Localization, Purification, and Biological Activity of a New Aldosterone-Stimulating Factor

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SUMMARY An aldosterone-stimulating factor (ASF) has been isolated from normal human urine and found to be a glycoprotein with a molecular weight of 26,000 daltons. ASF stimulated aldosterone production both in vivo and in vitro. ASF was found to be different from other known aldosterone secretagogues by the use of high performance liquid chromatography (HPLC). The retention time of ASF was different (17.0 minutes) from ACTH (retention time, 28.4 minutes), β-lipotropin (retention time, 20.5 minutes), and angiotensin II. Proteolytic enzyme digestion and purification of ASF by HPLC yielded a smaller molecule (retention time, 22.0 minutes) with a molecular weight of 4000 daltons. This smaller molecule also stimulated aldosterone production in vitro. This showed that the structural requirement for steroidogenesis may be residing in a smaller molecule. ASF failed to produce hypertension in adrenalectomized rats. By immunofluorescence (using fluorescein conjugated antibodies), ASF was found to be localized in the anterior lobe of the pituitary gland. Data suggest that ASF, a new aldosterone-stimulating hormone that has not been described before, is secreted by the pituitary gland, and the adrenal gland appears to be the target organ for the biological activities. (Hypertension 3 (suppl I): 1-81-1-86, 1981)

KEY WORDS • high pressure liquid chromatography • anterior pituitary • aldosterone • immunofluorescence • adrenal gland

We have reported the existence and partial purification of a hypertension-producing and aldosterone-stimulating factor from normal human urine.1,2 The compound has been purified to homogeneity and determined to be a glycoprotein with a molecular weight of 26,000 daltons.3 Because this compound stimulates aldosterone production by the adrenal glomerulosa cells both in vivo and in vitro, the compound has been named "aldosterone-stimulating factor (ASF)." More recently, Saito et al.4 defined the requirements for its steroidogenic activity and showed that ASF shares some similar requirements for steroidogenic activity with ACTH and angiotensin II (AI). We report its purity, its distinct structural difference from other known aldosterone secretagogues, conversion to a small fragment with steroidogenic activity, and localization by immunofluorescent antibodies.

Methods

Preparation of ASF

ASF was isolated and purified by the use of the method described previously.1,3

High Pressure Liquid Chromatography (HPLC)

Analytical separations were done in a liquid chromatograph (LC) using protein column 1-125, obtained from Waters Associates, Inc. This column separates protein molecules in the molecular weight range of 2000 to 80,000 daltons. Freshly prepared phosphate buffer, 0.1 M, pH 5.5, was used as the mobile phase, and the flow rate was maintained at 1 ml/min; the column was calibrated by using known molecular weight markers.

Preparation of Small ASF

Small ASF was prepared by isolating ASF as described earlier,6 without adding proteolytic enzyme inhibitors. After it stood at room temperature for 1 hour, 0.5 mg was applied on HPLC using the conditions described. The protein peak that emerged at a retention time of 22.5 minutes (approximate molecular weight 4000 daltons) was collected from five separate runs and pooled and lyophilized. The
residue was dissolved in 1 ml of water and rechromatographed by gel filtration using Sephadex G-25, and equilibrated with 0.05 M phosphate buffer pH 7.0. Finally, small ASF was partially desalted and concentrated by an Amicon ultrafiltration unit using UM05 membrane.

### Aldosterone Production by Small ASF In Vitro

Aldosterone production by ASF and small ASF in vitro was assayed by using collagenase-dispersed rat adrenal glomerulosa cells. The cells were harvested by crude collagenase as described previously. Approximately 100,000 cells in 0.9 ml medium 199 and either 0.1 diluent or 0.1 ml small ASF (10^{-4}M) were added to each vial for a final volume of 1 ml. They were incubated under 95% O_2 and 5% CO_2 atmosphere at 37°C for 2 hours. Aldosterone produced at the end of the incubation period was measured by the radioimmunoassay method described by Farmer et al.

### Preparation of Antibodies

Antibodies against ASF have been raised in a goat, and the specificity has been determined as described. ASF isolated from dog urine has been shown to cross-react with antihuman goat ASF. The antibodies did not cross-react with ACTH, renin, AII, and β-lipotropin.

### Labeling of Antibodies with Fluorescein Isothiocyanate

Immune serum was diluted 2 times with 0.9% NaCl for purification of antibodies by (NH_4)_2SO_4 fractionation. Four molar (4 M) (NH_4)_2SO_4 solution was added to a final concentration of 2 M. This was centrifuged at 5000 x G for 15 minutes, and the precipitate was washed 2 times with 2 M (NH_4)_2SO_4, dissolved in 0.1 M NaHCO_3, and dialyzed against 0.1 M Na_2CO_3 for 24 hours. The precipitated and dialyzed immunoglobulin fraction (0.5 g/10 ml) was mixed with 1 ml of 0.9% NaCl, 0.3 ml carbonate/bicarbonate buffer pH 9.2, and 750 µg fluorescein isothiocyanate dissolved in 0.25 ml dioxane and 0.25 ml acetone. The solution was stirred at 0°C for 18 hours and then dialyzed against 10 liters of 0.9% NaCl for 72 hours, with five changes of saline during that time period. The pH of the fluorescein-labeled solution was then adjusted to 7.0 with 1 N HCl and extracted with 2 volumes of ethyl acetate. The organic phase was discarded, and traces of ethyl acetate were removed under vacuum. For immunofluorescence control, preimmune serum immunoglobulins from the same goat were also purified and labeled as described above.

### Direct Immunofluorescence Technique

Normal dog tissue was obtained from different organs at autopsy immediately after death. These organs included the anterior and posterior pituitary gland, different areas of the brain (cerebral cortex, hypothalamus, and hypophysis), cerebellum, brain stem, nerves, parasympathetic and sympathetic ganglia, skeletal muscle, heart, lungs, liver, kidneys, gastrointestinal tract, spleen, thyroid, and adrenal glands. The samples were snap-frozen in liquid nitrogen and stored at -79°C.

Frozen sections of these tissues, 4 to 6 µm in thickness, were placed on glass slides, air dried, and incubated with fluorescein isothiocyanate-labeled goat antihuman ASF (1:20 dilution in PBS) in a humid chamber at room temperature for 20 minutes. The slides were then washed in three changes of phosphate buffer saline (pH 7.4), with an additional washing in the same buffer for 15 minutes, cover-slipped with buffered glycerin (1:10), and examined with a mercury lamp Carl Zeiss fluorescent microscope equipped with BG-12 and BG-38 excitation filters and 65 and 50 Carrier filters. The specimens were photographed with the same microscope using ethachrome-200 film.

For control, the same specimens were stained with fluorescein isothiocyanate-labeled goat non-immune serum (1:20 dilution in PBS), as described.

### Preparation of Adrenalectomized Rats

Twelve male Sprague-Dawley rats (180 to 200 g) were bilaterally adrenalectomized under ether anesthesia. They were maintained with oral hydrocortisone (200 mg/liter drinking water), approximately 12 mg/rat/day; blood pressure and body weights were determined daily during the experimental period. ASF was administered intraperitoneally daily in a dose of 6 µg/rat/day. Twelve male rats of the same age injected with the same volume of saline were used as normal controls.

### Results

#### Characterization of ASF by High Pressure Liquid Chromatography

A typical chromatogram of ASF on HPLC is shown in figure 1. As shown, high molecular weight ASF emerged from the column with a retention time of 16.9 minutes. This observation further confirmed our previous finding that ASF was homogeneous, since no other detectable peak was found during the 30-minute running time. The void volume of the I-125 column was 11.5 ml (Ferritin, mw 540,000). When compounds with known molecular weight (mw) were injected, and retention times were plotted against log mw, a linear curve was obtained (fig. 2). Using this plot, we calculated the molecular weight of the large ASF to be 22,000 daltons. By equilibrium ultracentrifugation method, we estimated the molecular weight of ASF to be 26,000 daltons. Equilibrium ultracentrifugation method, however, is a better method for accurate estimation of molecular weight.

We have shown that, when ASF was prepared and purified without proteolytic enzyme inhibitors at room temperature, it hydrolyzed to four smaller fragments (1 major and 3 minor). Using HPLC, we separated one small fragment with a retention time of 22.5 minutes, which was found to be biologically active in stimulating aldosterone production in vitro. The
molecular weight of this small fragment of ASF was estimated to be approximately 4000 daltons. The molecular weight of 4000 daltons may not be accurate, since the small ASF emerged close to the fully retained volume of the column. To compare ASF with the small fragment, when large ASF and small ASF were added in an equimolar quantity to isolated rat adrenal glomerulosa cells, they both stimulated aldosterone production. As shown in figure 3, when large ASF and small ASF were added in a dose of $10^{-7}$M, ASF produced $32.5 \pm 3$ ng/ml, whereas small ASF produced $21 \pm 3$ ng aldosterone/ml at the end of a 2-hour incubation period. This small fragment was 34% less potent than the larger molecule, assuming both molecules were equally stable. Furthermore, these data also suggest that the structural requirement for steroidogenesis of ASF may reside in a smaller fragment.

Separation of ASF and Its Fragment from Other Known Aldosterone Secretogogues by HPLC

With the use of HPLC, both ASF and its fragment have been shown to be structurally different from other known aldosterone secretogogues. A typical HPLC chromatogram is shown in figure 4. The retention time for ASF in this run was 17 minutes, the small ASF 22 minutes, β-lipotropin 20.5 minutes, and ACTH 28.4 minutes. This showed that both large ASF and small ASF were structurally different and can be readily separated from the other known aldosterone secretogogues.

Effect of ASF on Adrenalectomized Rats

When ASF was administered chronically for 10 days to control rats and adrenalectomized rats maintained with hydrocortisone (200 mg/liter), the adrenalectomized rats failed to develop hypertension. The data are shown in figure 5. The blood pressures of the control rats treated with ASF (6 µg/rat/day) were significantly elevated ($p < 0.01$), whereas in adrenalectomized rats blood pressure remained unchanged.
These data suggested that adrenal glands are necessary for the biological activities of ASF.

Histologic Localization of ASF

Direct immunofluorescence studies with goat antihuman ASF showed focal bright apple green cytoplasmic fluorescence in the cells of the anterior lobe of the pituitary gland (fig. 6) and not in any other tissue studied, including the posterior pituitary. Control studies with fluorescein-labeled goat non-immune serum were essentially negative; only a few pale brownish-yellow autofluorescence granules were observed (fig. 7).

Discussion

We reported the isolation and purification of ASF from normal human urine. In this study we have demonstrated that, in addition to the physical and immunological methods, ASF is also homogeneous by high pressure liquid chromatography. Saito et al. reported that ASF shares some similar requirements for steroidogenic activity with ACTH and AII. Like AII and ACTH, ASF has been shown to be highly dependent on potassium for its steroidogenic action. Furthermore, like AII, its ability to stimulate aldosterone biosynthesis is not dependent on cyclic AMP production. These observations suggested that ASF is indeed structurally different or not related to ACTH, AII, and β-lipotropin. In this study, by using HPLC, we have demonstrated that ASF is structurally different from all three aldosterone secretagogues, because the retention times were significantly different (fig. 4). In addition, there is evidence that ASF acts at a receptor site different from ACTH and AII. Saito et al. reported that specific competitive antagonists to ACTH and angiotensin II do not block the steroidogenic action of ASF. Results of these structural and physiological studies provide conclusive evidence that ASF is a new aldosterone secretagogue.

We reported that addition of a mixture of proteolytic enzyme inhibitor is very important for the purification process. Otherwise, the compound hydrolyzes to smaller fragments. By HPLC, we have been able to separate and prepare enough small ASF to study its steroidogenic action. The small ASF is 34% less potent than the parent molecule. When assayed in vitro, using collagenase dispersed rat adrenal glomerulosa cells, the addition of the small fragment in equimolar quantity (10⁻⁷M) as ASF produced 34% less aldosterone compared with ASF. The decreased potency of small ASF compared to ASF may be due in part to the overestimation of the weight because of the presence of some salt in the preparation of small ASF. Using Amicon ultrafiltration (UM05) membrane, complete desalting could
not be achieved. For the preparation of ASF (26,000 daltons) complete desalting was possible by dialyzing ASF against distilled water. Complete desalting of small ASF by dialysis could not be done due to the small size of the molecule (4000 daltons), and ultrafiltration was done instead. Work is in progress to use other biochemical methods to achieve complete purity. The biologically active small fragment will be extremely important if the structural requirement for the steroidogenic action resides in the small molecule, because it is much easier to synthesize and analyze amino acid sequence to define structure activity relationship of a smaller ASF (4000 daltons) than the parent molecule (26,000 daltons).

ASF has been isolated from human urine. To identify its source, an immunofluorescence technique was used to localize ASF. Using a direct immunofluorescence technique with goat antihuman ASF antibodies, ASF was found in the cytoplasm of cells of the anterior lobe of the pituitary gland only. This histologic localization correlates with the fact that ASF was not found in 24-hour urine samples from hypophysectomized patients (unpublished observation). The distribution of the anterior pituitary cells positively stained was focal, indicating that only a certain type of anterior pituitary cell is involved in the production of ASF.

In bilaterally adrenalectomized rats maintained on hydrocortisone, chronic administration of ASF failed to produce hypertension (fig. 5). This observation suggests that adrenal glands are required for the biological activities of ASF, and it appears that they mediate the hypertension-producing property of ASF.

It is generally accepted that the secretion of aldosterone is under the regulation of AII, ACTH, and potassium. However, there is evidence that indicates that other as yet unidentified factor(s) also may play a role. In a series of experiments, Farrell et al. in dogs, Abraham et al. and Denton et al. in sheep, and McCaa et al. in dogs, have reported evidence implicating the central nervous system as a possible source of an aldosterone-regulating factor. Recently, Brown et al. and Kem et al. reported the existence of ASF in normal human urine.

The results of this study provide additional evidence that ASF is structurally and physiologically different from other known aldosterone secretagogues; ASF is also present in the anterior pituitary gland. However, its role in the regulation of aldosterone remains to be determined.
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