Tumor Necrosis Factor Receptor 2 mRNA in Rat Models of Hypertension

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In human hypertension (HT) plasma tumor necrosis factor (TNF-α) and soluble TNF receptor 2 fragment (sTNF-R2) are increased, and the TNF-R2 gene (TNFRSF1B) has been implicated. Therefore, we measured Tnfr2 mRNA in kidney, adrenal, heart, and aorta from rats with ACTH-induced, corticosterone-induced, and spontaneous HT (SHR), and tested the effect of blockade of TNF-α by a recombinant TNF-R2 fragment (huTNFR:Fc) on development of HT in the ACTH model. Tnfr2 mRNA was quantified by real-time polymerase chain reaction, as were internal controls, β-actin, and glyceraldehyde-3-phosphate dehydrogenase mRNA. The results showed no differences in tissue Tnfr2 mRNA between HT and control rats. The ACTH-induced HT was not affected by huTNFR:Fc co-administration. The findings thus offer no support for altered Tnfr2 expression in the rat models studied. Am J Hypertens 2003;16:685–688 © 2003 American Journal of Hypertension, Ltd.

Key Words: Tumor necrosis factor receptor 2 mRNA, adrenocorticotropin-induced hypertension, corticosterone-induced hypertension, spontaneously hypertensive rat, real-time quantitative reverse transcriptase PCR, huTNFR:Fc.

Hypertension (HT) and other cardiovascular disorders exhibit inflammatory and immune system abnormalities that include increased plasma tumor necrosis factor (TNF) and soluble TNF receptor 2 fragment (sTNF-R2). The TNF mediates its effects by TNF-R1 and TNF-R2, which can trigger distinctive immunosuppressive and anti-inflammatory actions, some of which have cardiovascular significance. In contrast to TNFR1, TNFR2 is more involved in metabolic effects. Markers in the TNF-R2 gene (TNFRSF1B) have, moreover, shown linkage and association with essential HT. The TNF-α could be cardioprotective or anti-hypertensive, or could contribute to vascular damage by high inducible nitric oxide synthase.

TNF-R2 is coexpressed with TNF-R1 in most tissues, and sTNFRs (including recombinant TNF:Fc) neutralize TNF.

The aims of the present study were to measure Tnfr2 mRNA in ACTH, corticosterone, and SHR models of HT in the rat, and to determine the effect of huTNFR:Fc on development of HT in the ACTH model.

Methods

Animals

Male Sprague Dawley (SD) rats (Animal Resource Centre, Perth, Western Australia), Wistar-Kyoto (WKY), and spontaneously HT rats (SHR) (John Curtin School of Medical Research, Canberra, Australia) were housed in plastic cages. The facility is temperature controlled (21°C to 23°C) and maintains a 12-h light/dark cycle. Rats were given standard rat chow and tap water ad libitum. They were acclimatized for 2 weeks to their surroundings, water and food, handling and blood pressure (BP) measuring equipment before experimental procedures. The studies were approved by the Animal Experimentation Ethics Committee of ANU (Protocol nos. JHB 05.00 and JHB 06.00).

Corticosterone Treatment The SD rats were implanted subcutaneously with a 21-day slow release pellet (Innovative Research of America, Sarasota, FL) containing corticosterone (100 mg) (n = 4). Other rats were implanted with a placebo pellet (n = 3). Systolic BP (SBP) was measured three times/week for 4 weeks (1 week control


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period, then 3-week treatment). One rat from group B was not included due to wound complications.

**ACTH Treatment** Twenty SD rats were treated subcutaneously with either saline (1.0 mL/kg per day) (*n* = 10) or ACTH (Tetracosactrin, Synthacort Depot, generously provided by Novartis Pharmaceuticals, Sydney, Australia; 0.2 mg/kg per day; *n* = 10) for 12 days. The SBP was measured on alternate days. From each group we chose 4 rats randomly for *Tnfr* mRNA measurement. The BP data for these rats has been published.8

**SHR** The SBP of SHR and 5 WKY rats was measured on 3 separate days within a week to confirm elevation in SBP.

**HuTNFR:Fc Treatment** Groups of 8 SD rats were administered subcutaneously with saline (1.0 mL/kg per day, *n* = 6), huTNFR:Fc (1.5 mg/kg per day; kindly provided by Immunex Corporation, Seattle, WA), or ACTH (0.1 mg/kg per day) + huTNFR:Fc (1.5 mg/kg). This dose was deemed effective in lowering TNF, based on previous findings.9 –11 The SBP was measured on alternative days for 3 weeks (1 week of control readings and 2 weeks during treatment). The SBP data were compared with a previous study of saline versus ACTH on SBP in SD rats.12

**BP Measurement and Tissue Collection** The SBP was measured using tail-cuff plethysmography (HyperRat, SDR, Lane Cove, NSW, Australia) and daily value was average of four readings that were within 10 mm Hg of each other.12 At the end of each study, rats were anesthetized (pentobarbitone: 60 mg/kg), venous blood sample collected, and plasma stored at −80°C. Kidney, adrenal, thoracic aorta, and heart were removed, snap-frozen in liquid N₂, then stored at −80°C for subsequent mRNA assay.

**Quantitative Real Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

Total RNA was extracted using an RNA extraction kit (Qiagen, Hilden, Germany). The RNA samples were treated with DNase I (Life Technologies, Rockville, MD) to remove any genomic DNA. *Tnfr2* mRNA was reverse transcribed to cDNA using the Superscript First-Strand Strand Synthesis System for RT-PCR (Gibco-Invitrogen, Carlsbad, CA) and the cDNA then quantified by RT-PCR (Rotorgene, Corbett Research, Sydney, Australia) using for fluorescent label SYBR Green Nucleic Acid Stain (Molecular Probes, Eugene, OR), which is activated during the extension step and incorporated into the DNA minor groove. *Tnfr2* primers were: forward, 5′-CAAGGCCTGTCACAGGA-3′; reverse, 5′-GAAGAGAGATGCCACCCGT-3′. The PCR mixture comprised 25 μL of each primer (3 pmol), 0.2 mmol/L each dNTP, 0.1 U AmpliTaq Gold (PE Applied Biosystems, Norwalk, CT), 2 mmol/L MgCl₂, 56 mmol/L KCl, 11 mol/L Tris-HCl (pH 8.3), and SYBR Green 1:10000. After 94°C for 2 min, there were 50 cycles of 94°C (15 sec), 64°C (35 sec), and 72°C (50 sec), finishing with a melting step of 60°C to 99°C, at 0.05-sec increments. The latter distinguishes false-positive signals formed by primer-dimers from positive signals made by cDNA. The peak in the melt-curve graph represents amount of fluorescence recorded at the temperature at which the amplified PCR products were melted into single-stranded DNA. The mRNA level was based on threshold cycle (Ct). Each assay was repeated five times and results averaged.

Internal control involved quantification of mRNA for housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The β-actin primers were: forward, 5′-CTGTGGTGGTGGAAGCTGAG-3′; reverse, 5′-TCATGCCATCCTCGTCT-3′; and for GAPDH were: forward, 5′-GAACATCCTCCCTGCATTCCA-3′; reverse, 5′-ATGCCAGTGACCTCCCGTT-3′. Interassay variability for each was not significant (P > .05).

**Plasma sTNF-R2 Assay**

This involved an immunoenzymometric assay (sTNF-RII Elisa kit, BioSource Europe S.A., Fleurus, Belgium).

**Statistical Analyses**

All data were expressed as mean ± SE. Differences in mRNA values were tested by ANOVA using StatView (Abacus Concepts, Berkeley, CA). Student unpaired *t* test was used to test for significance of difference between treatment and control groups. The SBP between groups and within each treatment group for different days, was analyzed by repeated measures (RM)-ANOVA, with the Greenhouse-Geisser adjustment for multisample asphericity and Bonferroni correction for multiple comparisons (SPSS version 11.0, SPSS Inc., Chicago, IL). A *P* < .05 was considered significant.

**Results**

Values for β-actin and GAPDH mRNA did not differ between tissues. This contrasts with several reports of differential expression of housekeeping genes between tissues.13–16 *Tnfr2* mRNA was readily detected in each tissue, being lowest in aorta. Because extraction of RNA from the aorta was difficult, low RNA yield could explain this. Data on relative concentrations between each group of rats was nevertheless informative. The site of *Tnfr2* mRNA expression in each tissue77 was not investigated.
Corticosterone Rats

The three SBP values recorded during the control week were averaged to give a pooled value for each group. Rat weights on the day of pellet insertion were: placebo, 170 ± 2 g; pellet, 165 ± 8 g. In response to corticosterone, SBP increased significantly over the first week from 105 ± 4 mm Hg in pooled control to a treatment maximum of 156 ± 14 mm Hg (109 ± 2 and 134 ± 5 mm Hg, respectively, in pellet placebo; P = .035). There were no detectable differences in Tnfr2 mRNA between each group (Fig. 1a). The GAPDH mRNA was also similar.

ACTH Rats

The SBP increased in response to ACTH from 116 ± 4 mm Hg in pooled control to a treatment maximum of 147 ± 6 mm Hg (118 ± 5 mm Hg and 120 ± 4 mm Hg in saline control; P < .001). We saw no significant change in Tnfr2 mRNA concentration in any tissue, nor in housekeeping mRNAs (Fig. 1b).

SHR Versus WKY

The SBP in SHR (171 ± 4 mm Hg) was greater than in WKY (136 ± 4 mm Hg; P < .0005). Concentrations of Tnfr2 mRNA did not, however, differ (Fig. 1c).

HuTNFR:Fc Experiment

The coadministration of huTNFR:Fc with saline had no effect on SBP (pooled control 129 ± 3 mm Hg versus treatment maximum 129 ± 3 mm Hg), and SBP was similar to rats receiving saline alone (122 ± 1 vs 124 ± 1 mm Hg). The SBP in both ACTH-treated groups was significantly elevated compared to the saline-treated groups (RM-ANOVA: P < .001).

Plasma sTNF-R2

Plasma sTNF-R2 in saline and ACTH rats were similar: 4.8 ± 0.29 vs 5.5 ± 0.22 ng/mL, respectively (Fig. 2). In rats administered huTNFR:Fc in saline or with ACTH, values were 14.6 ± 0.4 and 14.3 ± 0.5 ng/mL, respectively (P < .0005) (Fig. 2).

Discussion

We developed a method for semiquantification of Tnfr2 mRNA and applied it to study Tnfr2 mRNA in HT. In every tissue in three models of HT no change was seen. Although Tnfr2 mRNA has not previously been mea-
sured in HT, sTNF-R2 has. It has been suggested that the ability to detect plasma TNF-α only in young SHR is because one source, the heart, may either lose its ability to secrete TNF-α with age or that during cardiac decompensation with age increased sTNFR shedding may decrease free TNF-α. We found no change in tissue Tnfr2 mRNA in any of the rat models studied, including the SHR. The latter finding argues against a genetic effect on Tnfr2 expression and the former suggests that steroid-induced HT is neither mediated by, nor has a secondary effect on Tnfr2 expression. Moreover, plasma sTNF-R2 level, measured only in the ACTH model, was unaltered, consistent with the Tnfr2 mRNA results. In contrast, in humans, plasma sTNF-R2 displays a strong positive correlation with BP and is influenced by TNFRSF1B genotype. It is thus possible that the involvement of TNF-R2 in HT is species or subtype specific.

In conclusion, the present findings offer no support for a role of altered expression of Tnfr2 in the onset of HT in the hormone-induced and genetic models studied.

References