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Angiotensin II receptors and renin release in rat glomerular afferent arterioles

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Angiotensin II receptors and renin release in rat glomerular afferent arterioles. The purpose of recent studies was to investigate the expression of angiotensin II (Ang II) receptor sites in afferent arterioles freshly isolated from the rat kidney, and the role of Ang II on renin release by these vessels. The method of isolation and purification of renal microvessels was based on iron oxide infusion into the kidneys and separation of the afferent arterioles from glomeruli and connective tissue with the aid of a magnetic field, successive passages through various sieves, and harvesting with collagenase. Ang II receptor characteristics were evaluated by radioligand binding studies using the non-peptide Ang II antagonists of AT_1 (Dup-753 and -532) and AT_2 (PD-123319 and CGP-42112) receptors. AT_1 antagonists displaced up to 80% of the Ang II binding with high affinity (3 nM), whereas the remaining 20% showed low affinity for the Dup compounds and CGP-42112 (>10 μM), and intermediate affinity for PD-123319 (12 μM). These data suggest the existence of two Ang II receptor subtypes in the renal vasculature of the rat. In separate experiments, renin release by isolated afferent arterioles in vitro was 9 ng/hr/mg under control conditions. Ang II (0.1 μM) inhibited renin secretion by 20%, whereas the adenylyl cyclase activator forskolin (10 μM) stimulated renin secretion by 50%. In arterioles isolated from rats chronically treated with a converting enzyme inhibitor (perindoprilate) to reduce endogenous formation of Ang II, renin release increased 20-fold under control conditions in vitro and was further stimulated by forskolin. These results demonstrate that this preparation is a useful tool to study the functional role of Ang II and the control of renin release in the afferent arterioles.

The renin-angiotensin system (RAS) plays a major role in the renal function. Although several investigations attempted to localize the exact sites of angiotensin II (Ang II) action on the renal vasculature, controversy still remains on whether Ang II can act on the preglomerular vessels [1]. Micropuncture and clearance techniques and studies on microdissected vessels suggest that Ang II constricts preferentially the efferent arteriole [2, 3] On the other hand, data obtained from *in vitro* visualization models (juxtaglomerular perfused nephron, hydronephrotic kidney) support the view that Ang II can act on afferent arterioles [4, 5]. Previous studies from our laboratories indirectly supported the latter hypothesis. Ang II receptors have been identified on isolated glomeruli and cultured mesangial cells, a structure that is thought to share several common characteristics with preglomerular vascular smooth muscle cells [6, 7]. Furthermore, injection of Ang II directly into the renal artery decreased renal blood flow by 50 to 60% [8]. Renal vasoconstriction of this magnitude is difficult to be achieved without participation of preglomerular vessels.

The purpose of the present study was to provide convincing evidence demonstrating the existence of functional receptor sites of Ang II in the preglomerular vasculature. For this reason we developed a technique to isolate *in vitro* pure and viable preparations of preglomerular vessels from the rat kidney, and we subsequently studied the regulation and expression of the renin-angiotensin system in these vessels.

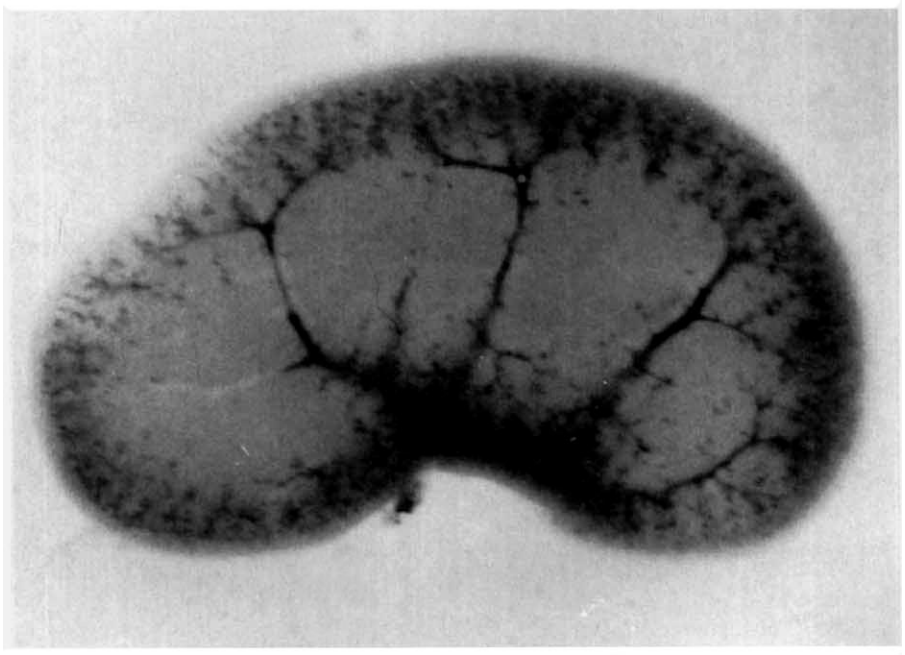
Methods

Isolation of afferent arterioles

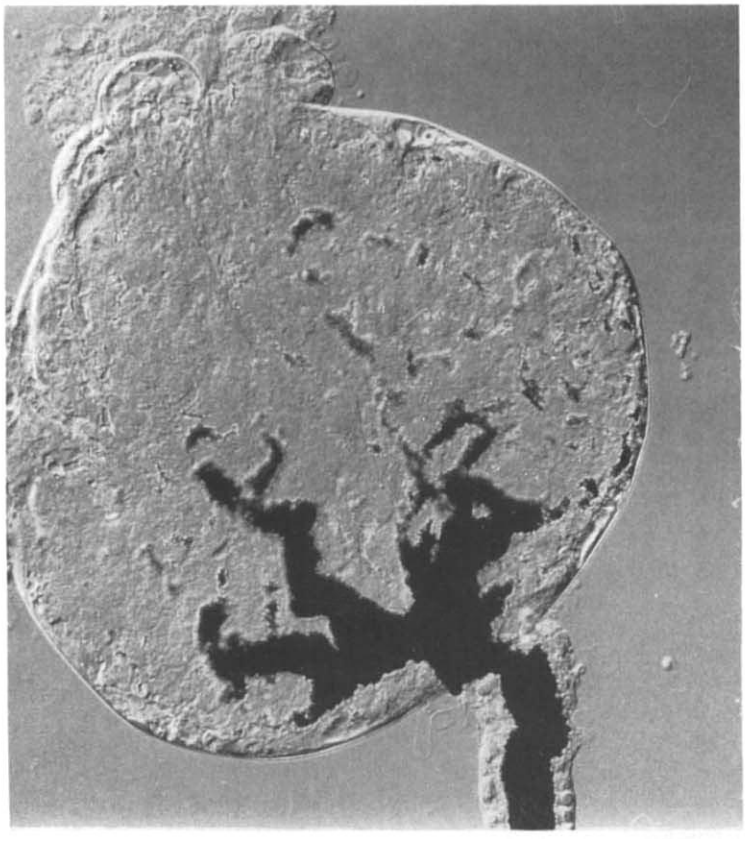
The technique to isolate afferent arterioles from the rat kidney was the same as previously described [9]. Briefly, a magnetized iron oxide suspension (1% Fe_3O_4 in isotonic saline) was perfused into the renal arteries of seven-week-old Wistar-Kyoto (receptor binding studies) or Sprague Dawley (renin release studies) rats (6 rats per experiment) (Fig. 1A). Thereafter the kidneys were removed, decapsulated, and the cortex was dissected from the medulla. Cortical tissue was homogenized with a Polytron homogenizer, and the iron oxide-loaded tissues (renal vessels and glomeruli) were removed from the crude homogenate with the aid of a magnet (Fig. 1B). Afferent arterioles were separated from larger vessels and glomeruli using repetitive passages through needles and sieves of decreasing sizes (20 to 23 gauge and 150 to 90 μm, respectively). The microvessels were recovered from the top of the 125 μm sieve and harvested with collagenase (55 U/ml) for 30 minutes at 37°C to remove the surrounding connective tissue. Only vascular preparations containing >90% of afferent arterioles were retained for subsequent experiments.

Receptor binding studies

Aliquots of microvessels (40 μg of protein) were incubated with increasing concentrations of ^{3}H-Ang II as previously described [9]. Nonspecific binding was determined in the presence of an excess of unlabeled Ang II (10 μm). In competitive inhibition studies, the AT_1 receptor antagonists Dup-753 and Dup-532 (DuPont) and the AT_2 receptor antagonists PD-123319 (Parke-Davis) and CGP-42112 (Ciba-Geigy) were used to displace ^{3}H-Ang II. Analyses of the data using the LIGAND program gave estimates of the maximum specific binding (B_{max}) and dissocation constant (K_d).



A



B

. *Longitudinal section of a kidney after* *perfusion.* **B.** Representative example
:rent arteriole attached to a
us. Note that iron oxide particles do
,letely fill glomerular capillaries.

Renin release evaluation

Aliquots of microvessels (150 to 200 μg of protein) were incubated at 37°C in a final volume of 1 ml DMEM/F12 (1:1) medium containing 1 mM Ca^{2+}. Incubations lasted one and three hours in the absence or presence of 10 μM of forskolin or 0.1 μM of Ang II. Renin release was measured by radioimmunoassay as the amount of Ang I produced by incubation of supernatants with an excess of rat renin substrate (plasma of binephrectomized rats)

[10]. Renin release was expressed in nanograms of Ang I generated per hour of incubation per milligram of protein of afferent arterioles.

Angiotensin converting enzyme (ACE) inhibition

In separate experiments, rats were treated with a converting-enzyme inhibitor (perindoprilate, 40 mg/kg/day) for five days to eliminate endogenous Ang II. Isolation of afferent arterioles and renin release assay were performed as described above.

Results

Receptor binding studies

The properties of Ang II receptor sites were characterized in afferent arterioles from seven-week-old rats using equilibrium radiolabeled techniques. Scatchard analysis revealed one class of high affinity receptors (K_d = 1 nM) with a density of 200 fmol/mg of protein.

To identify the Ang II subtype(s) expressed in these renal vessels, the AT_1 antagonists Dup-753 and Dup-532, and the AT_2 antagonists PD-123319 and CGP-42112 were used to displace unlabeled Ang II. The AT_1 antagonists displaced almost completely the Ang II binding. Analysis of the displacement curve by the LIGAND program indicated that a two-site model produced a significantly better fit than the one-site model ($P < 0.001$). In the two-site model, Dup-753 binds to one site with high affinity (3 nM), whereas it displays 500-fold lower affinity for the other site (1.5 μM; (Fig. 2A). The high-affinity site is the most abundant and occupies 80% of the total Ang II receptor sites. The low-affinity Ang II site occupies the remaining 20% of the total receptor sites.

The putative AT_2 antagonist PD-123319 displaced up to 25% of the Ang II binding, with a rather medium affinity (100 nM; Fig. 2B). The other AT_2 antagonist, CGP-42112, showed very low affinity to the Ang II receptor sites (> 10 μM).

To determine whether or not the high affinity site that Dup-753 bound to is distinct from the medium affinity site recognized by PD-123319, competitive inhibition curves were performed by adding a standard concentration of PD-123319 (1 μM) to increasing concentrations of Dup-753, and alternatively, by adding a standard concentration of Dup-753 (10 nM) to increasing concentrations of PD-123319. As shown in Figure 2A, PD-123319 provided an additive displacement (15 to 20%) that was uniform in all tested concentrations of Dup-753. Similarly, Dup-753 displaced an additional 20 to 25% of the sites in all PD-123319 tested concentrations (Fig. 2B).

Renin release studies

The viability of the afferent arterioles was checked by measurements of creatine kinase activity (CKA) in the medium. No changes in the release of CKA were observed between 0 and 6 hours after isolation of the vessels. On the contrary, CKA was increased eightfold at 24 hours compared to 0 hours. For this reason, renin release measurements were performed at 1 and 3 hours after isolation of afferent arterioles. During this period, the rate of secretion of renin was increased significantly with time. After 1 hour renin release in supernatants was 10 ± 2 ng/hr/mg, and after 3 hours it was 22 ± 2 ng/hr/mg (Table 1).

To investigate whether renin release can be regulated in our vascular preparation in vitro, we used forskolin and Ang II, two agents that are known to stimulate and inhibit renin release, respectively. Addition of forskolin (10 μM) increased renin release by 40% after 1 hour and 50% after 3 hours compared to control values. In contrast, when exogenous Ang II (0.1 μM) was added, renin release by afferent arterioles was decreased by 35% at 3 hours (Table 1).

To examine if *in vivo* regulation of the expression of the

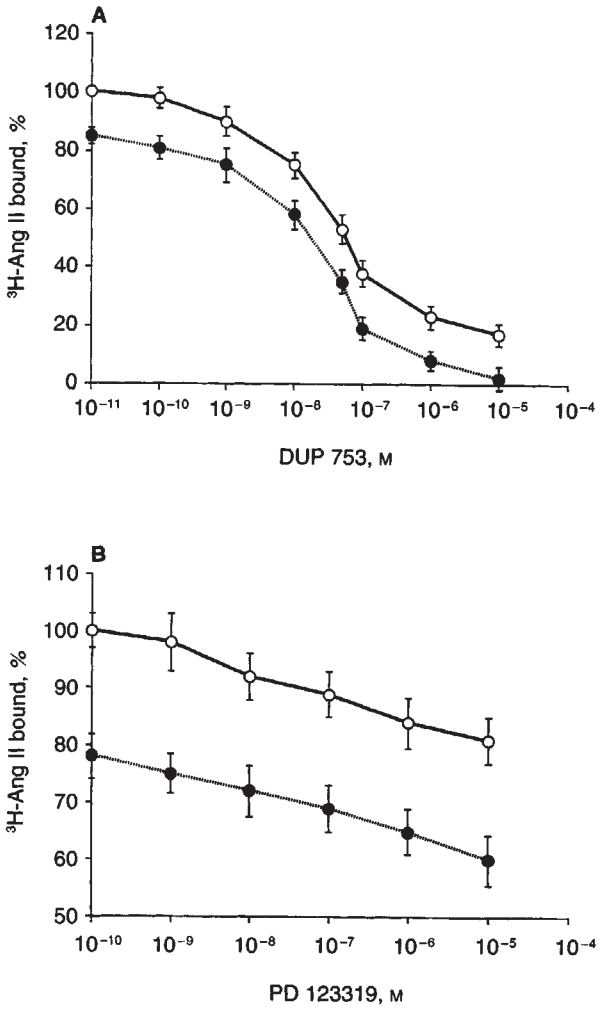


Fig. 2. *Displacement curves of ^{3}H-Ang II binding to preglomerular vessels by increasing concentrations of the AT_1 antagonist Dup 753 in absence and presence of the AT_2 antagonist PD 123319 (1 μM)* **(A)** *and inversely, by increasing concentrations of PD 123319 in absence and presence of Dup 753 (10 nM)* **(B).** Values are means ± SE for 4 experiments of each condition.

Table 1. Renin release from afferent arterioles freshly isolated from normal and perindoprilate-treated rats

	Normal		Perindoprilate	
	1 hr	3 hr	1 hr	3 hr
Control	10 ± 2	22 ± 2	153 ± 16	242 ± 19
Forskolin	15 ± 2[a]	34 ± 4[a]	217 ± 16[a]	394 ± 42[a]
Ang II	8 ± 1	15 ± 2[a]	125 ± 10	160 ± 14[a]

Values are expressed in ng/hr/mg and are means ± SE of 4 independent experiments.
[a] $P < 0.05$ vs. control

renin-angiotensin system can induce changes in renin release that can be detected in isolated afferent arterioles, a group of rats was treated chronically with a converting enzyme inhibitor (perindoprilate) to reduce production of endogenous Ang II. Afferent arterioles from rats treated with perindoprilate were found to release renin at a rate 15-fold higher than control. Addition of forskolin further increased renin release (50 and 70% above control at 1 and 3 hr, respectively), whereas addition of exogenous Ang II inhibited renin release (30% below control at 3 hr).

Discussion

In the present studies, we isolated afferent arterioles of high purity, and in sufficient quantity to perform several biochemical studies such as receptor characterization or responses to a variety of stimuli. This vascular preparation is viable *in vitro,* and it was used to study Ang II receptor sites in biochemical (binding characteristics) and functional (renin release regulation) terms.

The use of nonpeptide antagonists of Ang II indicated the existence of two subtypes of Ang II receptors in the afferent arterioles of rats. One site appears to be a typical vascular AT_1 subtype since it displays high affinity to the AT_1 antagonists Dup-753 and Dup-532 and very low affinity to the AT2 antagonists PD-123319 and CGP-42112. This subtype is predominant and occupies 80% of the total number of Ang II receptor sites. The other receptor subtype displays intermediate affinity to the AT_2 antagonist PD-123319 and low affinity to AT_1 antagonists. However, it does not appear to be the AT_2 subtype found in the adrenal gland, because it shows low affinity to CGP-42112. Several recent studies using molecular biology techniques indicated the expression of two AT_1 subtypes (designated as AT_{1a} and AT_{1b}) in the rat renal kidney [11, 12]. Of particular interest, use of quantitative PCR suggested that the relative ratio of the AT_{1a} versus AT_{1b} subtype in the renal cortex was 4:1 [12].

The characterization of Ang II receptor sites in freshly isolated afferent arterioles does not necessarily mean that these sites are functional. To verify the adequacy of this vascular preparation for functional studies, we investigated the responsiveness of the afferent arterioles to agents that control the expression of the renin-angiotensin system, such as Ang II and forskolin. In control rats, renin release was increased with time, it was inhibited by exogenous Ang II, and it was stimulated by forskolin. It would be interesting to investigate whether the two Ang II receptor subtypes found in our preparation influence renin release differently.

In rats chronically treated with an ACE inhibitor, baseline renin release was increased 15-fold. Such a large increase in renin release is in agreement with studies indicating that in response to ACE inhibition vascular smooth muscle cells of the renal vasculature from afferent arteriole up to interlobar and arcuate arteries express and release renin to a rate several times higher than the normal [13]. Interestingly, in the perindoprilate-treated rats, isolated afferent arterioles continued to be responsive to exogenous stimuli since renin release was increased or decreased following exposure to forskolin or Ang II, respectively (Table 1).

In conclusion, we have described a reproducible technique to isolate from the rat kidney afferent arterioles of high purity and in sufficient quantities to perform biochemical studies such as Ang II receptor characterization and regulation. The metabolic responsiveness of our vascular preparation to mediators of Ang II receptor expression and renin release suggests that this model is a useful tool to study the autocrine and paracrine regulation of the renin-angiotensin system at the cellular level.

Acknowledgments

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