Angiotensin II Receptor Subtypes on Adrenal Adenoma in Primary Hyperaldosteronism

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ABSTRACT
Patients with an aldosterone-producing adenoma (APA) characteristically fail to show an increase in plasma aldosterone (PA) concentration with maneuvers that increase angiotensin II (Ang II), yet they retain a brisk response of PA to adrenocorticotropic hormone. Therefore, adrenal Ang II receptor binding was characterized in a patient with APA who had a blocked PA response to Ang II infusion before adrenalectomy. The binding of [125I]Sar1, Ile8-Ang II in adrenal gland and tumor was fully displaced by excess Ang II. In the tumor, 98% of [125I]Sar1, Ile8-Ang II binding was displaced by the AT1 receptor antagonist losartan, yet only 5% was displaced by the AT2 receptor antagonist PD-123,319. Autoradiography of the adrenal gland itself showed a predominance of AT1 receptors in the cortex and AT2 receptors in the medulla. The tumor showed a predominance of AT1 receptors, but there was some evidence of a limited population of AT2 receptors. The tumor and adjacent adrenal contained high concentrations of Ang II. In conclusion, a defect in Ang II-stimulated aldosterone secretion in APA occurs despite high concentrations of Ang II in the adrenal and the presence of specific, high-affinity Ang II receptor binding sites.

Key Words: Hypertension, aldosterone, adrenocorticotropic hormone

Primary hyperaldosteronism is a rare secondary cause of hypertension the principal features of which include hypertension, hypokalemic alkalosis, and elevated plasma aldosterone (PA) concentration(s) with suppressed PRA. The two most common causes are aldosterone-producing adenoma (APA) in 80 to 90% and idiopathic hyperaldosteronism (IHA) due to bilateral micronodular or macronodular hyperplasia. There has been much interest in the differential pattern of aldosterone secretory response to angiotensin II (Ang II) in APA and IHA. Tests founded on this discriminant response help to distinguish between these two forms on the basis of the response of PA to change in posture, angiotensin-converting enzyme inhibition, or volume expansion (1). Characteristically, the responsiveness of PA to Ang II is preserved in patients with IHA, whereas patients with APA are relatively or totally unresponsive (2). In contrast, the aldosterone response to adrenocorticotropic hormone (ACTH) infusion may be supranormal in patients with both APA and IHA (3). The basis for the discrepancy between these responses to Ang II and ACTH has not been elucidated. It might relate to a loss of high-affinity receptors to Ang II on the adenoma or uncoupling of these receptors from the steroid response. At least two subtypes of Ang II receptors have been defined with antagonists such as losartan (DuP-753), an antagonist of Ang II type 1 (AT1) receptors, and PD-123,177, an antagonist of Ang II type 2 (AT2) receptors (4). Tofovic et al. (5) have shown that AT1 receptors mediate most of the recognized effects of Ang II, including vasoconstriction and aldosterone secretion. In vitro studies of patients with APA by Brown et al. (6) have demonstrated a decreased number of high-affinity Ang II receptors, but the presence of specific AT1 or AT2 receptors has not been studied. We have evaluated a patient with an APA by characterizing the PA response to infusions of Ang II and ACTH and examining the adenoma and adjacent adrenal tissue for specific AT1 and AT2 receptors.

CASE REPORT AND CLINICAL STUDIES

A 46-yr-old white man was referred for evaluation of refractory hypertension that had been present for 12 yr. He complained of thirst, nocturia, and paroxysmal palpitations. After 1 month without antihypertensive drugs, his supine blood pressure was 182/124 mm Hg with a heart rate of 76 beats/min and his standing blood pressure was 164/120 mm Hg.
with a heart rate of 92 beats/min. Funduscopic examination revealed Keith-Wagener Grade II hypertensive changes. There was no edema. Initial laboratory evaluation was significant for a serum potassium concentration of 2.9 mmol/L (normal range, 3.5 to 5.0), a measured serum total CO₂ concentration of 35 mmol/L, a random PRA of <0.03 ng/L-s (<0.01 ng/mL-h), and a PA concentration of 580 pmol/L (normal range. 30 to 440). A 24-h urine collection revealed a creatinine clearance of 1.65 mL/s, a potassium excretion of 64 mmol/24 h, a free cortisol excretion of 120 nmol/24 h (normal range, <140), and an aldosterone excretion of 135 nmol/24 h (normal range, 8 to 33). A computed tomographic scan of the abdomen detected a well-defined, 2-cm homogeneous left adrenal mass with a normal-appearing right adrenal.

Subsequent evaluations were performed in the Clinical Research Center, where he received a 2-g sodium diet. Serum potassium had been normalized by prior therapy for 6 wk with potassium chloride (120 to 160 mmol/24 h) and remained normal throughout this admission. After an overnight recumbency, a postural stimulation test was performed (7). This was followed by a saline suppression test (8). PRA was determined during these and the subsequent infusion studies; it was severely suppressed throughout (<0.03 ng/L-s). There was the expected fall in the serum cortisol concentration during the day. The PA before the postural stimulation test was 1,550 pmol/L at 8 a.m. and actually declined to 840 pmol/L after 4 h of ambulation. There were no significant effects on PA of stimulation or suppression of Ang II concentration by orthostasis or saline infusion, respectively. Saline infusion (1.25 L over 2 h) decreased PA only slightly from 870 to 730 pmol/L.

The PA responses to 30-min iv infusions of saline-5-Ang II (0.1, 0.3, 1.0, 3.0 and 10.0 ng/kg-min) and ACTH (6.25, 12.5, 25.0, 50, 100, 200, and 25,000 mU/30 min) were undertaken on separate days as described by Hollenberg et al. (2) and Kem et al. (3), respectively. There was a modest pressor response of 20 to 30 mm Hg with the higher doses of Ang II but no detectable changes in blood pressure with ACTH.

The patient subsequently underwent a left adrenalectomy. His blood pressure became normal without the need for antihypertensive drugs by the end of the third postoperative week. His serum potassium became normal without the need for supplementation. The left adrenal tissue was processed in the laboratory. He remains normotensive and normokalemic without any therapy 1 yr after adrenalectomy.

METHODS

Plasma levels of Ang II were measured by RIA as previously described (9). Briefly, plasma was extracted with acidic acetone; the extract was dried, redissolved in Tris buffer, and measured by RIA. Adrenal tissue was extracted with acetic acid; the Ang II was purified on SepPak C-18 cartridges (Waters Inc., Milford, MA) before RIA. For receptor studies, the adrenal gland and tumor were separated; both tissues were analyzed by receptor autoradiography and receptor binding methods. The studies of membrane binding were undertaken immediately after the adrenal gland was removed at the time of the operation. They were performed on fresh tissue that had not been frozen. Tissue for autoradiography was frozen on dry ice.

Receptor Binding

Pieces of adrenal cortex or adrenal tumor were homogenized in ice-cold Tris buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM Na₂EDTA, pH 7.4 at 4°C). To the buffer were added 0.1% BSA, 0.1% bacitracin, and 10 mM 1,10-phenanthroline to prevent the breakdown of Ang II. They were briefly centrifuged at 600 g at 4°C; the supernatant was centrifuged at 48,000 g, and the resulting pellet-containing membranes were resuspended in buffer. The adrenal cortex membrane suspension was incubated with 0.15 nM [125I]Ang II with increasing concentrations of losartan (100 pM to 1 μM) or PD 123,319 (10 nM to 10 μM). For comparison of receptor subtype density in this adrenal tumor membrane suspension, [125I]Ang II binding was displaced with maximal concentrations (10 μM) of losartan or PD-123,319. Non-specific binding was determined by displacement with 1 μM Ang II. The reaction was stopped after 45 min by the addition of ice-cold buffer; the mixture was then filtered onto polyethyleneimine-soaked Whatman GF/C filters (Whatman, Hillsboro, OR) under vacuum, washed, and counted in a β-counter.

Autoradiography

Monolabeled [125I]SI-Ang II (2.176 Ci/mmol by immunologic self-displacement analysis) was prepared by the chloramine T method (Hunter and Greenwood method; see reference 9 for details) with [125I]NaI (DuPont, NEN Research Products, Boston, MA; NEZ-33H) and Sar¹,Ile³-Ang II (Sigma Chemical Co., St. Louis, MO). The mono-[125I]-labeled peptide was purified by reverse-phase high-performance liquid chromatography.

Adrenal tumor tissue and adrenal gland tissue were cryostat sectioned into 20-μm-thick sections and thaw mounted onto chrom-alum-gelatin–coated slides in multiple sets of five. Sections were stored at –20°C until autoradiography was performed.

For autoradiography, slide-mounted tissue sections were thawed at 35°C and preincubated in assay buffer (150 mM NaCl, 50 mM NaPO₄, 1 mM EDTA, 0.1 mM bacitracin; pH 7.4) for 30 min before incubation with 500 to 600 pM [125I]SI-Ang II for 2 h in the presence of absence of 1 μM Ang II to define
nonspecific and total [125I]SI-Ang II binding, respectively. Adjacent sections were incubated with 1 μM losartan or 1 μM PD-123,319, dipped in two changes of distilled water, rinsed five times in assay buffer, and dipped a final two times in distilled water. The sections were dried under a stream of cool air for 3 to 4 min and mounted on cardboard. All steps were carried out at 22°C. Autoradiograms were generated by the apposition of the slide-mounted tissue sections with sheet film (SB-5; Kodak, Rochester, NY) in x-ray cassettes for 3 days.

RESULTS

Figure 1 depicts PA values during graded iv infusions of Ang II and ACTH. On both occasions, the basal PA concentrations were above the normal range. The PA levels were not changed consistently by Ang II infusion; however, PA levels were increased progressively by ACTH infusion above a threshold dose of 12.5 mU/30 min. Plasma Ang II concentration increased during Ang II infusion from 22.6 to 92.8 pg/mL at the highest dose but remained within the range of 18.8 to 31.5 pg/mL throughout the ACTH infusion. The Ang II concentration in the adrenal tissue was 873 pg/g, and in the adrenal tumor, it was 1,030 pg/g.

The competition between [125I]Ang II and increasing amounts of the antagonists for AT1 receptors (losartan) and AT2 receptors (PD-123,319) in the adrenal gland is shown in Figure 2A. There is a clear displacement by losartan to a maximum of 80% at 1 μM. The specific binding in the adrenal cortex was 6.82 ± 1 mU/mg. The IC50 for losartan was 23.1 nM. There was a lack of inhibition of binding by PD-123,319 in concentrations up to 10 μM. Therefore, the results demonstrate that the receptors in the adrenal cortex are predominantly the AT1 and not the AT2 subtype. Receptor binding inhibition in the adrenal tumor showed that the AT1 antagonist (losartan, 10 μM) produced 98% inhibition of [125I]Ang II binding. In contrast, the AT2 receptor antagonist PD-123,319 (10 μM) produced only 5% inhibition of binding (Figure 2B).

Figures 3 and 4 demonstrate autoradiography of the [125I]SI-Ang II binding to the adrenal gland and the adrenal tumor, respectively. In each figure, panel

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**Figure 1.** PA responses to iv infusions of Ang II (left panel) and ACTH (right panel). The normal range for PA concentration is 30 to 440 pmol/L.

**Figure 2.** Left, displacement of [125I]Ang II in the adrenal cortex by increasing concentrations of AT1 receptor antagonist (losartan; DuP-753; solid lines) or AT2 receptor antagonist (PD-123,319; broken lines). Right, displacement of [125I]Ang II from adrenal adenoma tissue by 10 μM concentrations of losartan or PD-123,319.
A depicts total [125I]SI-Ang II binding and panel B depicts the nonspecific binding that remains after displacement with Ang II. Panels C and D show [125I] SI-Ang II binding in the presence of the specific antagonists losartan and PD-123,319, respectively. Radioreceptor analysis by both the binding and autoradiography methods concur in showing that both adrenal gland and tumor tissue from the patient have specific angiotensin receptors. Considerable specific binding was apparent in both the cortex and the medulla of the adrenal gland (Figure 3A and B). The binding of [125I]SI-Ang II in the adjacent adrenal tissue (Figure 3B) or tumor (Figure 4B) was fully displaced by excess Ang II. The Ang receptors in the tissue of the adrenal cortex are predominantly AT1, because the binding is displaced by losartan (Figure 3C). In contrast, in the adrenal medulla, the receptors are predominantly AT2 because there is >98% displacement with PD-123,319 (Figure 3D). Although losartan displaced the [125I]SI-Ang II binding in the adrenal cortex, it displaced very little or none in the medulla (Figure 3C). This indicates a differential distribution of Ang II receptor subtypes in the two adrenal structures. With autoradiography, at a concentration of 1 μM, losartan appeared to displace about 80% of the [125I]SI-Ang II binding in the cortex, whereas PD-123,319 at a similar concentration displaced only about 10 to 20% (Figure 3C and D). Autoradiography confirmed that the tumor was rich with specific Ang-binding sites (Figure 4A and B). In the tumor, the predominant receptor was AT1, but there is also evidence for a limited population of AT2 receptors in the tumor and adjacent tissue, with a distribution shown in Figure 4C. PD-123,319 displaced little of the Ang binding (Figure 4D), confirming that the tumor displayed a marked predominance of AT1 receptors (Figure 2B).

DISCUSSION

Patients with APA characteristically lose the normal increment in PA during postural stimulation and the normal suppression during saline infusion. This was clearly seen in the patient presented here, whose aldosterone level actually fell after assuming the upright posture and remained strikingly elevated after saline infusion. Recently, Fontes et al. (10) reviewed
the diagnostic accuracy of the postural stimulation test in 146 patients with primary hyperaldosteronism. They found that the test had a sensitivity of 85% and a specificity of 81% for diagnosing APA. They concluded that this test, when associated with an abnormal computed tomographic or magnetic resonance imaging scan, provides a reliable method for the identification of all surgically correctable subsets of primary hyperaldosteronism. The postural test is based on a relative insensitivity of aldosterone secretion to small changes in plasma Ang II concentration in patients with APA. In this study, graded iv infusions of Ang II did not increase PA consistently, despite more than a doubling of plasma Ang II concentration; ACTH, however, caused a fourfold rise. These results confirm the insensitivity of the aldosterone response to Ang II in vivo in patients with APA and the preserved response to ACTH.

Our results demonstrate that this patient with APA had Ang II receptors in both the adrenal gland and the tumor. Brown et al. (6) detected Ang II receptors on specimens of adrenal adenomas from patients with APA. However, they found a loss of high-affinity Ang II receptors, but a preservation of low-affinity receptors, on the tumor and adjacent adrenal tissue. They further documented a blunted Ang II-dependent aldosterone secretion from aldosterone-producing adenoma tissue studied in vitro. They suggested that there was a defect in the aldosterone biosynthetic pathway. In the case presented here, a blunted aldosterone response to infused Ang II was found despite the presence of Ang II receptors, which were clearly detected in both the adrenal gland and the tumor.

Recently, Ang II receptors have been subclassified into AT1 and AT2 (4). Only AT1 receptors are implicated in the regulation of aldosterone secretion. The results from the study presented here show clearly that AT1 receptors are present throughout the adrenal adenoma and that most of the specific Ang II binding on the adenoma is due to the AT1, rather than to the AT2, receptor subtypes. In the adrenal gland, the cortex contained predominantly AT1 receptors, yet the medulla contained predominantly AT2 receptors. AT2 binding has not been reported in human adrenal medulla previously but has been reported in rat adrenal medulla (10). A single value of

Figure 4. Autoradiography of the [125I]Sl-Ang II binding to the adrenal tumor of the patient. A, total [125I]Sl-Ang II binding; B, nonspecific binding in the presence of 1 μM Ang II; C, binding in the presence of 1 μM losartan potassium; D, binding in the presence of 1 μM PD-123,319 (magnification, ×2).
IC50 for displacement of binding by losartan from the adrenal cortex is not sufficient to use for comparison with receptor affinity data obtained in other studies. A series of studies with normal and adenomatous adrenal glands would be required to determine whether there is a change in the IC50 for losartan displacement of Ang II binding to adenomatous tissue.

The finding of abundant AT1 receptors on the adrenal adenoma in a patient who has a severely blunted aldosterone response to Ang II infusion indicates that this blunted response is not likely due to defects in AT1 receptor number, affinity, or binding characteristics. Our studies have not identified the cause for this lack of aldosterone response to Ang II but are consistent with the hypothesis that there is a postreceptor defect. There were high levels of Ang II in the adrenal gland and tumor. High levels of Ang II in the adrenal gland have been found in rat adrenal (9), but this is the first report of Ang II in human adrenal and tumor tissue. It is interesting that there are high levels of Ang II in the adrenal gland and that AT1 receptors are present in the adrenal cortical tissue surrounding the tumor, yet there is no PA response to Ang II infusion. This indicates that whatever factor(s) the tumor is making affects normal as well as adenomatous tissue. This implies that the lack of aldosterone response is not due to simple dedifferentiation of the tumor tissue but rather that the tumor is making a metabolic inhibitor of normal adrenal glomerulosa function.

Patients with IHA have a retained, or even increased, aldosterone response to Ang II infusion. Therefore, it seems unlikely that the defective response to Ang II that is found in patients with APA is a consequence of prolonged aldosterone excess, hypertension, or volume expansion. Patients with APA characteristically have a considerable PA response to ACTH but not to Ang II. Because we found no defect in high-affinity Ang II receptor binding in APA, the second messenger system that is specific to Ang II may be impaired. Although the addition of Ang II, potassium, and ACTH to isolated zona glomerulosa cells activates the calcium second messenger system, only Ang II activates phosphoinositide hydrolysis. The responses of Y1 adrenal tumor cells appear analogous to the APA in this study. These tumor cells show impaired steroidogenic responses to Ang II, although they possess Ang II-binding sites (12). Ang II stimulation of these Y1 cells causes a subnormal accumulation inositol phosphates, which suggests that the coupling between Ang II and phospholipase C is impaired. Future studies will be required to characterize the defects in the postmembrane events that underlie the selective reduction in the responsiveness of aldosterone secretion to Ang II in patients with APA.

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