Systemic Infusion of Angiotensin II into Normal Rats Activates Nuclear Factor- κ B and AP-1 in the Kidney

Role of AT_1 and AT_2 Receptors

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Recent studies have pointed out the implication of angiotensin II (Ang II) in various pathological settings. However, the molecular mechanisms and the AngII receptor (AT) subtypes involved are not fully identified. We investigated whether AngII elicited the *in vivo* activation of nuclear transcription factors that play important roles in the pathogenesis of renal and vascular injury. Systemic infusion of Ang II into normal rats increased renal nuclear factor (NF)- κ B and AP-1 binding activity that was associated with inflammatory cell infiltration and tubular damage. Interestingly, infiltrating cells presented activated NF- κ B complexes, suggesting the involvement of AngII in inflammatory cell activation. When rats were treated with AT_1 or AT_2 receptor antagonists different responses were observed. The AT_1 antagonist diminished NF- κ B activity in glomerular and tubular cells and abolished AP-1 in renal cells, improved tubular damage and normalized the arterial blood pressure. The AT_2 antagonist diminished mononuclear cell infiltration and NF- κ B activity in glomerular and inflammatory cells, without any effect on AP-1 and blood pressure. These data suggest that AT_1 mainly mediates tubular injury via AP-1/NF- κ B, whereas AT_2 receptor participates in the inflammatory cell infiltration in the kidney by NF- κ B. Our results provide novel information on AngII receptor signaling and support the recent view of Ang II as a proinflammatory modulator. (Am J Pathol 2001, 158:1743–1756)

Angiotensin II (AngII), the main effector peptide of the renin-angiotensin system (RAS), plays a central role in the pathophysiology of cardiovascular and renal diseases and in the etiology of hypertension in humans. This vasoactive peptide is now considered to be a growth factor that participates in the regulation of cell growth and gene expression of various bioactive substances (ie, extracellular matrix components, growth factors, cytokines,

chemokines).^{1–4} Some studies have investigated the *in vivo* effects of systemic AngII infusion in the kidney, showing proliferation of renal cells, tubular atrophy, accumulation of extracellular matrix proteins (fibronectin and collagens),^{5–7} and induction of growth factors, such as transforming growth factor- β (TGF- β).⁸ Another feature of AngII-induced kidney damage is the presence of infiltrating inflammatory cells.^{5,9} However, the molecular mechanisms of AngII action in this setting still remain unclear.

Transcription factors are important mediators involved in signal transduction that bind to specific DNA sequences in gene promoters, and regulate transcriptional activity. In cultured cells, AngII activates various nuclear transcription factors, including the activator protein-1 (AP-1),¹⁰ STAT family of transcription factors,¹¹ cyclic adenosine monophosphate response element binding protein¹² and, as we have previously shown, nuclear factor- κ B (NF- κ B).^{3,13} Emerging attention has been focused on the regulation and function of transcription factors, such as NF- κ B and AP-1 during tissue injury.^{14,15} NF- κ B has special interest because it plays a pivotal role in the control of several genes, including cytokines, chemokines, adhesion molecules, NO synthase, and angiotensinogen, involved in the pathogenesis of inflammatory lesions, kidney damage, and hypertension.¹⁴ In several models of renal damage, an elevated tissular NF- κ B DNA binding activity that diminished in response to angiotensin-converting enzyme (ACE) inhibition has been found.^{3,16} In other pathological conditions associated with activated RAS, such as atherosclerosis, the increased tissular NF- κ B activity was also found to decrease by ACE inhibition.¹³ Double-transgenic rats overexpressing both renin and angiotensinogen genes exhibited increased NF- κ B activity in the heart and kidney. In these animals, the antioxidant pyrrolidine dithio-

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carbamate inhibits NF- κ B, ameliorates inflammation, and protects against AngII-induced end-organ damage.¹⁷ However, the *in vivo* effect of AngII on NF- κ B activation, and the potential receptor subtype involved, have not been elucidated.

Two pharmacologically distinct subclasses of AngII receptors (AT₁ and AT₂) have been described.^{18,19} The well-known AngII actions, such as the regulation of blood pressure and water-electrolyte balance, and growth-promoting effects, have been attributed mainly to the activation of various signal-transduction pathways via AT₁.^{18,19} AT₁ antagonists are currently used to treat patients with hypertension or heart failure. Treatment with AT₁ antagonists causes elevation of plasma AngII, which selectively binds to AT₂ and theoretically could exert clinically important, but yet undefined, effects.²⁰ The biological functions and the signal transduction pathway of AT₂ are primarily unknown. AT₂ regulates cell growth inhibition, blood pressure, diuresis/natriuresis, renal NO production and glomerular monocyte infiltration.^{9,21,22} The AT₂ mRNA is highly expressed in the fetal kidney, in lower levels in the adult, and is re-expressed in pathological situations involving tissue remodeling or inflammation, such as neointima formation, heart failure, and wound healing.^{21,23,24} Renal AT₂ may be activated during sodium depletion or AngII administration in the rat.^{21,25} Therefore, understanding of AT₂-mediated physiopathological actions may have important pharmacological implications.

To elucidate the molecular mechanisms implicated in the AngII-induced kidney damage we have investigated the renal activity of the transcription factors NF- κ B and AP-1, related to the pathological effects caused by systemic infusion of AngII, such as inflammatory cell infiltration and tubular damage. We have also determined the receptor subtype associated with these effects by using the specific receptor antagonists, losartan for AT₁ and PD123319 for AT₂.

Materials and Methods

Experimental Design

The *in vivo* effect of AngII was evaluated by systemic infusion of AngII (dissolved in saline) into female Wistar rats (subcutaneously by osmotic minipumps; Alza Corp., Palo Alto, CA), at the dose of 50 ng/kg/minute. Animals were sacrificed at 24, 48, and 72 hours (acute study), and at 7 days (chronic study). Then, tissue samples were immediately removed and further processed for histological studies and protein extraction. To determine the role of AngII receptor, a group of rats was treated with the AT₁ antagonist losartan (10 mg/kg/day in the drinking water) or the AT₂ antagonist PD123319 (30 mg/kg/day, subcutaneously by osmotic minipumps) from 24 hours before AngII infusion, and after an additional 72 hours. Losartan was kindly provided by MSD (Spain), and PD123319 was from Sigma (St. Louis, MO). The doses of losartan and PD123319 have previously demonstrated to cause an effective blockade of AT₁ and AT₂, respectively.^{26–29}

Control groups of animals of the same age, untreated or treated (AT antagonists and saline-infused), were also studied. Systolic arterial blood pressure was measured in conscious, restrained rats by the tail-cuff sphygmomanometer (NARCO Biosystems, CO). The blood pressure value for each rat was calculated as the average of three separate measurements at each session.

Cell Cultures

The murine tubuleepithelial cells (MCT cell line) were kindly donated by Dr. E. Neilson (University of Pennsylvania). The human monocytic cell line U937 (1593-CRL) was obtained from the American Type Culture Collection (Rockville, MD). U937 cells were grown in suspension and 5×10^6 cells were used in each experiment. These cell lines were cultured in RPMI medium with 10% fetal calf serum (FCS) (Gibco BRL, Paisley, Scotland, UK).

Analysis of Transcription Factor Activity

NF- κ B and AP-1 activity was evaluated by binding of 10 μ g of protein extracts from renal cortex or cells with an oligoconsensus NF- κ B or AP-1 labeled with [γ -³²P]-ATP and the complexes formed were analyzed by electrophoretic mobility shift assay (EMSA). Protein extraction were done as described.³ Growth-arrested MCT were incubated with AngII (10^{-7} , 10^{-9} and 10^{-11} mol/L) for 30, 60, and 120 minutes. As positive controls, 100 U/mL of tumor necrosis factor- α and 10^{-7} mol/L phorbol myristate acetate were used. For *in vivo* studies, frozen kidney pieces were pulverized in a metallic chamber and resuspended in a cold extraction buffer [20 mmol/L HEPES-NaOH (pH 7.6), 20% (vol/vol) glycerol, 0.35 mol/L NaCl, 5 mmol/L MgCl₂, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L dithiothreitol, 0.5 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 1 mg/L pepstatin A]. The homogenate was vigorously shaken for 30 minutes, and the insoluble materials precipitated by centrifugation at $40,000 \times g$ for 30 minutes at 4°C. For *in vitro* studies, cells were resuspended in extraction buffer (10 mmol/L HEPES, pH 7.8, 15 mmol/L KCl, 2 mmol/L MgCl₂, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1 mmol/L PMSF) and homogenized. Nuclei and cytosolic fractions were separated by centrifugation at $1000 \times g$ for 10 minutes, the nuclei were resuspended in extraction buffer to a final concentration of 0.39 mol/L KCl, and centrifuged at $100,000 \times g$ for 30 minutes. Supernatants dialyzed overnight against a binding buffer containing 20 mmol/L HEPES-NaOH (pH 7.6), 20% (v/v) glycerol, 0.1 mmol/L NaCl, 5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 0.5 mmol/L PMSF. The dialysates were cleared by centrifugation at $10,000 \times g$ for 15 minutes at 4°C and frozen at -80°C . Protein concentration was quantified by the bicinchoninic acid method (Pierce, Rockford, IL).

NF- κ B and AP-1 consensus oligonucleotides (5'-AGT-TGAGGGGACTTTCCAGGC-3' and 5'-CGCTTGAT-GAGTCAGCCGGA-3', respectively) were end-labeled with [γ -³²P]ATP (Amersham, Buckinghamshire, UK) and

T4 polynucleotide kinase (Promega). Nuclear extracts were equilibrated for 10 minutes in a binding buffer [4% glycerol, 1 mmol/L MgCl₂, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 50 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5, and 50 μ g/ml of poly(dI-dC)] (Pharmacia LKB, Uppsala, Sweden), then the labeled probe (0.35 pmol) was added and incubated for 20 minutes at room temperature. Negative controls without cellular extracts, and competition assays with a 100-fold excess of unlabeled NF- κ B, mutant NF- κ B, and AP-1 oligonucleotides, were performed to establish the specificity of the reaction. When competition assays were done, the unlabeled probe was added to this buffer 10 minutes before the addition of the labeled probe. HeLa cell nuclear extracts were used as a positive control (not shown). The antibodies to NF- κ B proteins were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Chemikon (Temecula, CA). For supershift assays, 1 μ g of anti-p50, anti-p65, or anti-c-Rel antibodies was added to protein extracts from cortex of AngII-infused rats, and incubated for 1 hour before the addition of the labeled probe. Supershift assays were also done with HeLa cell nuclear extracts. The supershift band was competed by a 100-excess of unlabeled specific (NF- κ B), but not by mutant NF- κ B probe (not shown). The specificity of the antibodies was confirmed by Western blot (not shown). Oligonucleotides were from Promega Corp. (Madison, WI). The reaction was stopped by adding gel-loading buffer (250 mmol/L Tris-HCl, 0.2% bromophenol blue, 0.2% xylene cyanol, and 40% glycerol) and protein-DNA complexes were separated on a nondenaturing, 4% acrylamide gel in Tris-borate. The gel was dried and exposed to X-ray film.

Determination of mRNA Expression of AT₂

In kidney samples and in 48-hour serum-deprived MCT cells, AT₂ mRNA expression was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR),²³ with specific primers 5'-CTGACCCTGAACATGTTTGCA-3' (sense) and 5'-GGTGTCCATTCTCTAAGAG-3' (antisense), yielding a 710-bp product. PCR amplification (1 minute at 58°C, 1 minute at 68°C, and 1 minute at 94°C) was linear up to 50 cycles, and data corresponding to cycle 35 were used for calculations. Control experiments were done with RNA samples, but without avian myeloblastosis virus reverse transcriptase. The DNA products were analyzed on 1.5% agarose gel and ethidium bromide staining.

Western Blot of AngII Receptors

Total proteins were obtained from homogenized cells in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% Triton X-100, 0.3% Nonidet P-40, 0.1 mmol/L PMSF, and 1 μ g/ml pepstatin A) and then separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, samples were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked in 0.01 mmol/L

Tris, pH 7.5, and 0.4 mol/L NaCl containing 0.1% Tween-20, 1% bovine serum albumin (BSA) and 5% dry skimmed milk for 30 minutes at 37°C, and then incubated in the same buffer with a specific AT₂ or AT₁ antibody for 18 hours at 4°C. After washing, detection was made by incubation with peroxidase-conjugated secondary antibody, and developed using an ECL chemiluminescence kit (Amersham). Samples from rat adrenal gland were used as positive control for AT₂ expression (not shown). The specificity of AT₁ and AT₂ antibodies was also determined by incubation with blocking peptides (not shown). The antibodies to AT₁ and AT₂ were from Santa Cruz, secondary horseradish peroxidase-conjugated antibodies were from The Binding Site (Birmingham, UK), and control rabbit IgG from Sigma.

Renal Histopathological Studies

The kidney samples were studied by staining with hematoxylin/eosin and Masson's trichrome technique, and examined by light microscopy. The inflammatory cell infiltration was evaluated by immunohistochemistry in formalin-fixed paraffin-embedded sections with an anti-rat CD43 antibody (Pharmingen). This monoclonal antibody reacts with an epitope of CD43 expressed in monocytes, macrophages, natural killer cells, and T cells. A specific monocyte/macrophage marker, an anti-rat ED1 antibody (Serotec) was also used. The presence of AT₂ in renal tissue was determined by immunohistochemistry with a specific goat anti-AT₂ antibody recognizing rat and human AT₂ (Santa Cruz; tested by Western blot). To localize DNA-binding activity of transcription factors Southwestern histochemistry was used.³⁰ The quantification of the infiltrating cells was performed as previously reported.³¹ The mean number of cells per glomerular cross-section was determined by evaluating 15 glomeruli and the whole interstitium from each animal. Semiquantitative determination was done for morphology, immunohistochemistry, and Southwestern, using the following score: 0, no staining; 1+, mild staining; 2+, moderate staining; 3+, marked staining. Histological studies were quantified by two independent observers in a blinded manner and the mean value was calculated for each rat.

Tissue Localization of CD43, ED-1, and AT₂ Immunoreactivity

Paraffin-embedded renal tissue sections (4 μ m) were mounted on poly-L-lysine-coated slides. The slides were deparaffinized with xylene and graded concentrations of ethanol and then rehydrated. The endogenous peroxidase was blocked by incubating in 3% H₂O₂/methanol (1:1) at 25°C for 30 minutes. The slides were subsequently incubated in phosphate-buffered saline (PBS) with 6% horse serum in 4% BSA for 1 hour at 37°C to reduce nonspecific background staining, and then incubated overnight at 4°C with anti-CD43, anti-ED1, or anti-AT₂ antibodies in PBS containing 1% serum and 4% BSA. After being washed with PBS, the sections were incubated with secondary anti-IgG HRP-conjugated anti-

Figure 1. Glomerular and interstitial morphology after systemic infusion of AngII. Animals were pretreated for 24 hours with the AT antagonists losartan (AT₁, 10 mg/kg/day in drinking water) and PD123319 (AT₂, 30 mg/kg/day, s.c.), and then infused with AngII for 72 hours (50 ng/kg/minute). Figure shows representative photomicrographs of the kidney from a rat infused with AngII for 72 hours alone (**A** and **B**) or treated with losartan (**C**) or PD123319 (**D**). Original magnifications: $\times 100$ (**A**, **C**, and **D**), $\times 200$ (**B**).

body diluted 1:100 in 4% BSA/PBS for 30 minutes, and after washing, they were stained with 0.05% 3,3'-diaminobenzidine (DAKO, Glostrup, Denmark) in 0.3% H₂O₂ for 10 minutes. The sections were counterstained with Mayer's hematoxylin and mounted in Pertex (Mediate). In each experiment, negative controls without the primary antibody, or using an unrelated antibody, were included to check for nonspecific staining.

Southwestern Histochemistry

Sections of renal tissue were dehydrated, rehydrated, fixed with 0.5% paraformaldehyde and treated with 5 mmol/L levamisole for 30 minutes. Then, sections were digested with 0.5% pepsin in 1 N HCl for 30 minutes at 37°C, washed twice with buffer HEPES-BSA (10 mmol/L HEPES, 40 mmol/L NaCl, 10 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 0.25% BSA), and treated with 0.1 mg/ml DNase I for 20 minutes. After that, samples were incubated overnight at 37°C with the NF- κ B and AP-1 oligonucleotide digoxigenin-labeled at a final concentration of 100 pmol/ml in HEPES-BSA with 0.5 μ g/ml of poly (dl-dC). After washing, samples were incubated with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany) for 1 hour at 37°C and were revealed with 0.4% 4-nitroblue tetrazolium chloride and 0.32% X-phosphate. The reaction was stopped with 10 mmol/L Tris, pH 8, 1 mmol/L EDTA. Preparations were mounted with glycerol. Synthetic consensus sense (NF- κ B: 5'-AGTTGAGGG-GACTTTCCAGGC-3'; AP-1: 5'-CGCTTGATGAGTCAG-CCGGAA-3'), and mutant sense (NF- κ B: 5'-AGTTAG-CGCTCCTTTCCAGGC-3'; AP-1: 5'-CGCTTGATAAAT-CAGCCGGAA-3') probes (Genosys Biotechnology, London, UK). Double-stranded DNA probes were labeled with digoxigenin (DIG oligonucleotide 3' end labeling, Boehringer). Preparations without probe were used as

negative controls, and mutant probes were used to test the specificity of the technique.

Statistical Analysis

Results are expressed as the mean $\pm$ SEM. Significance was established using *t*-test and analysis of variance when appropriate. Differences were considered significant if $P < 0.05$.

Results

Role of AT₁ and AT₂ Receptors in AngII-Induced Renal Damage

Systemic infusion of AngII (50 ng/kg/min, subcutaneously by osmotic minipumps) caused a slight increase in systolic blood pressure after 72 hours (121 ± 9 versus 100 ± 2 mmHg in controls, $n = 10$, $P < 0.05$), but within the normotensive range. Morphological lesions were examined by light microscopy. After 72 hours of AngII infusion, the majority of the glomeruli had a normal appearance. Interstitial inflammatory cell infiltration and mild tubular lesions were observed (Figure 1). The inflammatory cell infiltration was further examined by immunohistochemistry with a specific anti-CD43 antibody (Figure 2). In control animals, only few cells with positive anti-CD43 immunostaining were observed (Figure 2; A, C, E, and I). AngII-infused animals had some inflammatory cells in the glomeruli (Figure 2; B, F, and M). An important increase in the mean number of inflammatory cells expressing CD43 antigen was observed in the interstitium, distributed in a focal manner (Figure 2; D, J, and M). Some of these cells were monocytes/macrophages, as determined with an anti-rat ED1 antibody (data not shown). Our findings are similar to that observed with low

Figure 2. AngII infusion increases inflammatory cell infiltration in the kidney. Figure shows glomerular (A and B) and tubulointerstitial (C and D) areas of a representative animal of control (A and C) and AngII-infused (B and D) groups. Original magnification, $\times 1000$. Figure shows a representative animal of groups: control (E and I), infused with AngII for 72 hours (F and J), and treated with the AT₁ antagonist losartan (G and K) or the AT₂ antagonist PD123319 (H and L). Original magnifications: $\times 1000$ (A–D), $\times 200$ (E–H), $\times 100$ (I–M). Quantification of CD43+ cells (infiltrating inflammatory cells) in glomeruli and interstitium. Results are expressed as means $\pm$ SEM of positive cells per glomeruli or field of 6 to 10 animals from each group. *, $P < 0.05$ versus control; #, $P < 0.05$ versus AngII.

doses of AngII infusion, that also showed a slight increase in blood pressure, glomerular monocyte infiltration, and fibrosis.^{6,9} The animals treated with AngII (50 ng/kg/minute) for 7 days showed marked tubular injury (data not shown). In this sense, AngII infusion for 14 days, although at higher doses (200 ng/kg/min), resulted in moderate systolic hypertension associated with cell proliferation, fibrosis, and tubulointerstitial damage.^{5,6}

In 72-hour AngII-infused rats, losartan administration (AT₁ antagonist; 10 mg/kg/day) abolished the increase in blood pressure induced by AngII (99 ± 2 mmHg, $n = 6$, $P < 0.05$ versus AngII). AngII-infused rats that received a simultaneous infusion of PD123319 (AT₂ antagonist; 30 mg/kg/day) showed a rise in blood pressure similar to that of rats receiving only AngII (123 ± 16 mmHg, $n = 4$; $P = \text{ns}$ versus AngII; $P < 0.05$ versus controls). Losartan or PD123319 alone had no effect on blood pressure (not shown). Some differences were observed between the effect of AT₁ and AT₂ antagonists in AngII-induced renal

lesions. The AT₁ antagonist ameliorated tubular atrophy (Figure 1C), whereas the AT₂ antagonist diminished inflammatory cell infiltration (Figure 1D), presenting a marked reduction in CD43-positive cells both in glomerular and interstitial areas (Figure 2, H, L, and M). PD123319 also diminished glomerular ED-1-positive cells (monocytes/macrophages) (data not shown), as previously described.⁹

AngII Infusion Activates NF- κ B in the Kidney

AngII infusion increased renal NF- κ B DNA-binding activity, beginning at 24 hours, peaking at 72 hours (6.5-fold versus control, $n = 10$, $P < 0.05$), and declining at 7 days, but without reaching control values (3.7-fold, $n = 8$, $P < 0.05$ versus control) (Figure 3). Renal NF- κ B activity in saline-infused rats showed no difference versus that in healthy control rats ($n = 6$, $P = \text{ns}$, Figure 3A). The signal

Figure 3. Results of electrophoretic mobility shift assay for NF-κB activity in the kidney after systemic infusion of AngII. Rats were treated with AngII (50 ng/kg/minute) for 24, 48, and 72 hours, and 7 days. Protein extracts were collected and used for gel shift assays with the NF-κB probe. **A:** Representative EMSA experiment that shows two different animals from each group. Specificity of the reaction was demonstrated with a 100-fold excess of unlabeled NF-κB consensus sequence (specific competitor), mutant NF-κB, or unrelated oligonucleotide (AP-1). **B:** Densitometric analysis of the results expressed as arbitrary units of means $\pm$ SEM of 6 to 10 animals of each group. *, $P < 0.05$ versus control. **C:** Identification of NF-κB subunits in AngII-infused rats. The protein extracts of 72-hour AngII-infused rats were incubated in the presence of antibodies against the NF-κB subunits p50, p65, and c-rel. A supershifted band was observed with anti-p50 and anti-p65 antibodies. **D:** Detail of a longer exposition of the experiment, supershifted bands are marked by arrows. The position of the NF-κB complexes and free oligonucleotide is indicated. Figure shows a representative EMSA of three experiments.

of the retarded bands was only decreased with an excess of unlabeled NF-κB, but not mutant NF-κB or AP-1 (unrelated nuclear protein binding), showing the specificity of the binding (Figure 3B). To characterize the nuclear protein binding to the NF-κB motif, protein extracts from AngII-infused rats were preincubated with antibodies against the NF-κB subunits, p50, p65, and c-rel.¹⁴ In the presence of anti-p50 and anti-p65 antibodies a supershifted band appeared, whereas there was no changes in the intensity of the complexes (Figure 3, C and D). None of the complexes were inhibited by the antibody against c-Rel (Figure 3, C and D), thus this subunit does not seem to be present in AngII-induced NF-κB complexes, as observed in mesangial cells.³ These results suggest that in AngII-infused rats the activated NF-κB complexes contain p50 and p65 subunits.

We determined the renal cells involved in AngII-induced NF-κB activation *in vivo* by a digoxigenin-labeled NF-κB oligonucleotide that binds to nuclear active NF-κB complexes.³⁰ Control rats showed no nuclear staining for NF-κB (Figure 4; A, B, and C). In AngII-infused rats, there was an increase in positive nuclear staining, with activated NF-κB complexes located mainly in the glomeruli (mesangial, endothelial, and epithelial cells; Figure 4, D and E), and in tubulointerstitial areas (tubuloepithelial and mononuclear infiltrating cells; Figure 4F). Moreover, there was an increase in NF-κB staining in renal arteries, located in endothelial and vascular smooth muscle cells (Figure 4, H and I). The semiquantitative score of NF-κB staining of the different groups is shown in Figure 4J. Competition of binding by an excess of 100-fold unlabeled NF-κB oligonucleotide, incubation with a mutant digoxigenin-labeled NF-κB (100 pmol/L), or the absence of digoxigenin-labeled NF-κB, did not show any nuclear signal, (Figure 5) demonstrating the specificity of the binding. These controls were done in each experiment.

Treatment with AT₁ and AT₂ Antagonists Diminished NF-κB Activation in AngII-Infused Animals

We determined the effect of AT₁ and AT₂ antagonists in renal NF-κB DNA binding activity, in rats infused with AngII for 72 hours, by EMSA and Southwestern histochemistry. Both treatments partially diminished renal NF-κB activity in AngII-infused rats [losartan, 44% (inhibition versus AngII-infusion, $n = 6$, $P < 0.05$); and PD123319, 53% ($n = 4$, $P < 0.05$; EMSA)] (Figure 6). Neither losartan nor PD123319 alone affected renal NF-κB activity (not shown). By Southwestern histochemistry we observed that both treatments diminished nuclear NF-κB staining in the glomeruli (Figure 7, C and D). The effect of the AT₁ blocker was mainly observed in tubuloepithelial cells (Figure 7G), whereas AT₂ antagonist presented a marked effect on infiltrating cells (Figure 7H). The semiquantitative score of NF-κB staining in the glomeruli, infiltrating and tubular cells of the different groups is shown in Figure 7I.

Figure 4. Southwestern histochemistry in AngII-infused rats. Control animals did not present staining for NF- κ B (A–C). After AngII infusion for 72 hours a clear nuclear staining for NF- κ B was seen in some renal structures (D–F). There was a marked increase in the glomeruli, located in mesangial, epithelial, and endothelial cells (D and E). In the tubulointerstitial area there was an intense NF- κ B staining in infiltrating cells, and in some tubuloe epithelial cells (F). In renal arteries of control rats there was no NF- κ B signal (G), whereas after AngII infusion there was increased nuclear NF- κ B staining, located in endothelial and vascular smooth muscle cells (H and I). Original magnifications: $\times 1000$ (B, C, E–H, and J), $\times 200$ (A and D), and $\times 2000$ (I). **J:** Semiquantitative score of NF- κ B staining, scored as –, no staining; 1+, mild; 2+, moderate; 3+, intense. Results are expressed as means $\pm$ SEM of 6 to 10 animals per group. *, $P < 0.05$ versus control.

AngII Infusion Activates AP-1 Transcription Factor through AT₁ in the Kidney

In renal cortex from AngII-infused rats there was an increase in AP-1 DNA-binding activity at 72 hours (2.6-fold versus control, $n = 8$, $P < 0.05$) decreasing after 7 days (Figure 8, A and B). By Southwestern histochemistry we observed that AngII-infused rats for 72 hours presented positive nuclear staining for AP-1 in glomeruli and tubulointerstitial areas (Figure 8C). The specificity of the binding was evaluated by competition experiments (not shown). Treatment with AT₁ antagonist blocked the AngII effect on renal AP-1 binding activity (90% inhibition versus AngII-Infusion, $n = 4$, $P < 0.05$, EMSA), whereas AT₂ antagonist did not have any significant effect (Figure 9).

Localization of AT₂ in the Kidney

Many investigators have demonstrated the presence of AT₁ in the adult kidney and in cultured renal cells.^{1,21,32} However, there is still some controversial data about renal AT₂.^{9,21,24,33,34} For this reason, we investigated the

presence of AT₂ in the kidney, and in cultured renal cells by immunohistochemistry and Western blot (protein levels) and RT-PCR (gene expression). In renal sections of control rats, there was a positive immunostaining for AT₂ located mainly in tubuloe epithelial cells and, to a lesser extent, in resident glomerular cells (Figure 10A, a and b). Moreover, in rats with immune complex nephritis, which presented an activation of the local RAS,³¹ a strong glomerular staining with the anti-AT₂ antibody was observed (Figure 10A, c). Sections were incubated in the absence of primary antibody, as a negative control of the technique (Figure 10A, d). When total RNA from renal cortex was analyzed by RT-PCR, a band corresponding to the AT₂ mRNA was observed (Figure 10B). AT₂ was also detected in cultured mesangial cells from normotensive Wistar rats, but not from hypertensive rats,³⁴ in rat glomerular endothelial cells⁹ and rabbit tubuloe epithelial cells.³³ Murine tubuloe epithelial cells (MCT) were incubated for 48 hours in RPMI without serum. Total proteins were analyzed by Western blot and a band of 44 kd of apparent molecular weight corresponding to the predicted size was detected (Figure 10C). These cells also expressed AT₂ mRNA (Figure 10B). All these data are in agreement with previous studies showing detectable levels of AT₂ in the kidney in normal and pathological conditions.

AngII Activates NF- κ B in Cultured Renal Cells

We performed *in vitro* studies to further demonstrate that the *in vivo* AngII effect on NF- κ B could be because of a direct, nonhemodynamic, action. We have previously demonstrated that AngII activates NF- κ B in cultured glomerular mesangial cells.³ However, whether AngII regulated NF- κ B activity in tubuloe epithelial cells has not been evaluated. For this purpose, we used MCT cells that possess AT₁² and AT₂ (Figure 10).

In growth-arrested MCT AngII augmented NF- κ B DNA-binding activity, being maximal with 10^{-9} mol/L after 30 minutes (3.1-fold versus control, $n = 7$, $P < 0.05$) and declining at 1 hour (Figure 11B). AngII also increased AP-1 DNA binding in MCT (Figure 11B), showing a similar response to that in mesangial cells.³² To investigate the receptor subtype involved in AngII-induced NF- κ B and AP-1 activation growth-arrested MCT were preincubated for 30 minutes with losartan or PD123319 (range 10^{-5} to 10^{-7} mol/L), and then stimulated with 10^{-9} mol/L AngII for an additional 30 minutes. The AT₁ antagonist diminished in a dose-dependent manner the AngII-induced NF- κ B binding activity, with a maximal inhibitory effect at 10^{-5} mol/L (79% versus AngII alone, $n = 6$, $P < 0.05$) (Figure 11A), whereas the AT₂ antagonist only caused a slight diminution at 10^{-5} mol/L (35% versus AngII alone, $n = 6$) (Figure 11A). By contrast, only losartan blocked AngII-induced AP-1 binding activity (Figure 11B). All these data suggest that in cultured tubuloe epithelial cells AngII activated NF- κ B and AP-1 DNA binding mainly by AT₁, similar to that observed *in vivo* in tubulointerstitial areas.

Figure 5. Controls of the Southwestern histochemistry. In renal sections of AngII-infused rats several controls were done of the technique. Tissue was treated as described in Materials and Methods and incubated with digoxigenin-labeled NF- κ B alone (**A**) or in the presence of a 100-fold excess of cold NF- κ B oligonucleotide (competition study) (**B**). Other controls were done by incubation of renal samples with a mutant digoxigenin-labeled NF- κ B (100 pmol/L) (**C**) or in the absence of digoxigenin-labeled NF- κ B (**D**). Figure shows a representative animal of each group. Original magnification, $\times 200$.

Previous studies have demonstrated that AngII regulates NF- κ B DNA binding activity in human mononuclear cells.^{13,35} These cells possess all of the components of the RAS, including AT₁ and AT₂ receptors.³⁶ In human U937 mononuclear cells, pretreatment with AT₁ or AT₂ antagonists (10^{-5} to 10^{-7} mol/L) diminished in a dose-dependent manner the AngII-induced NF- κ B DNA binding activity (Figure 11C), suggesting that both receptors

are involved in the NF- κ B activation caused by AngII in mononuclear cells.

Discussion

In this study we have found that systemic AngII infusion into rats for 72 hours activated the transcription factors NF- κ B and AP-1 in the kidney being related to inflammatory cell infiltration and tubular damage. Elevated tissular NF- κ B and AP-1 has been observed in certain pathological conditions, associated to activated RAS, including experimental and human glomerulonephritis.^{3,14–17,37–39} Blockade of NF- κ B reduced renal damage.¹⁷ ACE inhibitors also diminished renal NF- κ B activity in experimental models of renal injury, associated or not with hypertension,^{3,16} suggesting that NF- κ B regulation maybe be a therapeutic target in kidney diseases. Our data provide the first *in vivo* evidence of NF- κ B activation by AngII, and identify the cell-specific localization of NF- κ B DNA binding. Thus, in AngII-infused rats a positive NF- κ B staining was observed in both resident renal cells and infiltrating mononuclear cells. Activated NF- κ B complexes were located mainly in the glomeruli (mesangial, endothelial, and epithelial cells), tubulointerstitial cells, and renal arteries (endothelial and vascular smooth muscle cells). Previous studies, besides ours, have shown that AngII activated NF- κ B in cultured cells, including glomerular mesangial cells.^{3,13,35,40} In the present study, we have also found that in cultured tubulointerstitial cells, AngII caused a rapid and transient, dose-dependent increase in NF- κ B DNA binding activity, with a similar potency to that of inflammatory cytokines. Although in AngII-infused rats there was a slight increase in systolic blood pressure (around 20 mmHg), the *in vitro* studies further demon-

Figure 6. Role of AT₁ and AT₂ receptors in AngII-induced NF- κ B activation. Animals were treated for 24 hours with the AT antagonists losartan and PD123319, and then infused with AngII for 72 hours **A**: Representative EMSA experiment that shows three different animals from each group. Specificity of the reaction was demonstrated with a 100-fold excess of unlabeled NF- κ B oligonucleotide. **B**: Densitometric analysis of the results expressed as arbitrary units of means $\pm$ SEM of four to six animals from each group. *, $P < 0.05$ versus control; #, $P < 0.05$ versus AngII-infusion.

Figure 7. Effect of AT₁ and AT₂ antagonists on the glomeruli and tubulointerstitial areas of AngII-infused rats. Figure shows a representative Southwestern experiment of an animal from each group: control (**A** and **E**), AngII-infusion (**B** and **F**), treatment with losartan (**C** and **G**), or with PD123319 (**D** and **H**). Both treatments showed a partial diminution in NF-κB staining in the glomeruli. Treatment with losartan only diminished NF-κB staining in tubular cells (**G**), whereas AT₂ blockade markedly diminished NF-κB-positive infiltrating interstitial cells (**H**). **I**: Semiquantitative score of NF-κB staining in glomeruli, infiltrating, and tubular cells. Results are expressed as means ± SEM of four to eight animals per group. *, *P* < 0.05 versus control; #, *P* < 0.05 versus AngII-infusion.

strate a direct, nonpressure-related AngII effect on the regulation of NF-κB activity in the kidney.

The potential role of AngII in the inflammatory response is a new and active field of investigation. AngII is involved

Figure 8. Systemic infusion of AngII activates renal AP-1 activity. Rats were treated with AngII for 24, 48, and 72 hours, and 7 days. Protein extracts were collected and used for gel shift assays with the AP-1 probe. **A**: Representative EMSA experiment that shows two different animals from each group. Specificity of the reaction was demonstrated with a 100-fold excess of unlabeled AP-1 consensus sequence (specific competitor), mutant AP-1, or unrelated oligonucleotide (NF-κB). The position of the AP-1 complexes and free oligonucleotide is indicated. **B**: Densitometric analysis of the results expressed as arbitrary units of means ± SEM of four to six animals from each group. *, *P* < 0.05 versus control. **C**: Southwestern histochemistry for AP-1 probe in AngII-infused rats. In control animals (**a**) there was no signal for AP-1, but after AngII infusion for 72 hours (**b**) a clear nuclear staining for AP-1 was seen, mainly in the glomeruli.

in the pathogenesis of immune-mediated renal diseases.^{3,41,42} We have found that AngII infusion causes inflammatory cell infiltration in the glomeruli and markedly in the interstitium associated with areas of focal inflammation, as previously reported.^{5,9} Several data support the idea of an important role of AngII in the inflammatory process. AngII causes monocyte chemotaxis and adhesion to mesangial and endothelial cells^{43–45} and NF-κB activation.^{3,35} We have observed in AngII-infused rats

Figure 9. AngII-infusion activates renal AP-1 through AT_1 . Animals were pretreated for 24 hours with the AT antagonists losartan and PD123319, and infused with AngII for 72 hours **A**: Representative EMSA experiment that shows three different animals from each group. Specificity of the reaction was demonstrated with a 100-fold excess of unlabeled AP-1 oligonucleotide. **B**: Densitometric analysis of the results expressed as arbitrary units of means $\pm$ SEM of four to six animals from each group. *, $P < 0.05$ versus control; #, $P < 0.05$ versus AngII-infusion.

that the infiltrating inflammatory cells exhibited activated NF- κ B complexes. Resident renal cells also respond to AngII stimulation with activation of NF- κ B and overexpression of related genes, such as MCP-1 and RANTES,^{3,9} that could be responsible for monocyte infiltration in the kidney. All these data suggest that the effect of AngII on inflammatory cell recruitment could be direct, through activation of mononuclear cells, and indirect, via chemokine production by renal cells, both processes mediated by NF- κ B activation. A common feature of all stages of atherosclerosis is inflammation of the vessel wall. Activation of NF- κ B has been observed in atherosclerotic lesions,⁴⁶ and in cultured vascular smooth muscle and endothelial cells stimulated with AngII.^{13,40} We have found that AngII-infusion activates NF- κ B in renal arteries, suggesting a more general feature not only reduced to renal parenchyma. On the whole, our *in vivo* data support the new view of AngII as a proinflammatory mediator.

The importance of AngII receptors *in vivo* has been demonstrated by treatment with specific receptor antagonists. For example, AT_1 participates in AngII-induced hypertension, cardiac hypertrophy, and extracellular matrix accumulation;^{27–29,47} whereas AT_2 mediates endothelium-dependent vasodilation,²¹ renal NO production⁴⁸ and its role in trophic effects on vascular smooth muscle cells is controversial.^{27–29} The receptor subtype involved in renal injury is not completely defined. AT_1 antagonists are currently used in the treatment of hypertension and proteinuria in patients with diabetic nephropathy.⁴⁹ In models of renal injury, AT_1 antagonists decrease proteinuria, matrix accumulation, and production of growth factors, such as TGF- β .^{21,50,51} The information provided by AT_2 blockers and AT_2 knockout mice supports an important role for the AT_2 in the physiopathology of the kidney.^{21,52} AT_2 might participate in blood pressure control, cGMP production in response to sodium

Figure 10. Demonstration of AT_2 in the kidney. **A**: Samples of renal tissue were incubated with an anti- AT_2 antibody and stained with immunoperoxidase. Figure shows the presence of AT_2 in control animals. As a positive control we studied a nephritic animal, and as negative control samples were incubated in the absence of primary antibody. Original magnifications: $\times 40$ (**a**), $\times 200$ (**b** and **c**), and $\times 100$ (**d**). **B**: Analysis of AT_2 mRNA expression by RT-PCR. Total RNA from renal cortex of a control rat and serum-starved murine tubulointerstitial cells (MCT) and human mononuclear cells (U937 cell line), expressed AT_2 mRNA. Figure shows one of four representative experiments of RT-PCR. **C**: Western blot analysis of the AT_2 in cultured samples of murine tubulointerstitial cells in quiescent state (after 48 hours of serum-starvation) or after treatment with 10% FCS. A representative immunoblot from three experiments showed a band of 44 kd of apparent molecular weight corresponding with the predicted AT_2 size.

depletion, in natriuretic response via bradykinin/NO, and in renal prostanoid production and metabolism.^{21,48,52,53} Unilateral obstruction in the knockout mice for the AT_2 gene caused accelerated renal interstitial fibrosis and collagen deposition.⁵⁴ In AngII-infused rats we have found that treatment with the AT_1 antagonist losartan restored blood pressure and improved tubular damage, whereas administration of the AT_2 blocker PD123319 decreased inflammatory cell infiltration. To further investigate the molecular mechanism of AngII-induced renal injury associated to each receptor activation, we evalu-

Figure 11. *In vitro* studies. Effect of AngII in NF-κB (A) and AP-1 (B) DNA binding in murine tubulointerstitial cells (MCT). Resting MCT cells were treated for increasing times with 10⁻⁹ mol/L AngII. After the incubation period, nuclear extracts were obtained and NF-κB or AP-1 activity was determined by binding assay to labeled NF-κB and analyzed by EMSA. Cells were preincubated for 30 minutes with losartan and PD123319 (10⁻⁵ to 10⁻⁷ mol/L), and then stimulated with 10⁻⁹ mol/L AngII for 30 minutes. Both antagonists at 10⁻⁵ mol/L had no effect on NF-κB/AP-1 activity in control cells (not shown). C: AngII increases NF-κB DNA in mononuclear cells via AT₁ and AT₂. Cells were preincubated for 30 minutes with losartan and PD123319 (10⁻⁵ mol/L), and then stimulated with 10⁻⁹ mol/L AngII for 30 minutes. The specificity of the reaction was established using competition assays with a 100-fold excess of unlabeled oligonucleotide. A representative EMSA is shown at the top and the bottom is shown the densitometric analysis of the results as arbitrary units of means ± SEM of six experiments. *, *P* < 0.05 versus control; #, *P* < 0.05 versus AngII-alone. The position of the NF-κB/AP-1-specific complexes and free oligonucleotide is indicated.

ated tissue levels and distribution of transcription factors. In AngII-infused animals, both AT₁ and AT₂ antagonists decreased AngII-increased renal NF-κB activity, whereas only AT₁ blocker decreased renal AP-1 activity. Some divergences in cellular distribution of NF-κB activated cells were observed in response to each receptor antagonist. Both antagonists decreased NF-κB-positive immunostaining in the glomeruli. Losartan markedly diminished NF-κB in tubular cells, and PD123319 did so mainly in inflammatory cells. In addition, AngII increased NF-κB and AP-1 DNA binding activity mainly by AT₁ in cultured tubulointerstitial cells, and activated NF-κB via both AT₁ and AT₂ in mononuclear cells.

One important finding of the present study is that the potential role of AT₂ in the renal inflammatory process may be mediated by NF-κB activation. Thus, in AngII-infused rats, the AT₂ blocker diminished both mononuclear infiltration and NF-κB DNA binding activity in inflam-

matory cells. Several experimental data support our hypothesis. In anti-thymocyte serum-induced nephritis two AT₁ antagonists caused a significant, but not a total reduction, in MCP-1, a chemokine under NF-κB control, and reduced glomerular macrophages/monocytes infiltration only around 30 to 50%.⁵⁵ In the model of ureteral obstruction nephropathy, monocyte/macrophage infiltration was reduced by ACE inhibitors, but not by AT₁ antagonists.^{50,56,57} In this model, both AT₁ and AT₂ antagonists partially diminished NF-κB activity.⁵⁸ ACE inhibitors diminished the renal activity of NF-κB and the expression of NF-κB-regulated genes, such as MCP-1 and the adhesion molecule VCAM-1,^{3,16,50} associated with the decrease in renal monocyte infiltration. In AngII-infused rats AT₂, but not AT₁ antagonists blocked glomerular monocyte infiltration and the expression of RANTES,⁹ another NF-κB controlled chemokine. From these data, we could hypothesize that differences in renal

monocyte recruitment between ACE inhibitors and AT₁ blockers could be because of the action of AngII via AT₂/NF- κ B pathway, and show a potential clinically important unresolved question, that needs to be addressed in human diseases. In cultured cells, AngII via AT₁/NF- κ B pathway up-regulates some genes, such as interleukin-6, VCAM-1, MCP-1, and angiotensinogen.^{59–62} In vascular smooth muscle cells, AngII increased gene and transactivated a NF- κ B-driven VCAM-1 promoter through AT₁.⁵⁹ Both AT₁ and AT₂ mediate AngII-induced NF- κ B DNA binding and transcription of a NF- κ B reporter gene.⁶² Recently, we have unraveled some of the intracellular signals elicited by AT₁ and AT₂. The potential mechanism of AT₁/NF- κ B/gene regulation could depend on redox-sensitive signals and activation of protein tyrosine kinases and mitogen-activated protein, as occurs with proinflammatory cytokines, whereas the AT₂/NF- κ B pathway seems to be mediated by ceramide production.⁶² All these data suggest that AngII could regulate a great variety of pathological genes through NF- κ B activation via AT₁ and/or AT₂, although the gene and receptor involved could be specific for each tissue or pathological condition.

Another important finding is the effect of AT₁ antagonist in AngII-induced renal damage. Losartan restored blood pressure, improved tubular damage, and decreased renal AP-1 activity. Interestingly, the effect of losartan on NF- κ B activity was mainly located in tubular cells. Many studies have demonstrated that the renal protection caused by ACE inhibitors or AT₁ antagonists are not only because of blood pressure control, but also to blockade of tissue RAS, in particular to the cellular actions of AngII.^{1,2} ACE inhibitors and AT₁ antagonists diminished profibrotic genes in experimental nephritis associated or not to hypertension^{1,2,31,50,51}. In AngII-infused rats, losartan diminished AngII-induced aortic fibronectin by a nonpressure-mediated mechanism.⁶³ We have observed that in cultured tubuloepithelial cells AngII, via AT₁, activated NF- κ B and AP-1. Although a limitation of our *in vivo* studies is that we cannot clearly dissociate between direct/pressure effect, our *in vitro* data support the idea that the beneficial effect of AT₁ antagonist could be attributed to a direct AngII/AT₁ action in tubular cells. In cultured renal cells, AT₁ regulates cell growth, matrix production, and the induction of profibrotic genes.^{1,2,8,32} Systemic AngII infusion into rats is characterized by cell proliferation and fibrosis,^{4,5} that are preceded by changes in the expression of some genes that are regulated by AP-1, such as c-fos, TGF- β , and fibronectin.^{4–8,57} Among these genes, TGF- β has a pivotal role in kidney diseases. In cultured renal cells, AngII increased TGF- β via AT₁.^{1,2,8,32} In tubuloepithelial cells, AngII causes hypertrophy by TGF- β production.² TGF- β is also involved in AngII-induced matrix production in cultured renal cells and in models of renal injury.^{1,2,31,32,50,51,64} AP-1 also regulates genes of the extracellular matrix proteins, such as fibronectin, that are induced by AngII via AT₁,^{1,51,64} and diminished by ACE inhibitors and AT₁ antagonists in various models of kidney injury.^{31,57} Injection of AngII induced the expression of c-fos/jun via AT₁ in the brain of spontaneously hyper-

tensive rats.⁶⁵ We have observed that AngII infusion caused activation of AP-1 via AT₁, suggesting that the beneficial effect of losartan treatment could be due, at least in part, to the regulation of AP-1 transcription factor. On the whole, these experimental data support the idea that losartan blocks AT₁-mediated cellular AngII actions, such as gene overexpression and activation of transcription factors (AP-1 and NF- κ B), and therefore ameliorates renal damage.

Although our studies have described several AngII-mediated effects, as well as the receptors involved, there are many open questions that need future research. AT₁ blockade leads to free ligand (AngII) that could theoretically bind to AT₂, and then exert some potential beneficial effects, including vasodilatation and antiproliferative/apoptotic responses,²¹ although there is little evidence of this mechanism in the kidney. On the other hand, AT₂ could exert pathological effects through the recruitment of mononuclear cells, although this point needs to be addressed in human diseases. In addition, AT₁ blockade stimulates aminopeptidase A activity,⁶⁶ an enzyme that degrades AngII to AngIII, suggesting a potential increase in AngIII that could bind to AT₂.¹⁸ AngIII causes proteinuria and increases growth-related, profibrotic and proinflammatory genes.^{44,67,68} Increased renal AngII production and aminopeptidase A expression have been described in pathological settings associated to tissue RAS activation, such as hypertension, diabetes mellitus, nephritis, and AngII infusion.^{69,70} These data indicate that although AngII is the main effector peptide of the renin angiotensin system other components of this system, such as AngIII, could play an active role in renal injury.

Our results suggest that the effects of AngII on renal structures take place through different receptors and transcription factors. AngII participates in inflammatory cell infiltration mainly mediated by NF- κ B activation via AT₂, whereas tubular damage seems to occur through AP-1 by AT₁. These results describe a novel signaling mechanism associated to AngII-induced renal injury, and may contribute to a better understanding of the pathological effects of this peptide in several diseases associated with activated RAS, as renal and cardiovascular disease.

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