Tumor Necrosis Factor-α Antagonist Etanercept Decreases Blood Pressure and Protects the Kidney in a Mouse Model of Systemic Lupus Erythematosus

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Abstract—Chronic inflammation has been implicated in the pathology of hypertension; however, the role for specific cytokines remains unclear. We tested whether tumor necrosis factor-α blockade with etanercept (Etan) reduces mean arterial pressure in a female mouse model of systemic lupus erythematosus (SLE). SLE is a chronic inflammatory disorder with prevalent hypertension. Thirty-week–old SLE (NZBWF1) and control mice (NZW/LacJ) received Etan (0.8 mg/kg SC weekly) for 4 weeks or vehicle. Mean arterial pressure (in millimeters of mercury) was increased in SLE mice (150±5 versus 113±5 in controls; P<0.05) and was lower in Etan-treated SLE mice (132±3) but not controls (117±5). Albuminuria (in milligrams per milligram of creatinine) was elevated in SLE mice (28 742±9032 versus 1075±883; P<0.05) and was lower in Etan-treated SLE mice (8154±3899) but not control animals (783±226). Glomerulosclerosis (in percentage of glomeruli) was evident in SLE mice (2.5±1.6 versus 0.0±0.0 in controls; P<0.05) and was ameliorated in Etan-treated SLE mice (0.1±0.1). Renal cortex CD68+ cell staining (in percentage of area) was elevated in SLE mice (4.75±0.80 versus 0.79±0.12 in controls; P<0.05) and was lower in Etan-treated SLE mice (2.28±0.32) but not controls (1.43±0.25). Renal cortex NADPH oxidase activity (relative light units per milligram of protein) was higher in SLE mice compared with controls (10 718±1276 versus 7584±229; P<0.05) and was lowered in Etan-treated SLE mice (6645±490). Renal cortex nuclear factor κB (phosphorylated and nonphosphorylated) was increased in SLE mice compared with controls and lower in Etan-treated SLE mice. These data suggest that TNF-α mechanistically contributes to the development of hypertension in a chronic inflammatory disease through increased renal nuclear factor κB, oxidative stress, and inflammation. (Hypertension. 2010;56:643-649.)

Key Words: systemic lupus erythematosus • hypertension • inflammation • TNF-α • oxidative stress • cytokine

A growing body of literature suggests that chronic inflammation plays an important role in the progression of several forms of hypertension. Plasma levels of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin 6, directly correlate with blood pressure and essential hypertension in humans. In recent studies, a response to the development of hypertension remains unclear. For example, the effect of TNF-α blockade in experimental models of hypertension varies, ranging from having no effect on pressure to delaying the progression or even completely ameliorating hypertension. Therefore, further studies to understand the contribution of this master immune regulator to the development of hypertension are warranted.

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory disorder of unknown etiology that predominantly affects young women. A loss of immunologic tolerance during SLE leads to the production of autoantibodies, of which anti-double-stranded DNA (anti-dsDNA) is the most common and is specific for the disease. Autoantibody production facilitates the formation of immune complexes that deposit in tissues and promote local inflammation and injury, with the kidneys being most commonly affected. Numerous inflammatory cytokines are implicated in the pathophysiology of SLE, including TNF-α. The potential mechanistic role of specific inflammatory cytokines, such as TNF-α, in the development of hypertension remains unclear. Therefore, further studies to understand the contribution of this master immune regulator to the development of hypertension are warranted.
flamboyant disorder SLE. This hypothesis will be tested by treating an established mouse model of SLE (female NZBWF1 mice) with etanercept, a clinically available recombinant TNF-α receptor that reduces the biological activity of TNF-α. We demonstrated previously that this model of SLE develops hypertension, and data from others show that renal TNF-α expression is increased in these mice. The findings from this study will further advance our understanding of the role for TNF-α in hypertension and have direct clinical relevance to patients with SLE.

Methods

Animals
Thirty-week-old female NZBWF1 (SLE) and NZW/LacJ (control) mice obtained from Jackson Laboratories (Bar Harbor, ME) were randomly assigned to receive a weekly subcutaneous injection of the TNF-α inhibitor etanercept (Enbrel, Wyeth, 0.8 mg/kg) or vehicle (saline) for 4 weeks. Urine was collected weekly and assessed for the presence of albumin, as described previously. A final dose of etanercept was administered the week of blood pressure recording and tissue harvest. Only mice with no sign of albuminuria at 30 weeks of age were included in the study. Mice were maintained on a 12-hour light/dark cycle in temperature-controlled rooms with access to chow and water ad libitum. Four groups of animals were included as follows: control-untreated (Ctrl + Veh), control treated with etanercept (Ctrl + Etan), SLE-untreated (SLE + Veh), and SLE treated with etanercept (SLE + Etan). All of the studies were performed with the approval of the University of Mississippi Medical Center Institutional Animal Care and Use Committee and in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Anti-dsDNA
Plasma anti-dsDNA antibodies were measured as described previously. Mice were considered anti-dsDNA positive at values ≥ 1 SD from controls. Only NZBWF1 mice with positive anti-dsDNA antibodies were considered to have SLE.

Blood Pressure Measurements
At the conclusion of the study, mean arterial pressure (MAP) was recorded using indwelling carotid artery catheters in conscious mice as described previously in our laboratory (2 consecutive days, 1.5 to 3.0 hours per day). Consistent with our previous studies, MAP was significantly lower in etanercept-treated SLE mice compared with control mice (n=7; conscious). Etanercept (Etan) significantly reduced MAP in SLE mice (n=10) but had no effect on MAP in control animals (n=9). Etan: *P<0.05 vs Ctrl + Veh and Ctrl + Etan. †P<0.05 vs SLE + Veh.

Renal TNF-α and Nuclear Factor-κB

Protein Expression
Renal cortical protein expression of nonphosphorylated (nuclear factor-κB [NF-κB]), phosphorylated (P) NF-κB, and TNF-α was determined using standard Western blot methods, as described previously. Blots were incubated with either a mouse monoclonal anti–TNF-α antibody (1:250 Santa Cruz Biotechnology), rabbit monoclonal anti-P–NF-κB p65 (Ser536; 1:1000; Cell Signaling), anti–NF-κB p65 (1:2000; Cell Signaling), and a mouse monoclonal anti–β-actin (1:5000; Abcam) as a loading control. Secondary antibodies were IR700-conjugated donkey antirabbit IgG and IR800-conjugated donkey antimouse IgG (1:2000; Rockland Immunocyticals). Antibody labeling was visualized using the Odyssey Infrared Scanner (LI-COR). Data are presented in arbitrary units of protein optical density band normalized to β-actin.

Chemokines and Endothelin 1
Urinary monocyte chemoattractant protein 1 (MCP-1) and urinary endothelin 1 (ET-1) were measured using commercial ELISAs (R&D Systems), as we reported previously.

Statistical Analysis
Data are presented as mean±SEM. Statistical analyses were performed using Graph Pad Prism 5 software. A 2-factor ANOVA was used to test for drug or group interactions. When a significant interaction was detected, a 1-way ANOVA with a Student-Newman-Keuls post hoc test was used to discern individual differences between groups. Significance was accepted at P<0.05.

Results

MAP and Body Weight
Consistent with our previous studies, MAP was significantly higher in vehicle-treated SLE mice compared with vehicle-treated control mice (Figure 1). SLE mice treated with etanercept for 4 weeks had significantly lower blood pressure compared with SLE + Veh. Etanercept treatment did not alter blood pressure in control animals. Body weight was greater in SLE mice compared with controls (Ctrl + Veh: 34±1 g, Ctrl + Etan: 35±1 g, SLE + Veh: 43±1 g, SLE + Etan: 46±1 g; P<0.0001 SLE versus control, 2-way ANOVA), as we reported previously. Body weight decreased over the course of the experiment in all of the groups; however, the weight
loss was greatest in SLE+Veh-treated mice. Because the change in body weight was greater in SLE+Veh than SLE+Etan mice, it is unlikely that the improved blood pressure can be attributed to weight loss. Please see the online Data Supplement at http://hyper.ahajournals.org (Figure S1).

**Autoantibodies**

Total plasma anti-dsDNA (IgG) antibodies were significantly greater in SLE mice compared with control mice (145±18 versus 20±14 ng/mL; *P*<0.05) as reported previously. Treatment with etanercept significantly increased the level of anti-dsDNA in SLE (291±54 ng/mL; *P*<0.05 versus SLE+Veh) and control animals (98±21 ng/mL; *P*<0.05 versus Ctrl+Veh).

**Renal Injury**

**Albuminuria**

Mice with SLE had higher urinary albumin compared with control mice (28 742±9032 versus 1075±883 μg/mg of creatinine; *P*<0.05). SLE mice treated with etanercept for 4 weeks had lower urinary albumin (8154±3899 μg/mg of creatinine; *P*<0.05 versus SLE+Veh). Urinary albumin was not different in control animals receiving etanercept (Ctrl+Etan: 783±226 μg/mg of creatinine).

**Renal Monocyte/Macrophage Infiltration**

Positive staining for CD68 was significantly higher in kidneys from SLE mice compared with control animals (Figure 2). CD68 staining was significantly reduced in SLE mice treated with etanercept but not in control mice.

**Glomerulosclerosis**

The percentage of glomeruli exhibiting a sclerotic area between 51% to 75% is shown in Figure 3. Glomerulosclerosis was greater in SLE mice compared with control mice. SLE mice treated with etanercept had significantly lower levels of sclerosis. Treatment with etanercept did not affect glomerulosclerosis in control mice. The complete analysis for glomerulosclerosis can be viewed in Figure S2.

**Renal Cortical NADPH Oxidase Activity**

NADPH oxidase activity was significantly higher in the cortex of SLE mice than in control animals, and mice treated with etanercept for 4 weeks had significantly reduced renal cortical NADPH oxidase activity in SLE. Treatment with etanercept had no effect on control mice (Figure 4). Urinary F2 isoprostanes were measured as described previously to assess whole body lipid peroxidation, and there were no differences between groups (please see Figure S3).

**Renal TNF-α and NF-κB Protein Expression**

TNF-α protein expression was increased in the renal cortex of SLE mice compared with controls (Figure 5A) and was not significantly reduced in mice treated with etanercept. Total NF-κB and P–NF-κB were significantly greater in the renal cortex of SLE mice compared with controls (Figure 5B). SLE mice treated with etanercept had a significantly lower level of P–NF-κB when compared with vehicle-treated SLE mice. The ratio of P–NF-κB:nonphosphorylated NF-κB protein expression, as an indicator of the relative level of NF-κB activity to the total amount, was significantly reduced in the renal cortex of etanercept-treated control and SLE mice (please see Figure S4).

**MCP-1 and ET-1**

Urinary levels of MCP-1 were measured, and although the means of each group were not statistically different, 3 of 5 samples from the vehicle-treated SLE mice were markedly higher compared with all of the other samples (please see Figure S5). Similar to our previous work, mice with SLE have increased urinary ET-1 (SLE+Veh 1.2±0.2 pg/mL versus Ctrl+Veh 0.50±0.2 pg/mL; *P*<0.05; *n*=5). ET-1 was not different between SLE and control mice treated with etanercept (SLE+Etan 1.3±0.7 pg/mL versus Ctrl+Etan 1.8±0.4 pg/mL; *n*=5).

**Discussion**

The major findings of the present study are as follows: (1) a model of chronic inflammatory disease treated with etanercept has lower blood pressure compared with vehicle-treated controls; (2) the lower blood pressure in etanercept-treated animals occurs despite the persistent elevation in anti-dsDNA autoantibody levels; (3) SLE mice treated with etanercept have less renal injury and inflammatory infiltrates than...
In experimental animal models, immunosuppression lowers blood pressure in rat models of salt-sensitive hypertension, but the treatment affords renal protection. In a model of angiotensin II and high-salt–induced hypertension in rats, treatment with etanercept delayed the development of hypertension, whereas hypertension was ameliorated in a fructose-fed model of insulin resistance and in a model of placental ischemia that mimics preeclampsia. The data from the present study suggest that TNF-α plays an important role in the development of hypertension during SLE, a disease with its origins in immune system dysfunction.

We showed recently that an agonist of peroxisome proliferator activated receptor-γ reduces blood pressure and protects against renal injury in mice with SLE. In that study, we proposed that the anti-inflammatory and renal protective effects of rosiglitazone were important mechanisms for the reduced blood pressure. The data in this study further advance the idea that chronic inflammation, and specifically inflammation caused by TNF-α, promotes hypertension during SLE. In addition to these proinflammatory effects, TNF-α may also have a role to prevent autoantibody production that occurs during SLE. For example, TNF-α blockade can promote inflammatory cell apoptosis leading to increased autoantibody production (although these antibodies may not be pathogenic). In the NZBWF1 model of SLE, IgG2a and IgG2b anti-dsDNA antibodies are closely associated with renal injury, whereas IgG1 and IgG3 antibodies are not. Because the currently available commercial assays do not distinguish between the IgG subclasses, it is possible that the increased autoantibodies observed here are nonpathogenic. This is indirectly supported by the fact that renal pathology is not exacerbated in either the control or SLE mice treated with etanercept. That etanercept treatment significantly increased autoantibody production in SLE mice, yet reduced blood pressure and protected the kidneys, is consistent with the effects of TNF-α blockade on autoantibody production in humans with SLE and suggests that downstream inflammation is an important mediator of hypertension.

The potential for factors other than TNF-α in the development of hypertension during SLE remains given that the

### Hypertension and Chronic Inflammation

Several lines of evidence suggest that inflammation may have an important role in the development of hypertension in both humans and experimental animal models. For example, circulating inflammatory cytokines, such as TNF-α and interleukin 6, directly correlate with blood pressure in humans. In experimental animal models, immunosuppression lowers blood pressure in rat models of salt-sensitive hypertension and mice lacking both B and T lymphocytes (RAG-1 knockout mice) are protected against angiotensin II–induced hypertension. However, the specific role of TNF-α in the development of hypertension is not as clear and varies depending on the experimental animal model studied. Angiotensin II hypertension (transgenic model) and deoxycorticosterone acetate-salt hypertension in rats are not attenuated by treatment with etanercept, although the treatment affords renal protection. In a

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**Figure 3.** Effect of etanercept on glomerulosclerosis in SLE and control mice. Representative kidney sections of Periodic acid–Schiff staining (top, ×40 magnification). The percentage of glomeruli exhibiting a sclerotic area between 51% and 75% was significantly higher in kidneys from SLE mice (n=6) than control mice (n=10; bottom). Treatment with etanercept for 4 weeks significantly reduced glomerulosclerosis in SLE mice (n=8) but had no effect in control animals (n=9). *P<0.05 vs Ctrl+Veh and Ctrl+Etan, †P<0.05 vs SLE+Veh.

**Figure 4.** Effect of etanercept on renal cortical NADPH oxidase activity in SLE and control mice. NADPH oxidase activity was significantly higher in the cortex of SLE mice (n=12) than in control animals (n=11), and treatment with etanercept for 4 weeks significantly reduced NADPH oxidase activity in the renal cortex of SLE mice only (n=10). *P<0.05 vs Ctrl+Veh, †P<0.05 vs SLE+Veh.
hypertension was not completely normalized in etanercept-treated mice. Extrarenal factors, such as changes in metabolic parameters or peripheral vascular or central nervous system impairment, may also contribute to the hypertension. We chose to focus on the kidneys because of their central role in the long-term control of blood pressure and because the NZBWF1 mouse is an established model of lupus nephritis. The concept that TNF-α can directly affect renal function is also supported by a recent study demonstrating that TNF-α infusion in anesthetized mice impairs renal hemodynamics (renal blood flow and glomerular filtration rate) and that this response is prevented by treatment with etanercept.30 Based on the present data, the possibility of a pressure-dependent effect to reduce renal injury cannot be ruled out; however, there are several factors that argue against this. First, the renal injury in SLE is known to be mediated by immune complex deposition and the downstream inflammation. Second, to our knowledge, there is no evidence for a direct blood pressure–lowering effect of etanercept as there is with rosiglitazone treatment from our earlier study (rosiglitazone is known to have direct vasodilatory effects through ion channel modulation31). Finally, several studies in both humans and experimental animal models of SLE, including the NZBWF1 model used here, demonstrate that simply lowering blood pressure is not sufficient to reduce renal injury.32–34 For example, blockade of the renin-angiotensin system with captopril and sympathetic blockade with bretylium cause the same drop in blood pressure in NZBWF1 mice, yet only captopril provides renal protection.32 These data strongly suggest that the renal injury during SLE is not simply pressure dependent.

**TNF-α and SLE**

Because of the varied actions of TNF-α (immunoregulatory versus proinflammatory), the precise role for TNF-α in the precise role for TNF-α in the pathogenesis of SLE remains controversial with some evidence suggesting that it is protective against SLE disease progression. A recently published retrospective study showed that patients receiving adalimumab or infliximab (monoclonal antibodies against TNF-α) developed lupus-like syndrome with generation of antinuclear and anti-dsDNA antibodies, malar rash, serositis, oral ulcers, and hematologic abnormalities, all of which resolved after discontinuation of treatment.35 To the contrary, some
patients with SLE respond well to TNF-α inhibition. Treatment with infliximab has been reported to decrease proteinuria and reduce SLE disease activity score as assessed by the SLE Disease Activity Index.36,37 These disparate effects of TNF-α inhibition in humans with SLE may be related to the time course of the treatment. Short-term administration of infliximab (over weeks) appears to have more favorable outcomes for lupus nephritis, whereas long-term treatments (over months) have deleterious renal effects.38 In the present work, we found that mice treated with etanercept have less renal damage, as measured by albuminuria, glomerulosclerosis, and renal macrophage infiltration. Therefore, the experimental protocol used in the present study mimics the short-term administration of TNF-α inhibition and demonstrates that this type of treatment protocol has implications for blood pressure control in patients with SLE.

TNF-α, Oxidative Stress, and Hypertension

Data from humans with SLE show that there is a direct correlation between superoxide generated by isolated polymorphonuclear cells and SLE disease activity.39 The importance of oxidative stress in the development of hypertension is also well known through effects on renal sodium reabsorption and vascular function.40–43 In this study, the data indicate that mice with SLE have increased renal cortex NADPH oxidase activity that is reduced by the 4-week treatment with etanercept. The multisubunit enzyme NADPH oxidase is a major cellular source of superoxide. Therefore, one cellular mechanism by which TNF-α inhibition may protect against hypertension during SLE is through decreased renal inflammation and a subsequent reduction in renal oxidative stress. The finding that F2 isoprostanes were not different among the groups is not necessarily surprising given that F2 isoprostanes represent a whole body measure of lipid peroxidation and arachidonic acid metabolism in vivo. Moreover, it is well known that the production of reactive oxygen species is highly compartmentalized in tissues and cells. We interpret these data as stronger evidence of a role for renal oxidative stress in SLE and SLE hypertension. Whether antioxidant therapy reduces blood pressure in SLE has yet to be explored, although there is evidence that antioxidants delay mortality and albuminuria in the NZBWf1 model.44

TNF-α, NF-κB, MCP-1, and ET-1

Etanercept is a recombinant receptor with a long half-life (4.35 days45) that binds to and, therefore, reduces the biological effectiveness of TNF-α. Consistent with previous studies,19 our data show that renal TNF-α protein expression is increased in mice with SLE compared with controls. Treatment with etanercept did not significantly affect TNF-α expression, which is consistent with the concept that etanercept stabilizes the TNF-α protein but decreases its biological activity.46

Because TNF-α protein expression was not altered after treatment with etanercept, we tested whether an important molecular downstream mediator of TNF-α signaling, NF-κB, was altered in the renal cortex of etanercept-treated mice. The ratio of P–NF-κB/NFκB reported in the online Data Supplement (Figure S4) can be used as an indicator of the relative amount of activated NF-κB of the total. Although the ratio was significantly reduced in both control and SLE mice treated with etanercept, it is important to note that the total amount of both NF-κB and P–NF-κB was significantly greater in SLE mice compared with controls and that SLE mice treated with etanercept had significantly reduced levels of P–NF-κB. These data cannot completely rule out the possibility that etanercept modulates the activity of other inflammatory pathways, including those activated by the Fc receptor or TNF-β46; however, they strongly implicate NF-κB as an important molecular mediator for the biological actions of TNF-α in the renal cortex during SLE.

We also measured urinary levels of MCP-1 and found that 3 of the 5 samples tested from vehicle-treated SLE mice had markedly increased urinary MCP-1 (≈8-, 24-, and 38-fold increases over control); however, the means from the groups were not statistically different. The failure to demonstrate a P value of <0.05 in this experiment is attributed to the very large variability in the data. This variability is created by the high levels of MCP-1 in the vehicle-treated SLE mice, whereas none of samples from etanercept-treated SLE mice exhibited these levels. These data suggest that at least some mice with SLE have increased urinary MCP-1 that may contribute to increased inflammatory cells in the renal cortex. Urinary ET-1 was assessed and, consistent with our earlier study, was found be higher in SLE mice when compared with controls.18 Urinary ET-1 levels were comparable between control and SLE mice treated with etanercept, suggesting that treatment prevented an increased ET-1 in SLE mice. The specific role of renal ET-1 during SLE hypertension after TNF-α blockade is not yet clear and will require comprehensive studies to evaluate receptor expression and localization, as well as hemodynamic effects of ET-1 blockade.

Perspectives

Chronic inflammation is increasingly recognized as an important pathophysiological mechanism leading to hypertension. Plasma TNF-α levels directly correlate with blood pressure in humans; however, the response to TNF-α inhibition in experimental animal models of hypertension has been varied. SLE is a chronic inflammatory disorder with a high prevalence of hypertension and renal disease. In the present study, using a model of disease with its origins in immune system dysfunction, we demonstrate that blockade of TNF-α reduces blood pressure, renal injury, and renal inflammation. Based on the data from this study, our current thinking is that TNF-α is mechanistically an important mediator of hypertension and renal injury likely by activating NF-κB–mediated pathways leading to increased oxidative stress in the renal cortex. Because etanercept is already clinically available for treatment of autoimmune diseases, these findings may have direct implications for the better treatment of SLE-related hypertension, as well as for the understanding of the role that TNF-α plays in the development of essential hypertension.

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Disclosures

None.

References

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The TNF-a antagonist etanercept decreases blood pressure and protects the kidney in a mouse model of systemic lupus erythematosus

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Figure S1. Percent change in body weight over the course of the experiment. Etanercept treatment did not affect body weight change in SLE or in CTRL mice. (Ctrl+Veh n=11, Ctrl+Etan n=12, SLE+Veh n=10, SLE+Etan n=9) * p<0.05 vs Ctrl+Veh and Ctrl+Etan, # p<0.05 vs Ctrl+Etan
Figure S2. Complete glomerulosclerosis data for all categories of scoring. This method was described previously (Reference 18) and divides the groups based on the percent area of the glomerulus that is sclerotic. The glomeruli with 51-75% sclerotic area is shown in the manuscript (Figure 3) because this data reached a statistically significant difference. *p<0.05 vs. Ctrl+Veh, † p<0.05 vs. SLE+Veh. (Ctrl+Veh n=6, Ctrl+Etan n=10, SLE+Veh n=8, SLE+Etan n=9)
Figure S3. Urinary F2-isoprostanes were measured using a commercially ELISA as a marker of whole body oxidative stress. There was no statistical difference between the groups. (Ctrl+Veh n=9, Crtl+Etan n=7, SLE+Veh n=8, SLE+Etan n=10)
Figure S4. The ratio of P-NFκB to non-phosphorylated protein expression (representing the relative activation of P-NFκB) was significantly reduced in the renal cortex of both etanercept-treated SLE mice and etanercept-treated control mice. *p<0.05 vs. Ctrl+Veh, # p<0.05 vs. SLE+Veh (n=4 per group)
Figure S5. Urinary MCP-1 levels in SLE and Ctrl mice treated with vehicle or etanercept. Due to the large variability in the amount of urinary MCP-1, a statistical difference was not observed. However, 3 out of 5 mice from SLE+Veh had increased MCP-1 whereas MCP-1 was not elevated from any of the other mice in this experiment. (Ctrl+Veh n=5, Ctrl+Etan n=5, SLE+Veh n=5, SLE+Etan n=5).