Urinary Prostasin
A Candidate Marker of Epithelial Sodium Channel Activation in Humans

Oliviero Olivieri, Annalisa Castagna, Patrizia Guarini, Laura Chiecchi, Gherardo Sabaini, Francesca Pizzolo, Roberto Corrocher, Pier Giorgio Righetti

Abstract—Prostasin is a serine peptidase hypothesized to regulate epithelial sodium channel (ENaC) activity in animals or on in vitro cultured cells. We investigated whether urinary prostasin may be a candidate marker of ENaC activation in humans. We studied 10 healthy volunteers and 8 hypertensive patients with raised aldosterone-to-renin ratio before and after spironolactone or saline/Florinef suppression test, respectively. Four healthy subjects were also studied before and after saline. Urinary prostasin was evaluated by SDS-PAGE, 2D maps, and Western blotting. Every sample of normotensive individuals was compared with the corresponding sample of urine collected after spironolactone or saline; every sample of hypertensive patients was compared with the corresponding sample of urine collected after saline or Florinef. Prostasin was detectable in all subjects regardless of gender, dietary sodium intake, and spironolactone treatment. Spironolactone (100 mg) increased urinary Na⁺/K⁺ ratio and decreased urinary prostasin in normotensives in whom the renin/aldosterone axis was activated by a low Na⁺ intake, but it was ineffective in individuals with high Na⁺ intake. Saline infusion also reduced prostasin in normotensive subjects. In contrast, prostasin paradoxically increased in urine of patients affected by primary aldosteronism after volume expansion. By 2D immunoblotting, several protein isoforms were observed, some of them being overexpressed after inhibition tests in patients with primary aldosteronism. In addition to a “basal” aliquot of prostasin, constitutively released in human urine regardless of sodium balance and aldosterone activation, there exists a second “aldosterone-responsive” aliquot modulated by Na⁺ intake and potentially suitable as candidate marker of ENaC activation. (Hypertension. 2005;46:683-688.)

Key Words: aldosterone ■ renin ■ sodium ions ■ mineralocorticoids ■ hypertension ■ sodium channels

Aldosterone, the principal human mineralocorticoid hormone, is increasingly recognized as playing a significant role in the pathophysiology of renal and cardiovascular disease states. In particular, after the widespread use of the aldosterone-to-renin ratio (ARR) as a screening test, primary aldosteronism was found to be much more frequent than suspected previously. Recently, an elevated ARR was observed in all subjects regardless of gender, dietary sodium intake, and spironolactone treatment. Spironolactone (100 mg) increased urinary Na⁺/K⁺ ratio and decreased urinary prostasin in normotensives in whom the renin/aldosterone axis was activated by a low Na⁺ intake, but it was ineffective in individuals with high Na⁺ intake. Saline infusion also reduced prostasin in normotensive subjects. In contrast, prostasin paradoxically increased in urine of patients affected by primary aldosteronism after volume expansion. By 2D immunoblotting, several protein isoforms were observed, some of them being overexpressed after inhibition tests in patients with primary aldosteronism. In addition to a “basal” aliquot of prostasin, constitutively released in human urine regardless of sodium balance and aldosterone activation, there exists a second “aldosterone-responsive” aliquot modulated by Na⁺ intake and potentially suitable as candidate marker of ENaC activation.

In the last few years, a novel mechanism modulating ENaC activity by serine proteases was identified. A new serine protease, called channel-activating protease 1 (CAP-1), was cloned from a renal cell line; subsequently, Vuagniaux et al isolated a cDNA clone of CAP-1 from a mouse cortical collecting duct cell line, suggesting that CAP-1 is an orthologous gene for prostasin. It was also demonstrated that murine CAP-1/prostasin activates ENaC when expressed in oocytes.

Prostasin is a membrane-bound serine protease discovered in human semen and abundant in prostate gland; in this connection, it was studied mainly as possible marker of high-grade prostate tumors by immunohistochemistry. In 2002, Narikiyo et al demonstrated that aldosterone substantially increases prostasin expression, with functional consequences on Na⁺ balance in vivo; moreover, they reported that urinary excretion of prostasin in 3 patients with aldosterone-producing adenoma was abnormally elevated and normalized after adrenalectomy. These findings suggested urinary prostasin as a possible marker of aldosterone-dependent ENaC activation at the renal level in humans.

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However, in 2003, Wang et al showed that expression of human prostasin by adenovirus-mediated gene delivery was associated with subsequent increase of aldosterone and, in turn, of blood pressure levels in rats. Elevated plasma aldosterone levels were detected at 3 days after gene transfer before the development of hypertension, indicating that adrenal stimulation of mineralocorticoid production by prostasin is the primary event and not vice versa.

Currently available data on prostasin can be therefore summarized as follows: (1) Prostasin seems to be involved in aldosterone-dependent ENaC activation at renal level; however, it is unclear whether aldosterone increases prostasin expression or, on the contrary, a preceding stimulation of prostasin determines adrenal secretion of aldosterone. (2) Available findings were generally obtained by studies on animals or on in vitro cultured cell lines, the only published data on humans being limited to 3 hypertensive patients with aldosterone-producing adenoma. (3) Urinary prostasin was never investigated in normotensive individuals in relation to urinary excretion and balance of Na/HK.

Based on these attractive but preliminary findings, urinary prostasin is far from being considered as a practically suitable marker of ENaC activation because basilar information is still lacking. The present article was therefore aimed at: (1) verifying whether prostasin is detectable in urine of healthy normotensive subjects; (2) clarifying the relationships between prostasin and aldosterone in healthy normotensive individuals by evaluating changes in urinary prostasin after spironolactone-induced blockade of mineralocorticoid receptor (MR) or acute volume expansion; (3) evaluating possible differences in spironolactone-dependent prostasin changes associated with sodium intake or gender (to specifically verify whether prostasin-dependent changes in aldosterone are gender-related); and (4) assessing urinary prostasin in hypertensive patients with inappropriate aldosterone secretion (ie, elevated ARR) before and after saline infusion or fludrocortisone suppression testing.

Methods

Normotensive Subjects

We studied 10 apparently healthy volunteers (24 to 26 years of age; 5 males and 5 females), students of the Faculty of Medicine in Verona, who presented normal blood pressure values in 30 days. Women had to have a negative pregnancy test and regular, predictable, cyclic menses to avoid menstrual bleeding during the study period (48 hours). After informed consent was obtained, each individual collected 24-hour urines before and after taking orally 100 mg spironolactone, the traditional aldosterone receptor inhibitor. At the same times, Na+, K+, creatinine, aldosterone, active renin, and 24-hour urinary excretion of sodium and potassium were also measured. No restricted diet was suggested before the assay, and individuals were invited to avoid relevant changes in their usual diet. No adverse drug-related effect (ie, hypotension) was recorded during the study.

In 4 healthy normotensive subjects (3 females and 1 male), urinary prostasin was also measured before and after saline infusion (2 L of saline IV, infused within 4 hours).

Hypertensive Patients

Urinary prostasin was studied in 8 hypertensive patients (6 females and 2 males; 37 to 65 years of age) in whom an elevated ARR was demonstrated previously. Four patients were investigated by saline and 4 by fludrocortisone suppression testing to definitely confirm or exclude primary aldosteronism. Patients included in the first group received saline infusion test (2 L IV in 4 hours), whereas the others received 4 days of administration of a high-sodium diet and of fludrocortisone acetate (Florinef 0.1 mg every 6 hours). Aldosterone was measured before and after 4 hours of saline infusion or 4 days after fludrocortisone treatment: urine for prostasin evaluation was collected at the same time. Primary aldosteronism was diagnosed if mineralocorticoid hormone did not fall at concentrations <50 pg/mL after saline infusion or Florinef treatment. The study was approved by our institutional review committee, and all subjects gave informed consent.

Sample Preparation

Urines were collected in the morning, immediately chilled on ice, and processed as follows.

After measuring the volume, the pH was adjusted to a value of 7.0 with 1 N NaOH, then the Complete protease inhibitor cocktail (Roche Diagnostics) was added to inhibit the activity of endogenous proteases present in the specimen. Urines were centrifuged at 4°C for 30 minutes at 4000 rpm, and finally filtered through a 0.45-μm Millipore filter. When not immediately concentrated, the samples were kept frozen at −30°C. The concentration was achieved by means of Centricron Plus-20 (M, cutoff 10 000; Millipore), and the final volume was accurately measured. The concentration ratio ranged between 1/10 and 1/40, and the same protein amount (50 μg) was obtained for all samples.

Protein Assay

Protein concentration in concentrated urine was determined with the DC Protein Assay (Bio-Rad) by bovine serum albumin as standard.

SDS-PAGE and Western Blot

An aliquot of concentrated urine was mixed with an equal volume of SDS—sample buffer 2×(0.5 mol/L Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2.5% β-mercaptoethanol, 10% SDS), and boiled for 5 minutes. The resolution was obtained in 10% SDS—polyacrylamide gels. An M, marker kit was used (Benchmark prestained Protein Ladder; Invitrogen).

The resolved proteins were then electrophotographed to a nitrocellulose membrane. After a rapid staining with Poncze Red to verify the transfer and a wash for 1 hour with quenching buffer containing 5% BSA, the membrane was incubated overnight at 4°C with the primary antibody (purified mouse anti-prostasin Mab; BD Biosciences). After washing, the membrane was incubated 1 hour with a secondary antibody (Ab anti-mouse Ig; Amersham Pharmacia Biotech.) conjugated with horseradish peroxidase. Bands were visualized by using a chemiluminescent substrate (ECL; Amersham Pharmacia Biotech.), followed by exposure to x-ray film (Hyperfilm ECL; Amersham Pharmacia Biotech). The antibody concentrations used were 1:375 and 1:10 000 for primary and secondary antibody, respectively.

Every sample of normotensive individuals was processed together with the corresponding sample of urine collected after spironolactone therapy or saline infusion; similarly, every sample of hypertensive patients was processed together with the corresponding sample of urine collected after saline or fludrocortisone suppression testing.

The band densities were measured by densitometry (Densitometer GS710; Bio-Rad), and the relative images were evaluated by means of a specific software (Quantity One; Bio-Rad).

2D-PAGE and Western Blot

The desired volume of each sample was subjected to protein precipitation in a cold mixture of acetone and methanol (v/v ratio 8:1), for removing lipids and salts and for regulating the concentration of protein samples, for 2 hours at −20°C; the solution was then...
TABLE 1. Spironolactone-Induced Changes in Healthy Normotensives

<table>
<thead>
<tr>
<th>Subjects*</th>
<th>Basal Urine Na⁺ (mmol/die)</th>
<th>Basal Active Renin (pg/mL)</th>
<th>Basal Plasma Aldosterone (pg/mL)</th>
<th>Basal Urine Na⁺/K⁺</th>
<th>After Spironolactone Plasma Aldosterone (pg/mL)</th>
<th>Basal Prostasin (OD)/Urinary Proteins†</th>
<th>After Spironolactone Urine Na⁺/K⁺</th>
<th>Basal Prostasin (OD)/Urinary Proteins†</th>
<th>After Spironolactone Prostasin (OD)/ Urinary Proteins†</th>
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<tr>
<td>Low sodium intake</td>
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<td></td>
<td></td>
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<tr>
<td>F1</td>
<td>115</td>
<td>16</td>
<td>178</td>
<td>1.5</td>
<td>150</td>
<td>2.1</td>
<td>0.36/35</td>
<td>0.05/28</td>
<td></td>
</tr>
<tr>
<td>F3</td>
<td>96</td>
<td>21</td>
<td>185</td>
<td>2.5</td>
<td>134</td>
<td>3.1</td>
<td>0.9/18</td>
<td>0.14/16</td>
<td></td>
</tr>
<tr>
<td>F5</td>
<td>76</td>
<td>23</td>
<td>133</td>
<td>2.3</td>
<td>90</td>
<td>3.7</td>
<td>1.38/26</td>
<td>0.2/34</td>
<td></td>
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<tr>
<td>M1</td>
<td>109</td>
<td>24</td>
<td>155</td>
<td>2.7</td>
<td>155</td>
<td>2.5</td>
<td>0.21/29</td>
<td>0.06/20</td>
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<tr>
<td>M5</td>
<td>142</td>
<td>24</td>
<td>350</td>
<td>1.5</td>
<td>300</td>
<td>2.5</td>
<td>1.31/38</td>
<td>0.5/22</td>
<td></td>
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<tr>
<td>High sodium intake</td>
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<tr>
<td>F2</td>
<td>217</td>
<td>3</td>
<td>93</td>
<td>1.7</td>
<td>116</td>
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<td>0.15/25</td>
<td>0.16/33</td>
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<tr>
<td>F4</td>
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<td>16</td>
<td>70</td>
<td>6.3</td>
<td>70</td>
<td>4.6</td>
<td>0.81/12</td>
<td>0.90/10</td>
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<tr>
<td>M2</td>
<td>238</td>
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<td>92</td>
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<td>145</td>
<td>2.8</td>
<td>0.10/36</td>
<td>0.28/42</td>
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<tr>
<td>M3</td>
<td>244</td>
<td>25</td>
<td>106</td>
<td>2.9</td>
<td>110</td>
<td>2.8</td>
<td>0.40/27</td>
<td>0.40/20</td>
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<tr>
<td>M4</td>
<td>187</td>
<td>8</td>
<td>130</td>
<td>2.4</td>
<td>144</td>
<td>2.4</td>
<td>0.50/32</td>
<td>0.56/34</td>
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</table>

*F indicates female; M, male; †OD, optical density/50 μg of urinary proteins; ‡total urinary proteins (mg/L).

Glycosylated Isoforms

To better characterize the different isoforms of prostasin, N-deglycosylation was performed on some samples by treating them with N-glycosidase F (peptide-N-glycosidase F) or with other deglycosylating enzymes according to manufacturer instruction (Prozyme; Roche Diagnostics) for 12 hours at 37°C before any electrophoretic separation.

Results

Spironolactone- and Saline-Induced Changes in Healthy Normotensives

Effects of a single dose of spironolactone (100 mg) were as expected; in particular, in urine, Na⁺ presented an increase of 8% against a decrease of 9% for K⁺. These findings were consistent overall with the cation handling at distal tubular level usually ascribed to the drug effect. However, spironolactone-induced changes differed in individuals according to their previous dietary Na⁺ intake, evaluated as 24-hour urinary Na⁺ excretion before taking the drug. In Table 1, the main features of the normotensive subjects included in the study are reported, grouped according to low (urinary Na⁺ <150 mmol/day) and high (urinary Na⁺ >150 mmol/day) dietary Na⁺ intake. Subjects with a low sodium intake had generally higher levels of plasma aldosterone (146±134 pg/mL) and active renin (21.6±3.3 pg/mL) than those observed in individuals with elevated sodium intake (98±22 and 9.4±9 pg/mL, respectively); however, ARR values were normal and similar in 2 groups (9.3±3.5 and 12.9±11 pg/mL, respectively). On average, spironolactone treatment increased urinary Na⁺/K⁺ ratio by 30% in subjects with low sodium intake (basal Na⁺/K⁺ ratio 2.1±0.56; after drug 2.8±0.6) but not in those with high sodium intake (basal Na⁺/K⁺ ratio 3.5±1.9; after drug 3.1±0.9).

Prostasin was clearly detectable in urine of all subjects regardless of gender, dietary sodium intake, total protein excretion, and spironolactone treatment (Table 1). After the drug treatment, an apparent density decrease of protein band compared with the corresponding basal sample was observed in the urines of all the individuals with low sodium intake but not in the urines of individuals who had high sodium intake (Table 1). A concomitant increase in Na⁺/K⁺ ratio and a decrease in urinary prostasin were therefore the main effects induced by spironolactone, but these effects appear to be limited to the group of subjects in whom the renin/aldosterone axis was activated by a relatively low sodium intake. Normotensive subjects with an elevated sodium intake and, in turn, suppressed renin/aldosterone axis resulted insensitive to spironolactone action in terms of cations and prostasin urinary excretion.

After acute volume expansion by intravenous infusion of saline, prostasin band density decreased in all the subjects...
investigated, regardless of total urinary protein excretion (Table 2).

**Hypertensive Patients**

Eight hypertensive patients, selected previously for having an elevated ARR (>30 pg/mL), were investigated before and after saline infusion or Florinef suppression test. Patients with a positive test (ie, in whom plasma aldosterone levels were still >50 pg/mL after inhibitory challenge) were considered to be affected by primary aldosteronism. In Figure 1, SDS-PAGE density bands of urinary prostasin before and after saline (NaCl) or Florinef test are shown for all patients, with positive or negative inhibition test. Five patients (3 subjects of the group investigated by saline infusion and 2 of that investigated by Florinef) had a positive test (Figure 1, **so that the diagnosis could be confirmed.** Urinary prostasin band in these patients paradoxically increased after inhibitory challenge compared with the corresponding basal density band (Figure 1, patients 1, 2, 5, 6, and 7). On the contrary, in patients presenting an adequate fall of aldosterone levels after suppression test (Figure 1, **), prostasin density bands were reduced or substantially unchanged after inhibition (Figure 1, patients 3, 4, and 8).

**2D Immunoblotting**

Urinary samples of normotensive and hypertensive subjects were also studied by 2D immunoblotting. In agreement with the literature data, prostasin focused at pH range of 5.0 to 6.3; 2D maps, obtained by antiprostasin immunostaining of urine of all individuals tested, showed a complex pattern of protein isoforms with a maximum of 7 apparently well-defined bands, with an apparent M, of 37 kDa (Figure 2).

In hypertensive patients with aldosteronism, 2D immunoblottings were performed before and after suppression test, and the relative patterns of prostasin isoforms were compared by means of PDQuest analysis. According to the software results, 4 isoforms were overexpressed after inhibition tests (Figure 3), confirming the overall increase in prostasin expression observed in the corresponding monodimensional blotting.

Considering this pattern of expression and the previously published information on the glycosylated nature of prostasin, the glycosylation pattern was also investigated, and 2D immunoblotting was performed after treating the samples with specific deglycosylating enzymes. In Figure 4, 2D PAGE immunoblots obtained after treatment with N-glycanase, O-glycanase, or Sialidase-A are reported. An apparent change in the set of protein isoforms was observed after N-glycanase and Sialidase-A treatment but not after treatment with O-glycanase, thus suggesting that N-linked, sialylated sugars characterize urinary prostasin in the investigated samples. Interestingly, some of the isoforms overexpressed in primary aldosteronism resulted to be sialylated and particularly rich in N-linked sugars (Figure 4; 2D PAGE).

**Discussion**

This work represents the first attempt at establishing urinary prostasin as a candidate marker of ENaC activation in vivo, thus extending the previous findings, obtained on animals or in vitro cultured cells, to the setting of human beings with or without hypertension. Based on this premise, it is clear that the value of the present results has to be considered as proof of preliminary feasibility in the perspective of further clinical investigations.

Prostasin was easily detectable in filtered, concentrated urine of all individuals examined, regardless of gender, age,
total protein excretion, and hypertensive or normotensive state. Considering that the same urinary protein concentration was loaded on the immunoblots of all samples, prostasin expression was not substantially different in subjects of both sexes (Table 1). However, keeping in mind that an immunoblotting method is not ideal for a precise quantification of the absolute concentration of a protein (we used it mainly for comparative purpose in the same individual), this finding ruled out substantial confounding effects because of the component of prostatic origin in males.

To clarify the physiological relationships linking prostasin, aldosterone, and ENaC function, we adopted a strategy based on the concept that a clinically tested dose of spironolactone (100 mg) could induce an effective competitive inhibition of aldosterone action at MR level and, in turn, of subsequent Na/H transport through the ENaC. Young students without any significant history of disease and in apparently good health, with proven normal blood pressure and normal plasma potassium, free of any hormone- or drug-related interferences, were therefore studied before and after spironolactone. In this context, the only other covariate able to influence the renin/aldosterone axis was dietary Na intake, which was checked by measuring 24-hour urinary Na. Actually, in these subjects, the extent of basal renin/aldosterone activation inversely reflected low (urinary Na+ <150 mmol/die) and high (urinary Na+ >150 mmol/die) dietary Na+ intake, with lower renin and aldosterone levels in individuals consuming high-Na diet and vice versa (Table 1). The biological consistency of this inverse relationship further confirmed such subjects as a physiologically appropriate model of study.

A single dose (100 mg) of spironolactone at the same time increased urinary Na+/K+ ratio and decreased urinary prostasin expression. However, these effects were not generalized but were restricted to the group of individuals in whom the renin/aldosterone axis was activated by a relatively low sodium intake (Table 1). Thus, in agreement with the expected inhibition of aldosterone and ENaC function, spironolactone-dependent changes in urinary Na+ and K+ excretion were proportional to the hormone levels, resulting relevant or null in subjects with high or low aldosterone, respectively. Similarly, a spironolactone-inhibited aliquot of prostasin was observed only in individuals with an activated renin/aldosterone axis.

Such results suggest that a “basal” aliquot of prostasin is constitutively expressed and released in human urine regardless of sodium balance and hormone activation, whereas a second “aldosterone-responsive” aliquot strictly reflects the modulation exerted by Na+ intake and urinary excretion. In agreement with such a view, it was demonstrated recently in rats that dietary sodium depletion and aldosterone infusion induced prostasin mRNA in colonic epithelial cells. Sodium repletion and volume expansion elicit an inhibitory response on adrenal synthesis and aldosterone circulating levels, and such physiological effect is exploited in clinical practice to confirm the diagnosis of primary aldosteronism in hypertensive patients with an elevated ARR. In addition, Iwashita et al demonstrated recently that an increase in urinary Na+ excretion was associated with a decrease in urinary prostasin excretion in rats. Based on these considerations, we hypothesized that volume expansion induced by saline or Florinef suppression testing could represent a useful model for investigating prostasin expression in normotensive subjects and hypertensive patients with suspected primary aldosteronism. A clear inhibitory effect of saline infusion on

Figure 3. Schematic representation of 2D immunoblotting in hypertensive patients (PDQuest analysis). The master map (synthetic representation) shows the differentially expressed isoforms; after inhibition tests, 4 isoforms were overexpressed (black arrows).

Figure 4. 2D immunoblottings of human prostasin, representative of all samples investigated: A, control; B, after N-glycanase treatment; C, after O-glycanase treatment; D, after Sialidase A treatment.
prostasin excretion was observed in normotensive individuals (Table 2), thus confirming the existence of a volume-dependent feedback mechanism modulating the physiological release of prostasin in urine. In contrast with such behavior, prostasin increased paradoxically in urine of patients affected by primary aldosteronism after volume expansion (Figure 1, patients 1, 2, 5, 6, and 7). On the contrary, prostasin remained unchanged or decreased in the patients who showed an adequate fall in aldosterone levels (and therefore were not diagnosed as having primary aldosteronism; Figure 1, patients 3, 4, and 8). These findings suggest that patients resistant to the physiological feedback mechanism of hormone inhibition, exerted by Na⁺ repletion, also present an abnormal regulation of urinary prostasin expression. Interestingly, the increase of urinary prostasin was not strictly related to the basal absolute levels of aldosterone and the type of inhibitory challenge (saline infusion or Florinef test). Because saline infusion yields a short-term (4 hours) and Florinef test a long-term (4 days) inhibition on aldosterone, the parallel modulation of prostasin seems to occur promptly and permanently over a period of several days.

The findings obtained on normotensive and hypertensive subjects suggested that at least 2 prostasin components could be detectable in human urine, but only 1 appeared to be responsive to spironolactone or modulated by Na⁺ intake and volume. For this reason, we performed 2D immunoblotting to identify possible isoforms of the protein specifically responsive to this modulation. At pH range of 5.0 to 6.3, a specific set of 7 isoforms with an apparent Mr of 37 kDa could be visualized in all samples. Four isoforms were overexpressed after inhibition tests in patients with primary aldosteronism (Figure 3); moreover, some of these isoforms resulted to be sialylated and particularly rich in N-linked sugars (Figure 4). Although further work is necessary, these data suggest that it is possible to separate and to characterize those isoforms of greater clinical interest and utility. Considering that urine is easily collected and represents a medium at relatively low number of interfering proteins, a precise quantification of aldosterone-dependent prostasin by more accurate methods should be feasible.

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References
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