Steroidogenic Characteristics of a New Aldosterone-Stimulating Factor (ASF) Isolated from Normal Human Urine

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SUMMARY The steroidogenic properties of a glycoprotein fraction (urinary ASF), isolated from normal human urine, were studied in collagenase-dispersed rabbit adrenal capsular cells to 1) define the requirements for its steroidogenic activity, and 2) assess its site and mode of action. When incubated with adrenal cell suspension at 37°C for 2 hours, urinary ASF induced dose-related increases in both aldosterone and corticosterone production. However, urinary ASF was less potent (ED50 = 10^-8 M) than either angiotensin II (ED50 = 8 x 10^-11 M) or ACTH (ED50 = 4 x 10^-11 M). Increases in cyclic AMP accompanied the steroidogenic response to ACTH but not to either urinary ASF or All. Deprivation of potassium in incubation media or the addition of ouabain (1 mM) during incubation completely inhibited the steroidogenic response to either urinary ASF, ACTH, or All. Like ACTH and All, urinary ASF increased conversion of corticosterone to aldosterone. Specific competitive antagonist of All (Sar1, Thr1) and ACTH ([Ile2]ACTH1-24) did not prevent the ASF-induced increase in aldosterone production. These results suggest that urinary ASF is readily distinguishable from ACTH. Although it shares similar steroidogenic properties with All, the inability of All antagonist to block its effects suggests that it acts at a separate receptor site.

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Key Words • adrenal capsular cells • aldosterone-stimulating factor • aldosterone biosynthesis • angiotensin II antagonist • ACTH antagonist • cAMP production

It is generally accepted that the secretion of aldosterone is under the regulation of four humoral factors; namely, angiotensin II (All), ACTH, potassium, and sodium. In addition, evidence has accrued indicating that other as yet unidentified factor(s) may also play a role. In 1959, Mulrow and co-workers isolated from human urine a substance that stimulated rat adrenocortical tissue. However, they dismissed their observation as nonphysiologic because the substance failed to produce a dose-related steroidogenic response. In a series of experiments, Farrell in the dog, Abraham et al. and Denton et al. in sheep, and McCaa et al. in the dog have reported evidence implicating the central nervous system as a possible source of an aldosterone-regulating factor. Nicholls et al. using the transplanted sheep adrenal gland as the bioassay system, reported that plasma from a patient with primary aldosteronism due to adrenal cortical hyperplasia possessed an aldosterone-stimulating activity that could not be attributed to either potassium, renin, or ACTH.

More recently, Sen and coworkers reported the isolation of a protein fraction from the urine of normal human beings that was found to produce hypertension in normal rats during chronic intraperitoneal administration. The hypertension was gradual in onset (7-10 days) and remitted within 5-7 days after the injections were discontinued. The hypertension appeared dependent on the adrenal glands since it was associated with hypervolemia and hyperaldosteronism and did not develop when the compound was given to adrenalectomized animals on maintenance doses of corticosterone.

Since that initial description, the compound has been more fully purified and characterized. It has now been determined to be a glycoprotein molecule with a molecular weight of 26,134 daltons. The present study was undertaken to define more fully the requirements for its steroidogenic activity and to assess its site and mode of action. The results suggest that its in vitro steroidogenic activity is readily distinguishable from either ACTH or All, and that it increases aldosterone production partly by promoting the conversion of corticosterone to aldosterone.
Materials and Methods

The procedure for the isolation, purification, and characterization of the protein fraction from urine has been described elsewhere. Its biochemical characteristics are as follows: 1) it is a glycoprotein with a molecular weight of 26,134 daltons; 2) it is completely destroyed by enzymatic hydrolysis with trypsin and by exposure to extremely low or high pH; and 3) its chromatographic and electrophoretic mobility are unlike that of either ACTH or All.

Alpha 1-24 ACTH (Cortrosyn) was a gift from Dr. Kumar. [Asp1, Ile8]ACTHa 1-24 was a gift from Dr. M. Khosla (Research Division, Cleveland Clinic). Medium 199 was obtained from Grand Island Biological Company, Grand Island, New York. 3-Isobutyl-1-methylxanthine and ouabain-octahydrate were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. Crude collagenase was obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

Preparation of Rabbit Adrenal Capsular Cell Suspension

Cell suspensions were prepared as described by Fredlund et al. Both adrenal glands were excised from rabbits (4-7 kg) under sodium pentobarbital anesthesia (30-50 mg/kg i.v.). The glands were cleaned of fat and bisected; then the core (inner portion of the fasciculata, reticularis, and medullary tissue) was extruded by squeezing the gland. The capsules were finely minced and washed in medium 199 containing bovine serum albumin (2 mg/ml) and potassium (4 mEq/liter). The minced capsular tissue was placed in a 50 ml polyethylene centrifuge tube suspended in 10 ml of media 199 containing collagenase (2 mg/ml), then incubated at 37°C under 95% O2 and 5% CO2 for 15 minutes, with shaking at 50 cycles per minute (rpm) in a Dubnoff metabolic shaker. The tissue was then allowed to settle, and the supernatant solution was discarded. The collagenase-treated tissue was resuspended in 10 ml of collagenase-free medium 199 and dispersed mechanically by drawing up repeatedly into a 15 cm section of 5 mm 1D polyethylene tube attached to a 30 ml syringe, and then it was allowed to settle. The supernatant containing the isolated cells was filtered through two layers of fine gauze into another polyethylene centrifuge tube. The remaining tissue was then reincubated with collagenase and treated as described above.

The pooled supernatant containing the isolated cells was centrifuged at 100 g for 10 minutes at room temperature. The supernatant was discarded, and the cell pellet was resuspended in 5 ml of medium 199. The number of harvested cells was estimated with a hemocytometer, then diluted to a final concentration of approximately 200,000 cells/0.9 ml of medium 199. Most cells were viable as less than 5% of them failed to exclude trypan blue stain. The harvested cells consisted of approximately 92% zona glomerulosa cells and 8% fasciculata-reticularis cells.

Incubation of Isolated Adrenal Capsular Cells

Incubation was performed in polyethylene scintillation vials (Beckman Poly Q II) at 37°C under 95% O2 and 5% CO2 for 2 hours in a Dubnoff metabolic shaker. Diluent for test substances was medium 199. Approximately 200,000 cells in 0.9 ml medium 199 and 0.1 ml of diluent with or without the test substance was added to each vial. Following 2 hours of incubation, media and cells were decanted into a polyethylene tube and centrifuged at 3000 X g for 15 minutes. Aliquots of the supernatant were assayed for aldosterone, corticosterone, and cAMP. In each experiment, duplicate or triplicate samples were run including "zero time" and "2-hour" control samples. Values reported are net changes (i.e., control values were subtracted from the vials with the test substance).

Comparative Studies

Comparative Effects of Urinary ASF, All, and ACTH Upon Aldosterone and Corticosterone Production

In seven separate experiments, the steroidogenic potency of urinary ASF was compared to those of All and ACTH. In these studies, all determinations were done in triplicate utilizing the same pool of cells. Doses of urinary ASF and All ranged from concentrations of 10-11 to 10-7 M and those of ACTH from 3.5 × 10-18 M to 3.5 × 10-8 M.

Relationship of the Steroidogenic Action of Urinary ASF to Cyclic AMP Production

This relationship was assessed in five separate incubation studies. Responses to varying concentrations (10-14 to 10-3 M) of urinary ASF were assessed. Determination of aldosterone and cAMP in media were made after 2 hours of incubation. Separate vials were employed for each point. In the vials for determination of cAMP, incubation proceeded in the presence of 1.0 mM of 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, to prevent destruction of generated cAMP by naturally occurring phosphodiesterase. No inhibitor was added to those vials for determination of aldosterone.

Comparative Effects of Varying Concentrations of Potassium in Media upon the Steroidogenic Response to Urinary ASF, All, and ACTH

The effect of urinary ASF (10-7 M) on aldosterone production was assessed in the presence of varying concentrations of potassium (0, 2, 4, and 8 mM) in five separate experiments. These responses were compared to those evoked by maximal stimulatory concentrations of All (10-7 M) and ACTH (3.5 × 10-8 M) under identical conditions. For these studies, the preparation of isolated cells and dilution of all agonists were carried out with potassium-free medium 199. Changes in medium concentration of potassium were made by
adding the appropriate amount of potassium chloride solution. Measurements were done following 2 hours of incubation.

Effect of Inhibiting Na-K ATPase Activity on the Steroidogenic Response to Urinary ASF

The ability of urinary ASF to stimulate aldosterone production was assessed in the presence of ouabain (1 mM), an inhibitor of membrane-bound Na-K ATPase activity, to determine if intracellular transport of potassium is essential for its steroidogenic action. Results were compared to those evoked by All and ACTH under identical conditions. Doses of urinary ASF ranged from concentrations of $10^{-11}$ to $10^{-7}$ M and those of ACTH from $3.5 \times 10^{-11}$ to $3.5 \times 10^{-4}$ M. The dose of ouabain used has previously been shown to reduce intracellular potassium by approximately 60%, and its inhibitory effect to be readily reversed by high concentrations of extracellular potassium. Potassium concentration in media was kept constant at 4.0 mM.

Effect of Urinary ASF on the Late Pathway of Aldosterone Biosynthesis (Conversion of Corticosterone to Aldosterone)

Adrenal capsular cells prepared as described above were resuspended in 5 ml of medium 199 with BSA (5 g/100 ml), then diluted to a final concentration of approximately 200,000 cells/ml. In these studies, WIN 19,578 (4α, 5-epoxy, 17β-hydroxy-3-oxo-androstane-2α-carbonitrile) was used to inhibit conversion of pregnenolone to progesterone. Then 0.1 ml of diluent, with or without test substance, 0.1 ml of WIN 19,578 (1.0 μM), and 0.1 ml of corticosterone (500 ng/ml) were added to the cells. After the preparation was incubated for 2 hours, as described above, the cells were sedimented by centrifugation and the supernatant assayed for aldosterone. Values are reported as percent increment of conversion.

Effects of Specific Competitive Antagonists of All and ACTH on Aldosterone Response to Urinary ASF

Aliquots of isolated cells were incubated with urinary ASF ($10^{-7}$ M) or All ($10^{-7}$ M) in the presence of [Sar1, Thr4]All ($10^{-8}$ M), a specific competitive antagonist of All. In a second series of experiments, the response to urinary ASF ($10^{-7}$ M) or ACTH ($3.5 \times 10^{-4}$ M) was assessed in the presence of equimolar concentrations of [Ile8]ACTH.

Analytical Procedures

Aldosterone concentration in media was determined by radioimmunoassay utilizing antibodies to aldosterone-γ-lactone, 3-carboxymethylxoxime coupled to BSA. The sensitivity of the assay is 0.1 ng/tube. The interassay and intraassay coefficient of variation is 10% and 7% respectively. Corticosterone concentration in media was determined by radioimmunoassay using antibodies to corticosterone-21-hemisuccinate. The sensitivity of the assay is < 0.1 ng/tube.

Cyclic AMP content was determined using the protein-binding assay described by Tovey et al. The sensitivity of the assay is < 0.05 pmole/tube, and the recovery is 76%. The interassay and intraassay variability of the method is 12% and 8% respectively.

Statistical Analysis

The data are presented as mean ± SE. Statistical evaluation of the results was made by unpaired t test.

Results

Comparative Effects of Urinary ASF, All, and ACTH upon Aldosterone and Corticosterone Production

Urinary ASF, All, and ACTH induced dose-related increases in both aldosterone and corticosterone production (fig. 1). In order of potency,
ACTH > AII > urinary ASF in stimulating steroidogenesis by isolated adrenal zona glomerulosa cells. The half-maximal concentration required to stimulate steroidogenesis (ED$_{50}$) was 4.0 × 10$^{-11}$ M for ACTH, 8.0 × 10$^{-11}$ M for AII, and 1.0 × 10$^{-8}$ M for urinary ASF. ACTH was a more potent stimulus for corticosterone production than either AII or urinary ASF.

Relationship of the Steroidogenic Action of Urinary ASF to Cyclic AMP Production

Unlike ACTH, urinary ASF failed to increase cyclic AMP production in concentrations that stimulate aldosterone biosynthesis significantly (fig. 2). Although not shown here, the response to AII was similar to that induced by urinary ASF.

Comparative Effects of Varying Concentrations of Potassium in Media upon the Steroidogenic Response to Urinary ASF, AII, and ACTH

Figure 3 depicts the effect of increasing concentrations of potassium upon aldosterone production in response to maximal doses of AII (10$^{-7}$ M), ACTH (3.5 × 10$^{-8}$ M) and urinary ASF (10$^{-7}$ M). In the complete absence of potassium, neither AII, ACTH, nor ASF was able to stimulate aldosterone production. Potassium, of itself, was a potent stimulus to aldosterone production; at a concentration of 8.0 mM, the increase in aldosterone achieved was not different from that produced when combined with maximal doses of either AII, ACTH, or urinary ASF. Of interest was the observation that ACTH and urinary ASF appeared to be less dependent on potassium concentration. Whereas the response to AII did not reach maximum until the potassium concentration was at least 4.0 mM, both ACTH and urinary ASF produced maximal responses with potassium concentrations of 2.0 mM.

Effect of Inhibiting Na-K ATPase Activity on the Steroidogenic Response to Urinary ASF

Ouabain, an inhibitor of membrane-bound Na-K ATPase activity, completely inhibited the aldosterone response to urinary ASF without inducing any changes in basal aldosterone production (fig. 4). Similar results were obtained with AII and ACTH.
TABLE 1. Effect of Urinary ASF, All, and ACTH on Conversion of Corticosterone (B) to Aldosterone

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>No. of experiments</th>
<th>Aldosterone (ng/2 × 10^6 cells/2 hrs)</th>
<th>% Conversion of B to aldosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>32.8 ± 4.8</td>
<td>6.55 ± 0.97</td>
</tr>
<tr>
<td>Urinary ASF (10^-7 M)</td>
<td>15</td>
<td>38.2 ± 5.3*</td>
<td>7.64 ± 1.06*</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>25.4 ± 3.1</td>
<td>5.08 ± 0.61</td>
</tr>
<tr>
<td>All (10^-7 M)</td>
<td>9</td>
<td>30.1 ± 3.1*</td>
<td>6.02 ± 0.62*</td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>29.7 ± 4.8</td>
<td>5.94 ± 0.96</td>
</tr>
<tr>
<td>ACTH (3.5 × 10^-9 M)</td>
<td>12</td>
<td>38.4 ± 5.0†</td>
<td>7.69 ± 1.00†</td>
</tr>
</tbody>
</table>

All studies were done using WIN 19,578 (1 pM) to inhibit 3β-hydroxysteroid dehydrogenase 44,5-isomerase and the addition of corticosterone (0.1 ml of a solution containing 500 ng/ml) as precursor steroid. All values are expressed as mean ± SEM. Differences are between control and agonist.

* p < 0.01.
† p < 0.001.

**Effect of Urinary ASF, ACTH, and All on the Conversion of Corticosterone to Aldosterone**

All three agonists significantly increased conversion of corticosterone to aldosterone (table 1). Percent increment of conversion for ASF, All, and ACTH were 17.4 ± 2.5, 19.1 ± 3.1, and 31.5 ± 4.56, respectively.

**Effects of Specific Competitive Antagonist of All and ACTH on Aldosterone Response to Urinary ASF**

In these studies, the response to ACTH, All, or urinary ASF alone was taken as 100%. [Sar^1, Thr^8]All produced no effect on the ability of urinary ASF to stimulate aldosterone production. However, the response to All was inhibited by approximately 70%.

Similarly, in the presence of [lle^9]ACTH, a specific competitive antagonist of ACTH, the response to ACTH was significantly inhibited (by 52%) while that to urinary ASF was left unchanged (fig. 5).

**Discussion**

These studies have demonstrated that the glycoprotein fraction isolated from the urine of normal human beings is a potent stimulus of aldosterone biosynthesis in isolated rabbit adrenal capsular cells, and confirm the earlier results obtained in the rat and cat. The maximal stimulatory effect of this ASF was almost similar to that of ACTH and greater than that of All; however, its threshold dose and ED₅₀ were higher than those of either ACTH or All.

The increase in corticosterone production probably reflects not only the response of zona glomerulosa cells but also of contaminating zona fasciculata cells. Although the response of pure fasciculata cells to urinary ASF was not assessed in these studies, it is likely that urinary ASF can stimulate fasciculata cells since in an earlier study utilizing cat adrenal cells we demonstrated that urinary ASF stimulates cortisol production. The ability to stimulate the conversion of corticosterone to aldosterone suggests that its site of action might partly be situated at the late step of steroidogenesis. In these studies, its effect at early steps was not evaluated.

Urinary ASF shares some similar requirements for steroidogenic activity as All and ACTH. Like All and ACTH, urinary ASF is highly dependent on potassium for its steroidogenic action. Increasing concentrations of potassium in medium augmented the aldosterone-stimulating capabilities of all three compounds. In the presence of ouabain, even maximal stimulatory doses of urinary ASF failed to stimulate aldosterone production. These results are compatible with the concept that all stimuli that increase aldosterone secretion require the presence of potassium.

The mechanisms by which urinary ASF stimulates aldosterone production are readily distinguishable from ACTH. In the first place, ASF-induced steroid responses are not associated with increased generation...
of cAMP, while those of ACTH are. Second, and more important, its ability to stimulate aldosterone production could not be inhibited by [Ile^9] ACTH, a specific competitive antagonist of ACTH.\textsuperscript{10}

Urinary ASF shares other similarities with All. Both are relatively weak agonists of corticosterone production. Both stimulate aldosterone biosynthesis without increasing cAMP production. However, the inability of [Sar\textsuperscript{1, Thr\textsuperscript{8}}] All, a specific competitive antagonist of All, to inhibit the steroidogenic activity of urinary ASF indicates that the two compounds act at separate receptor sites.

The present studies have provided evidence for the presence of an aldosterone-stimulating factor in the urine of human beings. In some respects, its steroidogenic activity is similar to that of All and ACTH. However, the finding that it does not stimulate cAMP and that specific competitive antagonists of All and ACTH do not inhibit its activity suggests that it is unlike any aldosterone-stimulating substance described thus far. Whether it is a circulating hormone and plays a major role in the regulation of aldosterone remains to be determined.

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