Adenovirus-mediated human prostasin gene delivery is linked to increased aldosterone production and hypertension in rats

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Wang, Cindy, Julie Chao, and Lee Chao. Adenovirus-mediated human prostasin gene delivery is linked to increased aldosterone production and hypertension in rats. Am J Physiol Regul Integr Comp Physiol 284: R1031–R1036, 2003.—Prostasin has been demonstrated to be an activator of epithelial sodium channels in cultured renal and bronchial epithelial cells. In this study, we evaluated the effects of adenovirus-mediated gene transfer of human prostasin on blood pressure regulation and sodium reabsorption in Wistar rats. Expression of human prostasin mRNA was identified in rat adrenal gland, liver, kidney, heart, lung, and aorta, and immunoreactive human prostasin was detected in the circulation and urine of rats receiving prostasin gene transfer. A single injection of adenovirus carrying the prostasin gene caused prolonged increases in blood pressure for 3–4 wk. Blood pressure increase was accompanied by elevated plasma aldosterone levels and reduced plasma renin activity. The increase in blood pressure and plasma aldosterone levels as well as the reduction of plasma renin activity correlated with the expression of human prostasin transgene. Elevated plasma aldosterone levels were detected at 3 days after gene transfer before the development of hypertension, indicating that stimulation of mineralocorticoid production is the primary target of prostasin. Prostasin gene transfer significantly reduced urinary K⁺ excretion but increased urinary Na⁺ and kallikrein excretion. Elevated renal kallikrein levels promote natriuresis, which may lead to sodium escape and prevent further increases of blood pressure after prostasin gene transfer. In summary, these results suggest that prostasin participates in blood pressure and electrolyte homeostasis by regulating the renin-angiotensin-aldosterone and kallikrein-kinin systems.

PROSTASIN, a membrane-bound serine proteinase, was recently discovered, purified, and cloned in our laboratory (34–36). We first identified the synthesis of prostasin in the prostate gland and its secretion into seminal fluid and thus designated this new human proteinase as “prostasin.” Prostasin displays trypsin-like activity on tripeptidyl fluorogenic substrate and is inhibited by several trypsin inhibitors, in particular, with a high affinity to aprotinin (34). Immunoreactive human prostasin and its mRNA were identified in epithelial tissues, including kidney, lung, colon, pancreas, salivary gland, and prostate gland (34, 35). Furthermore, prostasin is targeted to the epithelial membrane via the glycosylphosphatidylinositol anchorage (4). The structural characteristics of prostasin suggest that it is widely distributed as a membrane-anchored or secreted protein.

It has been shown that coexpression of prostasin and epithelial sodium channels (ENaC) increased epithelial sodium transport in renal and bronchial epithelial cells (5, 28). Cell-surface expression of channel-activating protease, the Xenopus counterpart of human prostasin, is required for the activation of ENaC (26, 27). ENaC represent the rate-limiting step of epithelial Na⁺ transport in the kidney, lung, and gastrointestinal tract (19). Therefore, it plays a pivotal role in the regulation of sodium balance, extracellular fluid volume, and blood pressure. Until now, ENaC are the only sodium transport protein for which there is genetic evidence for its involvement in the development of hypertension and hypotension (10). The activity of ENaC has to be tightly regulated with regard to the maintenance of sodium balance. These findings suggest that prostasin is involved in the control of sodium balance and blood pressure. However, there is still lack of evidence that prostasin is causally involved in the blood pressure regulation and electrolyte homeostasis. The aim of the present study is to explore effects of a continuous supply of prostasin via adenovirus-mediated gene delivery on blood pressure and electrolyte balance in rats. We showed that prostasin gene transfer caused a marked increase in blood pressure and electrolyte imbalance, which were accompanied by increased plasma aldosterone level, reduced plasma renin activity (PRA), and increased urinary kallikrein excretion. These findings suggest that prostasin plays an important role in blood pressure homeostasis and electrolyte balance via regulation of the renin-angiotensin-aldosterone and kallikrein-kinin systems.

MATERIALS AND METHODS

Preparation of adenovirus. Adenovirus Ad.CMV-hPRS, in which the expression of human prostasin cDNA was under the control of the cytomegalovirus (CMV) promoter/enhancer, was constructed and prepared by using a simplified
system as previously described (9). Adenovirus harboring the luciferase gene under the control of CMV promoter/enhancer (Ad.CMV-Luc) was used as control virus. Wistar rats (7 wk, male) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Rats were randomly divided into two groups and injected with adenoviruses Ad.CMV-hPRS or Ad.CMV-Luc (4 x 10^12 plaque-forming units) via the tail vein.

RT-PCR Southern blot analysis of human prostasin mRNA. Total RNA was extracted from fresh rat tissues at 4 days after intravenous injection of adenoviruses by using guanidine isothiocyanate-cesium chloride gradient ultracentrifugation (20). RT-PCR Southern blot analysis specific for human prostasin (5'-primer, 5'-GTC CAT GTG TGT GGT GG-3'; 3'-primer, 5'-TGG GTC TCG TGA GTT GG-3'; probe, 5'-CTC TCA GCT GCT CAC TGC-3') was performed as previously described (35). β-Actin was amplified as internal control (30).

Human prostasin ELISA. Immunoreactive human prostasin was measured in the plasma and urine of rats after gene transfer using ELISA specific for human prostasin as previously described (14).

Blood pressure measurement. Systolic blood pressure was measured with a photoelectric tail cuff device (Natsume, Tokyo, Japan) (29). This device requires minimal warming of rats (usually <15 min) before blood pressure determination and a brief period of restraint in a plastic cage. For each animal, the systolic blood pressure was represented as the mean of eight recordings.

Plasma collection and measurements of plasma aldosterone levels and PRA. Blood for aldosterone and PRA determination was collected in EDTA-coated tubes via cardiac puncture under pentobarbital sodium anesthesia (50 mg/ml ip) at 3, 14, and 21 days post gene transfer. Plasma aldosterone levels were determined by radioimmunoassay (Coat-A-Count Aldosterone kit, Diagnostic Products). PRA was determined from EDTA-plasma as described (13, 17). All samples were thawed once in an ice bath. Once thawed, 0.1 M maleate buffer, pH 5.6 and inhibitors were added to 100 μl of plasma to a total of 200 μl containing 5 mM dithmercaprol and 3 mM 8-hydroxyquinoline. Samples were divided into two tubes. Tube A was incubated at 0°C and tube B was incubated at 37°C for 1 h. ANG I released by renin was measured by radioimmunoassay using antiserum to ANG I (kindly provided by Professor Hilgenfeldt, University of Heidelberg, Germany). PRA was determined by subtracting the amount of ANG I generated at 0°C from the amount generated at 37°C.

Urine collection and measurement of urinary kallikrein excretion. Twenty-four-hour urine was collected by housing rats in metabolic cages with access to tap water but not food at 14 days after injection. The urine samples were subjected to sodium, potassium, and creatinine determinations. Uinary Na⁺ and K⁺ excretion was measured by flame photometry. Twenty-four-hour urinary kallikrein excretion was determined by radioimmunoassay specific for rat tissue kallikrein (22).

Statistical analysis. The results were expressed as means ± SE for five or six animals. The statistical significance of the difference in systolic blood pressure between control receiving Ad.CMV-Luc and rats receiving Ad.CMV-hPRS was determined by ANOVA. In addition, we used an unpaired Student’s t-test to assess the difference of physiological parameters between Ad.CMV-hPRS and Ad.CMV-Luc groups after gene delivery.

RESULTS

Expression of human prostasin mRNA in rats after gene delivery. Expression of human prostasin mRNA in rats after gene delivery was analyzed by RT-PCR followed by Southern blot using specific oligonucleotide probes for human prostasin. Human prostasin mRNA was detected mainly in rat liver and, to a lesser extent, in the adrenal gland, heart, lung, kidney, and aorta (Fig. 1, top). The expression of human prostasin mRNA was not detected in the corresponding tissues of control rat receiving adenoviral vector Ad.CMV-Luc (Fig. 1, top). Similar levels of β-actin mRNA were detected in tissues of both experimental and control groups, indicating the integrity of RNA (Fig. 1, bottom). The results showed that human prostasin is expressed in tissues relevant to cardiovascular and renal function following gene transfer in rats.

Time course of immunoreactive human prostasin levels in rats. Immunoreactive human prostasin levels in the plasma and urine of rats were analyzed by an ELISA specific for human prostasin at 3, 7, 14, and 21 days after injection of Ad.CMV-hPRS. Immunoreactive human prostasin reached the highest levels in the plasma (23.95 ± 2.20 ng/ml, n = 3) and urine (721.40 ± 353.07 ng/24 h urine, n = 3) at 3 days and was detectable at 21 days after gene delivery (Fig. 2, A and B). Linear displacement curves for immunoreactive human prostasin in the plasma and urine of rats were parallel with the standard curve of human prostasin, indicating their immunological identity (data not shown). Immunoreactive human prostasin was not detected in the plasma or urine of control rats receiving Ad.CMV-Luc. These results indicate that rabbit anti-human prostasin antibody is specific for human prostasin and it does not cross-react with endogenous rat prostasin.

Human prostasin gene delivery increases systolic blood pressure. Figure 3 shows the effect of human prostasin gene delivery on systolic blood pressure of rats receiving human prostasin adenovirus Ad.CMV-hPRS or control virus Ad.CMV-Luc. A single intravenous injection of the recombinant adenovirus harboring the human prostasin gene caused an increase of blood pressure that began 5 days postinjection and

![Fig. 1. Expression of human prostasin mRNA in Wistar rats after adenovirus-mediated prostasin gene delivery. Total RNA was isolated from liver, kidney, adrenal gland, aorta, and heart at 4 days post gene delivery. One microgram of RNA was used for RT-PCR followed by Southern blot analysis.](Image)
continued for 24 days. A maximal blood pressure increase was observed 12 days after human prostasin gene delivery compared with that of control rats injected with Ad.CMV-Luc (150 ± 2 vs. 136 ± 1 mmHg, n = 6, P < 0.01). At 26 days post gene delivery, there was no significant difference in systolic blood pressure of rats receiving Ad.CMV-hPRS (136 ± 1 mmHg, n = 6) and Ad.CMV-Luc (137 ± 2 mmHg, n = 6). No apparent changes in the body weight and heart rate were observed in rats injected with Ad.CMV-hPRS compared with control rats receiving Ad.CMV-Luc.

**Effects of prostasin gene delivery on plasma aldosterone level and PRA.** Figure 4 shows the effect of prostasin gene delivery on plasma aldosterone level and PRA in rats measured at 3, 14, and 21 days after gene delivery. Prostasin expression significantly increased plasma aldosterone level compared with control rats receiving Ad.CMV-Luc at 3 days (598.3 ± 78.3 vs. 248.3 ± 56.5 pg/ml, n = 6, P < 0.05) and 14 days (567.2 ± 57.5 vs. 204.9 ± 40.1 pg/ml, n = 5, P < 0.05) post gene transfer (Fig. 4A). Prostasin expression significantly reduced PRA compared with control rats receiving Ad.CMV-Luc at 3 days (5.96 ± 0.52 vs. 8.67 ± 0.84 ng ANG I·ml⁻¹·h⁻¹, n = 5, P < 0.05) and 14 days (7.10 ± 0.66 vs. 9.90 ± 0.10 ng ANG I·ml⁻¹·h⁻¹, n = 7 or 9, P < 0.05) post gene transfer (Fig. 4B). Plasma aldosterone levels (387.1 ± 54.8 vs. 243.6 ± 59.2 pg/ml, n = 6) and PRA (8.33 ± 0.41 vs. 9.48 ± 0.67 ng ANG I·ml⁻¹·h⁻¹, n = 5) were not significantly different in rats receiving prostasin gene or control virus at 21 days post gene transfer.

**Effects of prostasin gene delivery on urine excretion and electrolyte balance.** At 14 days post gene transfer urine excretion (5.9 ± 0.9 vs. 6.5 ± 0.6 ml·100 g body wt⁻¹·24 h⁻¹, n = 6) and water intake (5.2 ± 0.4 ml·100 g body wt⁻¹·24 h⁻¹, n = 6) were similar between control and Ad.CMV-hPRS groups. There is also no difference in urinary creatinine excretion between control and Ad.CMV-hPRS groups (2.89 ± 0.07 vs. 2.87 ± 0.18 mg·100 g body wt⁻¹·24 h⁻¹, n = 5). However, prostasin gene transfer reduced urinary potassium output (451.1 ± 16.3 vs. 525.4 ± 11.9 mmol·100 g body wt⁻¹·24 h⁻¹, n = 5, P < 0.05), but it increased urinary sodium output (171.7 ± 29.9 vs. 124.1 ± 14.4 mmol·100 g body wt⁻¹·24 h⁻¹, n = 4, P < 0.05) compared with control rats (Fig. 5, A and B). Prostasin gene delivery significantly increased urinary kallikrein excretion compared with control rats (37.98 ± 3.74 vs. 27.57 ± 1.09 μg/24 h, n = 6 or 5, P < 0.05; Fig. 5C).

**DISCUSSION**

This is the first study to demonstrate a link between prostasin expression and blood pressure regulation in the animal model. This study showed that elevated levels of prostasin resulted in a prolonged increase of blood pressure and electrolyte imbalance in normotensive rats by regulating the renin-angiotensin-aldosterone and kallikrein-kinin systems. The human prostasin was synthesized in rat tissues important for cardiovascular and renal function and secreted into
circulation and urine. Aldosterone production was increased and PRA was reduced before blood pressure increase in rats receiving prostasin gene delivery. These findings suggest that stimulation of aldosterone production is the primary target of prostasin. These results support the notion that prostasin regulates aldosterone synthesis/secretion, which might be critical for the development of hypertension after prostasin gene transfer. It has been shown that aldosterone treatment increased prostasin expression in cultured cortical collecting duct cells and urinary prostasin excretion in rats (15). Thus, it is likely that there is a positive feedback control by aldosterone in the regulation of prostasin expression.

Aldosterone is the most potent hormone regulating the body’s electrolyte balance. Aldosterone stimulates Na\(^+\) reabsorption on its target epithelia via activation of ENaC in the distal tubule. Recent studies have suggested that aldosterone contributes to elevated arterial pressures in essential hypertension patients with low PRA (6). In light of these findings, we speculate that the elevated plasma aldosterone may be a contributing factor for elevated arterial pressure, despite suppressed renin, in rats receiving prostasin gene delivery. These results suggest that prostasin may be involved in the control of blood pressure via regulation of aldosterone production, making it a candidate gene for the pathogenesis of hypertension.

Our previous studies showed that adenovirus-mediated gene transfer via intravenous injection resulted in a rapid increase of transgene expression at 2–3 days postinjection and lasted 3–4 wk (3, 33), which is consistent with the expression profile of human prostasin in this study. The transient nature of prostasin transgene expression in rats is most likely due to a host immune response to viral proteins and episomal expression of adenoviral vector. We showed that prostasin gene delivery is associated with significantly increased plasma aldosterone levels and reduced PRA at 3 and 14 days post gene transfer, whereas blood pressure was significantly increased in rats receiving prostasin gene transfer starting at 5 days post gene delivery. At 26 days post gene transfer, plasma aldosterone levels, PRA, and blood pressure were similar in rats receiving prostasin and control virus. Therefore, the prostasin transgene expression correlated with biochemical and physiological changes in these rats.

Although prostasin was originally isolated from seminal plasma, our previous studies and a recent study using microarrays demonstrated that human prostasin expression is widely distributed in various tissues (24, 34, 35). It has been shown that renal prostasin expres-

**Fig. 4.** Plasma aldosterone levels (A) and plasma renin activity (PRA; B) of Wistar rats receiving adenoviral vectors Ad.CMV-hPRS or Ad.CMV-Luc at 3 and 14 days post gene delivery. Data are expressed as means ± SE (n = 6). *P < 0.05, Ad.CMV-hPRS vs. Ad.CMV-Luc group.

**Fig. 5.** Urinary K\(^+\) excretion (A), urinary Na\(^+\) excretion (B), and urinary kallikrein excretion (C) of Wistar rats receiving adenoviral vectors Ad.CMV-hPRS or Ad.CMV-Luc. Twenty-four-hour urine was collected at 14 days post gene delivery. Data are expressed as means ± SE (n = 5). *P < 0.05, Ad.CMV-hPRS vs. Ad.CMV-Luc group.
sion is under the control of aldosterone. Plasma aldosterone is mainly generated by zona glomerulosa in the adrenal gland. Recently, several groups reported aldosterone synthase expression and activity in the heart and vessel wall (23, 25). Interestingly, prostasin is locally produced in the adrenal gland, heart, and vessel wall where synthesis and/or actions of aldosterone take place (24, 34). Thus, there is a potential association between prostasin expression and the aldosterone synthesis/secretion. The molecular mechanisms of prostasin-induced aldosterone production are not clear. Although it is generally accepted that aldosterone biosynthesis is mainly regulated by renin-angiotensin system and K+ (7), studies suggest that serine proteinases are implicated in the regulation of aldosterone production. It has been shown that serine proteinases are involved in aldosterone production as well as the compensatory growth response of adrenal gland (2, 18). Administration of the serine proteinase inhibitor aprotinin in rats before unilateral adrenalectomy inhibited compensatory growth of adrenal gland (2). In addition, serine proteinases increased aldosterone secretion via activation of phospholipase C signaling pathway (18). Whether these processes are involved in prostasin-induced aldosterone production awaits further investigation.

We were surprised to find that urinary sodium excretion increased at 14 days post prostasin gene transfer. The increased urinary sodium and reduced potassium excretion in rat receiving prostasin gene delivery were possibly due to the result of aldosterone escape. The effect of aldosterone on renal sodium retention can be overridden in some circumstances by the phenomenon of aldosterone escape (1). Several studies demonstrated that kallikrein excretion is induced by aldosterone. Administration of aldosterone significantly increased urinary kallikrein, whereas adrenalectomy reduced kallikrein excretion (8, 16). An increased kallikrein excretion was reported in patients with primary aldosteronism (12). Extensive studies have shown that renal kallikrein-kinin system prevents the development of hypertension through augmentation of urinary sodium excretion (11). In this study, both urinary kallikrein excretion and urinary sodium excretion increased, but urinary potassium reduced after prostasin gene transfer. Higher levels of kallikrein may function as a mechanism of escape from sodium retention by elevated aldosterone in these rats. Consistent with this notion, a recent study showed that urinary kallikrein excretion increased in response to elevated aldosterone levels in nonmalignant but not malignant hypertensive (mREN2)27 transgenic rats (32). Increased kallikrein levels made these nonmalignant hypertensive rats less susceptible to renal damage and premature death.

We evaluated the time course of urinary sodium, potassium excretion, and kallikrein levels at 3, 7, and 21 days postinjection and found that sodium, potassium, and kallikrein levels remained unaltered. Experimental studies have shown that sodium retention and volume expansion are short-term phenomena with aldosterone excess (1). The transient nature of aldosterone-induced sodium retention may account for the unaltered sodium excretion observed at 3 and 7 days post gene transfer. Prostasin transgene expression reached a peak at 3 days postinjection and gradually declined. At 21 days postinjection, plasma prostasin level was barely detectable and plasma aldosterone levels were similar between control and Ad.CMV-PRS groups. Consistent with these results, urinary sodium, potassium excretion, as well as urinary kallikrein levels were similar between control and Ad.CMV-PRS groups at 21 days postinjection. Urinary kallikrein excretion increased 16.5 and 40.7% in the Ad.CMV-hPRS group compared with control rats at 3 and 7 days postinjection, respectively. However, the difference was not statistically significant. In this study, plasma aldosterone levels increased at days 3 and 14 and returned to normal at day 21 postinjection while systolic blood pressure increased from day 3 to day 24 postinjection. The sustained increase of blood pressure in the setting of elevated aldosterone levels relates more to a raised vascular resistance in the long term (21, 31). It is likely that vascular resistance was still elevated, although plasma aldosterone levels returned to normal at 24 days postinjection.

It is not clear, at the present time, whether the endogenous prostasin is involved in regulating blood pressure and aldosterone production under physiological conditions. Analysis of genetically modified animals is likely to shed light on the potential role of prostasin in the cardiovascular system.

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