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## 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 pro-hypertensive actions on renal microvascular and tubular function. Whether oxidative stress contributes to the prevalent hypertension associated with autoimmune disease is not clear. We previously showed 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 mM) and apocynin (1.5 mM) in the drinking water for 4 weeks (T+A). 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. T+A-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.

### Keywords

pressure; immune; inflammation; oxidative stress; kidney; renal

### INTRODUCTION

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

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**DISCLOSURES** None.

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lowers blood pressure<sup>1,2,3,4</sup>. Early studies in humans show that auto-antibody levels, a clinical hallmark of and mechanistic contributor to autoimmune disease, are elevated in patients with essential hypertension<sup>5</sup>. 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<sup>6</sup>. 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 hypertension<sup>7,8,9,10,11,12</sup> for reasons that are not yet clear. We recently reported that an established female mouse model of SLE (NZBWF1) develops hypertension that can be ameliorated by anti-inflammatory therapy (i.e., TNF-alpha antagonism with etanercept)<sup>13</sup>. 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 this is abrogated in animals treated with etanercept. In the present study it was hypothesized that oxidative stress promotes 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 five week old female NZBWF1 and control (NZW/LacJ) mice were obtained from Jackson Laboratories (Bar Harbor, ME). As previously described, 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 as previously described<sup>14,13,15</sup>. All mice were maintained on a 12-hour light/dark cycle in temperature-controlled rooms with access to food and water ad libitum. All studies were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee (IACUC) and were in accordance with National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

### Auto-Antibody Production

The presence of plasma anti-double-stranded-DNA antibodies (dsDNA), a clinical hallmark of SLE, was measured as previously described by our laboratory<sup>14,13,15</sup>. Data is presented as antibody activity calculated according to the manufacturer's instructions.

### Experimental Protocol

Mice were administered a combination of tempol (2 mM) and apocynin (1.5 mM) 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 at least two consecutive days as previously described by our laboratory<sup>14,13,15</sup>. Animals were sacrificed 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<sup>14</sup>, 35% 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 Int, San Antonio, TX) as previously published by our laboratory<sup>14,13,15</sup>.

## Hydrogen Peroxide

Urinary and renal cortical hydrogen peroxide was measured as an index of oxidative stress according to manufacturer's instructions (Invitrogen, Eugene, OR). Urinary hydrogen peroxide is normalized to urinary creatinine. Data is presented as the percent 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 previously described<sup>16</sup>. A mouse monoclonal anti-nitrotyrosine, clone 1A6 antibody (1:1000; Millipore, Billerica, MA) and rabbit anti- $\beta$ -actin (1:2500, Abcam Inc, Cambridge, MA) were used. Proteins were visualized using an IR700-conjugated donkey anti-mouse IgG (1:4000) and IR800-conjugated donkey anti-mouse IgG (1:2000; Rockland Immunologicals, Gilbertsville, PA).

Renal cortical protein expression of antioxidant enzymes were determined using a sheep monoclonal anti-copper-zinc superoxide dismutase (CuZnSOD; 1:4000; Meridian Life Sciences), rabbit monoclonal anti-extracellular superoxide dismutase (ECSOD; 1:1000; Enzo Life Sciences, Plymouth Meeting, PA), and rabbit monoclonal anti-manganese superoxide dismutase (MnSOD; 1:1000; Enzo Life Sciences), each along with a mouse anti- $\beta$ -actin (1:5000, Abcam Inc, Cambridge, MA). Proteins were visualized using an IR700-conjugated donkey anti-sheep IgG (1:10000) (or IR700-conjugated donkey anti-rabbit for MnSOD and ECSOD) and IR800-conjugated donkey anti-mouse IgG (1:2000; Rockland Immunologicals, Gilbertsville, PA).

All 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  $\beta$ -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 previously described<sup>17,14</sup>. The following primer sequences were used: p22 phox (forward 5' aaagaggaaaagggtcca, reverse 5' taggctcaatgggagtccac), p47phox (forward 5' atactcaacggcctcatgg, reverse 5' ctgtcccgaactctctcg), gp91phox (forward 5' tccatttcttctctggagtg, reverse 5' cccaaccagtacagccact),  $\beta$  actin (forward 5' tgttaccactgggacgaca, reverse 5' ggggtgtgaaggctcaaa). Primer pairs were located in different exons spanning at least one intronic sequence. A product-melt 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 previously described<sup>17,14</sup>. Changes were calculated according to the  $\Delta/\Delta$  ct method.

## Statistical Analysis

Data are presented as mean  $\pm$  standard error of means (SEM). Statistical analyses were performed using Sigmaplot 3.0 software (Systat, Richmond, CA). A two-way ANOVA was used to assess group and treatment effects followed by a one-way ANOVA with a Holm-Sidak post-hoc test (Holm-Sidak) to determine individual difference. A student's t-test was used when comparing only two 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 dsDNA autoantibodies, is increased in SLE mice compared to controls ( $4.2 \pm 0.6$  vs.  $0.6 \pm 0.1$  Units;  $p < 0.001$ ). 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 to controls (Figure 1A;  $138 \pm 4$  vs.  $115 \pm 3$  mmHg;  $p < 0.001$ ). T+A treated SLE mice had significantly reduced blood pressure in ( $121 \pm 1$  mmHg;  $p = 0.005$ ), but the treatment did not affect pressure in control mice ( $114 \pm 2$  mmHg). As previously shown by us and others<sup>18,15,17,13</sup>, urinary albumin was increased in SLE mice compared to controls (Figure 1B;  $75404 \pm 41338$  vs.  $56 \pm 6$   $\mu$ g/mg creatinine). In T+A treated SLE mice the levels of urinary albumin were reduced ( $6740 \pm 6168$   $\mu$ g/mg creatinine) but treatment had no effect on urinary albumin in control mice.

### Antioxidant therapy reduces renal oxidative stress in SLE mice

In order 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 discernable on the blot at 15, 25, 42 and 50 kDa. Nitrosylation of the 15 kDa protein was significantly greater in the ( $22 \pm 4\%$ ,  $p < 0.05$ ) in SLE mice compared to controls; however, nitrosylation of this protein was not increased in SLE mice treated with T+A (Figure 2;  $-4 \pm 3\%$ ). 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 ( $29 \pm 13$  uM,  $n=3$ ) and control ( $32 \pm 13$  uM,  $n=3$ ,  $p=0.87$ ) mice, nor were there statistical differences between urinary hydrogen peroxide levels in vehicle treated SLE ( $16 \pm 9$  uM,  $n=4$ ) and control ( $37 \pm 3$  uM,  $n=3$ ,  $p=0.14$ ) 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- and antioxidant enzymes are altered in SLE

Renal cortical protein expression of MnSOD was increased by  $88 \pm 27\%$  in SLE mice compared to 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 ECSOD (Figure 4B) or CuZnSOD (Figure 4C) were detected among any of the experimental groups. In a subgroup of vehicle treated and T+A treated SLE mice, renal expression of the p22, p47, and gp91

phox subunits of the NADPH oxidase were measured by real time PCR (Figure 5). p47 phox expression was significantly increased in the kidneys from T+A treated SLE mice compared to vehicle treated SLE mice. The expression of p22 and gp91 was not different between the groups.

## 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 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<sup>19,20,21</sup>. 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<sup>22,23,24</sup>. 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, there are renal tubular actions that promote hypertension. For example, data shows that reactive oxygen species can promote an increase in thick ascending limb expression of the sodium potassium 2 chloride transporter (NKCC)<sup>25</sup> 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<sup>-/-</sup> mice replete with T lymphocytes from p47phox<sup>-/-</sup> mice<sup>26</sup>. In addition, recent work shows an important mechanistic role for renal interstitial inflammation and oxidative stress in salt-sensitive hypertension<sup>27</sup>. This combination of work has led to the common hypothesis that sustained hypertension results from an initial insult (i.e., angiotensin II, 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<sup>6</sup>.

### Hypertension, oxidative stress, and autoimmune disease

Data from the present study, further advances the idea that hypertension has autoimmune origins and tests whether oxidative stress is a contributing factor. Female NZBWF1 mice are a widely used and long established experimental model of SLE with hypertension. Our laboratory recently showed that NADPH oxidase activity is increased in the renal cortex from SLE mice and that treatment with etanercept, to block TNF-alpha activity, not only reduces blood pressure but also abrogates the oxidative stress<sup>13</sup>. This led to the question addressed in the present study of whether oxidative stress mechanistically contributes to hypertension during chronic autoimmune disease. The data show that SLE mice treated with antioxidant therapy have significantly lower blood pressure than vehicle-treated animals and strongly supports an important role for oxidative stress as a contributor to autoimmune- and inflammatory-induced hypertension.

Importantly, treatment with antioxidants in mice with SLE reduced two 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<sup>28,29,30</sup>. 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<sup>15,31</sup>. Therefore, the impaired renal hemodynamics associated with SLE hypertension is consistent with the current understanding of the potential effects that reactive oxygen species have on renal vascular function<sup>24,23,22</sup>.

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 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<sup>32</sup>.

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<sup>33,34</sup>. In addition, SLE disease activity scores positively correlate with the level of superoxide generated by polymorphonuclear cells isolated from patients with SLE<sup>33</sup>. 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.

### **Pro- and anti-oxidant 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<sup>13</sup>. However, local inflammatory processes within the renal cortex likely promote the generation of reactive oxygen species from vascular (endothelial, smooth muscle) and tubular sources 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<sup>13</sup>.

Superoxide is normally dismutated by superoxide dismutase to molecular oxygen and hydrogen peroxide, another reactive oxygen species. Therefore, we assessed renal cortical protein expression of CuZnSOD, ECSOD and MnSOD anticipating that expression of these antioxidant enzymes would be decreased during SLE. However, the data show that CuZnSOD and ECSOD expression was not different between the groups and MnSOD expression was significantly increased in the renal cortex from SLE mice. One interpretation of this data is that antioxidant enzymes like the SODs do not contribute to the initiation of oxidative stress in this model and that the increased renal cortical MnSOD is a compensatory change in response to elevated reactive oxygen species. The increased MnSOD expression may also provide some insight into the potential intracellular source of reactive oxygen species given that it is predominantly a mitochondrial enzyme. Ultimately, the conclusions from this data must be made with caution given that protein expression does not necessarily reflect the activity for these antioxidant enzymes. Detailed studies will be required to determine if there is altered mitochondrial function in the renal cortex of mice with SLE (as suggested by the MnSOD) and whether chronic treatment with scavengers of hydrogen peroxide also reduces blood pressure in SLE mice. Nevertheless, the data from this study suggest that oral antioxidants are able to reduce renal oxidative stress.

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 tempol/apocynin treated SLE mice. Somewhat unexpectedly, the expression of 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.

## 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 auto-antibodies, 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<sup>31</sup>, a physiological response that is mediated by oxidative stress in other experimental models<sup>23,22</sup>. Moreover, the data suggests a possible role of the mitochondria and the reactive oxygen intermediate, hydrogen peroxide, as important contributor to autoimmune hypertension. Therefore, these findings advance the field by providing insight into the mechanisms that promote autoimmune induced-hypertension and provides a basis from which to design further experiments.

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## NOVELTY AND SIGNIFICANCE

### What is known?

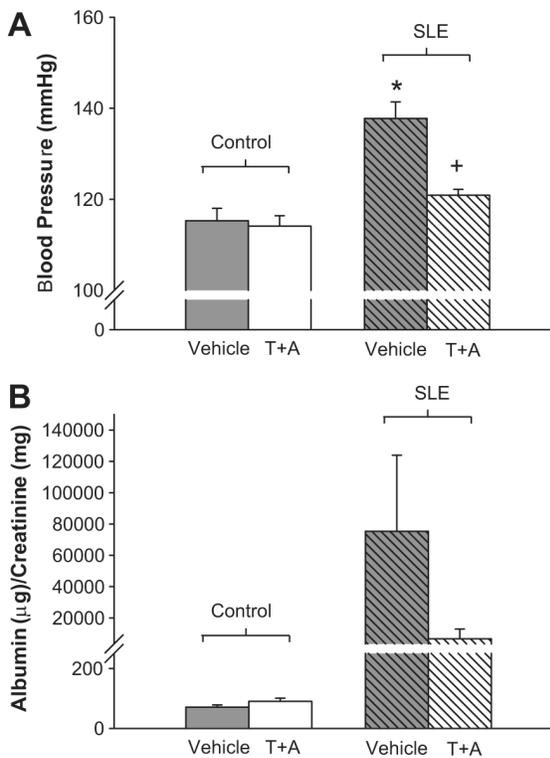
- Chronic inflammation and oxidative stress are strongly linked to the development of hypertension.
- Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder with prevalent hypertension for reasons that remain unclear.

### What is New?

- Oxidative stress is an important mechanism that contributes to the development of hypertension and albuminuria during autoimmune disease.
- Oxidative stress does not promote SLE disease activity, but rather likely occurs as a result of autoimmune-mediated local tissue injury and inflammation.

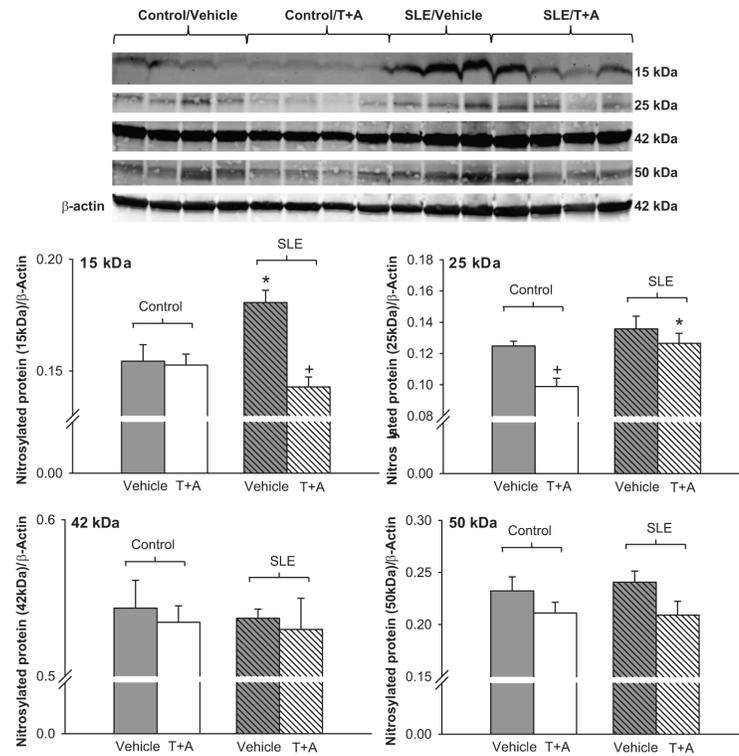
### What is Relevant?

- A growing body of evidence suggests that autoimmunity is an underlying factor that promotes the development of essential (primary) hypertension in humans.
- Therefore, understanding mechanisms that drive the high prevalence of hypertension during autoimmune disease may have direct relevance to understanding the underpinnings of essential hypertension.



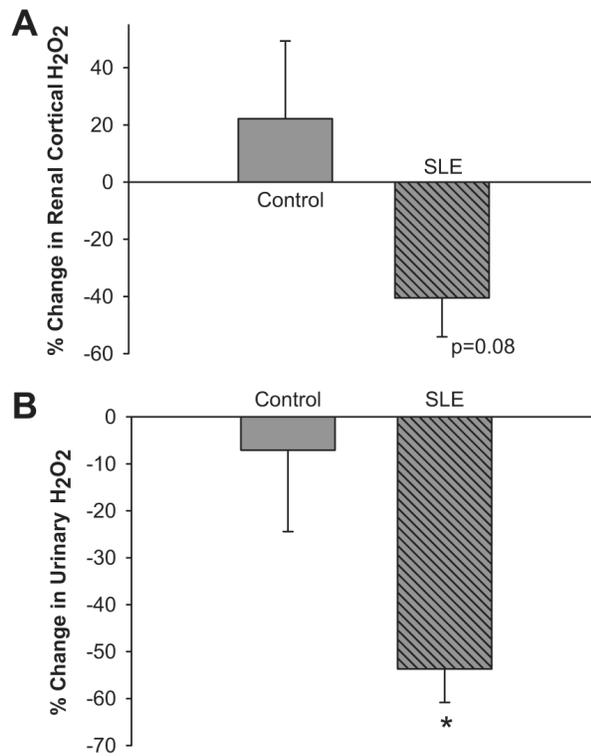
**Figure 1. Antioxidant therapy reduces blood pressure and albuminuria in SLE mice**

1A) Mean arterial pressure (mmHg) was significantly increased in SLE animals compared to controls (n = 6-20). Tempol + apocynin (T+A) decreased blood pressure in SLE animals, but not controls. \*p < 0.05 vs. corresponding Control; <sup>+</sup>p < 0.05 vs. SLE/Vehicle. 1B) Urinary albumin excretion (µg/mg creatinine) was increased in SLE animals compared to controls (n = 8-19). Tempol + apocynin (T+A) decreased albuminuria in SLE animals, but not controls.

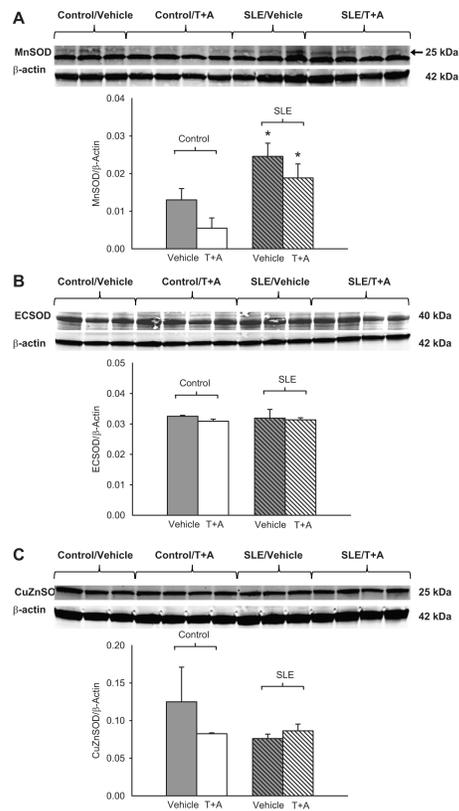


**Figure 2. Antioxidant therapy reduces renal oxidative stress in SLE mice**

Renal cortical protein expression of the 15 kDa nitrosylated protein (measured by Western blot) was increased in SLE mice compared to controls (n=3-4). Tempol + apocynin (T+A) decreased expression in SLE mice, but not controls. \*p<0.05 vs. corresponding Control; <sup>+</sup>p<0.05 vs. corresponding Vehicle.

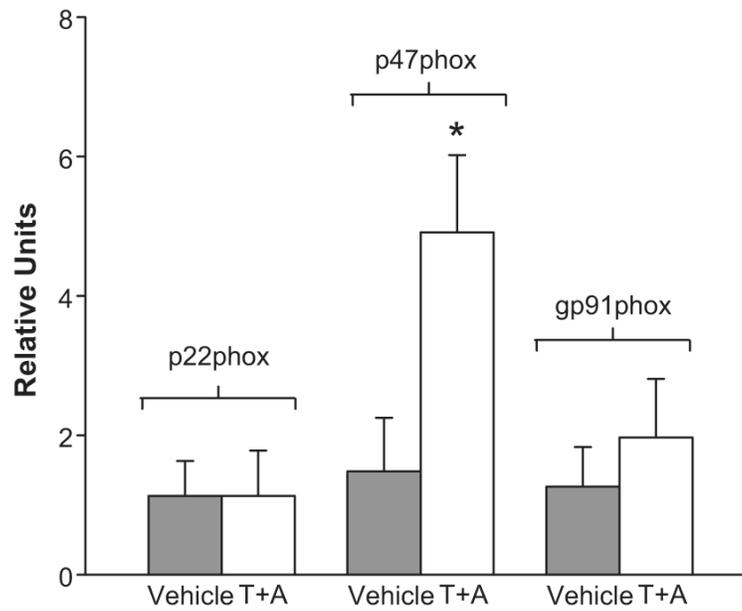


**Figure 3. Antioxidant therapy reduces hydrogen peroxide in SLE mice**  
3A and 3B) Renal cortical and urinary hydrogen peroxide was reduced in SLE mice treated with tempol and apocynin but was unchanged in control animals. \* $p < 0.05$  vs. Control.



**Figure 4. Antioxidants do not affect renal cortical expression of superoxide dismutases in SLE mice**

Renal cortical protein expression (measured by Western blot) of manganese superoxide dismutase (5A; MnSOD) was increased in SLE mice compared to controls (n=3-4), whereas expression of extracellular superoxide dismutase (5B; ECSOD) and copper zinc superoxide dismutase (5B; 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.



**Figure 5. Antioxidant therapy increases renal cortical mRNA expression of subunits of NADPH oxidase in SLE mice**

Renal cortical mRNA expression of p47phox (measured by RT-PCR) was increased in SLE mice treated with tempol + apocynin (T+A) compared to 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.