

# Regulation of Angiotensin II Receptor AT<sub>1</sub> Subtypes in Renal Afferent Arterioles during Chronic Changes in Sodium Diet

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## Abstract

Studies determined the effects of chronic changes in sodium diet on the expression, regulation, and function of different angiotensin II (ANG II) receptor subtypes in renal resistance vessels. Rats were fed low- or high-sodium diets for 3 wk before study. Receptor function was assessed in vivo by measuring transient renal blood flow responses to bolus injections of ANG II (2 ng) into the renal artery. ANG II produced less pronounced renal vasoconstriction in rats fed a low- compared with high-sodium diet (16% vs. 56% decrease in renal blood flow,  $P < 0.001$ ). After acute blockade of ANG II formation by iv enalaprilat injection in sodium-restricted animals, ANG II produced a 40% decrease in renal blood flow, a level between untreated dietary groups and less than high salt diet. Intrarenal administration of angiotensin II receptor type 1 (AT<sub>1</sub>) receptor antagonists losartan or EXP-3174 simultaneously with ANG II caused dose-dependent inhibition of ANG II responses. Based on maximum vasoconstriction normalized to 100% ANG II effect in each group, AT<sub>1</sub> receptor antagonists produced the same degree of blockade in all groups, with an apparent maximum of 80–90%. In contrast, similar doses of the angiotensin II receptor type 2 (AT<sub>2</sub>) receptor ligand CGP-42112 had only a weak inhibitory effect. In vitro equilibrium-saturation <sup>125</sup>I-ANG II binding studies on freshly isolated afferent arterioles indicated that ANG II receptor density was lower in the low- vs. high-sodium animals (157 vs. 298 fmol/mg,  $P < 0.04$ ); affinity was similar (0.65 nM). Losartan and EXP-3174 displaced up to 80–90% of the ANG II binding; fractional displacement was similar in both diet groups. In contrast, the AT<sub>2</sub> receptor analogues PD-123319 and CGP-42112 at concentrations  $< 10^{-6}$  M had no effect on ANG II binding. RT-PCR assays revealed the expression of both angiotensin II receptor type 1A (AT<sub>1A</sub>) and angiotensin II re-

ceptor type 1B (AT<sub>1B</sub>) subtypes in freshly isolated afferent arterioles, while there was very little AT<sub>2</sub> receptor expression. Total AT<sub>1</sub> receptor mRNA expression was suppressed by low sodium intake to 66% of control levels, whereas it was increased to 132% of control by high-sodium diet, as indicated by ribonuclease protection assay. Receptor regulation was associated with parallel changes in AT<sub>1A</sub> and AT<sub>1B</sub> expression; the AT<sub>1A</sub>/AT<sub>1B</sub> ratio was stable at 3.7. We conclude that AT<sub>1</sub> receptors are the predominant ANG II receptor type in renal resistance vessels of 7-wk-old rats. Chronic changes in sodium intake caused parallel regulation of expression and amount of receptor protein of the two AT<sub>1</sub> receptor genes that modulate receptor function and altered reactivity of renal vessels to ANG II. (*J. Clin. Invest.* 1997; 99:1072–1081.) Key words: kidney • renal circulation • vascular resistance • AT<sub>1</sub> receptor mRNA • losartan • AT<sub>2</sub> receptor • PD-123319 • CGP-42112 • receptor antagonist • sodium balance • renin-angiotensin system • vascular smooth muscle cell

## Introduction

Angiotensin II (ANG II)<sup>1</sup> is a potent vasoconstrictor that exerts its biological actions after binding to cell surface receptors and activating several signaling pathways that increase intracellular calcium concentration and stimulate excitation-contraction. Most of the effects of ANG II in the renal and cardiovascular systems are mediated by angiotensin II receptor type 1 (AT<sub>1</sub>) receptors (1–4). In the kidney, AT<sub>1</sub> receptors are present in preglomerular arteries and arterioles, glomeruli, vasa rectae, and proximal convoluted tubules. The major receptor in adult animals is the AT<sub>1</sub>, although a larger population of AT<sub>2</sub> receptors is observed in young, developing animals (5).

Our previous studies on ANG II receptors and vascular reactivity in rats on a normal salt diet indicate that ANG II primarily binds to AT<sub>1</sub> receptors and that such ligand-receptor interactions produce renal vasoconstriction. AT<sub>1</sub> receptors mediate a majority of the binding and functional response in the rat kidney (6). Other blood flow studies demonstrate that activation of AT<sub>1</sub> receptors produce contraction of renal resistance vessels by a combination of calcium entry through L-type calcium channels, calcium mobilization from internal stores, and activation of protein kinase C (7, 8).

Reactivity to ANG II in different vascular beds varies with sodium intake, activity of the renin-angiotensin system in healthy animals, and during different pathological states. It is

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1. Abbreviations used in the paper: ACE, angiotensin-converting enzyme; ANG II, angiotensin II; AT<sub>1</sub>, angiotensin II receptor type 1; AT<sub>1A</sub>, angiotensin II receptor type 1A; AT<sub>1B</sub>, angiotensin II receptor type 1B; AT<sub>2</sub>, angiotensin II receptor type 2.

well known that renal vascular reactivity to ANG II is exaggerated when endogenous ANG II plasma concentrations are low, and vice versa (9, 10). The reasons for the differences in vascular reactivity are likely to be multifaceted. Postulates include the abundance of cell surface receptors available to bind ligand, and efficiency of AT<sub>1</sub> receptor coupling with second messenger signals leading to tension and vasomotor tone.

ANG II receptors frequently, but not always, exhibit physiological adaptation, and respond to chronic changes in sodium diet and associated changes in renin-angiotensin system activity. Previous studies have used glomeruli and cultured mesangial cells as models of vascular smooth muscle cells. Reciprocal changes in salt intake and ANG II receptor density in rat glomeruli has been reported (11–13), although this is not a universal finding (14). The changes correlated with plasma ANG II concentration and similar responses are elicited by ANG II infusion, implicating ANG II as the important regulator (12, 15). A similar pattern of downregulation of ANG II receptors by ANG II or low-salt diet has been reported in vascular smooth muscle cells from the mesenteric artery (16–19). In marked contrast, a low-salt diet leads to an increase in ANG II binding to renal cortical basolateral membranes (presumably most of proximal tubular origin) freshly isolated proximal tubular cells, and also in cultured proximal tubular cells (2, 20, 21). Upregulation of ANG II receptors is also observed in the adrenal gland (18, 22, 23). In cultured mesangial cells, ANG II is reported to down-regulate ANG II receptors, although other investigators have found no effect on ANG II binding sites (24, 25). Little is known about the regulation of ANG II receptors in resistance vessels within the renal microcirculation—in particular the afferent arterioles—and small arterioles in other vascular beds. The reported changes in receptor density and binding characteristics may reflect changes involving synthesis of different subtypes and/or subsequent processing.

The present studies investigated the influence of chronic changes in sodium diet on the expression and function of ANG II receptors in renal resistance vessels. We used RT-PCR and ribonuclease protection assay and ANG II binding assay to quantify ANG II receptor gene and protein expression. The results indicate parallel increases in total AT<sub>1</sub> mRNA expression, and in AT<sub>1</sub> receptor density in afferent arterioles isolated from kidneys of animals maintained 3 wk on a high-sodium diet. The ratio of angiotensin II receptor type 1A (AT<sub>1A</sub>)/angiotensin II receptor type 1B (AT<sub>1B</sub>) mRNA was independent of salt intake, reflecting parallel change in response to salt-induced changes in the renin-angiotensin system. The functional correlate of receptor density was evaluated by assessing in vivo reactivity of renal arterioles to ANG II. Renal vascular reactivity to injection of ANG II into the renal artery was inversely related to salt diet. Inhibition with enalaprilat in low-sodium animals revealed that part of the attenuated response to administered ANG II was due to prior receptor occupation by endogenous ANG II in addition to the observed changes in receptor synthesis and binding sites.

## Methods

**Renal blood flow studies.** Experiments were performed on male, 7-wk-old anesthetized Wistar-Kyoto rats obtained from the Chapel Hill breeding colony. The animals were maintained for 3 wk on Harlan rat chow balanced in all respects except for a high-sodium (3.2%) or low-sodium (0.08%) content; tap water was allowed ad libitum.

An animal was deprived of food but not water the night before an experiment. Standard surgical techniques were employed (6–8, 26). Anesthesia was induced by an intraperitoneal injection of sodium pentobarbital (65 mg/kg body wt), and the animals were placed on a servo-controlled heating table that maintained body temperature at 37°C. A tracheostomy was performed to facilitate free breathing. The right carotid artery was cannulated to obtain blood samples, and to monitor arterial pressure (P23Db transducer; Statham Instruments, PR). The right jugular vein was cannulated for the administration of maintenance infusions, cyclooxygenase inhibitor, and supplemental doses of sodium pentobarbital. Isoncotic bovine serum albumin (4.7 g/dl) was infused intravenously, initially at a rate of 50  $\mu$ l/min to replace losses associated with surgery (1.25 ml/100 g body wt), and then at 10  $\mu$ l/min for the duration of an experiment to maintain hematocrit and plasma protein concentration at presurgical levels. Midline and subcostal incisions were used to expose the abdominal aorta and left kidney. A noncannulating electromagnetic flow probe (1.5 mm circumference; Carolina Medical Electronics, King, NC) was placed around the left renal artery to measure renal blood flow. A tapered and curved PE-10 catheter was introduced into the left femoral artery and advanced through the aorta until its tip was positioned approximately 1 mm into the left renal artery (26). Placement of the catheter in the renal artery did not affect renal blood flow. The renal arterial catheter was used for the local administration of ANG II, the AT<sub>1</sub> receptor antagonists losartan and EXP 3174 (DuPont-Merck Pharmaceutical Co., Wilmington, DE), or the AT<sub>2</sub> receptor antagonist CGP-42112 (Ciba Geigy Limited, Basel, Switzerland) (6–8). After completion of surgery, the animals were allowed to stabilize for 1 h before starting the measurements.

At the end of the stabilization period, indomethacin (5 mg/kg body wt) was administered intravenously to inhibit cyclooxygenase activity. Previous studies indicated that this dose of indomethacin produced a 60–80% decrease in the rate of urinary PGE<sub>2</sub> excretion for at least 3 h, as measured by radio-immunoassay (26). Throughout the experiment, a continuous infusion (5  $\mu$ l/min) of heparinized isotonic saline was administered via the renal arterial catheter. 1 min before the administration of ANG II, the rate of saline infusion was increased to 120  $\mu$ l/min so that the entire bolus of ANG II could be delivered to the kidney within 5 s. A Cheminert sample injection valve was used to introduce a 10  $\mu$ l bolus into the infusion line (Valco Instruments Co., Houston, TX). Previous studies indicated the bolus reached the kidney 12 s after its introduction into the infusion line (6–8, 26). Following recovery of renal blood flow to its baseline level (usually < 2 min), the infusion rate was returned to 5  $\mu$ l/min.

The following drugs were used: ANG II (Sigma Chemical Co., St. Louis, MO), losartan (DuP-753, DuPont-Merck Pharmaceutical Co., Wilmington, DE), EXP-3174 (DuPont-Merck), PD-123319 (Parke-Davis Pharmaceutical Co., Ann Arbor, MI), CGP-42112 (Ciba Geigy), enalaprilat (Merck), and indomethacin (Sigma). ANG II was injected at the dose of 2 ng into the left renal artery. This dose of angiotensin produces a reversible transient 50% decrease in renal blood flow (6–8, 26). Various doses of the ANG II receptor antagonists were injected as a mixture with 2 ng of ANG II. The time interval between injections was 15 min. The order of doses was randomly selected each day. To avoid treatment interactions, a single ANG II antagonist was used in each rat. To evaluate whether consecutive injections of an antagonist created an additive buffering effect, ANG II was injected alone at the beginning, middle, and end of an experiment. In all cases, ANG II produced a similar decrease in renal blood flow. To test if the buffering effect of ANG II antagonists was localized to the kidney, ANG II was injected intravenously before and during intrarenal injections of the agonists. In both conditions, ANG II caused a similar rise in arterial pressure.

The data acquisition system consisted of an IBM-compatible computer and a 12 bit A/D converter. The flow probe was interfaced to the data acquisition system using a Carolina Medical Electronics 500 electromagnetic flowmeter. A Hewlett-Packard 8805B carrier amplifier was used for the pressure sensor interface. The outputs of

the transducers monitoring arterial pressure and renal blood flow were sampled at a rate of 100 samples/s for a period of 2 min, which usually was sufficient time to allow flow to return to its baseline value after each injection of ANG II. Each recording was started when ANG II was introduced into the renal artery infusion line. Consecutive blocks of 100 data points were averaged to obtain second-by-second estimates of renal blood flow and arterial pressure, and these averages were used to calculate second-by-second estimates of renal vascular resistance. The renal blood flow, arterial pressure, and renal vascular resistance values were normalized and expressed as a percent of baseline values. The baseline was calculated separately for each injection using the mean values of the corresponding variables observed during the time between the introduction of ANG II into the infusion line and the onset of the renal vascular response. Plots of normalized arterial pressure, renal blood flow, and renal vascular resistance as a function of time were prepared using the SigmaPlot software package.

**Preparation of isolated afferent arterioles.** Afferent arterioles from the rat kidney were isolated by a method of Chatziantoniou and Arendshorst (6). Acute receptor binding studies were performed on vascular tissue isolated from male, 7-wk-old euvoletic rats maintained on three different levels of sodium diets (0.8, 1.2, and 3.4%) for 3 wk prior to study. The kidneys were infused with a magnetized iron oxide suspension (1% Fe<sub>3</sub>O<sub>4</sub> in saline) for 0.5–1 min at constant pressure (120 mmHg) and then excised and placed in a phosphate buffer solution (20 mM K<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 125 mM NaCl, 5 mM MgCl<sub>2</sub>, 4°C, pH 7.3). All subsequent steps of isolation were performed at 4°C. Cortical tissue was homogenized using a Polytron tissue mixer. Renal preglomerular vessels, glomeruli, and the surrounding connective tissue were removed from the crude homogenate with the aid of a magnet. The vascular tissue was passed through needles of decreasing size and sieved through a 125 µm sieve mesh screen to detach connective tissue, remove iron oxide from the large vessels, and to separate afferent arterioles from glomeruli. The vascular tissue remaining on the top of the 125 µm sieve consisting of afferent arterioles of high purity (> 95%) was used for the subsequent binding studies. The average yield of the arteriolar preparation was 200–400 µg protein for both kidneys of a 200-g rat.

**Receptor binding studies.** Aliquots of microvessels (40 µg of protein) were incubated at room temperature in a final volume of 0.25 ml buffer containing 0.3% bovine serum albumin (Sigma). Incubations lasted 45 min with 10 concentrations (0.05–1.5 nM) [<sup>125</sup>I]-[Sar<sup>1</sup>, Ile<sup>8</sup>] ANG II (NEN, DuPont, Boston, MA) under continuous gentle shaking. The protein concentration, binding conditions, and time for equilibrium were determined in preliminary experiments; the results agreed with those previously reported (6). Bound ligand was separated from the free ligand using centrifugation in a sucrose gradient. 0.2 ml of the samples were gently layered over 0.2 ml of assay-binding buffer containing 20% sucrose in 0.5 ml polyethylene microcentrifuge tubes, and were centrifuged at 5,000 g for 30 min in a free-angle rotor. The tubes were then frozen rapidly by insertion into dry ice, and the tips, containing the bound ligands, and the tops, containing the free ligands, were assayed in a gamma counter. Nonspecific binding was determined in the presence of an excess of unlabeled ANG II; it was < 10% of total binding. In competitive inhibition studies, the AT<sub>1</sub> receptor antagonists losartan (DuP 753), EXP 3174, or CGP-42112 were used to displace [<sup>125</sup>I]-[Sar<sup>1</sup>, Ile<sup>8</sup>] ANG II (0.375 nM). Analyses of the data using the LIGAND program gave estimates of the maximum specific binding (B<sub>max</sub>) and dissociation constant (K<sub>d</sub>) (6).

**RNase protection assay for AT<sub>1</sub>-receptor mRNA.** Total RNA was isolated from frozen isolated afferent arterioles according to standard methodology, and AT<sub>1</sub>-receptor mRNA was measured by RNase protection assay as described previously (27). Fragments of 298 bp (bp 139–437) from AT<sub>1A</sub> and 216 bp (bp 127–344) from AT<sub>1B</sub> cDNA were cloned into vector pSP65 (Promega-Serva, Heidelberg, Germany) (28, 29). Linearization with *S*tyI, followed by in vitro transcription with SP6 RNA polymerase, yielded a 98 bp protected cRNA fragment for both AT<sub>1</sub> receptor subtypes. Transcripts were continu-

ously labeled with α[<sup>32</sup>P]GTP (410 Ci/mM, Amersham International, Little Chalfont, UK) and purified on a Sephadex G50 spin column. For hybridization, total RNA from the kidneys was dissolved in a buffer containing 80% formamide, 40 mM piperazine-N,N'-bis (2-ethanesulfonic acid), 400 mM NaCl, and 1 mM EDTA (pH 8). RNA (30–200 µg) was hybridized in a total volume of 50 µl at 60°C for 12 h with 5 × 10<sup>5</sup> cpm each of radiolabeled AT<sub>1A</sub> and AT<sub>1B</sub> probe. RNase digestion with RNase A and T1 was carried out at room temperature for 30 min and terminated by incubation with proteinase K (0.1 mg/ml containing 0.4% sodium dodecyl sulfate) for 30 min at 37°C. The combination of protected AT<sub>1A</sub> and AT<sub>1B</sub> fragments was purified by phenol/chloroform extraction, ethanol precipitation, and subsequent electrophoresis on a denaturing 10% polyacrylamide gel. After autoradiography of the dried gel at –80°C for 1 d, the band representing a mixture of protected AT<sub>1A</sub> and AT<sub>1B</sub> fragments was excised from the gel, and radioactivity was counted with a liquid scintillation counter. GAPDH-mRNA was used as a standard RNA controlling the quality of RNA preparation.

**RT-PCR for AT<sub>1</sub> and AT<sub>2</sub> cDNA.** Reverse transcription was used to synthesize the first strand of cDNA. 2 µg of total RNA was mixed with 0.5 µg oligo dT in distilled water (final volume, 12.5 µl) and incubated at 70°C for 3 min. Then, the samples were placed on ice, and 10 µl of buffer (10.3 µl dNTP [25 mM], 20.6 µl 5× first strand buffer, 2.8 µl bovine serum albumin [20 mg/ml], 2.8 µl RNasin [40,000 U/ml, Promega Corp., Madison, WI], 10 µl DTT [0.1 M], and 4.7 µl MMLV reverse transcriptase [200 U/µl, GIBCO BRL, Gaithersburg, MD]) were added.

PCR for AT<sub>1</sub> cDNA was performed utilizing sense (5'-CCA AAG TCA CCT GCA TCA TC-3') and antisense primers (5'-CAC AAT CGC CAT AAT TAT CCT A-3') designed from the cDNA sequences common to rat AT<sub>1A</sub> and AT<sub>1B</sub> receptors (28, 29). These primers correspond to regions where no sequence divergence exists between AT<sub>1A</sub> and AT<sub>1B</sub>, and amplify a 305-bp cDNA fragment from position 723 to 1028 in the AT<sub>1A</sub> sequence, and from position 630–935 in the AT<sub>1B</sub> sequence (28, 29). For AT<sub>1</sub>-PCR, 10 pmol of the respective primers were mixed with 3 µl of cDNA obtained by the reverse transcription reaction described above and with 3 µl MgCl<sub>2</sub> (25 mM), 15.5 µl DEPC water, 2.5 µl 10× PCR buffer, and 3 µl deoxy [1',2',5'-<sup>3</sup>H]Cytidine 5'-triphosphate (1 µCi/µl).

To distinguish between AT<sub>1A</sub> and AT<sub>1B</sub> receptor amplification products, 1.5 µl (25 U/µl) was added to digest 20 µl of the PCR product obtained with the AT<sub>1</sub> primers. The mixture was incubated for 1 h at 37°C, a period known to be sufficient for complete digestion. The reaction was terminated at 94°C for 2 min. This digestion yielded fragments that matched the expected sizes of 128 and 177 bp characteristic of AT<sub>1A</sub> receptors. The digested PCR products were separated by polyacrylamide gel electrophoresis, visualized by ethidium bromide staining, excised from the gel, dissolved in 25 mM periodic acid, and quantified by liquid scintillation counting.

Rat β-actin cDNA was amplified with the sense primer (5'-CCG CCC TAG GCA CCA GGG TG-3') that spanned over the border of the second exon and second intron, and the antisense primer (5'-GGC TGG GGT GTT GAA GGT CTC AAA-3') that bound in the fourth exon (30). These primers identified a 286 bp DNA fragment. β-Actin was amplified from the same cDNA used for the AT<sub>1A</sub>/AT<sub>1B</sub> or angiotensin II receptor type 2 (AT<sub>2</sub>) amplification. Because of the higher abundance of actin mRNA, the cDNA obtained by the RT-reaction was diluted 1:1000 before adding 3 µl to the actin PCR, which was then performed under the same conditions as for AT<sub>1</sub> described above. The mixtures were heated at 94°C for 15 min, and then cooled on ice. PCR was started by the addition of 1 µl Taq polymerase (1 U/µl, Boehringer Mannheim) and 1 µl dNTP (25 mM). Denaturing, annealing, and extension reactions were performed at 94°C for 1 min, 52°C for 1 min, and 72°C for 20 s. As was done for the AT receptors, all 20 samples were analyzed in one assay to minimize inter-assay variability.

PCR products were separated by polyacrylamide gel electrophoresis and quantified. PCR products were visualized by ethidium

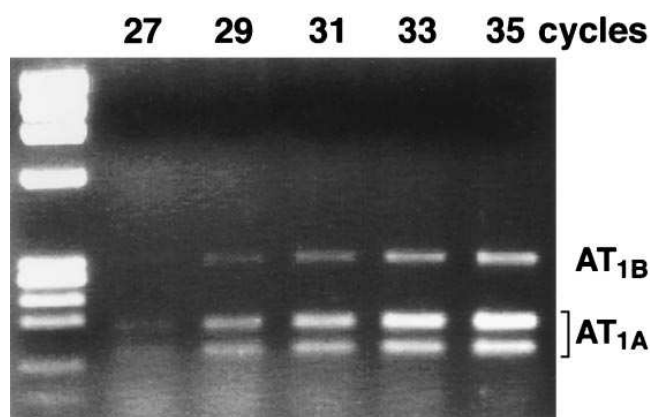


Figure 1. Dependence of amounts of AT<sub>1A</sub> and AT<sub>1B</sub> cDNA on PCR cycle number between 27 and 35 cycles.

bromide staining, excised from the gel, dissolved in periodic acid, and quantified by liquid scintillation spectrometer. The amounts of PCR products obtained for AT<sub>1A</sub> and AT<sub>1B</sub> receptor mRNA and for  $\beta$ -actin mRNA, were dependent on the PCR cycle number, and were linear between 27 to 35 cycles (Fig. 1) (31). Therefore, we used 30 cycles as the standard protocol for our determinations. To circumvent eventual contamination of genomic DNA in sample RNAs, direct PCR amplification of sample RNA without reverse transcriptase was performed routinely. No significant product was detected for up to 35 cycles.

PCR for AT<sub>2</sub> cDNA was carried out by reverse transcription of mRNA in a manner similar to that described above for AT<sub>1</sub> receptors. After PCR reaction with sense (5'-CGG GAT CCT TTG ATA ATC TCA AC- 3') and antisense primers (5'-GGA ATT CAA ACA CTT TGC CAT CAC- 3'), a 315-bp fragment was amplified (32). Primers were designed with a *Bam*HI site at the 5' and a *Eco*RI site at the 3' end for possible cloning experiments. Conditions for AT<sub>2</sub> PCR were as follows: 35–37 cycles with 30 s heating at 94°C were followed by a 1 min annealing step at 55°C and 30 s polymerization at 72°C. Previous studies showed linearity of the PCR reaction from 31 up to 37 cycles under these conditions (31).

**Statistical methods.** Statistical analyses were performed using a SigmaStat software package. Comparisons between two groups were analyzed using Student's unpaired *t* test. Larger data sets were tested with analysis of variance. Results with  $P < 0.05$  were considered statistically significant. All values reported are means  $\pm$  SE.

## Results

Baseline renal hemodynamic data are summarized in Table I. Experiments were performed on 7-wk-old animals that weighed  $\sim 220$  g. Animals maintained for 3 wk on either low- or high-sodium diets had similar values for mean arterial pressure, renal blood flow, and renal vascular resistance.

To evaluate effect of sodium intake on renal vascular reactivity, a bolus of ANG II (2 ng) was injected into the renal artery to elicit a transient decrease in renal blood flow. The animals were acutely treated with indomethacin to minimize ANG II interactions with vasoactive cyclooxygenase metabolites. Fig. 2 summarizes the group averages for the pattern of transient response to bolus injection of ANG II. The maximum renal vasoconstriction produced by ANG II was significantly reduced in the low-sodium group compared with responses in the high-salt animals ( $16 \pm 2$  vs.  $56 \pm 4\%$  decrease in renal blood flow,  $P < 0.001$ ). Similar group differences were noted in absolute changes in renal blood flow. Arterial pres-

Table I. Renal Hemodynamics in Euvoletic 7-Wk-Old Rats Maintained on Low- or High-Sodium Diets for 3 Wk

	Low-sodium diet	High-sodium diet
	<i>n</i> = 20	<i>n</i> = 19
Age (wk)	7 $\pm$ 1	7 $\pm$ 1
Body wt (g)	224 $\pm$ 6	211 $\pm$ 9
Mean arterial pressure (mmHg)	131 $\pm$ 3	132 $\pm$ 3
Renal blood flow (ml $\cdot$ min <sup>-1</sup> $\cdot$ min $\cdot$ g kidney wt <sup>-1</sup> )	8.3 $\pm$ 0.5	7.8 $\pm$ 0.4
Renal vascular resistance (mmHg $\cdot$ min <sup>-1</sup> $\cdot$ min $\cdot$ g kidney wt)	16.3 $\pm$ 0.8	17.8 $\pm$ 0.9
Hematocrit (ml $\cdot$ dl <sup>-1</sup> )	47.3 $\pm$ 0.9	47.5 $\pm$ 0.6

Values are means  $\pm$  SE. There were no statistically significant ( $P > 0.05$ ) differences between dietary groups.

sure was unaffected by the intrarenal injection of ANG II. Thus, the decreases in renal blood flow were mirrored by reciprocal increases in renal vascular resistance (data not shown).

To identify mediation of effects by ANG II receptor subtypes, the AT<sub>1</sub> receptor antagonist losartan was injected into the renal artery simultaneously with ANG II. The maximum ANG II-induced renal vasoconstriction was identified as a 100% response, and responses in low- and high-sodium groups were normalized to the maximum responses in their respective groups. As shown in Fig. 2, losartan inhibited the maximum decrease in renal blood flow in a dose-dependent manner. Clearly a large amount of this AT<sub>1</sub> receptor antagonist inhibited the majority of the renal vasoconstriction. The apparent maximum inhibition by 10  $\mu$ g losartan was about 80% of the renal response to ANG II (Fig. 3). This was the case for the normalized response in animals maintained on either low- or high-sodium diet. This observation is consistent with previous results for rats on a normal salt diet (6). Similar results were obtained when EXP-3174 was used to antagonize ANG II binding to AT<sub>1</sub> receptors. Increasing doses of EXP-3174 caused dose-dependent inhibition of ANG II effects. The same degree of inhibition was noted in rats on low- and high-sodium diets. Maximum inhibition was up to 90–95% of the ANG II-induced renal vasoconstriction (data not shown).

The AT<sub>2</sub> receptor antagonist CGP-42112 was much less effective (Fig. 3). Intrarenal injection of 1,000 ng CGP-42112 with 2 ng ANG II blunted less than 5% of the ANG II-induced renal vasoconstriction. The high doses of 5 and 10  $\mu$ g CGP-42112 blocked up to 30% of the renal hemodynamic response to ANG II. No differences were noted between sodium diet groups.

To evaluate the influence of endogenous levels of ANG II on vascular reactivity to administered ANG II, paired experiments were conducted in four low-sodium animals before and after intravenous injection of enalaprilat to inhibit angiotensin-converting enzyme (ACE). In the control period ANG II (2 ng) reduced blood flow by about 20%, as compared to a 40% decrease after administration of enalaprilat in animals consuming a low sodium diet. The twofold increase in vascular reactivity to the standard dose of ANG II after acute inhibition of ACE was significant ( $P < 0.02$ ). Losartan inhibited up to 80% of the renal vasoconstriction produced by exogenous

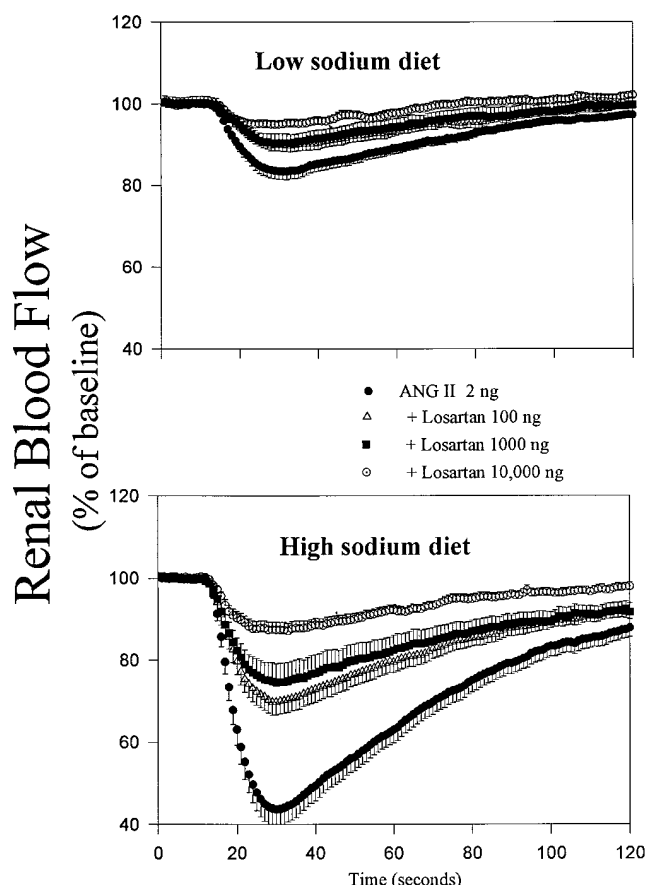


Figure 2. Dose-dependent inhibition by the AT<sub>1</sub> receptor antagonist losartan of angiotensin II-induced reductions in renal blood flow in rats consuming a low- or high-sodium diet for 3 wk prior to study. Angiotensin II was injected into the renal artery alone and in combination with different amounts of losartan. Means  $\pm$  SE for 20 low-salt rats and 19 high-salt animals.

ANG II whether or not ACE was blocked. In this regard, inhibition of ANG II production by enalaprilat converted the responses to exogenous ANG II to a pattern previously reported for rats maintained on their standard diet (6–8, 26). Thus, part of the attenuated vascular reactivity to exogenous ANG II could be attributed to prior receptor occupation by endogenous ligand.

In other studies, we determined ANG II receptor characteristics in preglomerular resistance vessels. Biochemical saturation ligand binding studies were performed on freshly isolated afferent arterioles from rats maintained on different sodium diets for 3 wk. Representative examples of ANG II binding under equilibrium conditions are presented in Fig. 4. Scatchard analysis revealed one predominant class of high affinity receptor in low-, normal-, and high-sodium diet groups (Hill coefficient = 0.9–1.0 for all groups). As the data in Table II show, there was no difference in the value of dissociation constant between the low- and high-sodium groups ( $K_d$  = 0.5 vs. 0.8 nM). On the other hand, there was a large almost two-fold difference in receptor density (Table II). Animals consuming the high-sodium diet had about twice as many receptor sites as the low-sodium animals ( $B_{max}$  = 298 vs. 158 fmol/mg). These results indicate that ANG II binding to afferent arteri-

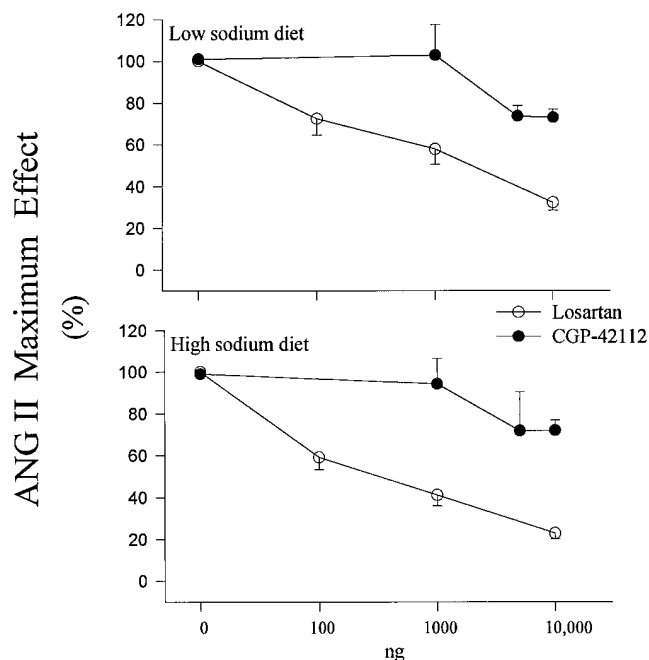


Figure 3. Summary of dose-dependent inhibition of the maximum angiotensin II-induced renal vasoconstriction produced by injection of either losartan or CGP-42112 simultaneously with angiotensin II. Means  $\pm$  SE for more than five animals in each group.

oles is regulated in response to variations in salt intake and activity of the renin–angiotensin system. Changes in receptor density probably participated in the observed differences in renal vascular reactivity to ANG II in sodium diet groups.

Other *in vitro* binding studies were performed to evaluate the effect of sodium diet on ANG II receptor subtype(s) expressed in this vascular preparation. Unlabeled ANG II, the AT<sub>1</sub> receptor antagonists losartan and EXP-3174, and the AT<sub>2</sub> ligands PD-123319 and CGP-42112, were used to displace 0.375 nM <sup>125</sup>I-ANG II from binding sites. The data are presented in Fig. 5, top and bottom panels, respectively. Expressed as a percent of ANG II specific binding, there were no

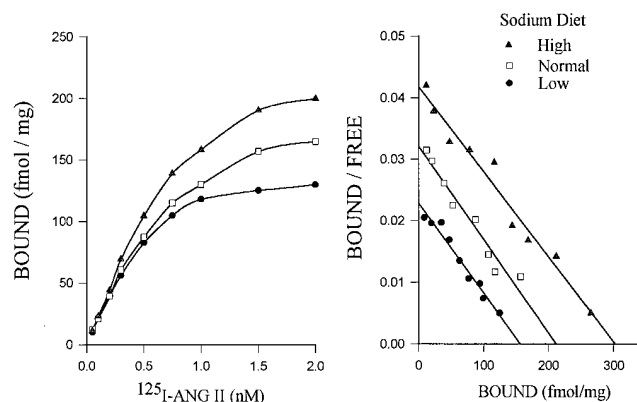


Figure 4. Representative examples of <sup>125</sup>I-angiotensin II binding to freshly isolated afferent arterioles obtained from rats on low-, normal-, and high-sodium intakes. Saturation binding curves are shown in left panel. Scatchard analysis is presented in the right panel.

**Table II. Characteristics of ANG II Receptor Binding in Afferent Arterioles Freshly Isolated From Kidneys of 7-Wk—Old Rats Maintained on Different Sodium Diets for 3 Wk**

Sodium diet	Density <i>fmol/mg protein</i>	Affinity <i>nM</i>
Low	158±20	0.5±0.1
Normal	202±3	0.5±0.1
High	298±45*	0.8±0.1

Values are means±SE of 3 experiments per group (4 rats/experiment). \**P* < 0.05 vs. low sodium diet.

major differences between the two groups on different sodium diets. Competitive inhibitory potencies of these agents showed ANG II > EXP-3174 ≥ losartan >> CGP-42112 ≥ PD-123319. Table III summarizes the *K<sub>i</sub>* values for each compound. Losartan was about two times less potent than EXP-3174 ligands (*P* < 0.002 for both diet groups), and more than ten times more potent than AT<sub>2</sub> receptor ligands. The Hill coefficient for losartan was less than unity for low- (0.75±0.02, *P* < 0.001) and high-sodium diets (0.80±0.03, *P* < 0.005), suggesting negative cooperativity, or multiple, noninteracting binding sites. In agreement with our previous findings (6), the most abundant ANG II binding sites showed high affinity for losartan, with a *K<sub>i</sub>* of 37 nM; this class accounted for up to 80% of the total population. The low-affinity site for losartan occupied the remaining 20% of the total ANG II receptor site with an 1,000-fold lower affinity than the majority, high affinity site. The AT<sub>2</sub> receptor analogue PD-123319 and CGP-42112 displaced <sup>125</sup>I-ANG II binding only at extremely high concentra-

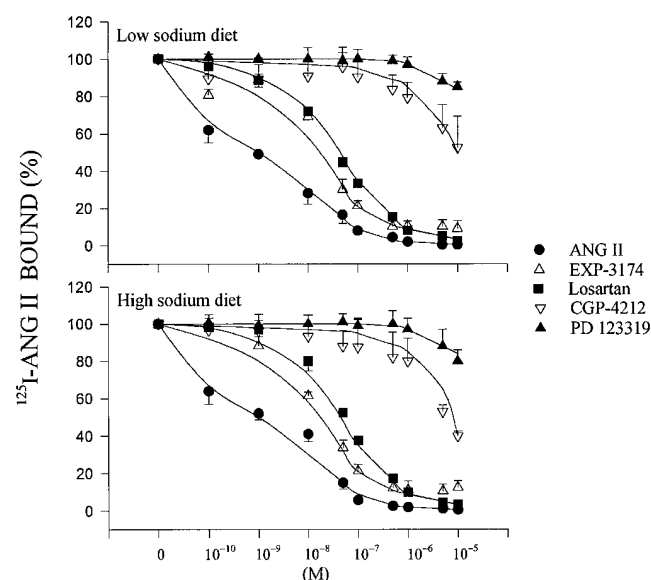
**Table III. Inhibitory Constant (*K<sub>i</sub>*) Values for Peptide and Nonpeptide Antagonists in Displacing <sup>125</sup>I-ANG II (0.375 nM) Binding to Fresh Afferent Arterioles Isolated from Kidneys of 7-Wk—Old Rats**

	<i>K<sub>i</sub></i> <i>nM</i>	<i>n</i>
ANG II	6.0±1.0	4
Exp 3174	9.5±1.5	9
Losartan	37±3.0‡	10
CGP 42112	490±141*§	6
PD-123319	> 10,000	7

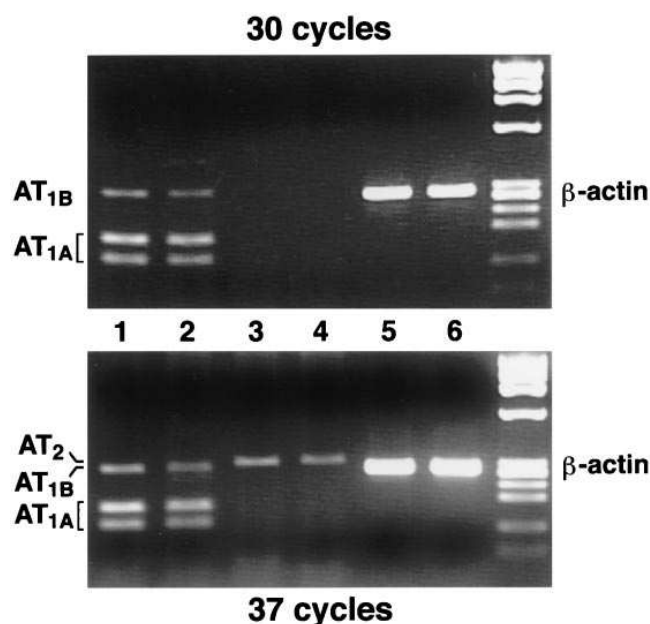
Values are means±SE. *n* = number of experiments; 4 rats/experiment. There were no differences between sodium diets, so the data were pooled. \**P* < 0.05 vs. ANG II, ‡*P* < 0.001 vs. ANG II, §*P* < 0.05 vs. losartan.

tions (5–10 μM). There was no statistically significant displacement when the AT<sub>2</sub> ligand's concentration was less than 1 μM (*P* > 0.1).

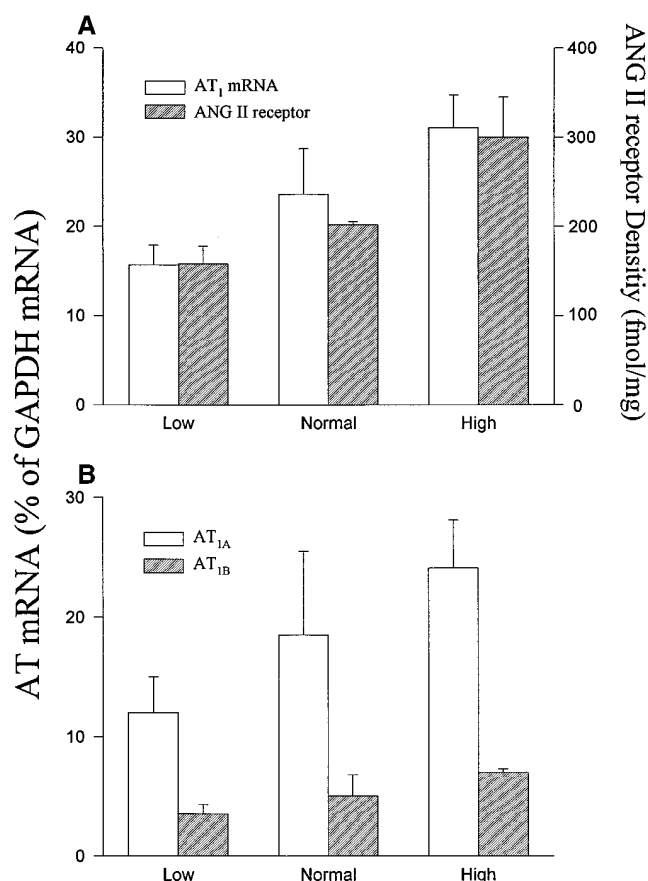
ANG II receptor gene expression was determined to explore the mechanism by which altered activity of the renin-angiotensin system (due to sodium diet) regulated ANG II receptor density. ANG II receptor mRNA levels in the afferent arterioles were determined by RNase protection assay. These resistance vessels expressed two, and perhaps three, subclasses of the ANG II receptors. Expression of AT<sub>1A</sub> and AT<sub>1B</sub> were clearly discernible (Fig. 6, top panel). The AT<sub>1</sub> receptor is the predominant subtype, whereas the AT<sub>2</sub> receptor message showed a much weaker signal. The AT<sub>2</sub> receptor mRNA relative to that of AT<sub>1</sub> was considerably less (~50–100 times less) and more difficult to document with any certainty. RT-PCR



**Figure 5.** Summary of displacement of <sup>125</sup>I-angiotensin II from freshly isolated afferent arterioles by unlabeled angiotensin II, AT<sub>1</sub> receptor antagonists (losartan and EXP-3174) and an AT<sub>2</sub> receptor ligands (PD-123319 and CGP-42112). Each curve is based on three or more experiments.



**Figure 6.** RT-PCR analysis of AT receptor cDNA in freshly isolated afferent arterioles. AT<sub>1A</sub> and AT<sub>1B</sub> receptor cDNA was evident at 30 cycles. An AT<sub>2</sub> receptor cDNA was detected at 37 cycles.



**Figure 7.** (A) The effect of chronic salt diet on the total AT<sub>1</sub> receptor mRNA expression normalized to GAPDH mRNA as determined by ribonuclease protection assay in afferent arterioles in groups consuming different sodium diets. There were parallel changes in ANG II receptor density in radioligand binding studies on afferent arterioles isolated from kidneys of rats consuming a low, normal, or high salt diet. Means  $\pm$  SE for three experiments in each group. (B) Changes in AT<sub>1A</sub> and AT<sub>1B</sub> mRNA in afferent arterioles as a function of sodium diet. Subtype levels were calculated from total AT<sub>1</sub> receptor mRNA determined by RNase protection assay and the ratio of AT<sub>1A</sub> to AT<sub>1B</sub> mRNA assayed by semiquantitative RT-PCR. Means  $\pm$  SE for three experiments in each group. \*Represents statistically significant difference between low- and high-salt groups.

analysis of the afferent arteriole eventually revealed the existence of an AT<sub>2</sub> mRNA signal. At least 35 cycles were required, however, as compared to a signal of similar strength for AT<sub>1</sub> mRNA with fewer than 30 cycles (Fig. 6, bottom).

Compared to the control diet, stimulation of the renin-angiotensin system by low-sodium diet decreased total AT<sub>1</sub> mRNA levels by 34%, but increased levels about 32% when the renin-angiotensin system was inhibited by high sodium intake. As is presented in Fig. 7 (top), quantitative assessment of total AT<sub>1</sub> mRNA levels in afferent arterioles by the RNase protection assay show that the changes in expression correlated with diet treatment. There was an approximate doubling of expression in high- vs. low-sodium diet. There was a close correlation between changes in AT<sub>1</sub> mRNA and ANG II receptor density in the three diet groups (Fig. 7, top). Both measures of receptor expression paralleled each other, exhibiting a twofold difference between low- and high-sodium diets.

To ascertain which AT<sub>1</sub> subtype was regulated during changes in sodium balance, the relative expression of AT<sub>1A</sub> and AT<sub>1B</sub> was analyzed by RT-PCR. The data show that mRNA for both AT<sub>1A</sub> and AT<sub>1B</sub> increased in parallel with diets of increasing sodium content (Fig. 7, bottom). Message for both subtypes increased twofold when high- and low-sodium diets are compared. The ratio of AT<sub>1A</sub>/AT<sub>1B</sub> mRNA averaged about 3.7, and was stable among different diet groups. Thus, the AT<sub>1A</sub> is primary receptor subtype expressed in the smallest preglomerular arterioles. Comparing the results in the lower with the upper panel in Fig. 7 demonstrates that the changes in both AT<sub>1A</sub> and AT<sub>1B</sub> expression were directly related to changes in total AT<sub>1</sub> mRNA and ANG II binding sites in response to salt diet induced changes in renin-angiotensin activity.

## Discussion

The present study provides new information about the expression and function of AT<sub>1</sub> receptor subtypes in the small diameter afferent arteriole, a resistance vessel that contributes significantly to the vascular response to ANG II in the kidney. Our investigation presents a comprehensive evaluation of receptor function incorporating molecular expression of mRNA, biochemical receptor characterization in isolated vascular smooth muscle cells, and functional assessment of vascular reactivity to receptor stimulation *in vivo*. Our blood flow results clearly demonstrate that the regulation of renal vascular reactivity varies directly with sodium intake, and indirectly with activity of the renin-angiotensin system. The chronic adjustments in vascular responsiveness to ANG II depend on at least three underlying mechanisms: receptor expression, receptor protein density, and prior receptor occupation by endogenous ANG II. PCR and RNase protection assays establish that the AT<sub>1A</sub> subtype is the predominant receptor in the afferent arteriole, and that changes in AT<sub>1</sub> receptor expression were accompanied by parallel regulation of AT<sub>1A</sub> and AT<sub>1B</sub> mRNA. In contrast, the AT<sub>2</sub> receptor is weakly expressed and is unaffected by salt diet. *In vitro* biochemical radioligand binding studies performed on freshly isolated afferent arterioles and interlobular arteries indicate increased ANG II receptor density with unchanged affinity during sodium loading. The opposite effects were elicited by activation of renin-angiotensin activity by a chronic low-sodium diet: mRNA for AT<sub>1A</sub> and AT<sub>1B</sub> receptors, ANG II receptor density, and vascular reactivity were downregulated in response to sodium deprivation.

Our animal blood flow studies confirm previous observations in the rat and other species including humans. Although vascular reactivity is known to vary in proportion to salt diet, the precise mechanisms mediating the changes are not clear. Renal blood flow studies indicate that the vascular response to exogenous ANG II is depressed in low-sodium diet rats, and enhanced in sodium-loaded animals (Fig. 2) (9). Part of the acute response to administered ANG II is modulated by high endogenous ANG II levels when the renin-angiotensin system is stimulated by low sodium diet. This was shown by administering enalaprilat acutely to block angiotensin-converting enzyme and subsequent production of ANG II. After suppression of endogenous ANG II formation, exogenous ANG II produced larger changes in renal blood flow in low-salt rats. Earlier studies had demonstrated that angiotensin-converting enzyme inhibition increased the systemic pressor effect of ANG II during sodium restriction, whereas only a small

change occurred in the salt-loaded rats (33). Thus, endogenous levels of ANG II contribute importantly to the difference in renal vascular reactivity to a standard dose of exogenous ANG II observed between animals on low- and high-salt intakes.

Our studies provide new information about the relative distribution of ANG II receptor subtypes in the afferent arteriole, and their regulation in response to differing salt intake. We present evidence demonstrating that the predominant subtype in the afferent arteriole from kidneys of 7-wk-old rats is the AT<sub>1A</sub> receptor, with a ratio of AT<sub>1A</sub> to AT<sub>1B</sub> receptor mRNA of almost 4. This compares with a ratio of 3 for renal tissue in general, and a ratio of 2 for aortic smooth muscle (34, 35). On the other hand, AT<sub>2</sub> receptor expression is considerably lower. Previous studies show that AT<sub>1</sub> mRNA is preferentially expressed in the rat kidney, and is found in glomeruli, juxtaglomerular cells, and epithelial cells (2, 21, 35–38). There is less convincing genetic evidence that the AT<sub>1</sub> receptor is localized to preglomerular resistance vessels or the afferent arteriole in particular. Several investigators failed to find significant expression of AT<sub>1</sub> receptors in preglomerular arteries and arterioles and efferent arterioles other than the localized region of juxtaglomerular cells (37, 39). On the other hand, radioligand binding data clearly demonstrate significant ANG II binding to AT<sub>1</sub> receptors that are antagonized by losartan in large and small preglomerular arteries and arterioles (Fig. 5) (6, 14, 40). Both afferent and efferent arterioles show a high density of AT<sub>1</sub> receptors when localized using antiserum directed against the NH<sub>2</sub> terminus of the receptor (41).

Two sets of our results indicate an increase in AT<sub>1</sub> receptor density in afferent arterioles of animals fed a high-salt diet. Our radioligand binding studies provide new information about renal resistance vessels, and demonstrate a  $B_{\max}$  value that is twofold larger during sodium loading. These findings are reinforced by results obtained using a RNase protection assay which indicates that total AT<sub>1</sub> mRNA expression was twofold greater in animals on a high-sodium diet. Our results for vascular reactivity in vivo lend further support to this view. We observed a similar degree of antagonism of AT<sub>1</sub> receptor by losartan and EXP-3174 in vessels isolated from low- and high-salt rats after the data were normalized for differences in total density. These new observations for the afferent arteriole agree with and extend previous results for renal glomeruli and nonrenal vascular smooth muscle cells. Activation of the renin–angiotensin system by low-sodium diet reduces the density of ANG II receptors in glomeruli, cultured mesangial cells, and mesenteric artery (12, 16, 19, 21, 42–44). A role for ANG II has been demonstrated by studies utilizing chronic infusions of ANG II and by chronic inhibition of ANG production using an ACE inhibitor (16, 19). Some reports, however, describe unresponsive glomerular receptors to low- and high-salt diets, and to administration of ANG II (14). Conflicting results also have been found in cultured rat glomerular mesangial cells. In one case, AT<sub>1</sub> mRNA was downregulated following an increase in ANG II, and the effect was antagonized by losartan (25). On the other hand, there are reports that ANG II has no effect on AT<sub>1</sub> receptor expression in the rat kidney or in human mesangial cells (24, 45).

Few studies have addressed the interesting question of AT<sub>1</sub> receptor subtype regulation in discrete cell types in the kidney. We observed that the ratio of AT<sub>1A</sub> and AT<sub>1B</sub> receptor mRNA in afferent arteriolar smooth muscle cells is unchanged by sodium dietary treatment. This results from parallel regulation of

message for both receptors in the afferent arteriole in response to changes in salt diet and renin–angiotensin activity (Fig. 7). A recent study suggests that one subtype may be regulated independently of another. Sodium deprivation was found to cause an increase in AT<sub>1A</sub> and a decrease in AT<sub>1B</sub> mRNA at the whole kidney level (46). In another report, Northern blot analysis showed that AT<sub>1A</sub> mRNA in renal tissue was increased by losartan treatment in normal and sodium-restricted animals (23). Results obtained by renal cortex or entire kidney tissue, however, should be interpreted with caution because of probable differential regulation in the multiple cell types. As a case in point, there are major differences in the regulation of AT receptors in vessels and tubules in the kidney. In contrast to the renal vasculature (8, 21, 25, 47), increased ANG II levels are associated with upregulation of AT<sub>1</sub> mRNA and ANG II receptor density in proximal tubular cells and isolated basolateral membranes, and also in the adrenal gland (2, 18, 21–23).

The present radioligand binding results provide another line of evidence for regulation of AT<sub>1</sub> receptors in the afferent arteriole. Afferent arterioles in rats maintained on low- or high-sodium diets, as well as those fed a standard, express AT<sub>1</sub> receptors antagonized by losartan (Fig. 5) (6). In all of the dietary groups, 80–90% of the ANG II binding sites display high affinity to AT<sub>1</sub> antagonists. Losartan also is reported to displace ANG II binding in larger preglomerular arteries (> 100  $\mu$ m) in normal rats (14, 40). The present study confirms previous reports from our laboratory showing that AT<sub>2</sub> receptor antagonists do not displace ANG II from vascular binding sites, and that they fail to affect the renal vasoconstriction elicited by ANG II in rats maintained on their standard sodium diet (6). The function of ANG II AT<sub>2</sub> receptor is unknown, but some studies found that AT<sub>2</sub> sites are abundant in rat fetus, suggesting that AT<sub>2</sub> receptors play a role during development (5, 48, 49). Other studies suggest AT<sub>1</sub> receptor(s) are critical to normal growth and development (48, 50).

Our blood flow results agree well with our receptor binding studies. As previously noted for animals on normal salt diet (6), the AT<sub>1</sub> receptor antagonist losartan inhibited up to 80% of the ANG II-induced renal vasoconstriction in vivo and 80% of the binding to isolated afferent arteriolar segments. Aside from the difference in absolute receptor number, percentage changes in inhibition of ANG II binding and vasoconstriction were similar in rats maintained on low- or high-sodium diets for 3 wk. Based on our earlier blood flow studies and ligand binding experiments, it is reasonable to conclude that losartan preferentially binds to and antagonizes the AT<sub>1</sub> receptor subtype in the afferent arteriole (6). The specific functions of AT<sub>1A</sub> and AT<sub>1B</sub> receptors in cells that normally express them in rodents is not known. Almost all known ANG II effects are thought to be mediated by the predominant receptor type, the AT<sub>1</sub> receptor, with a tacit assumption that physiological responses are caused largely if not exclusively mediated by the AT<sub>1A</sub> subtype. Currently available pharmacological ligands, however, do not distinguish between AT<sub>1</sub> receptor subtypes. Recent studies on mice with the AT<sub>1A</sub> receptor mutated by gene targeting suggest that the AT<sub>1B</sub> as well as the AT<sub>1A</sub> receptor may play a role in blood pressure control (51).

In summary, our data demonstrate the existence of two subtypes of AT<sub>1</sub> receptors, AT<sub>1A</sub> and AT<sub>1B</sub> in small-diameter, preglomerular renal resistance vessels. The AT<sub>1A</sub> subtype is predominant with expressed levels almost four times greater than those of the AT<sub>1B</sub> receptor mRNA in the afferent arteri-



ole. In contrast, there is a paucity of AT<sub>2</sub> receptors. Regulation of ANG II AT<sub>1</sub> receptor mRNA expression is associated with a change in sodium diet and presumed activity of the renin-angiotensin system. Variations in sodium intake produce similar changes in the AT<sub>1</sub> receptor in renal resistance vessels as evidenced by parallel message changes for AT<sub>1A</sub> and AT<sub>1B</sub> receptors during up and downregulation in response to salt diet. Changes observed in the binding density of ANG II receptors agreed well with those in total AT<sub>1</sub> expression; both indicated a twofold increase in high-sodium vs. low-sodium rats. A larger difference was suggested in blood flow studies, as the renal vasoconstriction elicited by administered ANG II was three to four times greater in the high-salt animals. After acute blockade of ANG II production by an angiotensin converting enzyme inhibitor, however, the ANG II-induced renal vasoconstriction increased in animals with an activated renin-angiotensin system such that the difference between groups was reduced to about 1.5 times. Thus, there was a close correlation between AT<sub>1</sub> receptor mRNA, AT<sub>1</sub> binding sites, and renal blood flow responses to injected ANG II during variations in sodium diet. Sodium restriction produces a reduction in ANG II AT<sub>1</sub> mRNA level, translating into reduced receptor protein synthesis, with the decreased density of cell surface binding sites responsible for attenuated vascular reactivity to administered ANG II.

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