was significantly increased in the brain, heart, liver, aorta, and remnant kidney of the CRF rats consuming a regular diet. Antioxidant therapy reduced nitrotyrosine abundance in the plasma and in all of the above tissues in CRF rats fed the vitamin E–fortified diet. In contrast, antioxidant therapy had no effect on nitrotyrosine abundance in the corresponding samples obtained from the sham-operated control groups (data not shown).

Tissue NOS Data

Data are illustrated in Figures 3 and 4. The untreated CRF group exhibited significant reductions of thoracic aorta eNOS and iNOS protein abundance when compared with the normal control group. Likewise, kidney tissue eNOS and iNOS and heart tissue eNOS were significantly reduced in the untreated...
CRF animals. In contrast, brain and kidney nNOS protein abundance was significantly increased in CRF rats fed the regular diet. Antioxidant therapy increased eNOS and iNOS in the aorta and kidney as well as eNOS in the heart toward values found in the normal control group. In addition, antioxidant therapy reversed the CRF-induced upregulation of brain nNOS.

Vascular Tissue NO Production Data
NO production by the vascular tissue as discerned from NO\textsubscript{X} recovered in the incubation medium was significantly reduced in the untreated CRF animals (1.55±0.16 nmole/mg protein/5 minutes) compared with that found in the control tissues (2.96±0.31 nmole/mg protein/5 minutes, P<0.05). Antioxidant therapy resulted in a significant improvement of vascular tissue NO production (2.4±0.16 nmole/mg protein/5 minutes, P<0.05) in CRF rats fed the vitamin E supplemental diet. NO production by the aorta tissues was directly related to aorta eNOS (r=0.81, P<0.01) and inversely related to aorta nitrotyrosine abundance in the study groups (r=−0.78, P<0.01).

Discussion
The CRF group showed a marked elevation of blood pressure and a significant increase in plasma lipid peroxidation product, MDA. The latter finding is consistent with several earlier studies in animals and humans with CRF\textsuperscript{12,2,4} and points to increased ROS activity in renal insufficiency. This was accompanied by widespread accumulation in various tissues of nitrotyrosine, which is the footprint of NO inactivation by ROS.\textsuperscript{10,11,13} Earlier studies have demonstrated ROS-mediated formation and accumulation of protein carbonylation and lipoxidation products, ie, AGE and ALE, in chronic renal insufficiency.\textsuperscript{8,9} The present study extends the latter phenomena to the ROS-mediated production and accumulation of nitrated proteins in CRF. We believe that ROS-mediated oxidation of NO and its widespread sequestration as nitrated proteins necessarily limits the bioavailability of NO in the CRF animals. This can, in turn, contribute to the genesis of the associated hypertension by compounding the effects of CRF-induced downregulation of renal, vascular, and cardiac NOS isoforms shown here, as well as, in our earlier studies of this model.\textsuperscript{25} Depressed NO production capacity in CRF animals was confirmed by the in vitro incubation experiments, which demonstrated a marked reduction in NO generation by isolated vascular tissues from the CRF animals. The observed reduction in NO production was not due to reduced substrate availability because plasma arginine concentration was normal in the CRF animals. Instead, it was
most likely caused by a combination of the reduced NOS abundance shown here and an accumulation of NOS inhibitors in CRF.

Antioxidant therapy with high-dose \( \alpha \)-tocopherol resulted in a marked rise in plasma tocopherol and a significant fall in plasma MDA concentration. This observation points to the efficacy of the given regimen in ameliorating the CRF-induced oxidative stress. Amelioration of oxidative stress with vitamin E supplementation in the CRF animals was accompanied by a partial reversal of CRF-induced downregulation of NOS isoforms in the remnant kidney, vascular, and cardiac tissues. This was associated with an expected rise in NO production capacity as demonstrated by the in vitro incubation experiments using isolated vascular tissues obtained from the antioxidant-treated CRF group. Despite increased NO production, tissue and plasma nitrotyrosine abundance declined significantly in the antioxidant-treated CRF animals, approaching values seen in the normal control group. This observation clearly points to the role of increased ROS activity in the pathogenesis of tyrosine nitration in the CRF animals. The combination of increased NO production capacity and decreased ROS-mediated NO inactivation can contribute to the amelioration of hypertension by enhancing NO availability, as seen in our antioxidant-treated CRF animals.

The CRF animals used in the present study exhibited a significant upregulation of brain nNOS protein abundance. This finding is consistent with the results of an earlier study in this model. Upregulation of brain nNOS represents a nonspecific compensatory response to hypertension and is observed in different models of hypertension. For instance, animals with lead-induced hypertension, uremic hypertension, aorta coarctation-induced hypertension, spontaneous hypertension, and Dahl salt-sensitive hypertension exhibit compensatory upregulation of brain nNOS. It is, therefore, intuitive that amelioration of the CRF-associated hypertension with antioxidant therapy could reverse the compensatory upregulation of brain nNOS as seen in this study. nNOS-derived NO in the brain has been shown to inhibit sympathetic outflow. Therefore, its upregulation in chronic renal failure and other hypertensive disorders cited above may represent a natural counterregulatory response to increased arterial pressure. In addition to the brain, nNOS abundance was significantly increased in the remnant kidney of our CRF animals. In the kidney, nNOS is abundantly expressed in the macula densa where nNOS-derived NO suppresses tubuloglomerular feedback–mediated afferent arteriolar vasoconstriction in response to luminal salt content. Therefore, upregulation of nNOS in the remnant kidney may facilitate glomerular hyperfiltration and salt excretion in the face of severe reduction of nephron mass in the CRF animals.
clear. However, patients employed in the former study had end-stage renal disease and received a greater amount of vitamin E than that used in the latter study.

In conclusion, CRF results in downregulation of renal and cardiovascular NOS isoforms and depressed NO production capacity. Acquired NO deficiency in CRF is compounded by ROS-mediated oxidation/inactivation of NO. These events contribute to the pathogenesis of CRF-associated hypertension and the formation of protein nitration products. Antioxidant therapy with high-dose vitamin E improves NO production, mitigates NO inactivation, decreases tissue nitrotyrosine abundance, and ameliorates hypertension in the CRF animals. These findings demonstrate the contribution of oxidative stress to CRF-induced hypertension and dysregulation of NO metabolism. The study further points to the potential usefulness of antioxidant supplementation in the overall management of CRF.

References

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