

Nuclear angiotensin II type 2 (AT₂) receptors are functionally linked to nitric oxide production

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Gwathmey TM, Shaltout HA, Pendergrass KD, Pirro NT, Figueroa JP, Rose JC, Diz DI, Chappell MC. Nuclear angiotensin II type 2 (AT₂) receptors are functionally linked to nitric oxide production. *Am J Physiol Renal Physiol* 296: F1484–F1493, 2009. First published February 25, 2009; doi:10.1152/ajprenal.90766.2008.— Expression of nuclear angiotensin II type 1 (AT₁) receptors in rat kidney provides further support for the concept of an intracellular renin-angiotensin system. Thus we examined the cellular distribution of renal ANG II receptors in sheep to determine the existence and functional roles of intracellular ANG receptors in higher order species. Receptor binding was performed using the nonselective ANG II antagonist ¹²⁵I-[Sar¹,Thr⁸]-ANG II (¹²⁵I-sartran) with the AT₁ antagonist losartan (LOS) or the AT₂ antagonist PD123319 (PD) in isolated nuclei (NUC) and plasma membrane (PM) fractions obtained by differential centrifugation or density gradient separation. In both fetal and adult sheep kidney, PD competed for the majority of cortical NUC (≥70%) and PM (≥80%) sites while LOS competition predominated in medullary NUC (≥75%) and PM (≥70%). Immunodetection with an AT₂ antibody revealed a single ~42-kDa band in both NUC and PM extracts, suggesting a mature molecular form of the NUC receptor. Autoradiography for receptor subtypes localized AT₂ in the tubulointerstitium, AT₁ in the medulla and vasa recta, and both AT₁ and AT₂ in glomeruli. Loading of NUC with the fluorescent nitric oxide (NO) detector DAF showed increased NO production with ANG II (1 nM), which was abolished by PD and *N*-nitro-*L*-arginine methyl ester, but not LOS. Our studies demonstrate ANG II receptor subtypes are differentially expressed in ovine kidney, while nuclear AT₂ receptors are functionally linked to NO production. These findings provide further evidence of a functional intracellular renin-angiotensin system within the kidney, which may represent a therapeutic target for the regulation of blood pressure.

nucleus; kidney; intracellular renin angiotensin system

ANG II, THE MAJOR BIOACTIVE component of the renin-angiotensin system (RAS) functions to induce a multitude of events that contribute to the regulation of blood pressure, body fluid volume, and electrolyte balance. The actions of ANG II include vasoconstriction, stimulation of tubular sodium reabsorption and aldosterone release, oxidative stress, and inflammation. The majority of these actions are thought to be mediated through the AT₁ receptor subtype. AT₁ receptor antagonists (ARBs) are now one of the major classes of blood pressure-lowering therapies associated with target organ protection. However, ANG II may also interact with the G protein-coupled AT₂ receptor, which generally opposes or attenuates the actions of the ANG II-AT₁ axis (6, 7). The role of the AT₂

receptor may be particularly relevant during ARB therapy, as the circulating levels of ANG II markedly increase due to the disinhibition of ANG II feedback on renin release.

The classic view by which ANG II exerts its cellular actions is by binding to receptors embedded in the plasma membrane, which induces a conformational change in the receptor and an ensuing cascade of downstream signaling events that may include activation of various kinases and phosphatases (12, 15, 59). This tenet for peptide hormones and their G protein-coupled receptors has undergone a revision as a number of studies have demonstrated a role for intracellular or “intracrine” ANG II (3, 4, 11, 13, 27–29, 64). Similarly, the localization of intracellular ANG II has been reported in several tissues, including heart and kidney (31, 33, 41, 60–61). Accordingly, recent studies in the rat have demonstrated that ANG II receptors in the kidney can be associated with the cell nucleus as well as the plasma membrane (39, 40, 48). Indeed, we reported the expression of nuclear AT₁ receptors in the medullary and cortical regions of the mRen2.Lewis hypertensive rat (48). However, the characterization of intracellular ANG II receptors in species more closely related to humans is important, particularly if an intracellular angiotensin system is relevant to cardiovascular disease. Moreover, the functional role of these intracellular receptors has yet to be established. Therefore, the objective of the current studies was to determine the expression and functional significance of intracellular ANG II receptor subtypes in the sheep kidney, an animal model in which renal development and nephrogenesis closely parallel that of humans (18).

MATERIALS AND METHODS

Animals. Tissues were obtained from 14 adult (8 males, 6 females; 1.5 yr of age) and 6 fetal (3 males, 3 females; 139 days gestation; term = 145–150 days) mixed-breed sheep housed in the Wake Forest University School of Medicine animal facility. Animals were fed a normal diet with access to water ad libitum, and maintained on a 12:12-h light-dark cycle. Kidneys were obtained from animals anesthetized with ketamine and halothane, dissected in saline on ice into renal cortex and medulla, then immediately frozen on dry ice and stored at –80°C or processed at 4°C for density gradient separation (see below). All procedures were approved by the Institutional Animal Care and Use Committee at Wake Forest University School of Medicine.

Preparation of nuclei and plasma membrane by differential centrifugation. Frozen tissue (~500 mg) was homogenized in buffer containing 25 mM KCl, 5 mM MgCl₂, 20 mM Tricine-KOH, and 25 mM sucrose (pH 7.8) utilizing a Polytron Ultra-Turrax T25 Basic (setting 4), followed by a Barnant Mixer (Series 10, setting 3). The homogenate was passed through a 100-μm mesh filter and centrifuged twice at 1,000 g (4°C) for 10 min to obtain the nuclear fraction. The

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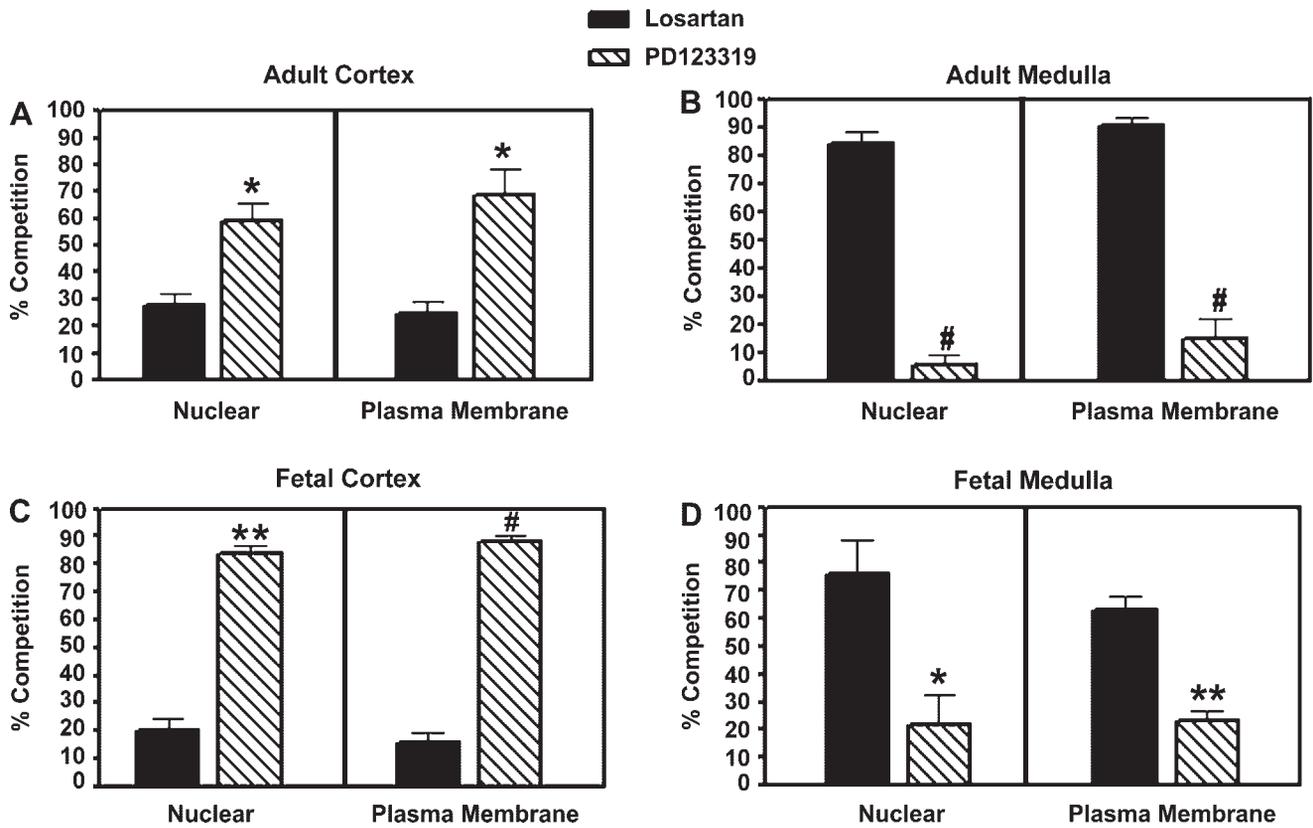


Fig. 1. Characterization of ovine renal angiotensin receptor subtypes in isolated nuclei and plasma membrane fractions of adult (1.5 yr of age; $n = 8$) cortex (A) and medulla (B) and fetal (139 days' gestation; $n = 4$) cortex (C) and medulla (D). Cellular fractions were derived by differential centrifugation of homogenates from equivalent wet tissue weight of fetal and adult cortices and medullas. Competition binding was carried out using 0.5 nM ¹²⁵I-sartran and receptor antagonists at a final concentration of 10 μ M. Values are means \pm SE. * $P < 0.01$ vs. losartan. ** $P < 0.001$ vs. losartan. # $P < 0.0001$ vs. losartan.

resultant supernatant was centrifuged at 25,000 g for 20 min (4°C), yielding the plasma membrane fraction.

Preparation of nuclei by OptiPrep density gradient separation. Apart from the crude preparation of nuclei obtained by differential centrifugation, an additional pure fraction of cortical and medullary nuclei was obtained by an isosmotic density gradient separation. As described above, renal cortices and medullas were homogenized and centrifuged at 1,000 g for 10 min (4°C), the pellet was resuspended in 20% OptiPrep solution (Accurate Chemical and Scientific, Westbury, NY) according to manufacturer's recommendations and layered on a discontinuous density gradient column. The columns, consisting of descending layers of 10, 20, 25, 30, and 35% OptiPrep solution to form the gradient, were centrifuged at 10,000 g for 20 min (4°C). The enriched fraction of isolated nuclei was recovered at the 30–35% layer interface (48).

ANG II receptor radioligand binding. Characterization of angiotensin receptor binding was performed as previously described (9, 48). Briefly, isolated nuclei and plasma membrane, as prepared above, were suspended in HEPES buffer supplemented with 0.2% BSA (pH 7.4) and coincubated with the radioligand ¹²⁵I-[Sar¹,Thr⁸]-ANG II (¹²⁵I-sartran) in the presence of losartan (the AT₁-receptor antagonist), PD123319 (the AT₂-receptor antagonist), or nonlabeled sartran. The final concentrations of all receptor antagonists used were 10 μ M. Sartran was radiolabeled with Na¹²⁵I using the chloramine T method and purified by HPLC as described (9).

These initial binding assays were carried out in fresh renal cortices and medullas. Frozen tissue paired to fresh tissue samples from each animal were similarly used in radioligand binding assays to assess the impact of freezing on receptor binding. After an identical receptor subtype profile between fresh and frozen tissues (data not shown) was

established, all subsequent experiments were conducted using tissue stored at -80°C.

Western blotting and immunodetection. Samples of renal homogenate were retained and assayed for protein analysis and Western blotting. Cellular fractions were suspended in PBS and added to Laemmli buffer containing mercaptoethanol. Proteins were sepa-

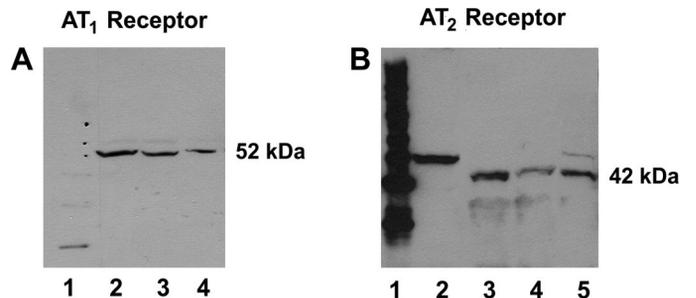


Fig. 2. Intracellular ANG II receptor subtype immunoreactivity of adult kidney cortex. A: ANG II type 1 (AT₁) receptor immunoreactivity using rabbit anti-human polyclonal antibody (1:5,000) and anti-rabbit IgG (1:5,000). The full-length gel represents molecular weight marker (lane 1) and kidney cortex nuclear fraction from 3 distinct animals (lanes 2–4). B: AT₂ receptor immunoreactivity utilizing rabbit anti-human polyclonal antibody (1:500) and anti-rabbit IgG (1:3,000). The full-length gel represents molecular weight marker (lane 1), adrenal plasma membrane (lane 2), kidney cortex nuclear fraction (lane 3), kidney cortex plasma membrane (lane 4), and adrenal nuclear fraction (lane 5). Differences in molecular weights between renal and adrenal AT₂ immunoreactivity may reflect varying degrees of glycosylation of this protein between tissue types.

Table 1. Renal ANG II receptor density and binding affinity

Kidney Region	K _D , nM	B _{max} , fmol/mg	R ²	n
Cortex				
Nucleus	2.67	24.05	0.88	4
Plasma membrane	10.79	96.95	0.91	4
Medulla				
Nucleus	7.63	337.30	0.76	4
Plasma membrane	2.92	36.65	0.87	4

Saturation binding and Scatchard analysis were performed on isolated nuclei and plasma membranes from the renal cortex and medulla using increasing concentrations of the specific angiotensin receptor antagonist ¹²⁵I-[Sar¹, Thr⁸]-ANG II (sarthran). Receptor density and affinity are defined as B_{max} and K_D, respectively. Nonspecific binding was obtained by the addition of 10 μM unlabeled sarthran.

rated on 10% SDS polyacrylamide gels for 1 h at 120 V in Tris-glycine buffer and electrophoretically transferred onto polyvinylidene difluoride membranes. Immunodetection was performed on blots blocked for 1 h with 5% dry milk (Bio-Rad) and Tris-buffered saline containing 0.05% Tween, then probed with antibodies against annexin II (1:5,000; BD Transduction Laboratories, San Diego, Ca), nuclear pore complex proteins (1:2,500; Abcam, Cambridge, MA), AT₁ (1:5,000; Alpha Diagnostics, San Antonio, TX), AT₂ (1:500; Life Span Biosciences, Seattle, WA), endothelial nitric oxide synthase (eNOS; 1:500; Upstate Cell Signaling Solutions, Lake Placid, NY), and soluble guanylate cyclase (sGC; 1:200; Cayman Chemical, Ann Arbor, MI). Reactive proteins were detected with Pierce Super Signal West Pico Chemiluminescent

substrates and exposed to Amersham Hyperfilm enhanced chemiluminescence (Piscataway, NJ).

Receptor autoradiography. A piece of kidney tissue taken at necropsy was frozen on dry ice, covered with Tissue Tek-Optimum Cutting Temperature (OCT) Embedding medium (Ft. Washington, PA) and stored at -80°C until use. Sections (14 μm) of kidney were treated with 5 μM receptor antagonists and incubated with 0.2 nM ¹²⁵I-sarthran. Nonspecific labeling was obtained by preincubation with unlabeled sarthran. Tissue slides were exposed against Kodak Biomax MR X-ray film, and quantification of autoradiograms was performed using an MCID image-analysis system (Micro Computer Imaging Device, Imaging Research, Ontario, Canada).

Measurement of nitric oxide production. Isolated cortical nuclei from adult sheep kidney, prepared by OptiPrep density gradient separation as described above, were preincubated with the fluorescence dye 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF; 5 μg/ml; Molecular Probes, Invitrogen) in buffer containing 140 mM NaCl, 14 mM glucose, 4.7 mM KCl, 2.5 mM CaCl₂, 1.8 mM MgSO₄, 1.8 mM KH₂PO₄, and 100 μM L-arginine (pH 7.4) for 30 min at 37°C. Nuclei were washed twice in HEPES buffer to remove any unbound dye, then incubated with 1 nM ANG II in the presence of losartan (the AT₁-receptor antagonist), PD123319 (the AT₂-receptor antagonist), the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME; 1 mM) or buffer alone. Increases in DAF fluorescence, indicative of nitric oxide (NO) production, were measured using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA) at wavelengths of 488 (excitation) and 510 nm (emission). DAF has a detection limit of 5 nM and does not react with other stable oxidized forms of NO, such as NO₂, or reactive oxygen species (36).

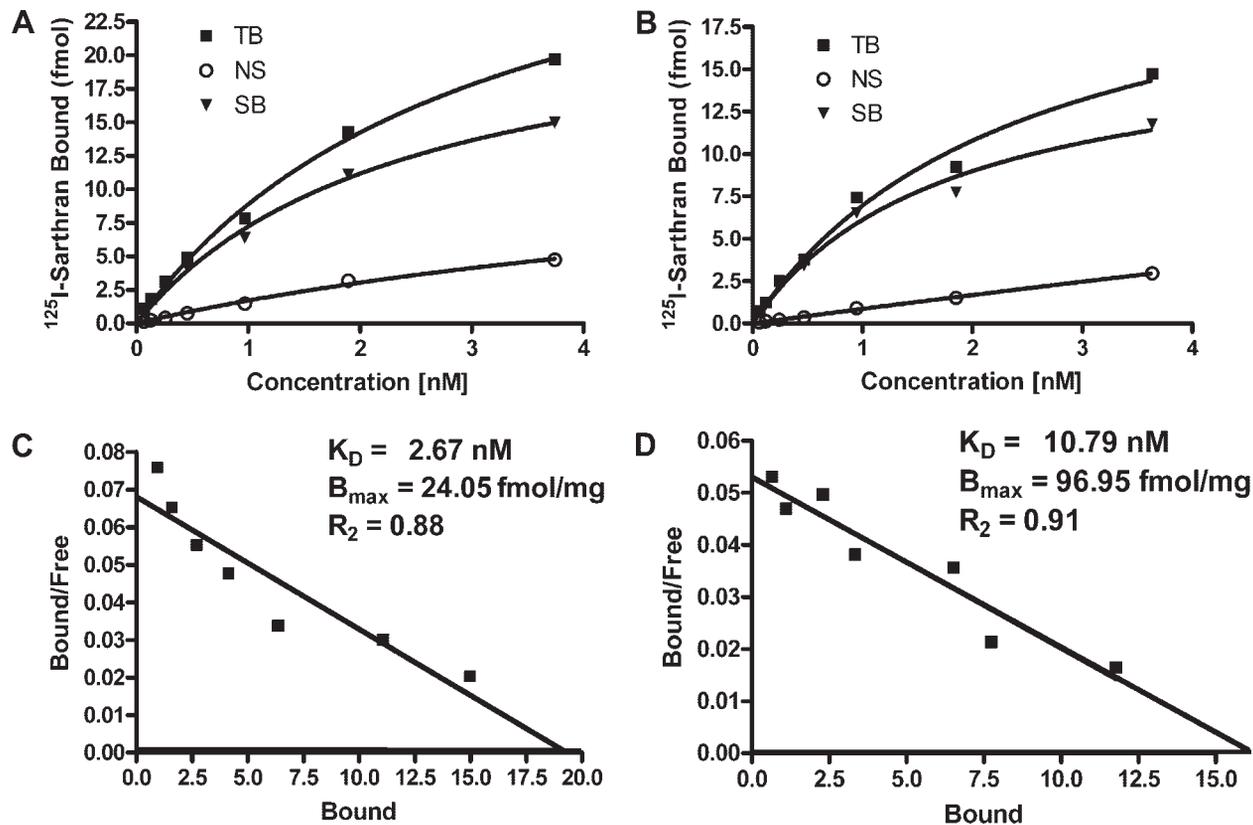


Fig. 3. Representative saturation binding for ANG II receptors in nuclear (A) and plasma membrane (B) fractions obtained by differential centrifugation of the renal cortex. Scatchard analysis for isolated cortical nuclei (C) and plasma membrane (D) is shown. Saturation binding and Scatchard analysis were performed with increasing concentrations of the specific receptor antagonist ¹²⁵I-sarthran. Nonspecific binding was determined by use of 10 μM unlabeled sarthran. The receptor density and affinity are defined as B_{max} and K_D, respectively.

Statistical analysis. Data are represented as means \pm SE. A paired Student's *t*-test, one-way ANOVA with Tukey's multiple comparison post hoc, and nonlinear regression were performed using GraphPad Prism 4.0 plotting and statistical software.

RESULTS

Determination of ANG II receptor subtypes. The relative proportions of ANG II receptor subtypes were determined by use of specific ANG II receptor antagonists. Both nuclei and plasma membrane fractions isolated from the cortex and medulla of the sheep kidney by differential centrifugation exhibit specific binding with the nonselective ANG II receptor antagonist ¹²⁵I-sartran. As shown in Fig. 1A, PD123319, the selective AT₂-receptor antagonist, competes for the majority of cortical ANG II receptors in both the nuclear and plasma membrane fractions of the adult kidney. Conversely, in the medulla, competition by the AT₁ antagonist losartan resulted in \sim 90% displacement of ¹²⁵I-sartran binding in both nuclei and plasma membrane fractions (Fig. 1B). To determine whether the distribution of ANG II receptor subtypes in adult ovine kidney (1.5 yr of age; approximately equivalent to an 18- to 20-yr-old human) differed significantly from that during fetal development, when AT₂ expression is thought to be greatest, the expression of ANG II receptors in fetal kidney (139-day gestation) was examined. Similar to the adult, competition by PD123319 represented $>$ 85% of ANG II receptor binding in the nuclei and plasma membranes of the fetal kidney cortex

(Fig. 1C), while losartan competition in the fetal medulla was 76 and 63% in the nuclei and plasma membranes, respectively (Fig. 1D).

To further illustrate the expression of intracellular ANG II receptors within the adult ovine kidney, immunoblots of isolated cortical nuclei were probed with an antibody directed against the AT₁ subtype and revealed a single 52-kDa band (Fig. 2A). Similarly, immunoblots of both cortical nuclei and plasma membranes were probed with an AT₂ antibody, which yielded a single band at \sim 42 kDa (Fig. 2B). Homogenates of sheep adrenal tissue, known to express a high density of AT₂ receptors, were used as a positive control for AT₂ immunoreactivity.

Receptor density and binding affinity. As indicated in Table 1, the density of ANG II receptors (B_{\max}) in the plasma membranes of the cortex was significantly greater than that of the nuclear membranes ($n = 4$; $P < 0.05$); albeit the cortical nuclear receptors demonstrate a higher binding affinity (K_D) than those of the plasma membranes ($P < 0.05$). Conversely, the medullary nuclei possess a 10-fold greater density of receptors than found in the medullary plasma membranes ($n = 4$; $P < 0.001$), while the affinity of these receptors (K_D) was lower than that of the plasma membrane receptors in this same region ($P < 0.05$). As demonstrated in the representative saturation binding curves and Scatchard analysis for the renal cortex (Fig. 3) and the renal medulla (Fig. 4), linear plots were

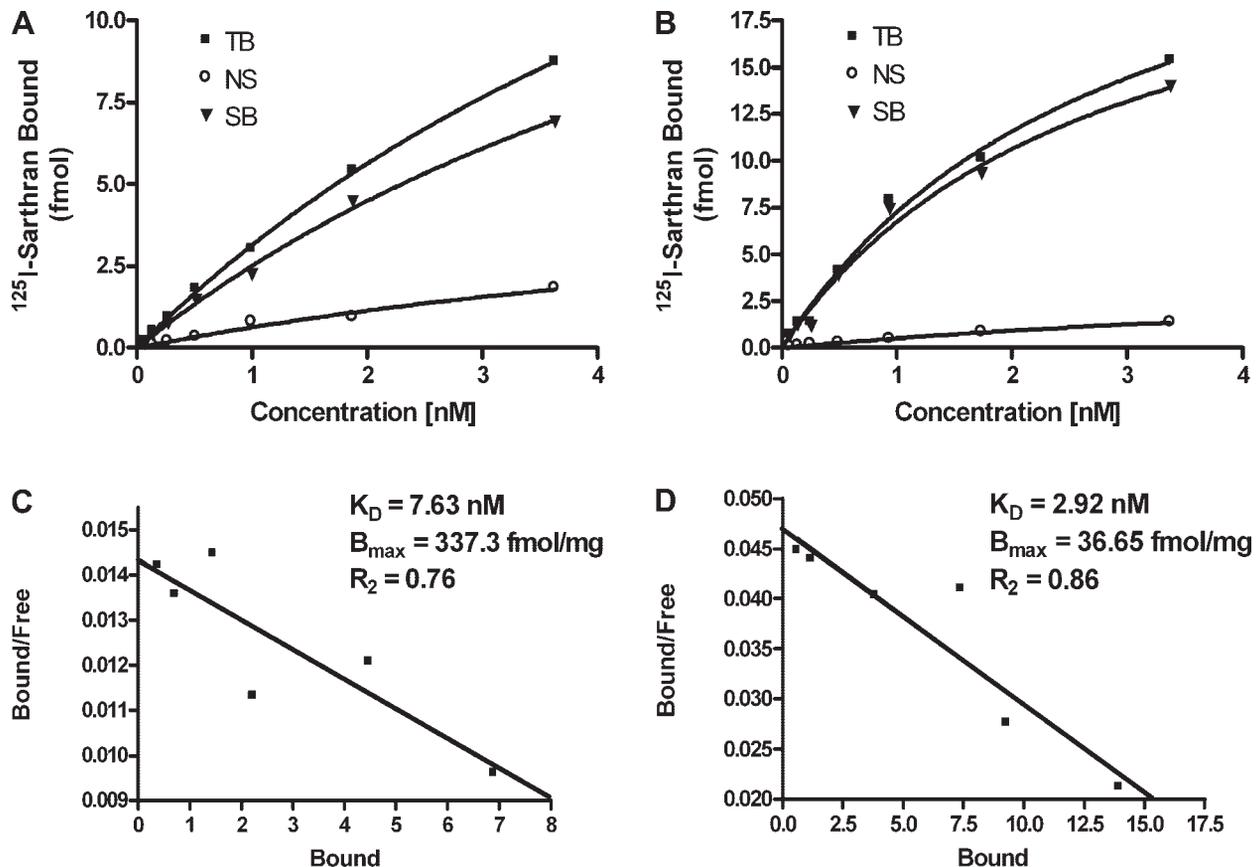


Fig. 4. Representative saturation binding for ANG II receptors in nuclear (A) and plasma membrane (B) fractions obtained by differential centrifugation of the renal medulla. Scatchard analysis of isolated medullary nuclei (C) and plasma membrane (D) is shown. Saturation binding and Scatchard analysis were performed with increasing concentrations of the specific receptor antagonist ¹²⁵I-sartran. Nonspecific binding was determined by use of 10 μ M unlabeled sartran.

obtained for both nuclear and plasma membrane fractions, indicating a single population of binding sites.

Autoradiographic analysis of ANG II receptor subtypes in sheep kidney. The distribution of ANG II receptor subtypes, as detected using ¹²⁵I-sartran in both fetal (Fig. 5A) and adult (Fig. 5B) sheep kidney, is similar. Autoradiograms show dense binding in the inner medulla and area of the vasa recta, the tubulointerstitial area, and glomeruli. Blockade of AT₁ receptors with the antagonist losartan reveals receptors remaining throughout the tubulointerstitial area of the cortex, consistent with the presence of AT₂ sites in this region. Binding in the inner medulla and area of the vasa recta is visibly reduced, suggesting predominant localization of AT₁ receptors to these regions. Incubation with the AT₂ antagonist PD123319 essentially abolishes binding in the tubulointerstitial area of the cortex, although there is evidence of remaining binding in glomeruli. Blockade of AT₂ receptors does not abolish binding in the medullary region or area of the vasa recta, the area of dense AT₁ receptors as indicated by nuclear/plasma membrane binding assays.

Nuclear ANG II receptor binding. To ensure the specificity of nuclear ANG II receptor binding, ¹²⁵I-sartran binding assays were performed with isolated nuclei derived from OptiPrep density gradients. Receptor binding utilizing isolated nuclei from OptiPrep medium yielded receptor profiles essentially identical to those observed in nuclei from differential centrifugation (Fig. 6A). Hematoxylin and eosin staining was

performed on enriched nuclei from the OptiPrep gradient, demonstrating intact nuclei (data not shown).

Immunoblot analysis of membrane fractions. To distinguish nuclear and plasma membrane receptor populations, anti-annexin II, a plasma membrane cellular marker, was employed to confirm the purity of the OptiPrep nuclear preparation. As shown in Fig. 6B, immunoblots revealed a single 36-kDa band for sheep kidney cortical plasma membranes. The nuclear fraction of the sheep kidney showed no immunoreactive band, indicating the absence of any contamination by plasma membrane receptors. Similarly, Fig. 6C demonstrates immunodetection of the nuclear pore complex, as indicated by a single 62-kDa band for cortical nuclei.

ANG II-mediated induction of NO through nuclear ANG receptors. To assess whether the nuclear AT₂ receptors are functional, nuclei were isolated from fresh adult renal cortices by OptiPrep density gradient separation and preincubated with the selective NO fluorescence detector DAF. Stimulation with 1 nM ANG II elicited a significant increase in DAF fluorescence, which was abolished by the AT₂-receptor antagonist PD123319 but not by the AT₁ antagonist losartan (Fig. 7A). In similar fashion, the ANG II-mediated production of NO was abrogated by the NO synthase inhibitor, L-NAME. Representative tracings of ANG II-stimulated DAF fluorescence and inhibition with antagonists are presented in Fig. 7, B–D.

Protein analysis showed the presence of endothelial NOS (eNOS/NOSIII, 135 kDa) (Fig. 8A) as well as soluble guany-

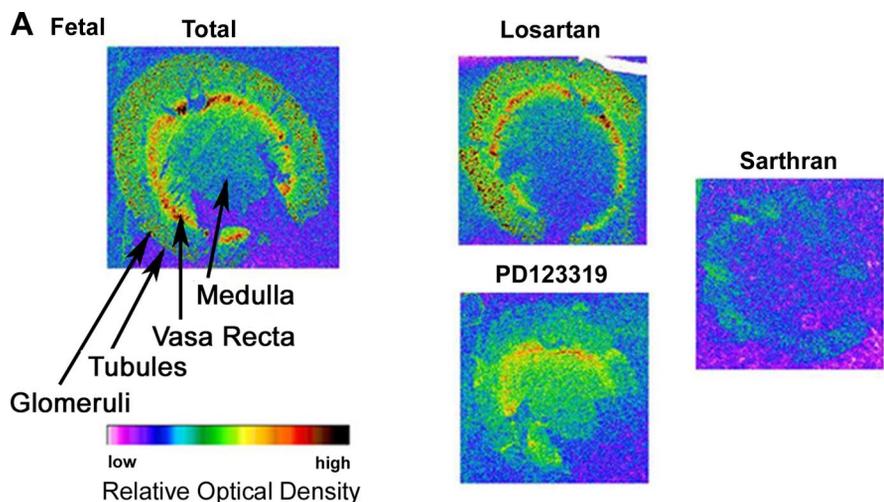
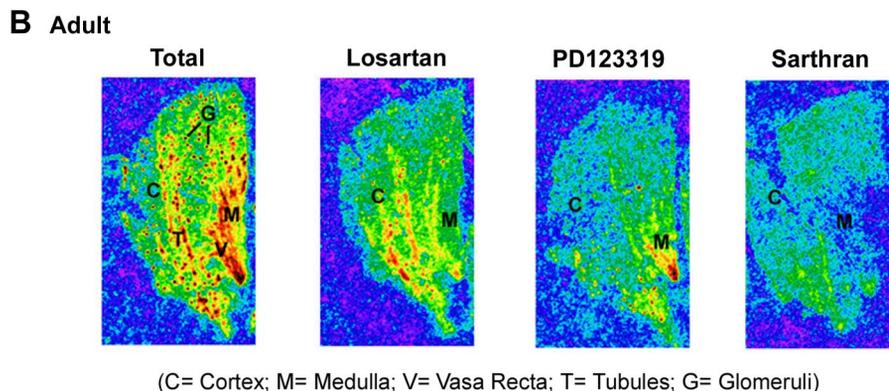


Fig. 5. Autoradiography of fetal (A) and adult (B) sheep kidney. Frozen-thawed kidney sections were incubated with 5 μ M receptor antagonists and probed with 0.2 nM ¹²⁵I-sartran. Nonspecific labeling was obtained by preincubation with unlabeled sartran.



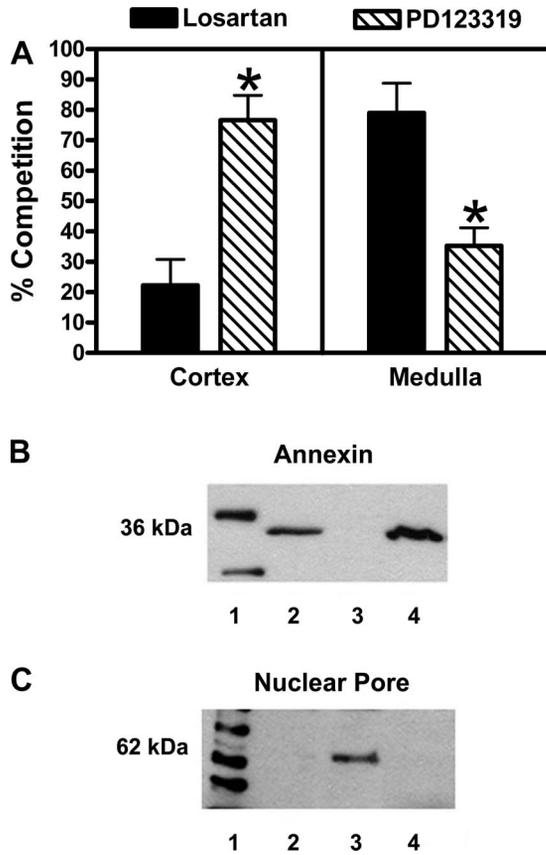


Fig. 6. Characterization of angiotensin receptor subtypes in isolated renal cortical and medullary nuclei derived by OptiPrep density gradient. *A*: competition binding was carried out using 0.5 nM ¹²⁵I-sartran and receptor antagonists at a final concentration of 10 μM. Values are means ± SE (*n* = 3; **P* = 0.02). *B*: immunodetection of cortical nuclei and plasma membrane fractions of fetal kidney. Annexin, a plasma membrane-specific cellular marker was used to distinguish nuclear and plasma membrane fractions: molecular weight marker (*lane 1*), rat kidney cortex plasma membrane (*lane 2*), sheep kidney cortex nuclear fraction (*lane 3*), and sheep kidney cortex plasma membrane (*lane 4*). *C*: nuclear pore complex protein immunoreactivity: molecular weight marker (*lane 1*), rat kidney cortex plasma membrane (*lane 2*), sheep kidney cortex nuclear fraction (*lane 3*), and sheep kidney cortex plasma membrane (*lane 4*).

late cyclase (70 kDa), the principal NO receptor (Fig. 8*B*), in full-length immunoblots of isolated cortical nuclei.

DISCUSSION

In the present study, we characterized angiotensin receptor expression in the sheep kidney and demonstrated the expression of both nuclear AT₁ and AT₂ receptors in fetal and adult kidney, where functional expression of nuclear AT₂ receptors in the cortex is linked to the production of NO. As revealed by ¹²⁵I-sartran binding and competition with selective ANG II-receptor antagonists, the AT₁ is the predominate receptor subtype expressed in the sheep renal medulla, while the renal cortex expresses primarily AT₂ receptors. To date, the majority of studies characterizing renal ANG II receptor expression have been conducted using either rat or mouse models, where very little AT₂ expression is observed in the kidney at adulthood (6, 7, 39, 40, 48). Indeed, we reported nearly complete competition of ¹²⁵I-sartran binding with the AT₁ antagonists losartan or candesartan, demonstrating AT₁ as the predominant

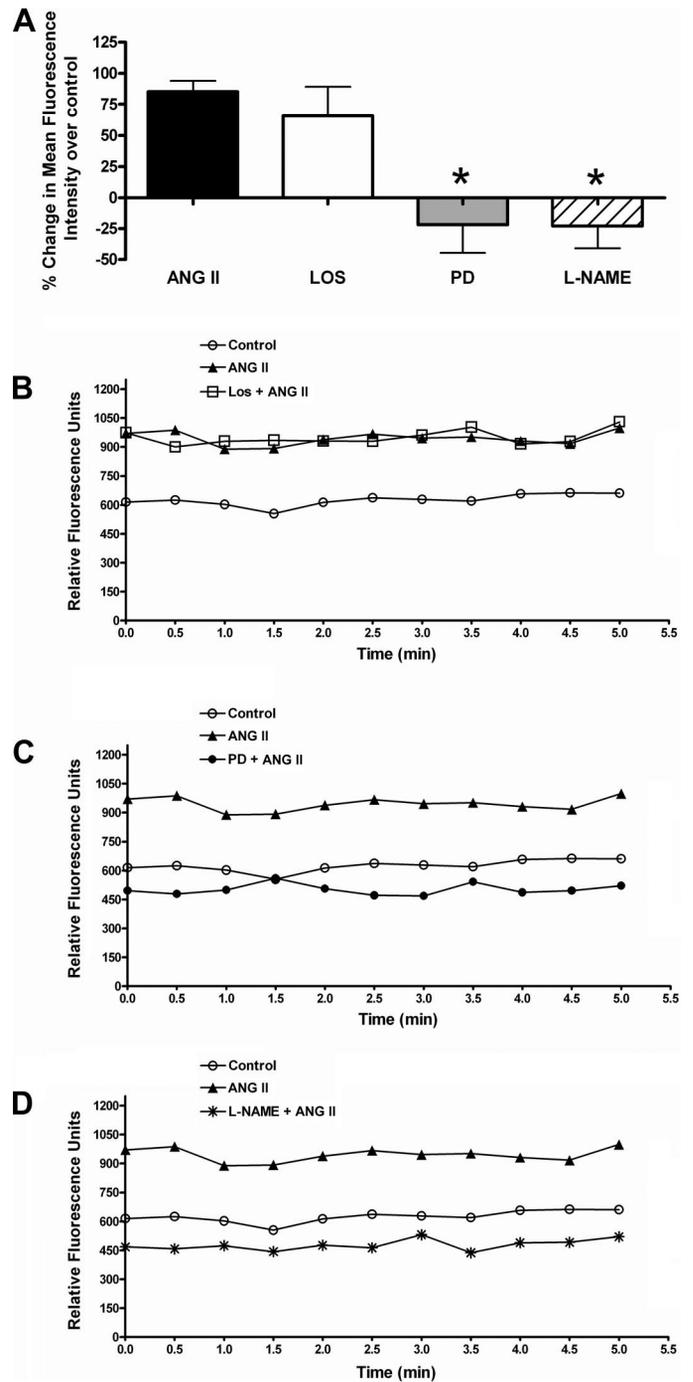


Fig. 7. ANG II-mediated production of nitric oxide (NO) through nuclear receptors. Fresh renal cortical nuclei were isolated from adult sheep kidneys by OptiPrep density gradient separation and preincubated with the selective NO fluorescence detector DAF. Values are expressed as % change in mean fluorescence intensity over control (baseline). *A*: nuclei were stimulated with ANG II (1 nM) in the presence of the AT₁ antagonist losartan (1 μM), the AT₂ antagonist PD123319 (1 μM), or the nitric oxide synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester (L-NAME; 1 mM). Values are means ± SE; *n* = 6, **P* < 0.05 vs. ANG II. Representative DAF fluorescence tracings over a 5-min time course in the presence of the AT₁ antagonist losartan (*B*), the AT₂ antagonist PD123319 (*C*), or the NOS inhibitor L-NAME (*D*) following a 2-minute prestimulation with 1 nM ANG II are also shown. Data are expressed as relative fluorescence units.

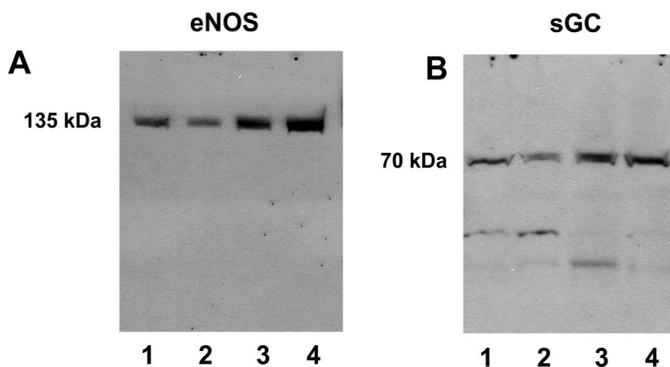


Fig. 8. Immunodetection of endothelial NOS (eNOS) and soluble guanylate cyclase (sGC). Isolated cortical nuclei were probed with antibodies raised against eNOS (A) and sGC (B). In each full-length gel, lanes 1–4 represent samples from 4 distinct animals.

receptor subtype in both the cortex and medulla of the Lewis and mRen2.Lewis rat kidney (48). In contrast, AT₂ sites represent the predominant receptor subtype in the large preglomerular vessels and perivascular region of the human (5, 21) and nonhuman primate (17) cortex. AT₂ receptor mRNA expression during fetal development in humans parallels that of sheep, in that AT₂ receptors are detected early in nephrogenesis and decline abruptly with the end of nephron differentiation (18, 50, 53), although a significant number of AT₂ receptors remain postnatally. Thus both sheep and human adult kidneys express significant AT₂ receptors, in marked contrast to the rat kidney.

The density of ANG II receptors was significantly greater in the cortical plasma membrane vs. the nuclear fraction; however, medullary nuclei expressed a larger number of receptors than the plasma membranes. The differences in receptor density between the nuclei and plasma membranes of the sheep kidney may reflect internalization and trafficking of the receptors into the cell, and ultimately to the nucleus. It is well established that the AT₁ receptor undergoes rapid internalization following binding of extracellular ANG II, which results in trafficking of both the peptide and receptor into intracellular compartments (31, 37). Importantly, the ANG II-AT₁ receptor internalization is thought to contribute to the functional effects of AT₁ activation (4, 34, 52, 58). Saturation binding and Scatchard analysis indicate the density of ANG II receptors in the medulla, consisting predominantly of AT₁, is greater in the nucleus than in the plasma membrane. In this regard, the ligand-induced internalization of the AT₁ may contribute to the greater density of intracellular receptors within the nuclei of the renal medulla. However, the density of cortical ANG II receptors, primarily AT₂, is greater in the plasma membrane fraction. These observations are not surprising, as the AT₂ receptor is not considered to undergo internalization and trafficking. Thus nuclear AT₂ receptors within the kidney cortex may entail the direct transport of these receptors to the nucleus. Salomone and colleagues (51) report that a cytosolic pool of AT₂ receptors may translocate and fuse to the plasma membrane in tubular epithelial cells following dopamine stimulation. Senbonmatsu and coworkers (54), however, reported colocalization of AT₂ with promyelocytic zinc finger protein, which promotes internalization of AT₂ receptors into the perinuclear space in Chinese hamster ovary K1 cells. This mechanism may contrib-

ute to the population of nuclear AT₂ receptors; however, the source(s) from which intracellular AT₂ arise remains to be determined. We employed antibodies raised against AT₁ and AT₂ receptors to further demonstrate the expression of nuclear ANG II receptors. Immunoblots revealed a single 52-kDa band for nuclear AT₁ and a ~42-kDa band in both nuclear and plasma membrane fractions for AT₂, suggesting a mature molecular form of both nuclear receptor subtypes. Various molecular forms of the AT₂ receptor have been reported, which likely reflect the extent of receptor glycosylation, as well as the epitope of the receptor the antibodies recognize.

Autoradiography using the sarthran radioligand further supports the differential expression of ANG II receptor subtypes within both fetal and adult sheep kidneys and reveals localization of AT₁ receptors to the medulla and vasa recta, while AT₂ receptors are found predominantly in the tubulointerstitial area, which would include the vasculature, interstitial cells, and tubule components. Glomeruli appear to possess both receptor subtypes. These findings are consistent with those of Butkus et al. (5), who reported AT₁ mRNA in the medulla and AT₂ mRNA in the glomeruli and interstitium of the mesonephros in fetal lambs up to 131 days' gestation. In the rat, the distribution of AT₂ receptors includes proximal and distal tubules, collecting ducts, glomeruli, and blood vessels (43, 45), although at a much lower expression. Moreover, AT₂ receptors in human kidney cortex are found predominantly in the large preglomerular vessels (21) and the adventitia of intralobular arteries (63), while both AT₁ and AT₂ subtypes are found in glomeruli and tubulointerstitial regions (21).

It is well known that ANG II is a powerful vasoconstrictor of both pre- and postglomerular vasculature in the rat (42). However, it has recently been demonstrated by Arima (1) that ANG II causes a much stronger constriction of efferent than afferent isolated microperfused rabbit arterioles, due to NO- and prostaglandin-induced effects on ANG II function in the afferent arterioles. Furthermore, this author reports that activation of AT₂ receptors causes endothelium-dependent vasodilation which modulates the vasoconstrictor action of the AT₁ receptor (1). These findings may account for the exaggerated vasoconstrictor actions of ANG II under conditions where there is a pathological reduction or impaired function of the AT₂, such as occurs in some models of hypertension. Thus the receptor distribution whereby AT₂ receptors are preglomerular and AT₁, postglomerular provides a rationale for reduced glomerular pressure with ARB treatments, and the effectiveness of ARBs to reduce vascular resistance and increase renal blood flow. Blockade of the AT₁ receptor may also increase the availability of circulating ANG II to cause AT₂-mediated vasodilation.

Recently, studies have been conducted to characterize the role of plasma membrane AT₂ receptors in arterial vasodilation and interaction with the bradykinin/NO/cGMP pathway (7). NO, a potent vasodilator, plays an important role in the maintenance of vascular tone. It is also well established that NO acts as a signaling molecule to modulate neurogenic control of blood pressure (62), baroreflex, and cardiopulmonary transmission in the nucleus of the solitary tract (14), as well as stimulating activation of nuclear transcription factor- κ B to mediate effects of cardiac protection (10). We report that ANG II generated significant increases in NO production in fresh isolated, intact renal cortical nuclei. Indeed, blockade

of AT₂ with PD123319 abolished ANG II-mediated production of NO, while blockade of AT₁ had no effect. Several reports suggest a direct link between AT₁ receptor blockade and NO release (8, 56); however, these studies were conducted in rodents, where AT₁ is the predominant receptor expressed. Our studies demonstrate that the AT₂ subtype comprises >85% of ANG II receptors in the ovine kidney cortex. Thus, blockade of the AT₁, a small proportion of total ANG II receptors, would likely have a negligible effect on NO production. Alternatively, AT₁ receptors on the sheep cortical nuclei may not be linked to NOS activation and NO formation.

In the kidney, NO release may originate from several different NOS isoforms (reviewed in Ref. 30). Both endothelial and tubular epithelial cells express eNOS (NOSIII) (46, 47), and NO production contributes to vascular tone and regulation of sodium reabsorption. Within the cortical nuclear fraction, we demonstrate a dense immunoreactive band for eNOS at ~135 kDa, indicating that the mature form of the enzyme is present within the nuclear compartment. Similarly, eNOS has been reported in nuclei of brown adipocytes (14), rat hepatocytes (20), and rat glioma cells (35), again demonstrating the functional expression of a nuclear NOS system. Moreover, we detected the presence of the heterodimeric hemoprotein sGC, the principal receptor for NO, which catalyzes the conversion of GTP to the second-messenger molecule cGMP. These findings indicate that the nucleus contains the necessary components for NO signaling through ANG II at the AT₂ receptor and are in accordance with those of Siragy and coworkers (57), who report that AT₂ inhibits the biosynthesis of renal renin through production of NO and cGMP.

AT₁ receptors on isolated nuclei from the liver and kidney are functional and capable of modulating gene expression (16, 39). Indeed, our preliminary data in isolated renal nuclei from both rat (49) and sheep (23) suggest that ANG II stimulates the generation of reactive oxygen species via the AT₁ receptor. Moreover, AT₁ antagonists invariably increase the circulating levels of ANG II such that the functional actions of ARB treatment may invoke activation of AT₂ receptors. Whether nuclear AT₂ receptors play a role in influencing gene transcription is yet to be determined. Nonetheless, the role of this receptor is becoming increasingly more important in the mechanisms involved in blood pressure maintenance and protection of the kidneys from renal injury and may represent a significant participation in kidneys of higher mammals.

Indeed, the functional significance of intracellular ANG II, as reviewed by Kumar et al. (37), together with the expression of functional nuclear ANG II receptors presented here, gives rise to the notion that the nucleus may be an important target of the RAS that would influence renal function and renal injury. However, the intracellular source for ANG II to bind to nuclear receptors has yet to be established. While Hammond and colleagues (22) suggest that in proximal tubules ANG II internalization is mediated by megalin, a membrane protein involved in receptor-mediated endocytosis, our preliminary findings suggest that intracellular ANG II may arise from the nucleus itself. We recently demonstrated in isolated nuclei from sheep kidney cortex, immunoreactivity for angiotensinogen, the ANG II precursor, and renin, its processing enzyme (23). Our findings are in agreement with those of Sigmund and colleagues (55), who demonstrate nuclear localization of angiotensinogen in astrocytes as well as an intracellular form of

active renin (38). Furthermore, we found significant activity for angiotensin-converting enzyme (ACE) as well as the homolog ACE2 within isolated sheep nuclei (23). Whether these RAS components are synthesized within the nucleus or are translocated to the nucleus after synthesis remains to be determined; however, their nuclear compartmentalization may provide a direct source for the production of intracellular ANG II and Ang-(1-7). Be that as it may, the precise signaling mechanisms by which ANG II activates intracellular receptors to elicit biological responses must be elucidated to develop therapies more effective at preventing or attenuating ANG II-induced organ damage in renal and cardiovascular diseases by blocking the intracellular or nuclear actions of ANG II.

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