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Angiotensin type 2 receptor is expressed in the adult rat kidney and promotes cellular proliferation and apoptosis

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Angiotensin type 2 receptor is expressed in the adult rat kidney and promotes cellular proliferation and apoptosis.

Background. Angiotensin II (Ang II) is associated with cell proliferation and apoptosis. The role of the angiotensin type 2 receptor (AT2R) in these processes remains controversial. Conventional radioligand binding of ^{125}I-Sar^{1}, Ile^{8} Ang II in adult kidney has failed to demonstrate the binding for the AT2R.

Methods. The presence of the AT2R was explored in adult rat kidney by in vitro and in vivo autoradiography using the selective AT2R radioligand ^{125}I-CGP 42112B. The roles of the angiotensin type 1 receptor (AT1R) and the AT2R in mediating cellular proliferation and apoptosis were assessed using selective AT1R or AT2R antagonists in Ang II-infused Sprague-Dawley (SD) rats.

Results. ^{125}I-CGP 42112B binding was demonstrated by in vitro and in vivo autoradiography techniques in the glomeruli and proximal tubules of SD rats. This binding could be displaced by Ang II and the AT2R antagonist PD123319 but not by the AT1R antagonist valsartan. Subcutaneous infusion of Ang II for 14 days in eight-week-old SD rats induced proliferation of proximal tubular epithelial cells, as assessed by a twofold increase in proliferating cell nuclear antigen (PCNA)-positive cells and apoptosis, as assessed by a threefold increase in terminal dUTP nick end labeling (TUNEL)-positive cells. The administration of the AT2R antagonist PD123319 or the AT1R antagonist valsartan was associated with attenuation of the increases in both PCNA- and TUNEL-positive cells following Ang II infusion. Ang II infusion was associated with increased osteopontin gene and protein expression, which could be reduced by treatment with either valsartan or PD123319.

Conclusion. These findings indicate that there is significant expression of the AT2R in the adult kidney, and that the AT2R has a role in mediating Ang II-induced proliferation and apoptosis in proximal tubular epithelial cells and expression of osteopontin.

At least two angiotensin receptor subtypes have been identified and classified: the angiotensin type 1 receptor (AT1R) and the type 2 receptor (AT2R). The functions of the AT1R in the kidney have been clarified, but the presence and function of the AT2R in the adult rat kidney remain controversial. The AT2R is a seven-transmembrane domain receptor, and the cDNA for this receptor has been cloned [1, 2]. It was shown that the AT2R was mainly expressed in fetal tissues [3] and neonatal kidneys [4, 5], which implies that the AT2R might have a role during kidney organogenesis. Expression of the AT2R in the kidney decreases after birth [3, 4] and only very low expression of the AT2R in adult rat kidney has been demonstrated by immunohistochemical staining techniques [5, 6]. The expression of the AT2R has also been shown in adult monkey [7] and in adult human kidney [8]. However, conventional radioligand binding studies using ^{125}I-Sar^{1}, Ile^{8} angiotensin II (Ang II) have failed to demonstrate binding for the AT2R in the adult rat kidney [7, 9, 10].

It has been suggested that the balance between cell proliferation and apoptosis plays an important role in the pathophysiology of various renal diseases [11–13]. Ang II has been clearly demonstrated to induce both cellular proliferation [14, 15] and apoptosis [16, 17]. It has been postulated that the AT1R and AT2R have opposing actions on proliferation and apoptosis, although this issue remains controversial [18–20]. The proliferative properties of Ang II are considered to be associated with the AT1R, whereas the AT2R is viewed to promote apoptosis [18, 19, 21]. However, other studies have suggested an association of the AT1R with apoptosis [22] and the AT2R with proliferation [20]. A further role of AT2R in the kidney has been recently suggested by a study in which Ang II-stimulated expression of the chemokine RANTES, which promotes glomerular influx of monocytes, is mediated by the AT2R rather than the AT1R [23]. It has been shown that osteopontin, an extracellular matrix protein that is associated with tubular injury, is stimulated by Ang II [22]. Whether the effect of Ang II on osteopontin expression is mediated by the AT1R or the AT2R remains unknown.

Key words: angiotensin II, receptor subtypes, autoradiography, CGP42112B peptide ligand, osteopontin expression.

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The aims of the present study were, first, to explore the localization of AT2R in the adult rat kidney by using in vitro and in vivo autoradiography with a selective AT2R ligand, CGP42112B [24], which has been used to demonstrate the presence of AT2R in rat brain [25], and second, to study the roles of the AT1R and AT2R on cell proliferation, apoptosis, and osteopontin expression in response to chronic Ang II infusion.

METHODS

Adult male Sprague-Dawley (SD) rats (Biological Research Laboratory, Austin and Repatriation Medical Center, Heidelberg, Australia) at the age of 8 ($N = 49$) and 24 weeks ($N = 15$) were used in this study. The protocols for animal experimentation and the handling of animals are in accordance with the principles set out by the National Health and Medical Research Council and the Animal Welfare Committee of the Austin and Repatriation Medical Center.

Protocol 1

Preparation of ^{125}I-CGP42112B. The AT2R peptide ligand CGP42112B (a generous gift from Dr. Marc de Gasparo, Novartis Pharma AG, Basel, Switzerland) was iodinated using the chloramine-T method [26] with some minor modifications. In brief, CGP42112B was combined at approximately 10 times molar excess with ^{125}I (ICN Pharmaceutical, Irvine, CA, USA) and 6.2 μg in 10 μL of 0.2 mol/L phosphate buffer (pH 7.2) containing 0.005 mol/L Na_2 ethylenediaminetetraacetic acid (EDTA). Sodium azide (0.1%) was added to 1 mCi in 10 μL of the iodine solution. The reaction was commenced with addition of 50 μL of chloramine-T and 0.5 mg/mL of iodination buffer and mixed continually on a custom-made micromixer for 60 seconds. The reaction was terminated with the addition of 50 μL of sodium metabisulfite 0.5 mg/mL in iodination buffer. The products were then loaded directly onto a μBondapak C18 3.9 × 300 mm high-performance liquid chromatography column (waters) and eluted with 24% acetonitrile in triethylamine phosphate (TEAP) buffer (pH 3.0) at 1 mL/min. The ^{125}I-monoiodinated CGP42112B was separated from free ^{125}I and nonlabeled peptide by this method, the specific activity for ^{125}I-monoiodinated CGP42112B being 2175 Ci/mmol (1602 μCi/μg).

In vitro CGP42112B binding and autoradiography. The kidneys of SD rats at the age of 8 ($N = 5$) and 24 weeks ($N = 5$) were harvested after the animals were anesthetized with intravenous injection of pentobarbitone sodium (60 mg/kg). The kidneys were removed, bisected, and snap frozen in liquid nitrogen-cooled isopentane and stored at −20°C. Embryonic tissue (E15, $N = 3$) was used as a positive control. Twenty micron sections were cut on a cryostat at −20°C for in vitro binding studies.

Autoradiographic localization of the AT2R was performed using in vitro binding techniques as previously described [27]. First, maximum specific binding of ^{125}I-CGP42112B in the adult kidney was explored by increasing the concentration of this radioligand from 3.8 to 45 pmol/L in the absence (total binding) or the presence (nonspecific binding) of 10^{-5} mol/L nonradioactive CGP42112B. Based on these pilot studies, ^{125}I-CGP42112B was used in subsequent experiments at a concentration that achieved >90% maximum specific binding, 15 pmol/L. To calculate the dissociation constant, ^{125}I-CGP42112B binding was performed in the presence of increasing concentrations of nonradioactive CGP42112B. The sections were preincubated for 15 minutes in 10 mmol/L sodium phosphate buffer (pH 7.4) followed by an hour of incubation in a fresh volume of the same buffer containing ^{125}I-CGP42112B, 0.1% bovine serum albumin (BSA), and 0.3 mmol/L bacitracin. After incubation, the sections were washed four times for one minute each in ice-cold buffer and air dried at room temperature.

Second, to assess the specificity of ^{125}I-CGP42112B binding, studies of ^{125}I-CGP42112B in the kidney sections were performed in the presence of 10^{-5} mol/L of Ang II (Auspep, Parkville, Victoria, Australia), the AT1R antagonist valsartan (Novartis Pharma AG), or the AT2R antagonist PD123319 (Parke-Davis, Ann Arbor, MI, USA) in the incubation buffer.

Third, to determine whether the ^{125}I-CGP42112B binding sites are G-protein–coupled receptors, the nonhydrolysable GTP analogue guanosine 5′-0-(3-thiotriphosphate) (GTP-γ-S; Boehringer Mannheim, Mannheim, Germany) was added to the binding buffer at a concentration of 1.25 mmol/L [28].

All slides, including a set of radioactive standards, were exposed to Kodak BioMax x-ray film at room temperature for five days [9]. Following exposure, the films were processed, and the optical densities were quantitated using a microcomputer imaging device (MCID Imaging system; St. Catherines, Ontario, Canada) connected to an IBM AT computer [9].

In vivo CGP42112B binding and autoradiography. In vivo ^{125}I-CGP42112B binding studies were performed using in vivo localization techniques as previously described [28]. In brief, 8- ($N = 10$) and 24-week-old ($N = 10$) SD rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (60 mg/kg). A midline incision into the abdomen was made, and the right kidney was ligated and removed. The abdominal aorta was cannulated with a 20-gauge needle. Following injection of 1 mL of phosphate-buffered saline (PBS) alone or PBS containing 10^{-5} mol/L of Ang II, valsartan, PD123319, or nonradioactive CGP42112B ($N = 4$/per group), approximately 6 pmol of ^{125}I-CGP42112B in 1 mL of 0.1 mol/L PBS (pH 7.4) was infused and circulated for 15 minutes. Perfusion with PBS was performed for one minute at 130 mm Hg and then followed by 10% neutral-

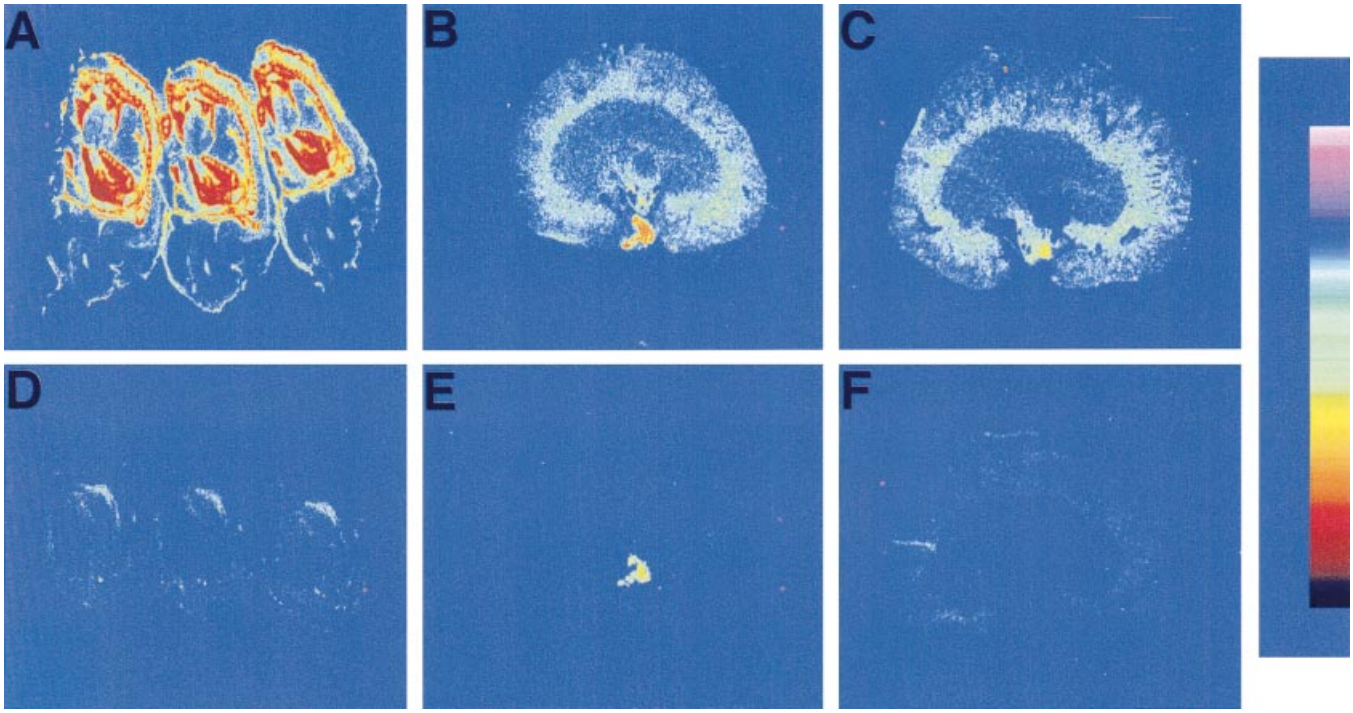

Fig. 1. Representative macroscopic autoradiographs of in vitro total binding for ^{125}I-CGP42112B in rat embryo (*A*, total binding; *D*, nonspecific binding) and the kidney sections from rats at the age of 8 (*B*, total binding; *E*, nonspecific binding) and 24 weeks (*C*, total binding; *F*, nonspecific binding). Reproduction of this figure in color was sponsored by Novartis Pharmaceuticals.

buffered formalin for one minute. The kidney was then removed and bisected. Half of the kidney was snap frozen, and 20 micron sections were cut and exposed to x-ray film. The other half of the kidney was fixed in 10% formalin and embedded in paraffin. Four micron sections were cut and then dipped in photographic emulsion (Ilford K5; Mt. Waverley, Victoria, Australia) and stored in the dark for two weeks. Slides were developed in phenisol developer (Ilford K5; Mt. Waverley) and fixed and stained with hematoxylin-eosin for subsequent light microscopy.

For identification of ^{125}I-CGP42112B binding in the proximal tubules, the kidney sections of the rats used in in vivo study were stained with a lectin (from Phaseolus Vulgaris PHA-E; Sigma Chemical Co., St. Louis, MO, USA) at a concentration of 1 μg/mL for one hour at room temperature [28]. Photographic emulsion was applied, and the sections were stored in the dark for two weeks. The slides were developed in phenisol developer and counterstained with hematoxylin.

Protocol 2

Eight-week-old male SD rats ($N = 34$) were anesthetized with enflurane (Ethrane; Abbott Australasia, Kurnal, NSW, Australia). An osmotic minipump (Model 2002; Alzet Corp., Palo Alto, CA, USA) filled with vehicle (0.15 mol/L NaCl, 1 mmol/L acetic acid) was subcutaneously implanted in the midscapular region. The minipump contained either vehicle (control, $N = 7$), human Ang II at a low dose of 12 ng/min (low-dose Ang II, $N = 6$), or Ang II at a high dose of 58 ng/min. Rats infused with high-dose Ang II were randomly allocated to Ang II infusion alone (high-dose Ang II, $N = 7$) or treatment with either valsartan (30 mg/kg/day by gavage, Ang II + valsartan, $N = 7$) or PD123319 (830 ng/min via an intraperitoneally implanted Alzet minipump, Ang II + PD123319, $N = 7$).

Systolic blood pressure (SBP) was measured by indirect tail-cuff plethysmography in prewarmed conscious animals as previously described [29]. Before the animals were sacrificed, glomerular filtration rate (GFR) was measured by using the ^{99m}Tc-DTPA method as previously described [30].

The animals were sacrificed after two weeks of treatment by intravenous injection of pentobarbitone sodium at a dose of 60 mg/kg body weight (Boehringer Ingelheim, Artarmon, NSW, Australia). The left kidney was removed and weighed and then fixed in 10% formalin and processed to paraffin. Four micron sections were cut and used for histologic assessment, immunohistochemistry, and in situ hybridization. To assess apoptosis morphologically, the right kidney was prepared for electron microscopy [31]. In brief, the right kidney was perfused in vivo at the rat's SBP via an intra-aortic cannula with saline, followed by 2.5% glutaraldehyde. One mm^3 cube was

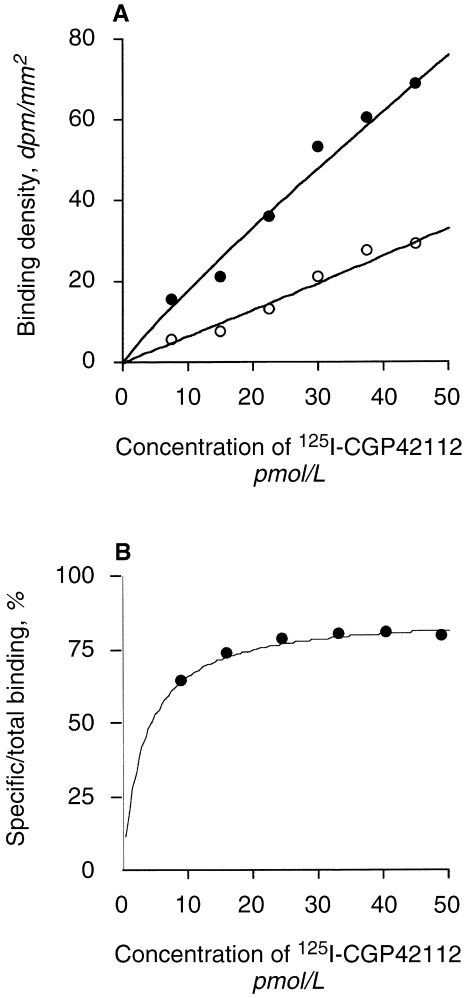

Fig. 2. Total binding (●) and nonspecific binding (○) for ^{125}I-CGP421-12B (*A*) and percentage of specific/total binding (*B*) in the adult kidney at increasing concentrations of the radioligand.

cut from deep areas of the renal cortex and fixed in 2.5% glutaraldehyde for 12 hours before washing in 0.1 mol/L phosphate buffer for 72 hours. Sections were processed by standard procedures [31]. Fifty nanometer sections were cut and viewed under a JEOL 1200EX transmission electron microscope for assessment of apoptosis.

Terminal dUTP nick end labeling

Apoptotic cells were identified by in situ labeling of fragmented DNA using terminal transferase (TdT)-mediated deoxy-uridine-5′-triphospate (dUTP) nick end labeling (TUNEL), which were then detected using immunohistochemical techniques [32].

Immunohistochemistry

For assessment of cell proliferation, kidney sections were labeled with a monoclonal antibody to proliferating cell nuclear antigen (PCNA, PC-10; Dako A/S, Copenhagen, Denmark). Biotinylated multilink swine anti-goat, mouse, rabbit immunoglobulin (Dako A/S) was used as a second antibody, followed by horseradish peroxidase (HRP)-conjugated streptavidin. Peroxidase activity was identified by reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co.) substrate.

For identification of proliferation (PCNA) and apoptosis (TUNEL) within the same section, a dual-colored immunohistochemical procedure was performed using a modified method described previously by Lan et al [33]. The sections were first stained with TUNEL and then incubated in 10 mmol/L sodium citrate buffer (pH 6.0) in a microwave oven at low power for 10 minutes [33]. The sections were treated with the endogenous alkaline phosphatase inhibitor levamisole to inhibit endogenous an antibody to PCNA and alkaline phosphate (AP)-conjugated streptavidin was applied. Detection was accomplished by using the Dako BCIP/NBT substrate system. Using this method, TUNEL-positive nuclei were stained brown, and PCNA-positive nuclei were stained dark blue.

Histomorphometry

Renal tubular damage was assessed in kidney sections stained with hematoxylin-eosin under a light microscope in a masked fashion (×400) using an Imaging Analysis System (AIS; Imaging Research, St. Catherines, Ontario, Canada) associated with a video camera and computer. Twenty fields of the tubulointerstitial area were scanned, and dilated tubulointerstitial areas were measured as a percentage of the total field. The number of cells positively stained with PCNA or TUNEL was counted manually in the renal cortical areas and expressed as the PCNA- or TUNEL-positive cell number per field (×400).

Osteopontin mRNA and protein expression

Osteopontin mRNA expression was determined by in situ hybridization as described previously [22, 34]. Osteopontin mRNA probes were generated from the rat smooth muscle osteopontin cDNA (a generous gift from Dr. Richard Johnson, formerly of the Department of Nephrology, University of Washington, Seattle, WA, USA, and currently Chief, Renal Section, Baylor College of Medicine, Houston, TX, USA) [35]. After in situ hybridization, kidney sections were exposed to x-ray film, and the densities of kidney sections on x-ray film were quantitated with the MCID system as previously described [36]. The slides were then dipped in photographic emulsion and prepared for observation under a microscope. Osteopontin protein expression was assessed by immunohistochemistry with a murine monoclonal antibody to osteopontin (also a generous gift from Dr. Johnson) as previously described [22].

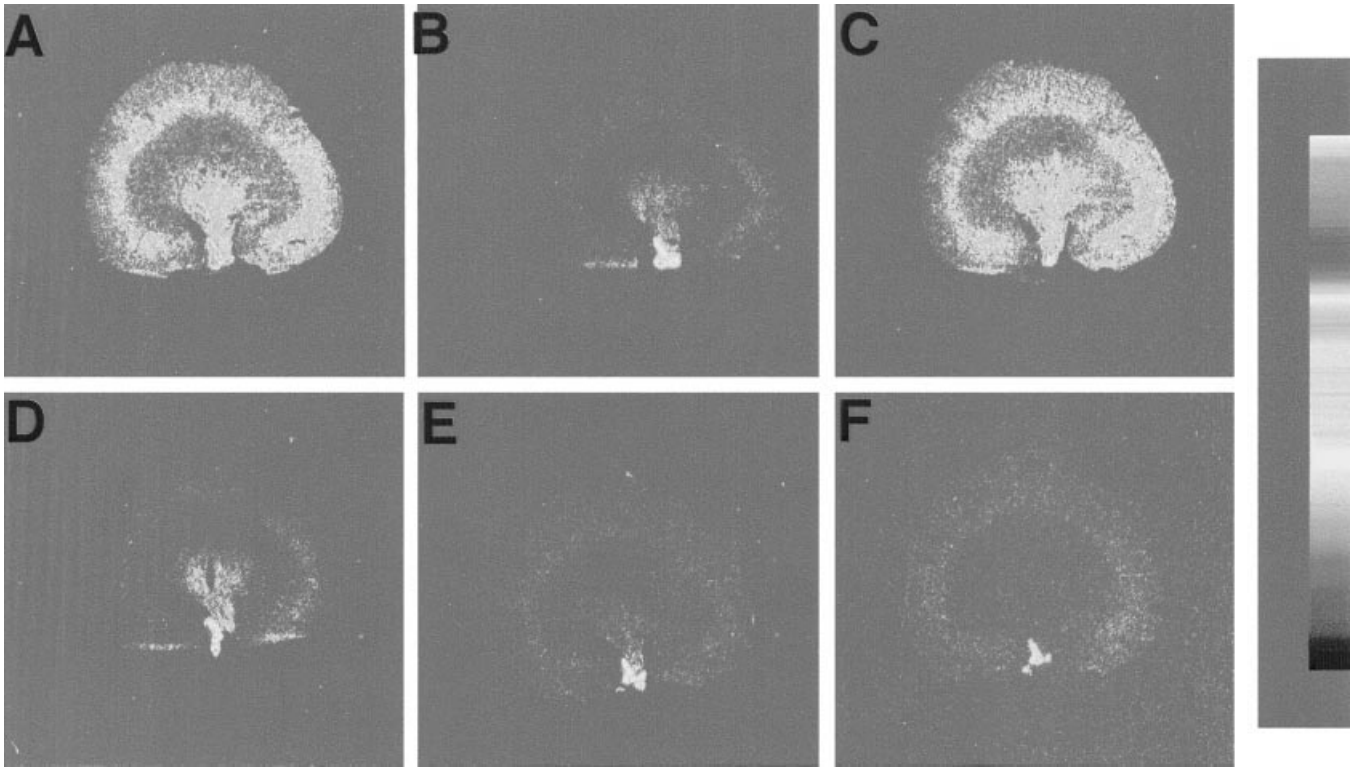

Fig. 3. Representative macroscopic autoradiographs of in vitro ^{125}I-CGP42112B binding. Sections were incubated with ^{125}I-CGP42112B (15 pmol/L) alone (*A*) or in the presence of 10^{-5} mol/L of Ang II (*B*), valsartan (*C*), PD123319 (*D*), nonradioactive CGP42112B (*E*), and GTP-γ-S (*F*).

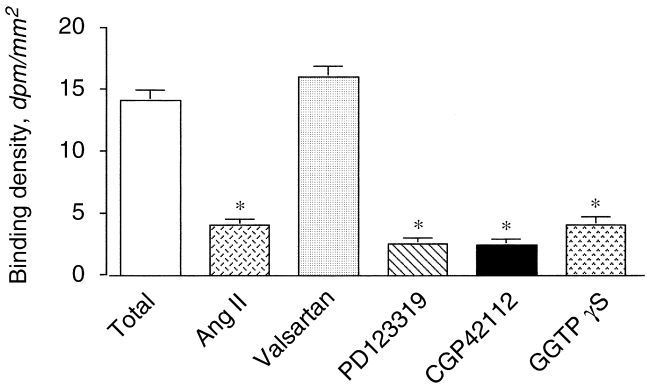

Fig. 4. Data of in vitro binding densities for ^{125}I-CGP42112B in the kidney cortex area were assessed using MCID system. $*P < 0.01$ vs. total binding.

Assessment of blockade of the AT1R and AT2R binding

To confirm blockade of the AT1R with valsartan and the AT2R with PD123319, binding densities for the AT1R and AT2R were determined in kidneys from rats administrated with high-dose Ang II alone or high-dose Ang II with either valsartan or PD123319. Ang II receptor binding density was assessed using an analogue of Ang II, ^{125}I-Sar^{1}, Ile^{8} Ang II [27]. Specific AT1R binding was determined by assessment of ^{125}I-Sar^{1}, Ile^{8} Ang II binding in the presence of the AT2R antagonist PD123319 (10^{-5} mol/L), whereas AT2R binding was determined by assessing the ^{125}I-Sar^{1}, Ile^{8} Ang II binding in the presence of the AT1R antagonist losartan (10^{-5} mol/L). Nonspecific binding was determined in parallel incubations in the presence of both 10^{-5} mol/L PD123319 and 10^{-5}mol/L losartan. Slides were exposed to x-ray film, and binding density was quantitated using the MCID system. Autoradiography using ^{125}I-Sar^{1}, Ile^{8} Ang II failed to demonstrate renal expression of the AT2R; therefore, autoradiography with ^{125}I-CGP42112B was performed to assess AT2R binding.

Statistical analysis

Data are shown as mean ± SEM and were analyzed by the program Statview SE (Brainpower, Calabasas, CA, USA) on a Macintosh G3 Computer (Cupertino, CA, USA). The dissociation constant (K_d) was calculated using the software MacLigand [37]. Comparisons of group means were performed by Fisher's least significant difference method. A P value of less than 0.05 was viewed as statistically significant.

RESULTS

Protocol 1

In vitro autoradiography. ^{125}I-CGP42112B binding was detected in the rat embryonic tissue and in kidney sections from rats aged 8 and 24 weeks (Fig. 1). In the kidney, the

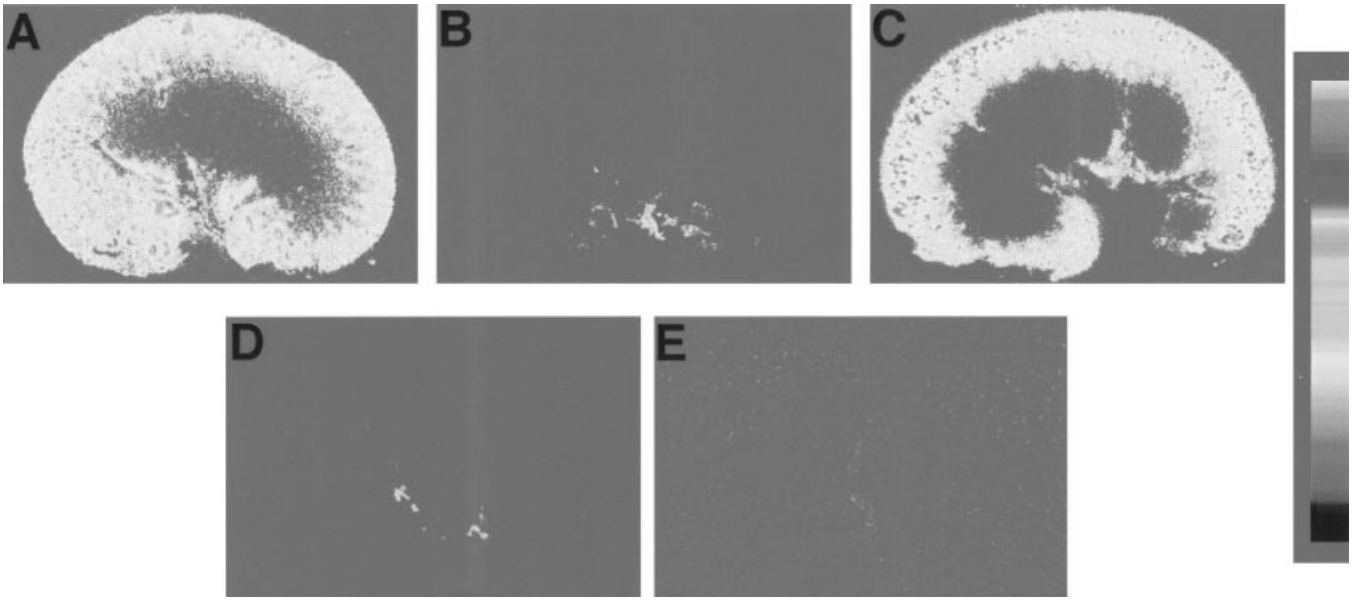

Fig. 5. Representative macroscopic autoradiographs of in vivo ^{125}I-CGP42112B binding. ^{125}I-CGP42112B was infused into rat aorta following injection of PBS (*A*, total binding) or PBS with 10^{-5} mol/L of Ang II (*B*), valsartan (*C*), PD123319 (*D*), nonradioactive CGP42112B (*E*).

binding for ^{125}I-CGP42112B was detected in the renal cortex with maximal binding in the inner cortex. The binding density for ^{125}I-CGP42112B increased with increasing concentrations of the radioligand (Fig. 2A). Ninety percent of specific binding was achieved at the concentration of 15 pmol/L (Fig. 2B).

The binding for ^{125}I-CGP42112B was reduced to levels equivalent to nonspecific binding in the presence of Ang II or PD123319, but not in the presence of valsartan (Figs. 3 and 4). ^{125}I-CGP42112B binding in the kidney was completely abolished in the presence of GTP-γ-S (Figs. 3 and 4). When compared with the binding of ^{125}I-CGP42112B in embryonic tissue (164 ± 5 dpm/mm^2), ^{125}I-CGP42112B binding density was much lower in the kidneys of 8- (18.2 ± 0.4 dpm/mm^2) and 24-week-old (17.6 ± 0.5 dpm/mm^2) rats ($P < 0.05$, respectively). Dissociation constants for^{125}I-CGP42112B binding in the embryo tissue (Kd 3.09 nmol/L) and in the kidneys at 8 (4.47 nmol/L) and 24 weeks (4.30 nmol/L) were all in the same nanomolar range.

In vivo autoradiography. In vivo autoradiography revealed that ^{125}I-CGP42112B binding was localized to the renal cortex, which was consistent with in vitro autoradiography (Fig. 5A). ^{125}I-CGP42112B binding was reduced in the context of concomitant administration of nonradioactive CGP42112B (Fig. 5E). As observed in the in vitro studies, ^{125}I-CGP42112B binding was reduced in the kidney sections from rats preinjected with Ang II (Fig. 5B) or PD123319 (Fig. 5D), but not reduced in kidney sections from rats preinjected with valsartan (Fig. 5C).

By emulsion autoradiography, the binding sites were detected in the glomeruli, proximal, and distal convoluted tubules and tubulointerstitium (Fig. 6A). This binding was not detected in the kidney sections from rats preinjected with Ang II, PD123319, or nonradiolabeled CGP42112B. Tissue sections from kidneys preinjected with ligand then sequentially stained with lectin for identification of proximal tubules and dipped in photographic emulsion revealed colocalization of the binding sites for ^{125}I-CGP42112B and lectin staining, consistent with a proximal tubular site for ^{125}I-CGP42112B binding (Fig. 6C).

Protocol 2

Systolic blood pressure, body weight, and kidney weight. Data for SBP, body weight, and kidney weight are shown in Table 1. Ang II infusion resulted in a dose-dependent rise in blood pressure. The increase in SBP induced by the high-dose Ang II infusion was attenuated by coadministration of valsartan but not of PD123319. Rats infused with Ang II at a high dose or combined with PD123319 gained less weight compared with other groups. Rats in these two groups had lower kidney weights than control rats; however, kidney-to-body weight ratios were higher because of reduced body weight as compared with other groups.

Glomerular filtration rate. Angiotensin II infusion at high dose was associated with reduced GFR when compared with that detected in control rats (Table 2). This was not observed in the low-dose Ang II groups. Administration of either valsartan or PD123319 was not associated with changes in GFR. The changes in GFR were consistent among rat groups whether filtration rate was

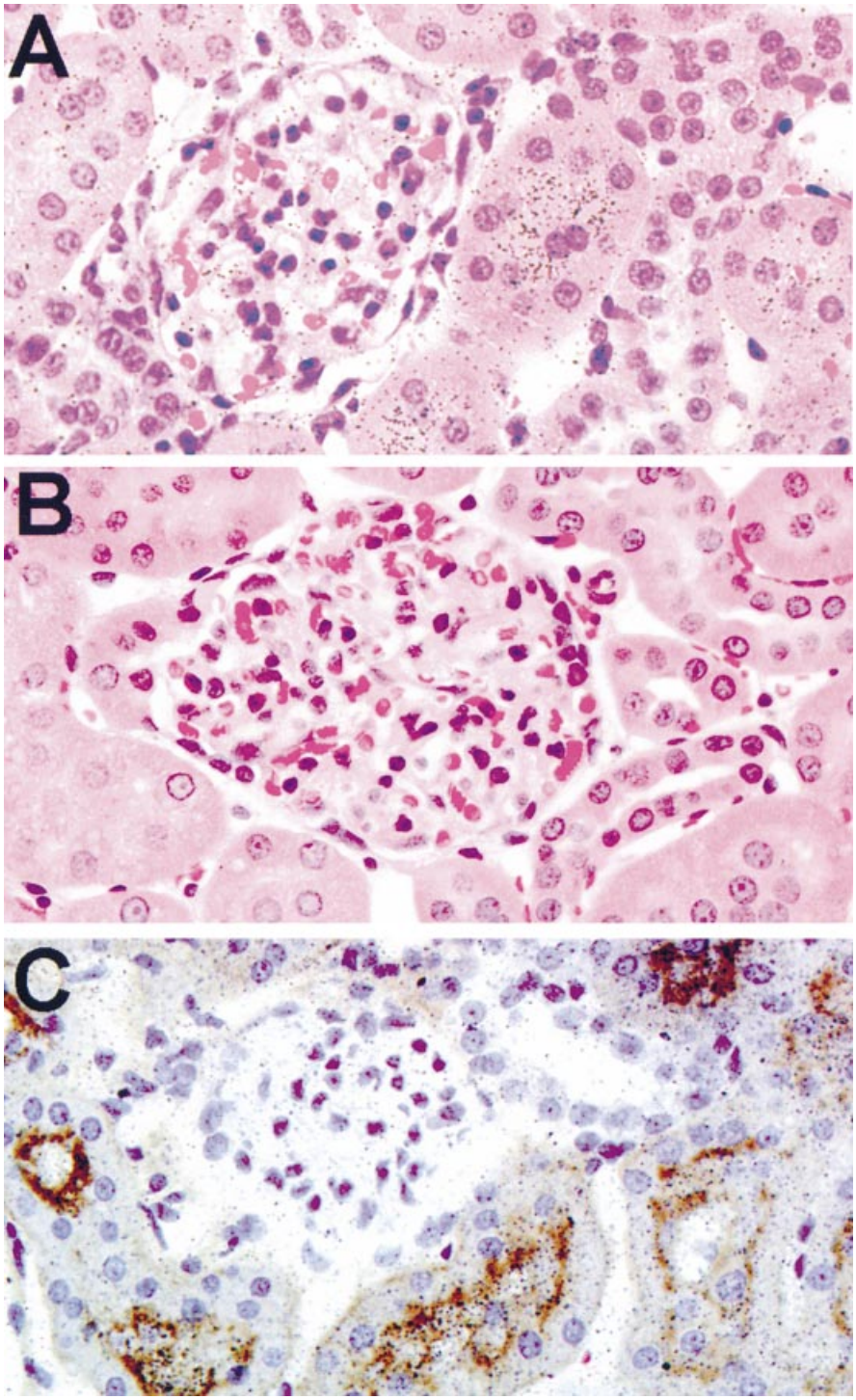

Fig. 6. Slides coated with photographic emulsion showing localization of ^{125}I-CGP 42112B in vivo binding sites. ^{125}I-CGP42112B was infused into rat aorta following injection of PBS (*A*, total binding) or PBS with 10^{-5} mol/L of nonradioactive CGP42112B (*B*, nonspecific binding). Silver grains (black dots) overlying cells in glomeruli and proximal convoluted tubules represent ^{125}I-CGP 42112B binding sites (A and *C*). The slide (C) shows double labeling of photographic emulsion (black dots, total binding) with lectin stain (brown) in the kidney section from a rat injected with radioligand. Sections are stained with hematoxylin-eosin (A and B) or hematoxylin with absence of eosin (C). Original magnification ×400. Reproduction of this figure in color was sponsored by Novartis Pharmaceuticals.

assessed as absolute GFR per minute or as GFR corrected for kidney weight (Table 2).

Histologic changes. Angiotensin II infused kidneys were associated with proximal tubular cell injury in a dose-dependent manner. Histologic changes included tubular dilation and atrophy and increased tubulointerstitial space (Table 1). At the electron microscopic level, the injured proximal tubular cells were shown to be vacuolated and atrophic and had condensed chromatin (data not shown). The administration of either valsartan or PD123319 was associated with reduced tubular injury, as assessed by attenuation of increased tubulointerstitial space (Table 1).

PCNA and TUNEL. Angiotensin II infusion resulted in an increase in PCNA-positive cells in proximal tubules.

Table 1. Data for systolic blood pressure, body weight and kidney weight in protocol 2

Group	N	SBP *mm Hg*	Body weight *g* week 0	Body weight *g* week 2	Kidney weight *g*	Kidney/body weight ratio *g/g × 100*	Tubulointerstitial space %
Control	7	124 ± 2	265 ± 6	336 ± 6	1.25 ± 0.03	3.77 ± 0.07	5.8 ± 0.4
Low-dose Ang II	6	158 ± 6[ab]	256 ± 5	343 ± 14[b]	1.27 ± 0.03[b]	3.73 ± 0.15[b]	10.1 ± 1.0[a]
High-dose Ang II	7	199 ± 5[a]	262 ± 5	270 ± 9[a]	1.14 ± 0.03[a]	4.30 ± 0.14[a]	13.2 ± 1.2[a]
Ang II + valsartan	7	129 ± 3[b]	256 ± 8	319 ± 8[b]	1.30 ± 0.06[b]	4.05 ± 0.12	6.0 ± 1.4[b]
Ang II + PD123319	7	207 ± 2[a]	269 ± 5	259 ± 8[a]	1.11 ± 0.06[a]	4.30 ± 0.12[a]	8.5 ± 0.8[b]

[a] $P < 0.01$ vs. control
[b] $P < 0.01$ vs. high-dose Ang II

Table 2. Glomerular filtration rate (GFR) in protocol 2

Group	N	GFR *mL/min*	GFR *mL/min/ 100 g kidney*
Control	7	3.07 ± 0.14	2.41 ± 0.07
Low-dose Ang II	6	3.19 ± 0.13[b]	2.53 ± 0.14[b]
High-dose Ang II	6	1.95 ± 0.21[a]	1.90 ± 0.11[a]
Ang II + valsartan	3	2.26 ± 0.07[a]	1.87 ± 0.11[a]
Ang II + PD123319	3	2.03 ± 0.25[a]	1.79 ± 0.21[a]

[a] $P < 0.01$ vs. control
[b] $P < 0.01$ vs. high-dose Ang II

This was also dose dependent (Figs. 7 and 8). Treatment with either valsartan or PD123319 was associated with reduced PCNA-positive cells when compared with rats with high-dose Ang II infusion without treatment. In fact, valsartan treatment was associated with a reduction in PCNA-positive cells in renal tubules to a level similar to that seen in the kidneys from control rats. The number of PCNA-positive cells in PD123319-treated rats was still higher than seen in control or valsartan-treated animals.

Terminal dUTP nick end labeling-positive cells were increased in kidney sections from rats with Ang II infusion, both at the low and high doses, when compared with control animals (Fig. 8). Treatment with either valsartan or PD123319 reduced TUNEL-positive cell numbers to a level similar to that observed in control kidney. Using double labeling, both PCNA- and TUNEL-positive cells were found in damaged renal tubules, with some cells positively stained for both PCNA and TUNEL in the high-dose Ang II infused rat (Fig. 9).

Gene and protein expression of osteopontin

In control rats, osteopontin mRNA expression was observed in the medulla with very little expression in the cortex. Following Ang II infusion, osteopontin mRNA was also expressed in the renal cortex, particularly in damaged tubulointerstitial areas, as well as in the Bowman's capsule (Figs. 10–12). Osteopontin expression, as assessed by immunohistochemical staining, was consistent with the expression of osteopontin mRNA. Increased osteopontin protein expression after Ang II infusion was observed in proximal tubules (Fig. 12). The elevation of both mRNA and protein expression of osteopontin after Ang II infusion was reduced by treatment with either valsartan or PD123319.

Assessment of AT1R and AT2R binding

Total Ang II binding as assessed by autoradiography with ^{125}I-Sar^{1}, Ile^{8} Ang II was down-regulated following Ang II infusion (Fig. 13A). Concomitant treatment with valsartan, but not PD123319, further reduced Ang II binding density. Ligand binding densities for the AT1R subtype were also down-regulated in Ang II-infused rats, and was further reduced by coadministration of valsartan but not of PD123319 (Fig. 13A). Binding to the AT2R was not detected by autoradiography with ^{125}I-Sar^{1}, Ile^{8} Ang II. However, using the radioligand ^{125}I-CGP42112B, binding to the AT2R following Ang II was similar to that detected in control rats. The binding density of AT2R was reduced in kidneys from rats treated with PD123319, but was not reduced in rats treated with valsartan.

DISCUSSION

The status of renal AT2R in the adult rat kidney has been an area of controversy. Initial studies suggested that there was no gene expression for the AT2R in adult kidney [7]. However, using immunohistochemical techniques, it was shown that AT2R protein was present, albeit at low levels, in glomeruli and tubules in rat kidney [5, 6]. Recently, using microdissection and reverse transcription-polymerase chain reaction (RT-PCR) methodology, AT2R mRNA has been reported to have a widespread distribution within the rat kidney [38]. In the current study, we have not only demonstrated the presence and binding characteristics of the AT2R in the adult rat kidney, but have also assessed its role in mediation of Ang II-induced cellular proliferation, apoptosis, and osteopontin expression.

In the present study, the AT2R was demonstrated in adult rat kidney using the selective AT2R ligand CGP42112B. Compared with rat embryonic tissue, which had high expression of the AT2R [3], CGP42112B binding in adult rat kidney was much reduced. However, the binding characteristics for such as the dissociation

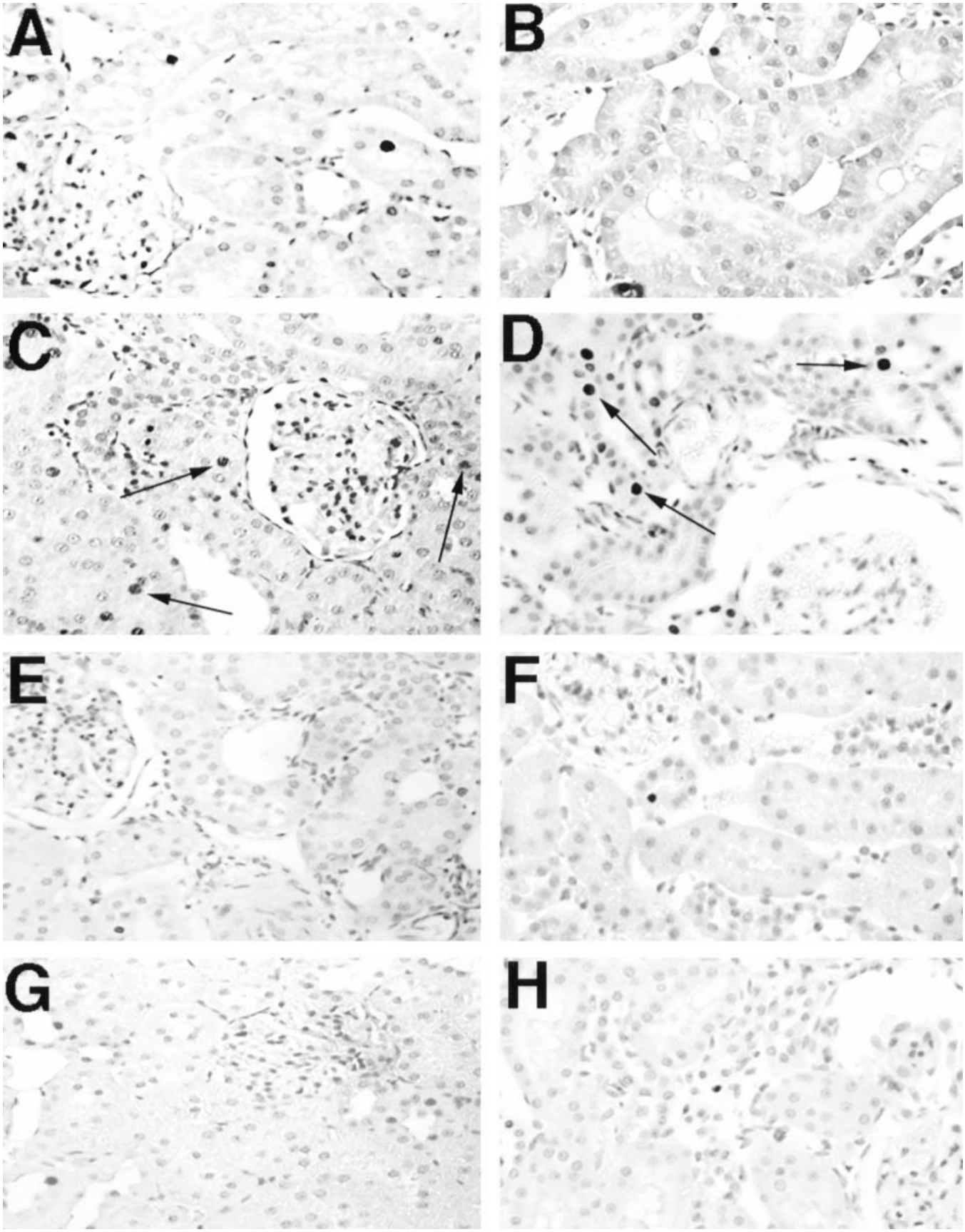

Fig. 7. PCNA- and TUNEL-positive cells in proximal tubules were identified as brown in nuclei. Sections showed PCNA-positive cells in control (*A*) and high-dose Ang II (*C*), Ang II + valsartan (*E*) or Ang II + PD123310 (*G*), and TUNEL-positive cells in control (*B*), high-dose Ang II (*D*), Ang II + valsartan (*F*) or Ang II + PD123319 (*H*). Sections were counterstained with hematoxylin. Original magnification ×400.

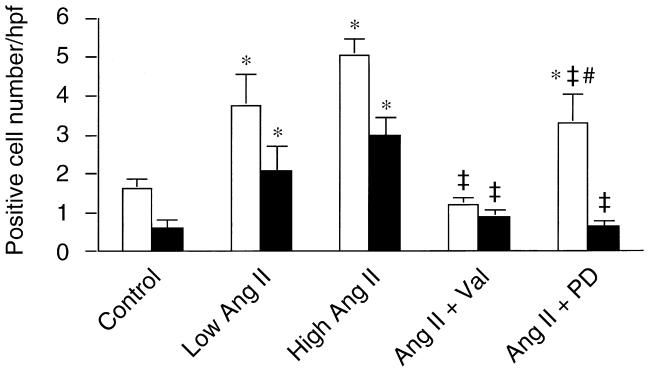

Fig. 8. PCNA- (□) and TUNEL- (■) positive cell numbers in the proximal tubules following Ang II infusion, and treatment with valsartan or PD123319. $*P < 0.01$ vs. control; $\dagger P < 0.01$ vs. high-dose Ang II; $\#P < 0.05$ vs. Ang II + valsartan.

constants were similar for both groups to those previously reported for the AT2R [39]. The findings from in vitro and in vivo autoradiographic studies were similar and revealed that CGP42112B binding was displaced by Ang II and the selective AT2R antagonist PD123319, but not by the AT1R antagonist valsartan. This indicates that the binding of CGP42112B was specific for the AT2R. Emulsion autoradiography localized these binding sites to the glomeruli and proximal tubules. These findings are similar to those reported using immunohistochemical staining with a specific antibody to the AT2 receptor [5, 6]. Furthermore, the CGP 42112B binding density was diminished in the presence of GTP-γ-S, consistent with the renal AT2R receptor being a G-protein–coupled receptor [40].

Using in vitro autoradiography, we demonstrated that Ang II infusion was associated with down-regulation of the AT1R, but there was no significant change in the AT2R. Similar effects of Ang II on AT1R and AT2R regulation have been demonstrated using immunohistochemistry with specific antibodies to the AT1R and AT2R [6]. Previous autoradiography studies using the nonselective Ang II agonist ^{125}I-Sar^{1}, Ile^{8} Ang II failed to reveal the presence of an AT2R in the adult rat kidney [27]. This may be due to the relatively low level of expression of the AT2R subtype in the adult kidney and autoradiography using ^{125}I-Sar^{1}, Ile^{8} Ang II not having adequate sensitivity to detect this low binding for the AT2R. In homogenized tissue, it appears that about 10% of Ang II receptors are of the AT2R subtype [41]. A similar portion of AT2R has been demonstrated in adult human kidney by in situ hybridization [8].

In the present study, the Ang II-induced increases in PCNA-positive cells in the proximal tubules and tubulointerstitium occurred in a dose-dependent manner with these proliferative effects of Ang II most likely mediated by both AT1R and AT2R. These results are consistent with previous findings, which have shown that both the AT1R and AT2R mediate Ang II-induced collagen synthesis [42] and increase in nuclear calcium [43]. Furthermore, it has been shown that Ang II-stimulated proliferation in cultured mouse spleen lymphocytes is mediated by both AT1R and AT2R [15]. Recently, our group reported that the proliferative and trophic effects of Ang II on the mesenteric arterial tree were reduced by either valsartan or PD123319, suggesting that both AT1R and AT2R may be involved in these processes [44].

Angiotensin II was also shown, in a dose-dependent manner, to induce apoptosis in renal tubular cells. This was demonstrated by the immunohistochemical TUNEL technique and was confirmed by electron microscopy. Both the AT1R and the AT2R antagonists attenuated this effect of Ang II on apoptosis. A number of studies have suggested that the AT2R rather than the AT1R promote apoptosis [18, 21, 45]. However, a recent study has suggested that the AT1Rs promote apoptosis in cultured adult ventricular myocytes [46]. These inconsistencies between studies may be due to the presence and relative distribution of angiotensin receptor subtypes in the in vitro and the in vivo contexts, or could be related to organ specificity of these receptors.

The in vivo blockade of the AT1R and AT2R demonstrated that both receptors have similar effects on proliferation and apoptosis in the Ang II infusion model. These findings are consistent with the hypothesis that there may be "cross-talk" between AT1R and AT2R, which coordinates the biological effects of Ang II. The mechanism for "cross-talk" between these receptor subtypes remains unknown. Based on the present study, it is possible that the AT1R and AT2R may have opposite actions with regard to blood pressure [47], but similar effects on proliferation [15, 42–44], and apoptosis [18, 21, 46] in certain experimental contexts.

Although both AT1R and AT2R antagonists reduced PCNA staining, PCNA-positive cell numbers in rats treated with PD123319 were still higher than those in rats treated with valsartan. This may indicate that the AT1R plays a more important role in mediating Ang II-induced proliferation that the AT2R. However, one must be cautious in interpreting the data, since antiproliferative effects of the AT2R antagonist were observed in persistent hypertension, whereas the effects of the AT1R antagonist occurred in the presence of reduced systemic blood pressure.

Following Ang II stimulation, proximal tubular cells underwent both proliferation and apoptosis. Using double-labeling immunohistochemistry, we found both PCNA- and TUNEL-positive cells in damaged proximal tubules. We demonstrated that in some cells there was positive staining for both PCNA and TUNEL after continuous infusion of Ang II. This provides immunohistochemical evidence showing that both Ang II-stimulated proliferation and apoptosis may occur in the same cell. The concept of cells undergoing both proliferation and apoptosis

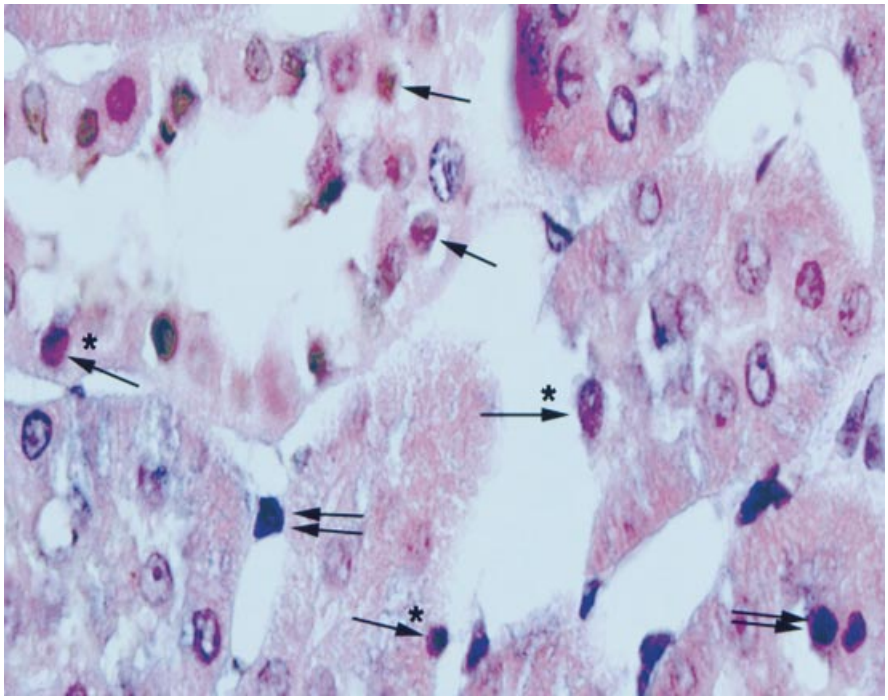

Fig. 9. Double labeling of TUNEL with PCNA in the renal tubules after Ang II infusion. TUNEL positive cells were identified as brown in nuclei (↑) and PCNA as dark blue (⇑); some cells stained positively for both (*↑), counterstain with eosin. Original magnification ×1000. Reproduction of this figure in color was sponsored by Novartis Pharmaceuticals.

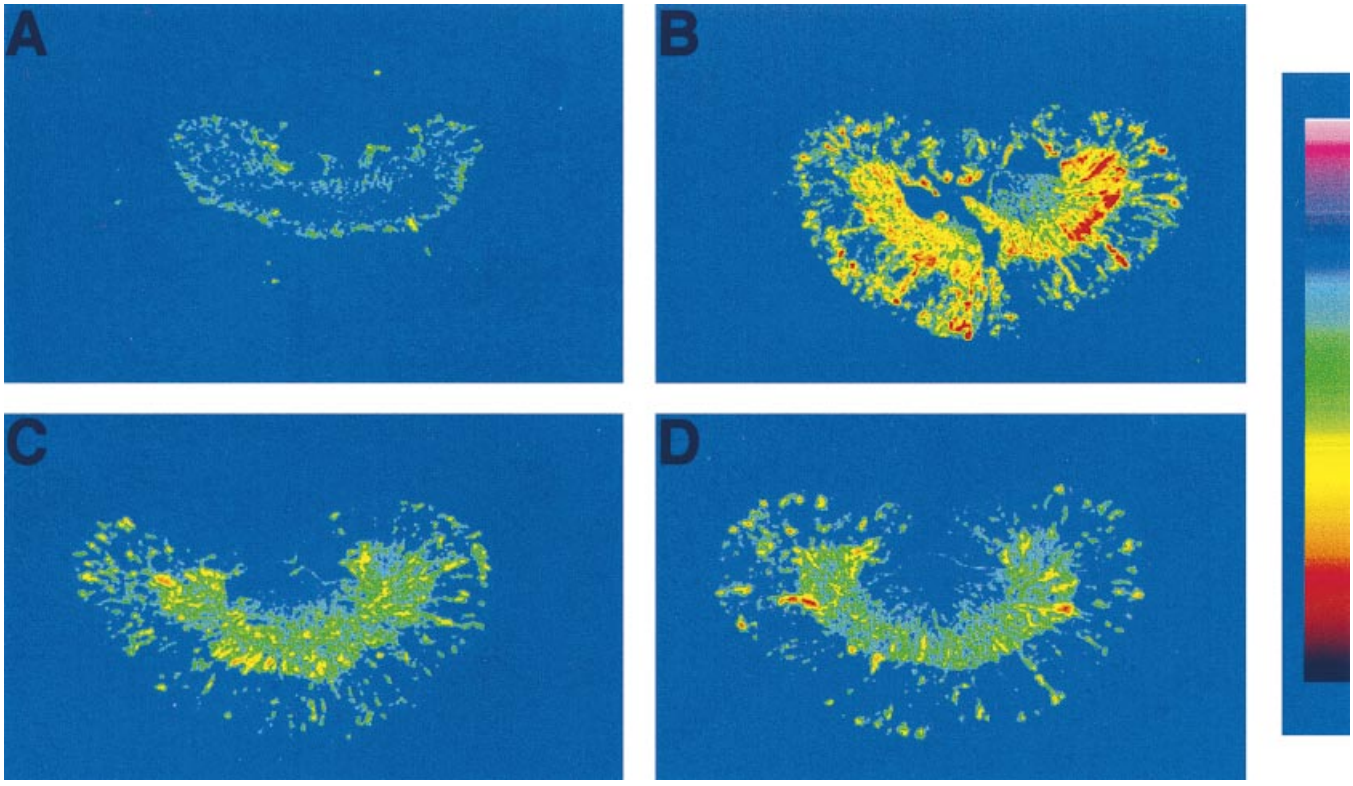

Fig. 10. Representative macroscopic autoradiographs of gene expression of osteopontin by in situ hybridization in control (*A*), high-dose Ang II (*B*), Ang II + valsartan (*C*), and Ang II + PD123319 (*D*). Reproduction of this figure in color was sponsored by Novartis Pharmaceuticals.

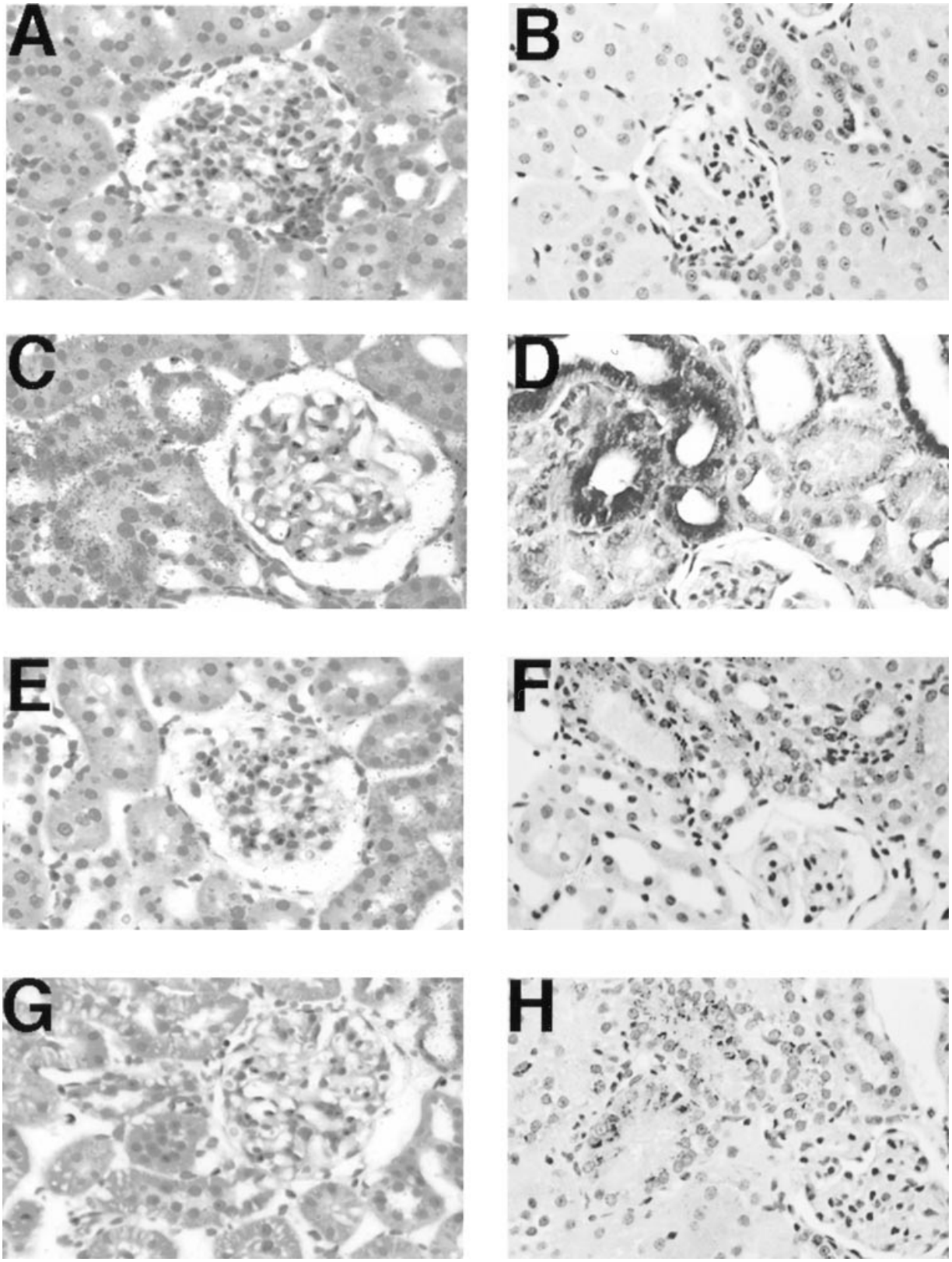

Fig. 11. Slides coated with photographic emulsion showing osteopontin mRNA expression by in situ hybridization in control (*A*), high-dose Ang II (*C*), Ang II + valsartan (*E*), and Ang II + PD123319 (*G*); and immunohistochemical staining of monoclonal antibody to osteopontin in control (*B*), high-dose Ang II (*D*), Ang II + valsartan (*F*) Ang II + PD123319 (*G*). Original magnification ×400.

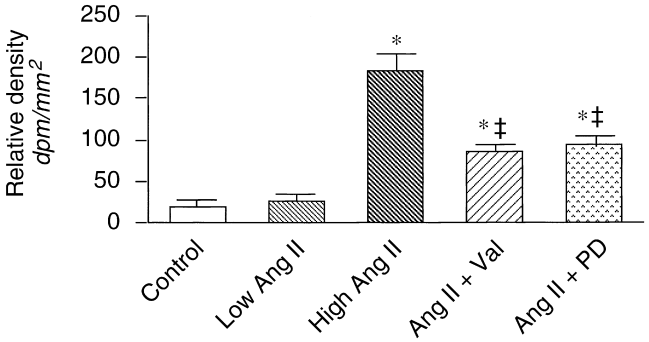

Fig. 12. Data for gene expression of osteopontin was assessed with MCID system following Ang II infusion or coadministration of valsartan or PD123319. *$P < 0.01$ vs. control; †$P < 0.01$ vs. high-dose Ang II.

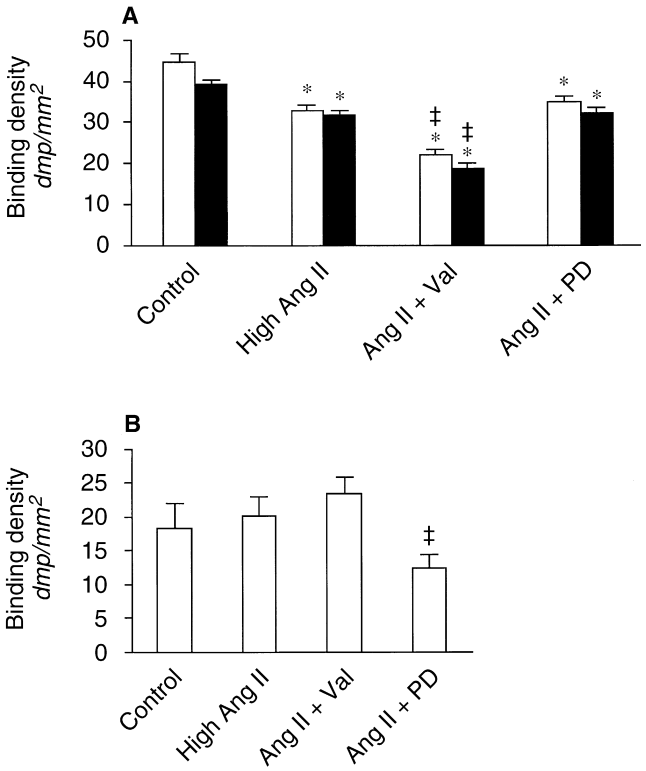

Fig. 13. Binding densities of Ang II (□) and AT1R (■) by autoradiography with ^{125}I-Sar1, Ile8 Ang II (*A*) and binding density of the AT2R (□) by autoradiography with ^{125}I-CGP42112B (*B*). *$P < 0.01$, vs. control; ‡$P < 0.05$ vs. high-dose Ang II.

has been reported in a range of pathophysiological situations, including prolactin-induced involution of the corpus luteum [48], malignancy [49], and vascular injury [50]. Furthermore, the proliferative and apoptotic responses to injury may share common cell cycle pathways, which could explain why these processes are often closely linked in disease states including renal injury [51]. It has been suggested that this phenomenon occurs as result of abnormalities in cell cycle signaling [48] and may reflect a response to tissue injury. In a porcine model of percutaneous transluminal coronary angioplasty, concomitant proliferation (PCNA) and apoptosis (TUNEL) were detected in the neointima [50].

The ability of both AT1R and AT2R antagonists to attenuate renal injury following Ang II infusion suggests that both receptors may mediate tissue injury in this context, possibly via a common pathway. It has been shown that overexpression of osteopontin is associated with tubulointerstitial injury [22, 52]. Expression of osteopontin is also increased by administration of Ang II [22]. In the present study, these findings have been confirmed with up-regulation of both gene and protein expression by Ang II infusion. Intervention with either valsartan or PD123319 was associated with a reduction in osteopontin expression. This suggests that osteopontin is regulated by both the AT1R and AT2R. However, osteopontin mRNA expression was only reduced by either of AT1R or AT2R antagonist to levels that were consistently above those detected in normal kidneys. The effects of dual blockade of both AT1R and AT2R on osteopontin expression in Ang II-infused model warrants further investigation. Increased osteopontin expression has been observed in several models of renal injury and is associated with morphological regeneration [52] and apoptosis [53]. A role for the AT2R in influencing expression of the chemokine RANTES has been reported and this provides additional evidence of a role for the AT2R in mediating cellular processes, including cell recruitment in the kidney [23].

The present study has focused on the potential role of the AT2R in influencing cellular proliferation and apoptosis. However, it has been suggested that the AT2R may have other effects. For example, some studies have shown involvement of the AT2R in sodium retention, pressor sensitivity [54, 55], vasodilation [56], and production of renal nitric oxide [57]. Recent studies have demonstrated that AT2R knockout mice had increased pressor and antinatriuretic sensitivity in response to Ang II stimulation when compared with AT2R wild-type mice [58]. The diverse effects of the AT2R must be explored in more detail, since there is widespread use of angiotensin-converting enzyme inhibitors and AT1R antagonists as renoprotective drugs, and these different inhibitors of the RAS may have disparate effects on the AT2 receptors.

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APPENDIX

Abbreviations used in this article are: Ang II, angiotensin II; AP, alkaline phosphatase; AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; GFR, glomerular filtration rate; GTP-γ-S, guanosine 5′-O-(3-thiotriphosphate) gamma; HPLC, high pressure liquid chromatography; K_d, dissociation constant; PCNA, proliferating cell nuclear antigen; RANTES, regulated upon activation, normal T cell expressed and secreted; SBP, systolic blood pressure; TUNEL, terminal transferase-mediate deoxy-uridine-5′-triphosphate nick end labeling.

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