Involvement of Tumor Necrosis Factor-α in Angiotensin II–Mediated Effects on Salt Appetite, Hypertension, and Cardiac Hypertrophy

Srinivas Sriramula, Masudul Haque, Dewan S.A. Majid, Joseph Francis

Abstract—Hypertension is considered a low-grade inflammatory condition induced by various proinflammatory cytokines, including tumor necrosis factor (TNF)-α. Recent studies have implicated an involvement of TNF-α in the development of salt-sensitive hypertension induced by angiotensin II (Ang II). To understand further the relationship between TNF-α and Ang II, we examined the responses to Ang II in TNF-α knockout (TNF-α-/-) mice in the present study. A continuous infusion of Ang II (1 µg/kg per minute) for 2 weeks was given to both TNF-α-/- and wild-type (WT) mice with implanted osmotic minipumps. Daily measurement of water intake, salt intake, and urine output were performed using metabolic cages. Blood pressure was monitored continuously with implanted radiotelemetry. Ang II administration for 2 weeks caused increases in salt (0.2±0.07 to 5.6±0.95 mL/d) and water (5.4±0.34 to 11.5±1.2 mL/d) intake and in mean arterial pressure (115±1 to 151±3 mm Hg) in wild-type mice, but these responses were absent in TNF-α-/- mice (0.2±0.04 to 0.3±0.09 mL/d, 5.5±0.2 to 6.1±0.07 mL/d, and 113±2 to 123±3 mm Hg, respectively). Cardiac hypertrophy induced by Ang II was significantly attenuated in TNF-α-/- mice compared with wild-type mice. In a group of TNF-α-/- mice, when replacement therapy was made with recombinant TNF-α, Ang II induced similar responses in salt appetite, mean arterial pressure, and cardiac hypertrophy, as observed in wild-type mice. These results suggest that TNF-α plays a mechanistic role in mediating chronic Ang II–induced effects on salt appetite and blood pressure, as well as on cardiac hypertrophy. (Hypertension. 2008;51:1345-1351.)

Key Words: angiotensin II ■ TNF-α ■ salt appetite ■ cardiac hypertrophy ■ hypertension

Angiotensin (Ang) II, the effector peptide of the renin-angiotensin system (RAS), plays a key role in regulation of body fluid homeostasis, the development of hypertension, and the maintenance of cardiovascular function.1,2 Ang II is widely recognized for its vasoconstrictor effect, thereby regulating vascular tone and systemic blood pressure,3 and exerts its actions by binding to G protein–coupled receptors, angiotensin type 1 (AT1), and angiotensin type 2 (AT2), located on the plasma membrane of target cells throughout the body.4,5 The AT1 plays a predominant role in the central regulation of arterial blood pressure and cardiovascular remodeling.6,7 Ang II has been shown to have both central and peripheral effects. In the peripheral vasculature, it normally acts to raise arterial pressure by AT1-mediated vasoconstrictor effects. This pressor response of Ang II administration is also known to be partially modulated by the concomitant release of endothelin, prostaglandins, NO, superoxide, and other free radicals from endothelial cells.6 Ang II also contributes to cardiac and vascular remodeling through its direct effect on the heart and the blood vessels.8,7,8 In addition, Ang II stimulates aldosterone, which acts on the renal distal tubules and collecting ducts to retain sodium and water, thereby raising blood pressure.9 Centrally, Ang II plays an important role in regulating the salt appetite and thirst mediated by AT1.10–12 Apart from these, Ang II also acts as a growth factor and stimulator of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin-6, and chemokines.13–15 TNF-α is a multifunctional cytokine that plays an important role in diverse physiological and pathophysiological processes, such as inflammation, cell survival, growth, differentiation, and apoptosis.16,17 Because inflammation is a key component in the pathogenesis of hypertension and cardiovascular disease, the interaction between Ang II and TNF-α may play an important role in the modulation of hypertensive response. Several in vitro and in vivo studies suggest the existence of cross-talk between the RAS and TNF-α.13,14–20 For instance, Ang II treatment induces the production of TNF-α in cultured cardiomyocytes and fibroblasts.13,21 In addition, TNF-α treatment increased AT1 mRNA levels in neonatal rat fibroblasts.22 Administration of the AT1 receptor antagonist valsartan inhibited the expression of TNF-α in a murine model of arterial injury.23 In

Received October 2, 2007; first decision October 17, 2007; revision accepted March 6, 2008.
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Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.107.102152

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patients with hypertension or heart failure, chronic blockade of AT1, resulted in a significant decrease in the circulating levels of TNF-α. More importantly, blockade of TNF-α by etanercept has been shown to prevent renal damage in genetically hypertensive rats and to lower blood pressure in rats with hypertension induced by Ang II and salt, suggesting a role for TNF-α in blood pressure regulation and renal injury. A recent study showed that mice treated with etanercept prevented the hypertension and blunted the increase in superoxide production in response to Ang II. However, the functional importance of TNF-α in Ang II–induced responses is not yet clearly defined. Therefore, in the present study, we examined the role of TNF-α in the mediation of Ang II–induced responses, particularly its effects on salt appetite, thirst, and blood pressure, as well as its role in myocardial cell growth. The effects of chronic administration of Ang II have been evaluated in TNF-α knockout mice and compared with those responses in wild-type (WT) control mice to dissect out the role played by TNF-α in the Ang II–induced effects.

**Methods**

An expanded Methods section can be found in the online data supplement, available at http://hyper.ahajournals.org.

**Experimental Animals**

Twelve-week-old male B6129S-Tnfα<sup>−/−</sup>/J TNF-α knockout (TNF-α<sup>−/−</sup>) mice and control B6129SF2/J (WT) mice (Jackson Laboratories, Bar Harbor, Maine) weighing between 25 and 30 g were used in this study. The mice were housed in a temperature-controlled room (23 ± 2°C) with a 12:12 hour light-dark cycle from 7AM to 7PM in the animal quarters. The studies were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Louisiana State University Institutional Animal Care and Use Committee. They were randomly divided into different groups according to chronic treatment with or without Ang II. Osmetic minipumps were implanted SC to deliver Ang II (1 μg/kg per minute; Sigma Chemical) for 14 days. The control animals were implanted with sterile saline pumps. These groups are as follows: (1) WT (sham-operated control); (2) WT + Ang II (WT treated with chronic Ang II); (3) TNF-α<sup>−/−</sup> (sham-operated control); (4) TNF-α<sup>−/−</sup> + Ang II (knockout mice treated with chronic Ang II); and (5) TNF-α<sup>−/−</sup> + Ang II + TNF-α (TNF-α–replaced knockout mice treated with chronic Ang II). In this group of 6 TNF-α<sup>−/−</sup> mice, along with Ang II infusion, human recombinant TNF-α was given IP at a dose of 10 ng/g per day for 14 days. It should be noted here that we have not seen any sign of increasing susceptibility to infection in these TNF-α<sup>−/−</sup> mice in preoperative or postoperative periods of surgical intervention for telemetry probes and minipump implantations.

**Blood Pressure Measurements**

In 1 set of experiments, mean arterial pressure in conscious mice was measured using a radiotelemetry system with carotid arterial catheters (Model TA11PA-C10, Data Sciences Intl). Mice were allowed to recover from the surgery for 7 to 10 days before experiments were begun. Data were collected, stored, and analyzed using Dataquest A.R.T. software (Data Sciences Intl).

**Metabolic Cage Study**

In another set of experiments, mice were individually housed in specially designed metabolic cages that prevented food and fecal contamination of urine samples. Food and water were available ad libitum. Mice were given both water and salt (1.8% sodium chloride) solution in 2 separate receptacles and were allowed to adapt to the metabolic cages for 7 days. After the acclimatization period, daily water intake, salt intake, and urine volume were measured at baseline and during the 14-day Ang II infusion period. At the end of 14 days, the mice were euthanized and the organs were weighed, and the hypothalamic and left ventricular tissues were collected for mRNA and protein measurements.

**Echocardiography**

Transesophageal echocardiography was performed on mice under isoflurane anesthesia using a Toshiba Aplo SH770 (Toshiba Medical) fitted with a 12-MHz transducer at baseline, and after 14 days of Ang II infusion. Left ventricular internal dimensions at end systole and end diastole (LVS and LVD), and interventricular septal wall thickness at the end of systole and at the end of diastole were measured digitally on the M-mode tracings and averaged from ≥3 cardiac cycles. Left ventricular fractional shortening was calculated as follows: (LVD − LVS/LVD) × 100.

**Protein Analysis by Western Blot**

The protein expression in the heart and hypothalamus was analyzed by Western blot with the use of anti-AT, antibody (Santa Cruz). Protein extracts (25 μg) were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore) by electroblotting. Immunoreactive bands were visualized through the use of enhanced chemiluminescence and quantified by VersaDoc MP 5000 System (Bio-Rad).

**Analysis of mRNA Expression by Real-Time PCR**

Total RNA was isolated from left ventricular tissue and the hypothalamus using TRizol reagent (Invitrogen) according to the manufacturer’s specifications. cDNA was synthesized from 1 μg of RNA with an iScript cDNA synthesis kit (Bio-Rad). Real-time PCR amplification reactions were performed with IQ SYBR Green Super mix with ROX (Bio-Rad) in triplicate using the ABI Prism 7900 real-time PCR machine (Applied Biosystems). Gene expression was measured by ΔΔCT method and was normalized to 18S ribosomal RNA or GAPDH mRNA levels. The data are presented as the fold expressions of the gene interest relative to their control animals.

**Statistical Analysis**

Data were analyzed by ANOVA, followed by Student t test. P<0.05 was considered significant.

**Results**

**Blood Pressure Measurements**

Continuous radiotelemetry recordings of arterial pressure showed that there were no significant differences in baseline blood pressure measurements among the groups (Figure 1). Ang II infusion for 14 days significantly increased mean arterial pressure in WT mice from 115±1 to 151±3 mm Hg (P<0.001) but not in TNF-α<sup>−/−</sup> mice (113±2 to 123±3 mm Hg). However, when TNF-α<sup>−/−</sup> mice were given replacement therapy with human recombinant TNF-α, Ang II administration caused an increase in mean arterial pressure (109±1 to 153±3 mm Hg; P<0.001), similar to that noted in WT mice.

**Metabolic Parameters**

At baseline, there were no significant differences in salt and water intakes and urine volumes between the WT and TNF-α<sup>−/−</sup> groups. As illustrated in Figure 2, Ang II infusion for 14 days in WT mice caused significant increases in salt and water intake, as well as urine output, the increases of which were seen as early as day 5 of infusion. However, the salt and water intakes and urine output remained unchanged in the TNF-α<sup>−/−</sup> mice infused with Ang II (Figure 2).
In contrast, the TNF-α increased the ratio between the heart weight and body weight. Mice, Ang II infusion had increased heart weight and also response in TNF-α (Figure 3A). To further verify the attenuated hypertrophic cardiac function was well preserved in TNF-α mice, mRNA levels of atrial natriuretic peptide were measured by RT-PCR. Although the infusion of Ang II significantly increased the expression of atrial natriuretic peptide in the left ventricle, this upregulation of atrial natriuretic peptide was significantly attenuated in TNF-α mice (Figure 3B).

Cardiac Hypertrophy Responses to Ang II Infusion
To evaluate changes in the cardiac weight in these WT and TNF-α/− mice, the hearts were harvested and weighed at the end of the 2-week experimental period. The ratio of heart weight:body weight was calculated. Figure 3 illustrated these results on cardiac weight. There were no differences in vehicle-treated TNF-α/− and WT mice. In WT mice, Ang II infusion had increased heart weight and also increased the ratio between the heart weight and body weight. In contrast, the TNF-α/− mice with Ang II infusion did not show any increase in the heart weight:body weight ratio (Figure 3A). To further verify the attenuated hypertrophic response in TNF-α/− mice, mRNA levels of atrial natriuretic peptide in the left ventricle were measured by RT-PCR. Although the infusion of Ang II significantly increased myocardial levels of atrial natriuretic peptide in WT mice, this upregulation of atrial natriuretic peptide was significantly attenuated in TNF-α/− mice (Figure 3B).

Gene Expression Studies
To determine whether the infusion of Ang II alters the expression of AT1 receptors, we examined the mRNA levels of AT1 in the heart and hypothalamus by real-time PCR. AT1 mRNA expression was also assessed in the samples collected from WT (n=5 to 6) and TNF-α/− mice (n=5 to 6) before Ang II infusion. The basal level of AT1 mRNA expression was not significantly different between the groups. The AT1 mRNA expression in both heart and hypothalamus was significantly increased in Ang II–infused WT mice, whereas that in Ang II–infused TNF-α/− mice remained unchanged (Figure 4). These results were confirmed at protein levels by the Western blot analysis (Figure 5). Because both Ang II and TNF-α have been shown to act through the nuclear factor κB (NF-κB)–mediated pathways, we analyzed the P50 subunit of NF-κB mRNA expression using real-time PCR. Ang II infusion significantly increased the NF-κB mRNA expression in WT mice but not in TNF-α/− mice (Figure 4C). To determine which TNF receptor is involved in Ang II infusion, the expression of TNF type 1 and type 2 in the left ventricle
Table. Echocardiographic Analysis of Cardiac Hypertrophy and Function

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>WT+Ang II</th>
<th>TNF-α−/−</th>
<th>TNF-α−/−+Ang II</th>
<th>TNF-α−/−+ANG II+TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
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<tr>
<td>IVSD, mm</td>
<td>0.45±0.03</td>
<td>0.54±0.02*</td>
<td>0.40±0.03</td>
<td>0.44±0.02</td>
<td>0.67±0.06†</td>
</tr>
<tr>
<td>IVSS, mm</td>
<td>0.60±0.04</td>
<td>0.81±0.08*</td>
<td>0.65±0.03</td>
<td>0.70±0.03</td>
<td>0.87±0.05†</td>
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<tr>
<td>LVD, mm</td>
<td>3.50±0.10</td>
<td>4.28±0.22*</td>
<td>3.14±0.07</td>
<td>3.17±0.06</td>
<td>3.69±0.07†</td>
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<tr>
<td>LVS, mm</td>
<td>2.55±0.08</td>
<td>3.03±0.14*</td>
<td>2.22±0.05</td>
<td>2.27±0.06</td>
<td>2.73±0.12†</td>
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<tr>
<td>PWD, mm</td>
<td>0.50±0.03</td>
<td>0.57±0.02*</td>
<td>0.42±0.05</td>
<td>0.46±0.02</td>
<td>0.60±0.04†</td>
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<tr>
<td>PWS, mm</td>
<td>0.63±0.07</td>
<td>0.74±0.03*</td>
<td>0.60±0.04</td>
<td>0.67±0.04</td>
<td>0.80±0.10†</td>
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<tr>
<td>FS, %</td>
<td>29.18±1.36</td>
<td>23.57±0.62*</td>
<td>27.77±0.90</td>
<td>27.84±1.06</td>
<td>24.40±0.75†</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>422±12</td>
<td>470±33</td>
<td>455±27</td>
<td>423±7.5</td>
<td>472±20</td>
</tr>
</tbody>
</table>

Values are means±SEMs. IVSD and IVSS indicates interventricular septal thickness at end diastole and end systole, respectively; LVD and LVS, left ventricular internal diameter at end diastole and end systole, respectively; PWD and PWS, posterior wall thickness at end diastole and end systole, respectively; HR, heart rate. *P<0.05 vs WT group and †TNF-α−/− group.

Discussion

The present study demonstrated that the responses to chronic Ang II administration on salt and water intake, blood pressure, and cardiac function were markedly attenuated in mice lacking the gene for the proinflammatory cytokine, TNF-α (TNF-α−/− mice). However, these Ang II responses had been restored in TNF-α−/− mice when these mice were given a replacement therapy with human recombinant TNF-α. In addition, it was also observed that the mRNA levels of the AT1 receptor, as well as NF-κB mRNA expressions in the heart and hypothalamus, were increased in response to chronic Ang II in WT but not in TNF-α−/− mice. These results suggest that a concomitant generation of TNF-α is involved in the complete expression of Ang II-induced salt appetite and hypertensive, as well as cardiac hypertrophic responses, possibly via the TNF-α–induced upregulation of AT1 receptors, as well as activation of NF-κB activity.

Most of the known physiological actions of Ang II, such as vasoconstriction, increased aldosterone secretion, increased sympathetic nerve activity, and increased water and sodium intake, are mediated by the activation of AT1 receptors, which are widely distributed in all organs, including the liver, adrenal glands, brain, lung, kidney, heart, and blood vessels.4,29 AT1 receptors in the brain are linked to vasopressor responses, along with regulation of salt appetite, thirst, and modulation of vasopressin release.12,30,31 Salt appetite and thirst are central nervous system phenomenon. Injection of Ang II into the brain or into the periphery increases salt appetite and thirst in rodents.10,32,33 Ang II is a relatively large peptide, and it does not readily pass the blood-brain barrier. The central nervous system effect of the Ang II could be via the circumventricular organs, where the blood-brain barrier is weak or absent.12 These include the organum vasculosum lamina terminalis, subfornical organ, and area postrema. Ablation of the area postrema or organum vasculosum lamina terminalis regions attenuates the Ang II–induced response on salt appetite and thirst.12 Thus, all of the components of the RAS system are expressed within the central nervous system, thereby facilitating some of the Ang II–induced effect observed centrally.

Activation of the RAS and the subsequent increase in the local production of Ang II is one of the main mechanisms responsible for hypertension and the progression of cardiovascular disease. Ang II has been shown in many reports to increase the expression of various cytokines and chemokines that induce cardiac hypertrophy, inflammation, and vascular remodeling that result in the long-term regulation of blood pressure.2,3 Several studies have shown that blockade of the RAS by ANG-converting enzyme inhibitors or by AT1 receptor blockers attenuates hypertensive response and end organ damage, as well as inflammatory markers, in many
It can be argued that the attenuated cardiac hypertrophy induced by Ang II in TNF-α−/− mice could be the result of reduced blood pressure response in those animals and, thus, poses a potential limitation to data interpretation in the present study. Further studies are needed to examine the pressure-dependent and -independent components of the attenuated hypertrophic response in these TNF-α−/− mice. However, accumulating evidence from clinical and experimental studies indicates that there is a functional crosstalk between Ang II and several proinflammatory cytokines, including TNF-α and interleukin-6, in the regulation of cardiovascular function.13,18–20 Although there is considerable evidence from previous studies supporting a role for AT1 activation in Ang II–mediated hypertension, there have been very limited studies that examined the functional importance of TNF-α in Ang II–induced hypertension and AT1 expression. The results from our present study demonstrated a more clear assessment of the functional involvement of the proinflammatory cytokine TNF-α in the chronic Ang II–induced effects, particularly on salt appetite, blood pressure, and cardiac hypertrophy. However, it may be argued that the component of RAS may be altered, which may influence the responsiveness of Ang II in these TNF-α−/− mice. However, this possibility may be unlikely, because we have observed that the basal tissue AT1 mRNA expression in both the heart and hypothalamus was not different between these TNF-α−/− mice and WT mice. Further studies are required to define the various components of RAS in TNF-α−/− mice.

It is well known that Ang II, by its direct effect on the activation of immune cells, induces the production of inflammatory mediators, such as TNF-α, and contributes to tissue damage in hypertensive response.13–15 Recently, blockade of TNF-α using etanercept has been shown to prevent renal damage in genetically hypertensive rats and to lower blood pressure in rats with hypertension induced by Ang II and salt, suggesting a role for TNF-α in blood pressure regulation and renal injury.26,27 Thus, an interaction between Ang II and TNF-α has been suggested to play an important role in hypertensive response.26,28,37 A recent study by Guzik et al28 showed that Ang II infusion caused infiltration of T lymphocytes in the aortic adventitia and periaortic fat, increased T-lymphocyte production of TNF-α, increased vascular superoxide production, and led to hypertension in mice. Treatment with the TNF-α antagonist etanercept prevented the hypertension and the increase in vascular superoxide caused by Ang II.29 Collectively, these data suggest the notion that chronic Ang II caused infiltration of T lymphocytes in various organ systems, including the cardiovascular and central nervous system, that facilitates the production of TNF-α. This enhancement of TNF-α production resulted in upregulation of AT1 receptors to further enhance the direct actions of Ang II in the target organs. In addition to these direct actions of Ang II, there are also effects of TNF-α–mediated enhanced oxidative stress induced by activation of reduced nicotinamide-adenine dinucleotide phosphate oxidase, possibly via activation of NF-κB activity.13,28

Figure 4. Effect of Ang II on mRNA expression. A, AT1 receptor in left ventricle. B, AT1 receptor in hypothalamus. C, NF-κB in left ventricle. Values are means ± SEMs. *P<0.05 vs WT group and #TNF-α−/− group.

Figure 5. Effect of Ang II on protein expression of AT1 receptor (AT1R). Representative Western blot and densitometric analysis of AT1R protein in left ventricle (A) and hypothalamus (B). *P<0.05 vs WT group.
However, earlier studies had reported differing results regarding the role of TNF-α in the regulation of blood pressure. It had been shown that TNF-α opposed the vasoconstrictor effects of phenylephrine in rat aortic ring preparations.38 Ferreri et al demonstrated that the administration of anti–TNF-α antiserum causes additional increases in mean arterial pressure in a model of Ang II–induced hypertension, indicating that TNF-α may oppose the pressor actions of Ang II. However, a study by Alexander et al showed that infusion of TNF-α at a dose of 50 ng/d for 5 days into virgin rats had no significant effect on blood pressure, but it produced a hypertensive response in pregnant animals. A recent study showed that mice treated with etanercept prevented the hypertension and blunted the increase in superoxide production in response to Ang II. These results also suggest that an interaction between TNF-α and other factors, including oxidative stress, is required for full expression of this cytokine-induced hypertensive response.

Interaction between the RAS and TNF-α in vivo in cardiac hypertrophy was apparent when losartan, an AT1 blocker, was given to transgenic mice overexpressing TNF-α in the heart. Losartan prevented the development of hypertrophy, whereas vehicle treatment produced a significant increase in the heart weight:body weight ratio and LV wall thickness in transgenic mice overexpressing TNF-α.40 Ang II has been shown to induce TNF-α biosynthesis in the heart by activating NF-κB, which, in turn, induces various proinflammatory cytokines and chemokines, including TNF-α.13–41 Sustained application of TNF-α induces an increase in AT1 mRNA levels in cardiac fibroblasts, which is dependent on NF-κB activation.22,42 Ang II, on binding with its receptor, becomes internalized, resulting in the activation of its intracellular signaling mechanism. AT1 receptor–mediated cellular signaling events have been postulated to occur via the Gq protein mechanism. Interestingly, one of the downstream signaling mechanisms of Gq also involves NF-κB activation. Similarly, TNF-α production also involves NF-κB translocation into the nucleus resulting in the perpetuation of TNF-α and other proinflammatory cytokines. In support of the present finding, it has been shown that NF-κB inhibition attenuates hypertensive response and end-organ damage in spontaneously hypertensive rats.43 Clearly, further studies are needed to understand the molecular mechanism involved in the TNF-α and RAS interaction.

The present results may provide a beneficial therapeutic implication of the TNF-α blocker in hypertensive patients who are also suffering from arthritis. At this moment, no direct clinical report is available that may indicate that treatment with a TNF-α blocker may cause a decrease in blood pressure in arthritic patients or have any additive hypotensive effects with the blockers of RAS. Obviously, more comprehensive studies would be required to examine the therapeutic benefit of a TNF-α blocker in the management of hypertension and/or arthritis in patients.

In conclusion, the results from the present study suggest that a concomitant generation of TNF-α is required for the full expression of chronic Ang II–induced effects, such as salt appetite, hypertension, and cardiac hypertrophy, possibly via its action in upregulating AT1 receptors, as well as enhancing NF-κB activity.

**Perspectives**

The findings of the present study emphasize an important mechanistic role of TNF-α in the mediation of hypertensive and cardiac hypertrophy responses induced by chronic Ang II administration. These results demonstrate an existence of a crosstalk between the RAS and the proinflammatory cytokines in the regulation of cardiovascular and other organ functions. However, the specific mechanisms and the downstream signaling pathways by which these 2 systems interact with each other are not yet clearly defined. These present findings provide an important clue in our quest in understanding the pathophysiology of hypertension and other cardiovascular diseases. Thus, it is imperative that further emphasis should be focused on complete elucidation of the interactive role of the RAS and proinflammatory cytokines to increase our understanding of cardiovascular diseases that are linked with inflammatory process.

**Sources of Funding**

These studies were supported by National Heart, Lung, and Blood Institute grant HL-80544 and Louisiana Board of Regents Louisiana Education Quality Support Fund grant to J.F. and by National Heart, Lung, and Blood Institute grant HL-66432 to D.S.A.M.

**Disclosures**

None.

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Hypertension. 2008;51:1345-1351; originally published online April 7, 2008;
doi: 10.1161/HYPERTENSIONAHA.107.102152

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INVOLVEMENT OF TNF-α IN ANGIOTENSIN II MEDIATED EFFECTS ON SALT INTAKE, BLOOD PRESSURE AND CARDIAC HYPERTROPHY

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Running Title: TNF-α in ANG II mediated effects

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DATA SUPPLEMENT

EXPANDED METHODS

**Blood Pressure Measurements Using Telemetry Probes**

Blood pressure in conscious mice was measured using a radio telemetry system with carotid arterial catheters (Data Sciences Intl, DSI; MN). For the implantation of the radiotransmitter, mice were anaesthetized with a ketamine-xylazine mixture (90 and 10 mg/kg, ip). A midline skin incision 2cm long from chin to manubrium was performed to isolate the carotid artery. The catheter portion of the telemetric probe (Model TA11PA-C10, DSI) was inserted into the ascending aorta through the left carotid artery, and the body of the probe was placed subcutaneously on the right flank. Mice were placed on a 12-hour light/dark cycle and received food and water ad libitum throughout the study. Mice were allowed to recover for 7-10 days before experiments were begun. Data was collected, stored, and analyzed using Dataquest A.R.T. software (DSI). Only animals giving stable records were included in the final analysis.

**Protein Analysis by Western Blot**

Protein was extracted from heart and hypothalamus tissue with lysis buffer. The total protein concentration in samples was measured by Bio-Rad Dc protein assay. Protein extracts (25µg) were combined with an equal volume of 2X Laemmli loading buffer, boiled for 5 minutes, and separated by electrophoresis on 10% SDS-Polyacrylamide gels. Then proteins were transferred from the gel to PVDF membranes (Immobilon-P, Millipore, Bedford, MA) by electroblotting. Membranes were blocked with 1% casein in PBS-T for 1 hour and then incubated with anti-AT₁ antibody or anti-TNFR1 antibody (Santa Cruz) overnight at 4°C. Membranes were washed four times in wash buffer (1X TBS, 0.1% Tween-20), followed by
incubation with peroxidase-labeled anti-rabbit IgG for 1 h at room temperature. Membranes were washed four times with wash buffer at room temperature before the antigen-antibody complexes were detected by an enhanced chemiluminescence kit (ECL Plus, Amersham). Autoradiographs were scanned and analyzed by densitometry using VersaDoc MP 5000 System (Bio-Rad). Protein expression of GAPDH was used to check equal loading. All primary antibodies were used at a dilution of 1:1000 and secondary antibodies were used at a dilution of 1:10,000.

**Analysis of mRNA Expression by Real Time PCR**

Total RNA was isolated from left ventricular tissue and the hypothalamus using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s specifications. The RNA concentration was calculated from the absorbance at 260 nm and RNA quality was assured by 260/280 ratio. The RNA samples were treated with DNaseI (Ambion) to remove any genomic DNA. First strand cDNA was synthesized from 1µg RNA with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real Time PCR amplification reactions were performed with iTaq SYBR Green Super mix with ROX (Bio-Rad) in triplicate using the ABI Prism 7900 Real time PCR machine (Applied Biosystems, Fostercity, CA). The primer sequences were used as follows: ANP forward 5’-TGC CGG TAG AAG ATG AGG TC-3’; ANP reverse 5’-AGC CCT CAG TTT GCT TTT CA-3’; BNP forward 5’-AGG GAG AAC ACG GCA TCA TT-3’; BNP reverse 5’-GAC AGC ACC TTC AGG AGA T-3’; AT1R forward 5’-CTG CGT GTT CTG AGG TG-3’; AT1R reverse 5’-ACT GGT CCT TTG GTC GTG AG-3’; NF-κB p50 subunit forward 5’-CGA GGC AGC ACA TAG ATG AA-3’; NF-κB p50 subunit reverse 5’-AGG TCC TTC CTG CCC ATA AC-3’; TNFR1 forward 5’-AAT ATC ATC TCT GAG GCT CTG AGA-3’; TNFR1 reverse 5’-ATG TAC ACC AAG TTG GTA GC-3’; TNFR2 forward 5’-AGC CCA GGG CGG GAT A-3’; TNFR2 reverse 5’-GGT AAT TCT GGG AAG CCG
Gene expression was measured by ∆∆CT method and was normalized to 18S ribosomal RNA or GAPDH mRNA levels. The data are presented as the fold expression of the gene of interest relative to their control animals.

Statistical Analysis
Results are presented as mean values ± SEM. One-way analysis of variance (ANOVA) was used for comparisons of results from more than two groups, where as Student t-test was used to analyze differences between two groups. For repeated measurements analysis a two-way ANOVA followed by Bonferroni post hoc test was used. GraphPad Prism version 4.03 software (GraphPad Software, San Diego, CA) was used for the analysis. Differences were considered significant at a value of $P < 0.05$.

Legend to Supplemental Figure
Figure S1. Effect of angiotensin II infusion on the expression of TNF receptor type 1 (TNFR1) and type 2 (TNFR2) in the left ventricle. A. TNFR1 mRNA expression. B. TNFR2 mRNA expression. C. Representative western blot and densitometric analysis of TNFR1 protein in Left ventricle. Values are mean ± SEM (n=5-6 animals per group). $P < 0.05$ compared with WT group (*) and TNF-α−/− group (#).
Figure S1:

A

Fold increase

B

Fold increase

C

TNFR1

GAPDH

WT+Saline

TNF-α⁻/⁻+Saline

WT+ANG II

TNF-α⁻/⁻+ANG II

TNF-α⁻/⁻+ANG II+TNF-α

Arbitrary units