Oxidative Stress Promotes Hypertension and Albuminuria During the Autoimmune Disease Systemic Lupus Erythematosus

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Abstract—Several lines of evidence suggest that essential hypertension originates from an autoimmune-mediated mechanism. One consequence of chronic immune activation is the generation of oxygen-derived free radicals, resulting in oxidative stress. Renal oxidative stress has direct prohypertensive actions on renal microvascular and tubular function. Whether oxidative stress contributes to the prevalent hypertension associated with autoimmune disease is not clear. We showed previously that female NZBWF1 mice, an established model of the autoimmune disease systemic lupus erythematosus (SLE), develop hypertension associated with renal oxidative stress. In the present study we tested the hypothesis that oxidative stress contributes to autoimmune-mediated hypertension by treating SLE and control (NZW/LacJ) mice with tempol (2.0 mmol/L) and apocynin (1.5 mmol/L) in the drinking water for 4 weeks. Although the treatment did not alter SLE disease activity (assessed by plasma double-stranded DNA autoantibodies), blood pressure and renal injury (urinary albumin) were reduced in the treated SLE mice. Tempol plus apocynin–treated SLE mice had reduced expression of nitrosylated proteins in the renal cortex, as well as reduced urinary and renal cortical hydrogen peroxide, suggesting that treatment reduced renal markers of oxidative stress. These data suggest that renal oxidative stress plays an important mechanistic role in the development of autoimmune-mediated hypertension. (Hypertension. 2012;59:673-679.)

Key Words: pressure ■ immune ■ inflammation ■ oxidative stress ■ kidney ■ renal

Immune system activation and inflammation are now commonly considered as important mediators in the development of hypertension. This is supported in part by studies showing that the treatment of a variety of experimental animal models, or essential hypertensive human patients with the immunosuppressive therapy mycophenolate mofetil, significantly lowers blood pressure.1–4 Early studies in humans show that autoantibody levels, a clinical hallmark of SLE, are elevated in patients with essential hypertension.5 This suggests that there are potential autoimmune origins to the development of hypertension, an idea that is also recently suggested in rodent models of salt-sensitive hypertension.6 The mechanisms by which autoimmunity can promote hypertension are not clearly defined.

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disease that predominantly affects young women. SLE in humans is associated with a high prevalence of hypertension7–12 for reasons that are not yet clear. We reported recently that an established female mouse model of SLE (NZBWF1) develops hypertension that can be ameliorated by anti-inflammatory therapy (ie, tumor necrosis factor-α antagonism with etanercept).13 In these studies, we also showed that oxidative stress (assessed by NADPH oxidase activity) is increased in the renal cortex from mice with SLE and that oxidative stress plays an important mechanistic role in the development of hypertension during SLE. This hypothesis was tested by measuring blood pressure in SLE and control mice chronically treated with an antioxidant therapy.

Methods

Animal Model

Three-to 5-week–old female NZBWF1 and control (NZW/LacJ) mice were obtained from Jackson Laboratories (Bar Harbor, ME). As described previously, 30-week–old NZBWF1 (and control) mice without any signs of renal injury (urinary albumin: <100 mg/dL by dipstick) were included in these studies.13–15 All of the mice were maintained on a 12-hour light/dark cycle in temperature-controlled rooms with access to food and water ad libitum. All of the studies were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Autoantibody Production

The presence of plasma ant double-stranded DNA antibodies, a clinical hallmark of SLE, was measured as described previously by our laboratory.13–15 Data are presented as antibody activity calculated according to the manufacturer’s instructions.

Experimental Protocol

Mice were administered a combination of tempol (2.0 mmol/L) and apocynin (1.5 mmol/L) in the drinking water for 4 weeks (T+A).
Mice were randomly divided into 4 groups, control mice administered vehicle (control/vehicle) or combined antioxidant therapy (control/T+A) and SLE mice administered vehicle (SLE/vehicle) or combined antioxidant therapy (SLE/T+A). Preliminary studies using these therapies separately showed a trend for a reduction in blood pressure that did not reach statistical significance (data not shown).

**Blood Pressure**

At 34 weeks of age, catheters were implanted into the left carotid artery, and animals were allowed 24 hours to recover from surgery. Blood pressure was recorded in conscious, unrestrained mice via pressure transducers on ≥2 consecutive days, as described previously by our laboratory.13–15 Animals were euthanized, and tissues were harvested at the end of the 34th week.

**Albuminuria**

Urinary albumin was monitored weekly throughout the study using dipstick analysis of 24-hour urine samples collected from mice placed in metabolic cages. Mice were considered positive for urinary albumin at 100 mg/dL (2+). Consistent with our previous work,14–15 55% of SLE/vehicle mice developed albuminuria over the course of the study. Only 25% of the SLE/T+A mice and none of the control mice developed albuminuria during the study. At the conclusion of the study, urinary albumin was measured by ELISA (Alpha Diagnostic Inc, San Antonio, TX), as published previously by our laboratory.13–15

**Hydrogen Peroxide**

Urinary and renal cortical hydrogen peroxide was measured as an index of oxidative stress using the Amplex red assay according to the manufacturer’s instructions (Invitrogen, Eugene, OR). Urinary hydrogen peroxide is normalized to urinary creatinine. Data are presented as the percentage of change between treated and untreated control and SLE mice.

**Immunoblots**

Renal cortical protein expression of nitrosylated proteins was determined using standard Western blot methods, as described previously.13 A mouse monoclonal antinitrotyrosine, clone 1A6 antibody (1:1000; Millipore, Billerica, MA) and rabbit anti–β-actin (1:2500, Abcam Inc, Cambridge, MA) were used. Proteins were visualized using an IR700-conjugated donkey antimouse IgG (1:4000) and IR800-conjugated donkey antirabbit IgG (1:2000; Rockland Immunologicals, Gilbertsville, PA).

Renal cortical protein expressions of antioxidant enzymes were determined using a sheep monoclonal anticopper-zinc superoxide dismutase (1:4000; Meridian Life Sciences), rabbit monoclonal antitryptophanase superoxide dismutase (1:1000; Enzo Life Sciences, Plymouth Meeting, PA), and rabbit monoclonal antimanganese superoxide dismutase (MnSOD; 1:1000; Enzo Life Sciences), each along with a mouse anti–β-actin (1:5000, Abcam Inc). Proteins were visualized using an IR700-conjugated donkey antitrypsin IgG (1:10000) (or IR700-conjugated donkey antirabbit for MnSOD and extracellular superoxide dismutase) and IR800-conjugated donkey antimonouse IgG (1:2000; Rockland Immunologicals, Gilbertsville, PA).

All of the blots were analyzed using the Odyssey Infrared Scanner (LI-COR Biosciences, Lincoln, NE). Data are presented as a ratio of densitometry units of protein, based on band optical density, and normalized to β-actin.

**Real-Time PCR**

Renal cortical mRNA expression of the NADPH oxidase subunits p22phox, p47phox, and gp91phox was measured using RNA isolation, reverse transcription, and real-time RT-PCR methods, as described previously.14–16 The following primer sequences were described previously.14,16 The following primer sequences were used: p22 phox (forward 5′atacttcatcggagtctga, reverse 5′tctgtcctggttacctctc), p47phox (forward 5′tctggttacctcctggttac, reverse 5′ctggttacctcctggttac), gp91phox (forward 5′taggttacctcctggttac, reverse 5′taggttacctcctggttac), and β-actin (forward 5′ctggttacctcctggttac, reverse 5′ctggttacctcctggttac). Primer pairs were located in different exons spanning ±1 intronic sequence. A product-specific melting curve was generated at the end of the experiment to confirm the presence of a single PCR product. Data are presented as the degree of change relative to control expression levels calculated as described previously.14–16 Changes were calculated according to the ΔΔCt method.

**Statistical Analysis**

Data are presented as mean±SEM. Statistical analyses were performed using SigmaStat 3.0 software (Systat, Richmond, CA). A 2-way ANOVA was used to assess group and treatment effects, followed by a 1-way ANOVA with a Holm-Sidak post hoc test (Holm-Sidak) to determine individual differences. A Student t test was used when comparing only 2 groups. Values were considered statistically different at P values <0.05.

**Results**

**Antioxidant Therapy Does Not Alter Progression of SLE**

SLE disease activity, as measured by plasma levels of characteristic double-stranded DNA autoantibodies, is increased in SLE mice compared with controls (4.2±0.6 versus 0.6±0.1 U; P<0.001). Treatment with T+A therapy did not alter this activity (data not shown).

**Antioxidant Therapy Reduces Blood Pressure and Albuminuria During SLE**

Blood pressure was increased in SLE mice compared with controls (Figure 1A; 138±4 versus 115±3 mm Hg; P<0.001).
A-treated SLE mice had significantly lower blood pressure (121 ± 1 mm Hg; \(P = 0.005\)), but the treatment did not affect pressure in control mice (114 ± 2 mm Hg). As shown previously by us and others, urinary albumin was increased in SLE mice compared with controls (Figure 1B; 75 ± 404 g/mg of creatinine vs. 56 ± 6 g/mg of creatinine). In T\(A\)-treated SLE mice, the levels of urinary albumin were reduced (67 ± 6168 g/mg of creatinine), but treatment had no effect on urinary albumin in control mice.

Antioxidant Therapy Reduces Renal Oxidative Stress in SLE Mice

To assess the efficacy of the antioxidant treatment on renal oxidative stress, we measured protein nitrosylation in the renal cortex by Western blot. Four major proteins were easily discernible on the blot at 15, 25, 42, and 50 kDa. Nitrosylation of the 15-kDa protein was significantly greater (22 ± 4%; \(P < 0.05\)) in SLE mice compared with controls; however, nitrosylation of this protein was not increased in SLE mice treated with T\(A\) (Figure 2; -4 ± 3%). The remaining nitrosylated proteins analyzed (25, 42, and 50 kDa) were not significantly increased in mice with SLE, although the expression of the 25-kDa protein was reduced in control mice treated with T\(A\). Taken together, these data show that the antioxidant treatment was successful in reducing renal oxidative stress during SLE.

Hydrogen peroxide measurements were made in the renal cortex and urine as a second method to demonstrate that T\(A\) effectively reduced renal oxidative stress. Baseline renal cortical hydrogen peroxide levels were not significantly different between SLE (29 ± 13 μmol/L; n = 3) and control (32 ± 13 μmol/L; n = 3; \(P = 0.87\)) mice, nor were there statistical differences between urinary hydrogen peroxide levels in vehicle-treated SLE (16 ± 9 μmol/L; n = 4) and control (37 ± 3 μmol/L; n = 3; \(P = 0.14\)) mice. However, treatment with T\(A\) reduced renal cortical (Figure 3A; \(P = 0.08\)) and urinary hydrogen peroxide (Figure 3B) in SLE mice but not controls (\(P < 0.05\)).

Renal Pro-Oxidant and Antioxidant Enzymes Are Altered in SLE

Renal cortical protein expression of MnSOD was increased by 88 ± 27% in SLE mice compared with controls but was unchanged in T\(A\)-treated mice of either group (Figure 4A; \(P = 0.044\)). No significant differences in renal cortical expression of extracellular superoxide dismutase (Figure 4B) or copper-zinc superoxide dismutase (Figure 4C) were detected among any of
there are renal tubular actions that promote hypertension. For example, data show that reactive oxygen species can promote an increase in thick ascending limb expression of the sodium potassium 2 chloride transporter, leading to increased sodium and water reabsorption.

One mechanism by which oxidative stress is generated in the kidneys is through immune system activation and inflammation. This is directly supported by studies showing that angiotensin II–induced hypertension is blunted in adaptive immune-deficient RAG1−/− mice replete with T lymphocytes from p47phox−/− mice. In addition, recent work shows an important mechanistic role for renal interstitial inflammation and oxidative stress in salt-sensitive hypertension. This combination of work has led to the common hypothesis that sustained hypertension results from an initial insult (ie, angiotensin II and high salt) that causes local injury, the production of neoantigens, and subsequent activation of adaptive immunity. The identity of these antigens is not yet clear, although, at least in salt-sensitive hypertension, some evidence suggests that antigenic heat shock proteins may underlie the autoimmune origin for hypertension.

**Hypertension, Oxidative Stress, and Autoimmune Disease**

Data from the present study further advance the idea that hypertension has autoimmune origins and test whether oxidative stress is a contributing factor. Female NZBWF1 mice are a widely used and long-established experimental model of autoimmune disease. The data show that SLE mice treated with antioxidant therapy have significantly lower blood pressure than vehicle-treated animals and strongly support an important role for oxidative stress as a contributor to autoimmune- and inflammatory-induced hypertension. Importantly, treatment with antioxidants in mice with SLE reduced 2 markers of oxidative stress in the renal cortex. First, the increased protein nitrosylation in the renal cortex is ameliorated in SLE mice treated with antioxidants. Protein nitrosylation blots from renal homogenates is commonly used as a marker for oxidative stress. Increased renal cortical oxidative stress can directly affect renal vascular function, and our published work suggests that hypertension during SLE is mediated, in part, through a renal vascular mechanism. This is based on our data showing a parallel rightward shift in the chronic pressure-natriuresis relationship and that renal vascular resistance is increased in mice with SLE. Therefore, the impaired renal hemodynamics associated with SLE hypertension are consistent with the current understanding of the potential effects that reactive oxygen species have on renal vascular function.

The second marker of oxidative stress reduced by antioxidant treatment in mice with SLE was renal cortical and urinary hydrogen peroxide. The possibility that hydrogen

### Figure 3. Antioxidant therapy reduces hydrogen peroxide in systemic lupus erythematosus (SLE) mice.

**A** and **B**, Renal cortical and urinary hydrogen peroxide was reduced in SLE mice treated with tempol and apocynin but was unchanged in control animals. *P*<0.05 vs control.

### Discussion

In the present study, we investigated whether antioxidant therapy reduces blood pressure and protects the kidneys in a model of autoimmune disease with hypertension. The major new findings of this study are as follows: (1) oxidative stress is an important mediator of autoimmune-mediated hypertension; (2) renal oxidative stress is reduced in mice treated with antioxidant therapy; (3) antioxidant therapy reduces urinary albumin excretion in mice with SLE; and (4) SLE disease activity is not altered by treatment with antioxidants.

**Oxidative Stress and Inflammation Promote Hypertension**

The generation of oxygen free radicals in the kidneys is widely recognized as a mechanistic contributor to the development of hypertension. Oxidative stress in the kidney promotes hypertension both by vascular and tubular mechanisms. For example, superoxide directly contributes to increased afferent arteriolar sensitivity to angiotensin II and promotes the myogenic response in renal microvessels. The net effect of this is to increase renal vascular resistance, a key mechanism in the development of hypertension. In addition to direct renal vascular effects of oxidative stress,
Peroxide is mechanistically involved in the development of SLE hypertension is intriguing, because previous studies in NZBWF1 mice show that N-acetylcysteine (hydrogen peroxide scavenger) reduces renal injury and delays mortality. Importantly, the data derived from experimental mouse models of SLE are consistent with evidence for oxidative stress in human SLE. For example, patients with SLE have increased serum levels of malondialdehyde and decreased serum levels of antioxidants, such as glutathione peroxidase. In addition, SLE disease activity scores positively correlate with the level of superoxide generated by polymorphonuclear cells isolated from patients with SLE. Therefore, clinical and animal studies suggest that oxidative stress promotes SLE disease activity, and the present work indicates that it is likely to contribute to the prevalent hypertension found in patients with SLE.

Figure 4. Antioxidants do not affect renal cortical expression of superoxide dismutases in systemic lupus erythematosus (SLE) mice. Renal cortical protein expression (measured by Western blot) of manganese superoxide dismutase (A; MnSOD) was increased in SLE mice vs controls (n=3–4), whereas expression of extracellular superoxide dismutase (B; ECSOD) and copper zinc superoxide dismutase (C; CuZnSOD) were unchanged. Tempol+apocynin (T+A) had no effect on any of the superoxide dismutases in either SLE or control animals. *P<0.05 vs corresponding control.
Pro-Oxidant and Antioxidant Enzymes During SLE Hypertension

Although the cell-specific source of oxidative stress in the kidneys during SLE remains unclear, increased macrophage infiltration in the renal cortex suggests a role for immune cells. However, local inflammatory processes within the renal cortex likely promote the generation of reactive oxygen species from vascular (endothelial, smooth muscle) and tubular as well. Regardless of the cell types contributing to oxidative stress in the renal cortex, our previously published work suggests that NADPH oxidase activity is increased.

Superoxide is normally dismuted by superoxide dismutase to molecular oxygen and hydrogen peroxide, another reactive oxygen species. Therefore, we assessed renal cortical protein expression of copper-zinc superoxide dismutase, extracellular superoxide dismutase, and MnSOD, anticipating that expression of these antioxidant enzymes would be decreased during SLE. However, the data show that copper-zinc superoxide dismutase and extracellular superoxide dismutase expressions were not different between the groups, and MnSOD expression was significantly increased in the renal cortex from SLE mice. One interpretation of these data is that antioxidant expression was significantly increased in the renal cortex, our previously published work suggests that NADPH oxidase activity is increased.

In addition to antioxidant enzymes, we measured renal cortical mRNA expression of the p22, p47, and gp91 phox subunits of the NADPH oxidase in vehicle and T+A-treated SLE mice. Somewhat unexpectedly, the expression of the p47phox subunit was significantly increased in SLE mice treated with antioxidant therapy. We interpret this to suggest that the reduced oxidative stress facilitated by antioxidant treatment creates a sink leading to a compensatory increase in components of the NADPH oxidase. Therefore, the treatment with antioxidants in this study is most likely acting as a scavenger for reactive oxygen species but not changing the underlying factors driving the oxidative stress.

Figure 5. Antioxidant therapy increases renal cortical mRNA expression of subunits of NADPH oxidase in systemic lupus erythematosus (SLE) mice. Renal cortical mRNA expression of p47phox (measured by RT-PCR) was increased in SLE mice treated with tempol+apocynin (T+A) vs vehicle-treated SLE mice (n=3–4). Expression of p22phox and gp91 phox was not altered in mice treated with T+A. *P<0.05 vs SLE/vehicle.

Perspectives

The NZBWF1 mouse is a widely used model of autoimmune disease that closely mimics many of the characteristics of SLE in humans, including the generation of autoantibodies, increased markers of oxidative stress, prevalent hypertension, and renal damage. The data here show that oxidative stress plays an important mechanistic role in the prevalent hypertension associated with SLE. Given the speculation that hypertension has autoimmune origins, SLE is a particularly relevant and timely disease model to consider for advancing the understanding of immune mechanisms that promote hypertension. Although carefully controlled studies will be required to better define the mechanisms by which oxidative stress promotes hypertension during SLE, the present study provides some provocative leads. For example, our recently published work shows that renal vascular responses to angiotensin II are enhanced in this model, a physiological response that is mediated by oxidative stress in other experimental models. Moreover, the data suggest a possible role for the mitochondria and the reactive oxygen intermediate, hydrogen peroxide, as an important contributor to autoimmune hypertension. Therefore, these findings advance the field by providing insight into the mechanisms that promote autoimmune-induced hypertension and provide a basis from which to design further experiments.

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Disclosures

None.

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