TNF-α inhibition reduces renal injury in DOCA-salt hypertensive rats

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Elmarakby AA, Quigley JE, Imig JD, Pollock JS, Pollock DM. TNF-α inhibition reduces renal injury in DOCA-salt hypertensive rats. Am J Physiol Regul Integr Comp Physiol 294: R76–R83, 2008. First published November 7, 2007; doi:10.1152/ajpregu.00466.2007.—Studies suggest that the inflammatory cytokine TNF-α plays a role in the pathogenesis of end-stage renal diseases. We previously showed that TNF-α inhibition slowed the progression of hypertension and renal damage in angiotensin II-sensitive hypertension. Thus, we hypothesize that TNF-α contributes to renal inflammation in a model of mineralocorticoid-induced hypertension. Four groups of rats (n = 5 or 6) were studied for 3 wk with the following treatments: 1) placebo, 2) placebo + TNF-α inhibitor etanercept (1.25 mg·kg−1·day−1 sc), 3) deoxycorticosterone acetate + 0.9% NaCl to drink (DOCA-salt), or 4) DOCA-salt + etanercept. Mean arterial blood pressure (MAP) measured by telemetry increased in DOCA-salt rats compared with baseline (177 ± 4 vs. 107 ± 3 mmHg; P < 0.05), and TNF-α inhibition had no effect in the elevation of MAP in these rats (177 ± 8 mmHg). Urinary protein excretion significantly increased in DOCA-salt rats compared with placebo (703 ± 76 vs. 198 ± 5 mg/ day); etanercept lowered the proteinuria (514 ± 64 mg/day; P < 0.05 vs. DOCA-salt alone). Urinary albumin excretion followed a similar pattern in each group. Urinary monocyte chemoattractant protein (MCP)-1 and endothelin (ET)-1 excretion were also increased in DOCA-salt rats compared with placebo (MCP-1: 939 ± 104 vs. 43 ± 7 ng/day, ET-1: 3.30 ± 0.29 vs. 1.07 ± 0.03 fmol/day; both P < 0.05); TNF-α inhibition significantly decreased both MCP-1 and ET-1 excretion (409 ± 138 ng/day and 2.42 ± 0.22 fmol/day, respectively; both P < 0.05 vs. DOCA-salt alone). Renal cortical NF-κB activity also increased in DOCA-salt hypertensive rats, and etanercept treatment significantly reduced this effect. These data support the hypothesis that TNF-α contributes to the increase in renal inflammation in DOCA-salt rats.

deoxy corticosterone acetate; renal inflammation; blood pressure; tumor necrosis factor-α; etanercept; nuclear factor-κB

METHODS

Experiments were conducted with male Sprague-Dawley rats (200–250 g, Harlan Laboratories, Indianapolis, IN). Animal protocols were in accordance with National Institutes of Health guidelines and were approved by the Medical College of Georgia Institutional Animal Care and Use Committee. Rats were fed a normal rat chow diet (Harlan Teklad Global Diets, Wilmington, DE) throughout the experiment and were housed under conditions of constant temperature and humidity with a 12:12-h light-dark cycle. Rats were allowed to adapt to these conditions for several days before starting any experimental procedures.

Rats were anesthetized with 100 mg/kg ketamine-20 mg/kg xylazine (ip). A right nephrectomy was performed via a retroperitoneal incision. Rats were implanted subcutaneously with 60-day time-release DOCA or placebo pellets (200 mg; Innovative Research of America, Sarasota, FL). Systolic arterial pressure was recorded with the tail cuff method (n = 5 or 6/group) as previously described (30). Tail cuff pressure was measured before implantation of DOCA pellets and once a week until the end of the experiment 3 wk later. After recovery from surgery, rats were classified into four groups: 1) placebo, 2) placebo plus the TNF-α inhibitor etanercept, 3) DOCA treatment, and 4) DOCA plus etanercept. In addition, DOCA rats were also given 0.9% NaCl to drink. Etanercept inhibits TNF-α binding to the TNF-α receptor (11). Etanercept was delivered subcutaneously in Alzet miniosmotic pumps (Cupertino, CA) at the time of pellet implantation at a dose of 1.25 mg·kg−1·day−1. This dose was shown previously to reduce renal damage effectively in angiotensin II hypotensive hypertension. Therefore, we hypothesized in the present study that TNF-α contributes to hypertension and its associated renal inflammation in mineralocorticoid-dependent hypertension.

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persistence (9, 24). After a 3-wk period, rats were placed in metabolic cages for 24-h urine collection. Rats were then anesthetized with pentobarbital sodium (65 mg/kg ip; Abbott Laboratories), and a terminal blood sample was taken from the abdominal aorta for determination of plasma 8-isoprostanate (Cayman Chemical, Ann Arbor, MI). Renal cortex was also collected, subdivided into three tubes, and snap frozen in liquid nitrogen for NF-κB analysis, real-time polymerase chain reaction (PCR), and Western blotting. Urinary protein concentration was determined by bicinchoninic acid protein assay (Pierce, Rockford, IL), and albumin concentration was measured with a highly sensitive immunoassay SPI-BIO kit (Cayman Chemical). Urinary MCP-1 concentration was also determined with a commercially available immunoassay kit (BD Biosciences, San Jose, CA), and urinary immunoreactive endothelin concentration was measured by radioimmunoassay (Amersham Pharmacia Biotech, Arlington Heights, IL).

NF-κB transcription factor assay. Whole cell lysates were obtained from kidney cortex from the above-mentioned groups with a nuclear extract kit (Acti Motif, Carlsbad, CA). Protein concentrations were determined by bicinchoninic acid protein assay (Pierce). Twenty micrograms of whole cell extract was used for the determination of NF-κB activity with the TransAM NF-κB p65 transcription factor assay kit (Acti Motif).

Real-time polymerase chain reaction. Total RNA was also isolated from 100 mg of kidney cortex with ultrapure TRIzol reagent according to the procedure described by the manufacturer (GIBCO-BRL, Grand Island, NY); RNA concentration was determined, and equal amounts of total RNA (3 μg) were used in the reverse transcription (RT) with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was used to quantify the mRNA expression of NF-κB, ICAM-1, VCAM-1, TNF-α, and transforming growth factor (TGF)-β. Amplification was performed with iQ Supermix and the iCycler iQ Real-Time Detection System according to the manufacturer’s instructions (Bio-Rad Laboratories). A standard curve was generated for each primer pair and probe to determine PCR efficiency. Mean threshold cycle (Ct) values for each sample were normalized to GAPDH and calibrated to the control group to obtain the threshold cycle difference (ΔΔCt), with 2^−ΔΔCt being the fold change relative to the control group.

Probes and primer sequences were used as follows: NF-κB probe 5′-GAC TCT TGG CAT GAT GAC CCG GAT CGC G-BHQ-3′; NF-κB forward 5′-GTA TGG CTT CCC CCA CTA TGG-3′; NF-κB reverse 5′-TCG CTC TTT GCA CAA TCT C-3′; ICAM-1 probe 5′-FAM-CCT CCT CCT GAG CCT TCT GTA ACT TGT A-BHQ-3′; ICAM-1 forward 5′-GTA CTG ATT GGC GGC TT-3′; ICAM-1 reverse 5′-GGG GCT TGT ACC TTG AGT TT-3′; TGF-β probe 5′-FAM-AGC CCT GTA TTC CGT CTC CTG GGTT TC-BHQ-3′; TGF-β forward 5′-AGT CAA CTG TGG AGC AAC AC-3′; TGF-β reverse 5′-AGC AGA GCA GGA G-BHQ-3′; TNF-α forward 5′-AGT GAC AAG CCC GTA GCCC-3′; TNF-α reverse 5′-GGG TGA GGA GCA CCTG AGT C-3′; GAPDH probe 5′-FAM-ACT CCA CGA CAT ACT CAC CAG CAG CAG-3′; GAPDH forward 5′-CAC GCC AAG TTC AAC GGC-3′; GAPDH reverse 5′-GGT GAA GAC GCC GGC AGT-3′.

Homogenization of renal cortex for protein expression. Kidney cortex was homogenized in homogenization buffer containing protease inhibitors, and VCAM-1, ICAM-1, TNF-α, and TGF-β protein expression were determined by Western blotting as previously described (12). The primary antibodies used were rabbit anti-VCAM-1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rat ICAM-1 (1:500; R&D Systems, Minneapolis, MN), rabbit anti-TNF-α (1:200, Cell Signaling), rabbit anti-TGF-β (1:2,000, Santa Cruz Biotechnology), and mouse anti-β-actin (1:10,000, Sigma, St. Louis, MO). Goat anti-rabbit (Santa Cruz Biotechnology) was used as a secondary antibody for VCAM-1, TNF-α, and TGF-β, donkey anti-goat was used as a secondary antibody for ICAM-1 (Santa Cruz Biotechnology), and goat anti-mouse was used as a secondary antibody for β-actin (Santa Cruz Biotechnology). Band intensity was measured densitometrically with Scion Image software, and the values were normalized to β-actin.

Telemetry experiment. To more accurately detect any subtle change in blood pressure, telemetry transmitters (Data Sciences) were implanted in a separate series of rats 2 wk before DOCA pellet implantation according to the manufacturer’s specifications under pentobarbital sodium anesthesia as previously described (29). In brief, a midline incision was used to expose the abdominal aorta, which was occluded to allow insertion of the transmitter catheter. The catheter was secured in place with tissue glue, and the transmitter body was

Fig. 1. Effect of the TNF-α inhibitor etanercept on arterial pressure in deoxycorticosterone acetate (DOCA)-salt- and placebo-treated rats. A: systolic blood pressure (tail cuff pressure, TCP) in placebo and DOCA-salt rats with or without etanercept treatment for 3 wk. B: 12-h average mean arterial blood pressure (MAP) in DOCA-salt hypertensive rats with or without etanercept treatment for 3 wk. Values are means ± SE (n = 5 or 6/group). *P < 0.05 vs. baseline values.
sutured to the abdominal wall along the incision line as the incision was closed. The skin was closed with staples that were removed 7 days later, after the incision had healed. Rats were allowed to recover from surgery and were returned to individual housing. A baseline arterial pressure was recorded for 1 wk before DOCA pellet implantation. A right nephrectomy was performed, and rats were implanted subcutaneously with DOCA pellets with or without etanercept treatment for 3 wk as described above (n = 6/group). Mean arterial blood pressure (MAP) was continuously recorded in the last two groups for 3 wk.

Statistical analysis. All data are presented as means ± SE. Arterial blood pressure data were analyzed by analysis of variance (ANOVA) for repeated measures combined with post hoc contrasts. Other data were analyzed by one-way ANOVA followed by Tukey’s post hoc test for multiple group comparisons. Differences were considered statistically significant at \( P < 0.05 \) compared with the control. Analyses were performed with GraphPad Prism version 4.0 software.

RESULTS
Systolic blood pressure increased in DOCA-salt hypertensive rats compared with the placebo group, and TNF-α inhibition with etanercept treatment did not significantly affect blood pressure in either placebo or DOCA-salt rats (Fig. 1A). Consistent with the tail cuff pressure data, DOCA-salt treatment significantly increased MAP measured by telemetry (180 ± 5 mmHg on day 21 vs. 107 ± 3 baseline; \( P < 0.05 \)), and etanercept treatment did not affect the increase in MAP in DOCA-salt hypertensive rats (Fig. 1B).
Excretion of protein, albumin, and MCP-1 was used as an indirect measure of renal injury. Urinary protein excretion increased 3.5-fold in DOCA-salt hypertensive rats compared with the placebo group, and etanercept treatment tended to lower protein excretion in DOCA-salt rats, although this change was not significant (Fig. 2A). A similar pattern was observed in albumin excretion (Fig. 2B). Urinary MCP-1 excretion increased roughly 10-fold in DOCA-salt hypertensive rats, and TNF-α inhibition significantly attenuated this increase (Fig. 2C). Urinary endothelin excretion also increased 10-fold in DOCA-salt hypertensive rats, and again etanercept treatment significantly lowered endothelin excretion in DOCA-salt rats (Fig. 2D).

TNF-α mRNA and protein expression increased in the kidney cortex of DOCA-salt hypertensive rats compared with placebo, and etanercept treatment reduced these changes, although it was only significant for mRNA (Fig. 3A). Renal cortical TGF-β mRNA expression also increased in DOCA-salt hypertensive rats, and etanercept treatment produced a modest decrease in TGF-β expression. However, we did not observe any significant difference in renal cortical TGF-β protein expression among the four groups (Fig. 3B).

Plasma 8-isoprostanate concentrations, a measure of oxidative stress, were significantly higher in DOCA-salt hypertensive rats compared with placebo rats (32 ± 5.1 vs. 21 ± 0.14 pg/ml; \( P < 0.05 \)). Etanercept treatment had no effect on the
DOCA-salt-induced increase in plasma 8-isoprostane (35 ± 9 pg/ml). Renal cortical NF-κB mRNA expression was increased in DOCA-salt hypertensive rats compared with placebo rats (Fig. 4A). TNF-α inhibition had no significant effect on NF-κB mRNA expression in DOCA-salt rats, although there was a trend toward reduced expression. Similar to expression, renal cortical activity of NF-κB also increased in DOCA-salt hypertension compared with placebo (1.4 ± 0.4 vs. 0.3 ± 0.08 pg/ng protein; \( P < 0.05 \)). Etanercept treatment significantly lowered the renal activity of NF-κB in DOCA-salt hypertension (0.6 ± 0.08 pg/ng protein) (Fig. 4B).

We also determined the effect of TNF-α inhibition on expression of cell adhesion molecules in DOCA-salt rats. Renal cortical ICAM-1 mRNA and protein expression increased in DOCA-salt hypertension, and etanercept treatment reduced the increase in mRNA but not protein expression (Fig. 5A). However, we did not see any changes among these groups in either renal cortical VCAM-1 mRNA or protein expression (Fig. 5B).

**DISCUSSION**

Compelling evidence supports the hypothesis that inflammatory cytokines play a critical role in the development of hypertension and end-stage renal disease (7). Therefore, we proposed that blocking TNF-α would decrease renal inflammation in the DOCA-salt model of hypertension. TNF-α inhibition with etanercept treatment reduced most indexes of inflammation independent of any blood pressure-lowering effect, as evidenced by the ability of etanercept to reduce MCP-1 excretion in DOCA-salt hypertensive rats. Etanercept also lowered renal cortical NF-κB activity in DOCA-salt hypertension. Renal cortical ICAM-1 and TNF-α expression also decreased in DOCA-salt hypertensive rats on etanercept treatment along with reductions in urinary ET-1 excretion. Together these data suggest that TNF-α contributes to renal inflammation associated with mineralocorticoid hypertension.

Although our previous finding (9) showed that etanercept was able to slow the progression of blood pressure elevation in angiotensin II hypertension, our present study demonstrates that etanercept did not affect systolic blood pressure in placebo and DOCA-salt hypertensive rats. Our initial observation with the tail cuff was confirmed by the use of telemetry to determine whether any subtle change in blood pressure on etanercept treatment could account for changes in other measured variables. Consistent with our tail cuff pressure data, DOCA-salt treatment increased MAP and etanercept failed to lower MAP in this model. Our data are also consistent with the previous finding of Muller et al. (24) in which etanercept treatment attenuated albuminuria in double transgenic rats harboring both human renin and angiotensinogen (dTGR) without lowering blood pressure. Thus, the data in the present study provide evidence that etanercept treatment protects the kidney from damage, and this effect was not dependent on the ability of etanercept to lower blood pressure or even slow the progression of hypertension in DOCA-salt-treated rats.

Albumin excretion is an important marker for the progression to end-stage renal disease and was found to positively correlate with renal TNF-α levels in a rat model of diabetic nephropathy (25). In our study, DOCA-salt treatment significantly increased urinary albumin and protein excretion. Although not statistically significant, etanercept treatment reduced urinary excretion of albumin and protein in DOCA-salt hypertensive rats. These trends are consistent with our previous finding (9) that etanercept reduced albumin and protein excretion in angiotensin salt-sensitive hypertension. Since these changes are not significant in our study, it is quite possible that much of the albuminuria and proteinuria in the DOCA-salt hypertensive model is driven by the elevations in renal perfusion pressure and mediators aside from TNF-α.

TNF-α is a monocyte- and macrophage-derived inflammatory cytokine and can produce vasodilation, increased vascular permeability, and platelet activation (26). For example, infla-
tion of TNF-α induced inflammation of glomerular capillaries in rabbits (3). Etanercept binds to TNF-α and prevents it from interacting with TNF-α receptors (11). Thus blocking TNF-α may reduce the incidence of cardiac and renal vascular inflammation associated with hypertension. In the present study, renal TNF-α expression increased in DOCA-salt hypertension, and inhibiting TNF-α with etanercept reduced renal TNF-α expression and renal damage in the DOCA-salt model. A previous study showed that plasma and tissue TNF-α levels increased in DOCA-treated rats, and this effect was blocked by the mineralocorticoid antagonist spironolactone (10). These data suggest that TNF-α is involved in the inflammatory response and renal damage in mineralocorticoid-dependent hypertension and suggest to us that TNF-α inhibition could be a potential therapy to reduce hypertension-induced renal inflammation.

There is substantial evidence that TNF-α stimulates release of ET-1, which is a potent vasoconstrictor peptide that contributes to the progression of renal diseases (6, 34). For

Fig. 5. Cell adhesion molecule expression in kidney cortex of DOCA-salt hypertensive rats: ICAM-1 mRNA (left) and protein (right) expression (A) and VCAM-1 mRNA (left) and protein (right) expression (B) in placebo and DOCA hypertensive rats with or without etanercept treatment (n = 5 or 6/group). Values are means ± SE. *P < 0.05 vs. placebo group; #P < 0.05 vs. DOCA-salt group.
example, Marsden and Brenner (21) previously demonstrated that TNF-α increased ET-1 release and preproendothelin mRNA content in vitro in bovine renal artery and glomerular capillary endothelial cells. Infusion of TNF-α in vivo was found to increase arterial pressure and preproendothelin in kidney, placenta, and aorta of normal pregnant rats, and ETₐ receptor blockade attenuated the increase in blood pressure produced by TNF-α infusion (17). Similarly, ETₐ receptor blockade also lowered MAP and renal injury in the DOCA-salt model (23). In our study, urinary ET-1 excretion was increased in DOCA-salt hypertensive rats. Interestingly, the increase in ET-1 excretion was significantly reduced with etanercept treatment in DOCA-salt hypertension. These data suggest that the TNF-α-induced inflammatory response and renal injury could be, at least in part, through the stimulation of renal ET-1 production in DOCA-salt hypertension. However, the relationship between endothelin and TNF-α needs further investigation given the contrasting actions of ETₐ versus ETₐ receptors to increase and decrease blood pressure, respectively (35).

TNF-α has been shown to activate the nuclear factor NF-κB, which modulates gene expression of many inflammatory genes such as cytokines, chemokines, and growth factors (12, 14, 36). Normally, NF-κB is composed of two subunits that are present in the cytoplasm as inactive heterodimers (2). Once stimulated, NF-κB translocates to the nucleus and regulates the transcription of cell adhesion molecules and chemokine-activating factors including MCP-1, leading to vascular inflammation and organ damage (2, 37, 38). To explore whether TNF-α-mediated renal damage may operate through a NF-κB-dependent mechanism in DOCA-salt hypertension, we measured renal cortical NF-κB expression and activity in each of the treatment groups. Both NF-κB expression and activity were increased in DOCA-salt rats, and etanercept treatment tended to reduce the increase in expression, but, more importantly, renal cortical NF-κB activity was significantly reduced by etanercept in the kidneys of DOCA-salt hypertensive rats. Previous findings demonstrated that renal NF-κB was increased in DOCA-salt hypertensive rats (4, 31). Muller et al. (24) also showed that NF-κB activity increased in DTGR, and this effect was reduced with etanercept treatment. Clinically, NF-κB activity was reduced in epidermis of psoriasis patients on etanercept treatment (19). These data suggest that downregulation of NF-κB is a potential mechanism of action of TNF-α inhibitors.

Because TNF-α-induced NF-κB activation can also increase production of downstream inflammatory markers such as adhesion molecules and MCP-1 (22, 31), we also determined renal expression of TGF-β, ICAM-1, and VCAM-1 as well as urinary MCP-1 excretion. Our results suggest that TNF-α contributes to the increase in renal TGF-β and ICAM-1 expression in DOCA-salt hypertension, and this effect was reduced with etanercept treatment; however, we did not see any changes in VCAM-1 or TGF-β expression among the various treated groups. Urinary MCP-1 excretion also was increased in DOCA-salt hypertension, and this effect was reduced with etanercept treatment. Previous studies have shown that TGF-β, adhesion molecules, and MCP-1 are increased in kidney and heart of DOCA hypertensive rats (16, 27, 28, 31). Rodriguez-Llurbe et al. (32, 33) also showed that the increase in NF-κB activity was accompanied by increased ICAM-1 and MCP-1 mRNA expression in kidney of spontaneously hypertensive rats (SHR), and these effects were reduced with NF-κB inhibition. These data suggest that downregulation of NF-κB with etanercept treatment protects the kidney from at least some of the factors stimulated by NF-κB, such as ICAM-1 and MCP-1.

In conclusion, our data indicate that TNF-α contributes to the progression of renal damage in DOCA-salt hypertension and this effect is independent of blood pressure lowering. Overall, these results suggest that reduction of the TNF-α-mediated inflammatory response may be a potential therapeutic strategy for the prevention of renal inflammation and the development of end-organ damage.

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