Tumor necrosis factor-α inhibits renin gene expression

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Todorov, Vladimir, Markus Müller, Frank Schweda, and Armin Kurtz. Tumor necrosis factor-α inhibits renin gene expression. Am J Physiol Regul Integr Comp Physiol 283: R1046–R1051, 2002—Renin, produced in renal juxtaglomerular (JG) cells, is a fundamental regulator of blood pressure. Accumulating evidence suggests that cytokines may directly influence renin production in the JG cells. TNF-α, which is one of the key mediators in immunity and inflammation, is known to participate in the control of vascular proliferation and contraction and hence in the pathogenesis of cardiovascular diseases. Thus TNF-α may exert its effects on the cardiovascular system through modulation of renal renin synthesis. Therefore we have tested the effect of TNF-α on renin transcription in As4.1 cells, which represent transformed mouse JG cells, and in native mouse JG cells in culture. Renin gene expression was also determined in mice lacking the gene for TNF-α (TNF-α knockout mice). TNF-α inhibited renin gene expression via an inhibition of the transcriptional activity, targeting the proximal 4.1 kb of the renin promoter in As4.1 cells. TNF-α also attenuated forskolin-stimulated renin gene expression in primary cultures of mouse JG cells. Mice lacking the TNF-α gene had almost threefold higher basal renal renin mRNA abundance relative to the control strain. The general physiological regulation of renin expression by salt was not disturbed in TNF-α knockout mice. Our data suggest that TNF-α inhibits renin gene transcription at the cellular level and thus may act as a modulator of renin synthesis in (physio)pathological situations.

As4.1 cells; juxtaglomerular cells; knockout mice

Regulation of arterial blood pressure is one of the fundamental processes of homeostasis. Blood pressure in mammals is under tight control of various neural and humoral factors, which act in concert to ensure optimal values for the function of the organism according to the environmental changes. The plasma renin-angiotensin-aldosterone-system (RAAS) is among the principal humoral regulators of arterial blood pressure. Although ANG II is the main effector of the system, renin is the key factor that determines the amount of generated ANG II and hence the activity of the RAAS in most mammalian species, including humans. Renin found in circulation is exclusively produced in the juxtaglomerular (JG) cells of the kidneys (10, 32). Renin production in the JG cells is precisely regulated at different checkpoints, starting with the transcription of renin gene, going through the cleavage of the precursor renin molecule, and ending with the processing and storage of renin in secretory granules. The rate of renin gene expression is the first limiting step in the synthesis of renin. Therefore renin gene expression is under the strict control of various factors, including sodium load, blood pressure, sympathetic renal nerve activity, macula densa signal, as well as catecholamines, ANG II, prostaglandins, nitric oxide, and endothelins (36, 37). All these factors interact to determine the actual renin production under both physiological and pathophysiological conditions (29).

During the past decade increasing evidence has been accumulated that inflammatory cytokines also are involved in the regulation of renal renin transcription. Thus it was found that IL-1 and oncostatin M inhibit renin gene expression in the JG-like cell line As4.1 (3, 11, 25). Transient transfections in the mouse adrenocortical tumor cell line Y-1 have shown that human renin promoter is responsive to TNF-α (5). On the other hand, inflammatory cytokines and particularly TNF-α, which is an archetypal representative of the cytokine family, seem also to be engaged in blood pressure homeostasis (7, 15). Thus TNF-α was found to be involved in the control of vascular contraction and proliferation (4, 26). Although blood mononuclear cells represent the main source of TNF-α, it is also reported to be produced in other cell types, including mesangial and proximal tubule cells of kidneys (2, 38). Remarkably, TNF-α is also produced in the medullar thick ascending limb of Henle’s loop (mTALH) under baseline conditions (20). Therefore it would not be unlikely if TNF-α could play an important role in the regulation of renal functions and in the control of renin production in JG cells in particular. To test the hypothesis that TNF-α may be a relevant regulator of renin transcription, we have studied renin gene expression in primary cultures of mouse JG cells and in As4.1 cell line. The As4.1 cells were isolated from mouse kidney after SV40/T-antigen transformation (28). They are believed to derive from JG cells, because they carry the most characteristic feature of native JG cells, namely to produce renin in a regulated manner. As4.1 cells are a well-established model for studying renin transcription (3, 11, 25, 34). We have also studied the effect of genetic knockout of TNF-α on renin gene expression in vivo.

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MATERIALS AND METHODS

The experiments were performed according to the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiology Society (1).

Cell Cultures

As4.1 cells were obtained from the American Type Culture collection (ATCC-CRL-2193). The cells were cultured in Dulbecco’s modified essential media (Biochrom KG) supplemented with 10% fetal calf serum, 1-glutamine and Na-pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C in a humidified atmosphere containing 10% CO₂. At the beginning of the experiments cells were confluent.

Primary cultures of JG cells were established from C57Bl/6 mice. For a cell preparation, two male mice (4–6 wk old) were killed by cervical dislocation. The kidneys were extirpated, decapsulated, and minced with a razorblade at 4°C. The minced tissue was incubated under gentle stirring for 90 min at 37°C in 50 ml buffer I (in mmol/l): 130 NaCl, 5 KCl, 2 CaCl₂, 10 glucose, 20 sucrose, and 10 Tris, pH 7.4 supplemented with 0.25% trypsin (Sigma) and 0.05% collagenase A (Boehringer). Next the tissue was sieved through a 22.4-μm screen. The sieved cells were collected, washed, and resuspended in 4 ml of buffer I. The cell suspension was divided into two tubes each containing 30 ml 30% isosmotic Percoll (Pharmacia) in buffer I. After 30 min of centrifugation at 4°C and 25,000 g, four cellular layers with different specific renin activity were obtained. The cellular layer (density = 1.07 g/ml) with the highest specific renin activity was used for cell culture. These cells were resuspended in 4 ml Dulbecco’s modified essential media (Biochrom KG) supplemented with 2% fetal calf serum, 1-glutamine and Na-pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were aliquoted at 0.5 ml into a 24-well plate and were incubated at 37°C in a humidified atmosphere supplemented with 5% CO₂. Experiments were started 24 h after plating.

Animal Experiments

Male TNF-α /− / mice and their control wild-type strain B6129SF2/J, 4–6 wk old, were purchased from Jackson Laboratory. Three groups of mice each composed of 16 animals (8 wild-type and 8 TNF-α /− / mice), were used for experiments. Animals in group 1 received no treatment and served as controls. Animals in group 2 were kept on low-salt diet (0.02% wt/wt) for 10 days. During the last 3 days of the period, the mice from this group received additionally the angiotensin-converting enzyme (ACE) inhibitor ramipril (10 mg·kg⁻¹·day⁻¹) via the drinking water. Animals in group 3 were fed with high-salt diet (8% wt/wt) for 10 days. At the end of the experiments animals were killed by cervical dislocation and the kidneys were extirpated, decapsulated, and snap-frozen at −80°C until total RNA was isolated.

RNA Isolation

Total RNA was isolated from As4.1 cells and kidneys according to the method of Chomczynski and Sacchi (6). Total RNA from cultured JG cells was isolated from each well separately in 30-μl end volume using Qiagen RNeasy Spin Columns.

RT-PCR

RT-PCR was performed using standard protocols as described (12). Five microliters total RNA extract from primary JG cultures or 1 μg total RNA from As4.1 cells was reverse transcribed in a total volume of 20 μl. The primers used for amplification of specific mouse renin and β-actin cDNA fragments were already described (12). A 289-bp TNF-receptor type I (TNF-RI) cDNA fragment was amplified using forward primer 5’-CGG GAT CCT CTC ACA GGA ATA CTA TG-3’ and reverse primer 5’-GGA ATT CTG TCG ACA GGT CCC AGA AT-3’. A 218-bp TNF-receptor type II (TNF-RII) cDNA fragment was amplified using forward primer 5’-CGG GAT CCT CAC TGG ACT AGT CCC TT-3’ and reverse primer 5’-GGA ATT CAC ACT GCC TGA GGT AAT T-3’. Two (for β-actin, TNF-RI, and TNF-RII fragments) or three (for renin fragment) microliters cDNA were amplified in a total volume of 20 μl. Thirty-five cycles were used for the reactions with specific primers for renin, TNF-RI, and TNF-RII cDNAs, while for the reactions with specific primers for the β-actin sequence, 28 cycles were used. PCR products were separated on a 2% agarose and viewed with ethidium bromide.

RNase Protection Assay

RNase protection assays for mouse renin and β-actin (used as an internal control) were performed as described previously (11, 34).

Transient Transfection

The mouse renin promoter/luciferase constructs were produced by amplifying the 5’-flanking sequence of the mouse renin gene from a commercially available mouse genomic DNA (Clontech) using the expanded long template PCR system (Boehringer Mannheim). Using the primers 5’-GGCTG-CATGTGGTGTACATG-3’ and 5’-GAGACTGAAAGTG-CAAGG-3’, a 4.1-kb fragment (~4071 to +98) of the mouse renin promoter (GenBank accession no. L78789) was generated and inserted in the polynucleotide site of vector pGL3 Enhancer (Promega), which contains a modified coding region of the firefly luciferase. As4.1 cells were split 24 h before transfection in 24-well culture plates with a density of 1 × 10⁵ cells/well. Transfection was performed using 6 μl Fugene 6 transfection reagent (Roche). For each transfection, 1 μg of DNA construct was transfected. To correct luciferase activity to the transfection efficiency, As4.1 cells were cotransfected with 0.01 μg of a plasmid containing an SV40 promoter driving Renilla luciferase (pRL-SV40 Renilla, Promega). The medium was replaced 12 h after transfection, and the cells were incubated with the test substances.

Luciferase activity was measured with the Dual Luciferase Assay Kit from Promega, according to the manufacturer’s instructions. Light production was measured 20 s first for firefly and then for Renilla luciferase activity in a Lumat LB 9507 luminometer (Berthold). The relative luciferase activity was calculated as firefly luciferase-to-Renilla luciferase ratio.

Renin Radioimmunoassay

Active renin was measured using the ANG I radioimmunoassay kit of Sorin Biomedica.

As4.1 cells. As4.1 cells were split in 24-well culture plates with a density of 1 × 10⁵ cells/well. Cells were incubated overnight with 10 ng/ml TNF-α, and then medium was changed and cells were treated with 1 ng/ml TNF-α for 20 h. Control cells were incubated just with standard medium. (Pro)renin activity in supernatants was measured after trypsin activation of prorenin to renin as described (21).

JG cells. Experiments on renin secretion in primary cultures of mouse JG cells were performed throughout 20 h of incubation. Supernatants were then collected and spun at
10,000 g to remove cellular debris. Five-microliter aliquots were taken for the determination of the amount of secreted active renin.

Mice. Wild-type (B6129SF2/J) and TNF-α−/− mice were killed by decapitation to minimize stress-induced variations of the plasma renin content, which could be up to 100-fold. Blood was collected and plasma was obtained according to Ref. 19, and plasma renin activity (PRA) was determined.

RESULTS

As4.1 Cells and Native JG Cells Express Specific Receptors for TNF-α

TNF-α exerts its effects through interacting with two specific receptors on the cellular surface, namely TNF-RI and TNF-RII (17, 18, 30). Using RT-PCR, we checked whether As4.1 cells and JG cells express mRNA for these receptors. TNF-RI and TNF-RII were found in As4.1 and JG cells. Moreover, the expression pattern of the TNF receptors was identical in both cell types, namely the expression of TNF-RII was higher than the expression of TNF-RI (Fig. 1).

TNF-α Inhibits Renin Gene Expression and Renin Promoter Activity in As4.1 Cells

Having found a morphological basis for a possible action of TNF-α on As4.1 cells, we studied the effect of TNF-α on renin gene expression. TNF-α suppressed renin mRNA formation at all concentrations tested, with a maximal inhibition at 100 ng/ml (4-fold) (Fig. 2A). Time chase experiments on the effect of TNF-α on renin gene expression revealed that TNF-α significantly decreased renin mRNA abundance after 8-h incubation. The maximum inhibition was observed between the 16th and the 20th hour of incubation (Fig. 2B). To check whether changes in the transcription rate of renin gene was responsible for the changes in the renin mRNA levels during incubation with TNF-α, we performed transient transfections with a 4.1-kb proximal promoter fragment of the mouse renin gene in As4.1 cells. Renin promoter activity was also effectively suppressed by TNF-α. This suppression was ∼30% after 16 h and almost threefold after 20 h of treatment with TNF-α (Fig. 3), implying that changes in the transcription of the gene rather than posttranscriptional modifications are mainly responsible for the effect of TNF-α on renin mRNA synthesis.

![Fig. 1. TNF-α-specific receptors are expressed in As4.1 and in native juxtaglomerular (JG) cells. RT-PCR was performed as described in MATERIALS AND METHODS with total RNA isolated from JG (lanes 1 and 2) or from As4.1 cells (lanes 3 and 4). Using specific primers, TNF-receptor type I (lanes 1 and 3) or TNF-receptor type II cDNA was amplified (lanes 2 and 4). St, length standard. Data are representative of 3 independent experiments.](http://ajpregu.physiology.org/)

![Fig. 2. Concentration and time dependency of the effect of TNF-α on renin gene expression in As4.1 cells. A: control and TNF-α-treated As4.1 cells were incubated for 16 h. TNF-α was added in increasing concentrations (0.1–100 ng/ml). Twenty micrograms total RNA were analyzed for renin, and 5 μg total RNA were analyzed for β-actin by RNase protection assay. B: time course of the effect of TNF-α on renin gene expression. Control and TNF-α (10 ng/ml)-treated As4.1 cells were incubated for up to 20 h. Twenty micrograms total RNA were analyzed for renin, and 5 μg total RNA were analyzed for β-actin by RNase protection assay. Data are means ± SE of 3 experiments. *P < 0.05.](http://ajpregu.physiology.org/)

![Fig. 3. Renin promoter activity. As4.1 cells were transiently transfected with the proximal 4.1 kb of the mouse renin promoter. TNF-α was applied in concentration of 10 ng/ml. Cells were incubated for 16 or 20 h, and relative luciferase activity was estimated. Data are means ± SE of 4 experiments. *P < 0.05.](http://ajpregu.physiology.org/)
Under such experimental conditions, TNF-α lead to a decrease in renin mRNA abundance also in native JG cells as shown by RT-PCR (Fig. 4A). Densitometric measurements of amplified renin cDNA bands showed that forskolin stimulated renin gene expression ~3-fold and TNF-α attenuated the forskolin-stimulated renin transcription by >30% (Fig. 4B).

Effect of Genetic Knockout of TNF-α on Renin Gene Expression In Vivo

We searched for evidence if TNF-α could inhibit renin transcription in vivo, too (Fig. 5). Because the known pharmacological inhibitors of TNF-α production belong to the group of phosphodiesterase blockers, which modify the synthesis of per se, and because prolonged treatments of mice with TNF-α or with neutralizing antibodies against TNF-α cannot yet be conducted, we characterized renin gene expression in mice lacking TNF-α (TNF-α −/− mice). These genetically knockout mice turned out to have almost threefold higher basal expression of renin mRNA compared with their corresponding wild-type strain. When stimulated with low-salt diet plus ACE inhibitor (ramipril), renin gene expression increased with a factor of 10 in both wild-type and TNF-α knockout mice. Renin gene transcription was twofold inhibited both in wild-type mice and in TNF-α −/− mice by a high-salt diet. Thus TNF-α may be a negative modulator of renin expression in vivo, without being involved in the physiological control of renin expression by salt intake.

As4.1 cells. As4.1 cells are known to secrete renin constitutively, rather than through a regulated pathway (16). As >90% of the renin secreted is in the form of inactive prorenin (14), we have converted prorenin to active renin by trypsin incubation. Due to the slow decline of renin mRNA in response to TNF-α, we have pretreated As4.1 cells with TNF-α overnight. Then the medium was changed and the cells were treated with TNF-α for another 20 h, during which prorenin secretion was determined. We found that TNF-α suppressed the synthesis of (pro)renin in As4.1 cells down to 10% of the basal level (Fig. 6A).

JG cells in vitro and in vivo. In primary cultures of mouse JG cells, renin secretion reflects the regulated exocytosis of stored active renin, rather than the de novo synthesis rate of (pro)renin, which is very low in the cultured native JG cells. As shown in Fig. 6B, TNF-α did not significantly change renin release from primary cultures of mouse JG cells. Finally, we also determined PRA as an indirect measure for renin secretion in TNF-α −/− mice and their wild-type controls. The PRA values in both strains displayed a rather broad scatter, which did not allow us to detect a possible minor difference between the two strains (Fig. 6C).

**DISCUSSION**

TNF-α is a cytokine known predominantly for its role in immune responses (35). However, TNF-α seems also to have important functions for the cardiovascular system. Changes in blood pressure could change TNF-α production, and conversely TNF-α could induce changes in arterial blood pressure. TNF-α mRNA and protein are markedly increased after hypertensive stress, and also TNF-α secretion from blood monocytes...
is increased in patients with essential hypertension (7, 15). On the other hand, TNF-α is a well-established stimulator of the expression of inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells (VSMCs), and hence of NO production, leading to a vasodilatation and fall in blood pressure (24, 33). The synthesis of ceramide, which is one of the second messengers in TNF-α signaling, was impaired in VSMCs of spontaneously hypertensive rats (13). TNF-α is also a relevant depressant of cardiac function (22). However, much less is known about the action of TNF-α on the systematic regulators of blood pressure. Therefore, we have studied the effect of TNF-α on renal renin gene expression. Renin, produced in the JG cells of the kidneys, is the limiting factor that sets the activity of the RAAS system, which in turn plays a pivotal role in

In summary, in this study we provide evidence that TNF-α is a potent and specific downregulator of renin gene expression in normal and immortalized JG cells in vitro, acting through inhibition of the renin promoter. Because TNF-α is a mediator, which is produced in vivo and in vitro, the RAAS system, which in turn plays a pivotal role in the regulation of blood pressure. We found that TNF-α inhibits renin transcription via suppression of renin promoter activity in the transformed JG-cell line As4.1. Downregulation of renin gene expression by inflammatory cytokines (IL-1, oncostatin M) in the juxtaglomerular cell line As4.1 was already reported by others and ourselves (11, 25). However, it was also found in this context that IL-1 had no effect on renin gene expression in native JG cells in culture (11), consistent with the fact that there were no IL-1-specific receptors identified on JG cells (31). These findings raised the possibility that the downregulation of renin transcription by inflammatory cytokines in As4.1 cells may be an “artefact” of their transfection with the SV40/T-antigen and the subsequent immortalization.

In the present study we show that TNF-α inhibits renin gene expression not only in As4.1 cells but also in cultured native JG cells and that both As4.1 cells and native JG cells express TNF-RI and TNF-RII, which are the specific receptors for TNF-α. These results suggest that TNF-α can in principle directly affect renal renin production via inhibition of renin gene transcription, indicating a novel pathway in the cellular control of renin gene expression.

Our observation that renal renin mRNA levels were substantially increased in TNF-α −/− mice would fit with the concept of an inhibitory effect of TNF-α on renin gene expression also in vivo. We are aware, however, that the data obtained with TNF-α −/− mice cannot prove a regulatory function of TNF-α for renin synthesis. The physiological impact of TNF-α in the control of renin synthesis and the situations in which it becomes active remain therefore to be clarified in future studies.

The findings that TNF-α inhibited prorenin synthesis in As4.1 cells, but did not affect regulated exocytosis of active renin from native JG cells, suggest that TNF-α acts predominantly on the slow regulation of (pro)renin synthesis rather than on the rapid regulation of exocytosis of active renin. The quite normal PRAs found in TNF-α −/− mice would fit with this conclusion. It should be mentioned in this context, however, that PRA in mice is considered not as an ideal indicator of renal renin secretion in vivo as in other species.

The possible interactions between TNF-α and the RAAS are not restricted just to renin. TNF-α was reported to inhibit ACE activity and expression in endothelial cells (23, 27). TNF-α production is significantly increased by ANG II in mTALH in vitro and also in mTALHs of ANG II-dependent hypertensive rats (8, 9). Thus downregulation of renin gene transcription by TNF-α could be an important mechanism in the compensatory activities of the organism against elevated blood pressure, and also it could contribute to a negative-feedback loop in the RAAS.

Fig. 6. Renin release in vivo and in vitro. A: renin release from As4.1 cells. Data are means ± SE; n = 4. *P < 0.05. B: renin release from mouse native JG cells in culture. JG cells remained untreated (control) or were incubated with 10 μM forskolin or with 10 μM forskolin plus 100 ng/ml TNF-α for 20 h; n = 4. *P < 0.05 relative to control. C: plasma renin activity in mice in vivo; n = 5. WT, wild-type.
principally under pathological conditions, the significance of the novel TNF-α effect on renin gene expression during physiological but also under pathophysiological conditions in vivo remains to be elucidated in further experiments.

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