Urinary Prostin: A Possible Biomarker for Renal Pressure Natriuresis in Black Adolescents

HAIDONG ZHU, JULIE CHAO, DEHUANG GUO, KE LI, YING HUANG, KIMBERLY HAWKINS, NIKKI WRIGHT, INGER STALLMANN-JORGENSEN, WEILI YAN, GREGORY A. HARSHFIELD, AND YANBIN DONG

Department of Pediatrics [H.Z., D.G., K.L., Y.H., K.H., N.W., I.S.-J., W.Y., G.A.H., Y.D.], Medical College of Georgia, Augusta, Georgia 30907; Department of Biochemistry and Molecular Biology [J.C.], Medical University of South Carolina, Charleston, South Carolina 29425

ABSTRACT: Prostin is a membrane-bound/secretive serine protease interacting with aldosterone and the epithelial sodium channel in the kidney. We and others have previously proposed the concept of stress-induced pressure natriuresis (SIPN) where increased urinary sodium excretion ($U_{Na}V$) is coupled with elevated blood pressure (BP) in response to behavioral stress in normotensive adolescents. This study thus aimed to test the relationship between prostin and pressure natriuresis using the SIPN model. A cohort of 102 normotensive black adolescents (mean age: 17.0 ± 1.2 y; 56% females) were placed on a controlled sodium (4000 ± 200 mg/d) and potassium (2600 ± 200 mg/d) diet for three days before testing. The SIPN protocol consisted of a 1-h baseline period, a 1-h stress period (competitive video game), and a 1-h recovery period. During the stress period, BP elevation was coupled with an increase in $U_{Na}V$. Urinary prostin concentration had more than a 2-fold reduction from baseline (38.4 ± 32.7 ng/mL) to stress (17.2 ± 16.0 ng/mL), and further declined during recovery (12.1 ± 16.2 ng/mL) ($p < 0.001$). Urinary prostin was inversely correlated with $U_{Na}V$ during stress ($r = -0.43$, $p = 0.0001$), even after being normalized by urinary creatinine. Our data suggest that urinary prostin could be a novel biomarker and/or mechanism for renal pressure natriuresis in normotensive black adolescents. (*Pediatr Res* 65: 443–446, 2009)

Renal pressure natriuresis, one of the key mechanisms of blood pressure (BP) homeostasis (1), is critically dependent upon the activity of sodium channels and transporters expressed in the nephron. Chief among these are the epithelial sodium channels (ENaC) consisting of three subunits, α ENaC, β ENaC, and γ ENaC in the cortical collecting duct (2,3). Although sodium reabsorption by ENaC accounts for only a relatively small proportion (less than 5%), it constitutes the rate-limiting step and the final renal adjustment to sodium balance (4).

Multiple mechanisms such as hormones, intracellular factors, and accessory regulatory proteins regulate ENaC function (2,3). Aldosterone stimulates ENaC expression and activates an ENaC-regulating protein kinase, serum, and glucocorticoid-regulated kinase 1 (SGK1). SGK1 inhibits ENaC ubiquitination by an ubiquitin ligase, the neural precursor cell expressed developmentally down-regulated 4-like protein (Nedd4L), and subsequently prevents channel degradation.

A series of studies previously indicated that numerous proteases such as prostin, a glycosylphosphatidylinositol (GPI)-anchored serine protease expressed in the distal nephron, are involved in the proteolytic processing and activation of ENaC subunits in *Xenopus* oocytes and a mouse cortical collecting duct cell line (5–16). Prostin was thought to induce cleavage of an inhibitory peptide from γ ENaC to activate the channel fully in the cell (16–18). Of interest, aldosterone enhanced the expression of prostin mRNA and protein in the cultured mouse cortical collecting duct cell line and in rats (19). In contrast, a single injection of adenovirus carrying the human prostin gene caused a prolonged (3–4 wk) increase in BP and plasma aldosterone concentration in rats, indicating that prostin may cause BP elevation via the stimulation of aldosterone (20). Furthermore, a decrease in urinary prostin excretion by a synthetic prostin inhibitor resulted in an increase in urinary sodium excretion in Sprague-Dawley rats (21). These findings suggest that prostin may participate in tubular sodium reabsorption and pressure natriuresis via either direct effects on ENaC or interactions with aldosterone.

Comparable data in humans are scarce. Therefore, the purpose of this study was to examine the relationship among prostin and renal pressure natriuresis in normotensive adolescents by using our stress-induced pressure natriuresis (SIPN) model (22–27). This model examines changes in pressure natriuresis induced by mental stress, which consists of a 1-h baseline period, a 1-h stress period (competitive video games), and a 1-h recovery period. The SIPN hypothesis postulates that a stress-induced increase in BP leads to a corresponding increase in urinary sodium excretion ($U_{Na}V$). Impaired stress-induced sodium regulation and resultant extracellular volume expansion may lead to the future development of hypertension and its sequelae. The second goal of this study was to examine the interaction between aldosterone and prostin during SIPN. We examined healthy normotensive black adolescents because 1) healthy normotensive adolescents may not have yet developed significant target organ damage, which can mask or compromise the normal response pattern; 2) the pressure natriuresis concept is particularly appealing owing to the greater sensitivity of BP to sodium in

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Correspondence: Yanbin Dong, M.D., Ph.D., Georgia Prevention Institute, Department of Pediatrics, Medical College of Georgia, 1120 15th Street, HS-1640, Augusta, GA 30912-3715; e-mail: ydong@mcg.edu

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Abbreviations: BP, blood pressure; ENaC, epithelial sodium channel; SIPN, stress-induced pressure natriuresis; $U_{Na}V$, urinary sodium excretion

blacks compared with their white counterparts (28,29); and 3) urinary prostaticin could be used as a possible biomarker to screen adolescents at risk of BP elevation.

METHODS

Subject recruitment. The protocol was approved by the Human Assurance Committee of the Medical College of Georgia. A sample of 102 healthy normotensive black adolescents including 45 males and 57 females were recruited from local public high schools in the Augusta-Richmond County area via school announcements, flyers, handout, or word of mouth. Written informed parental consent and subject assent were obtained before testing. Black race (African American) was identified by self-report of each subject and by parent if the subject was less than 18 y. Normotension was based on BP screening, namely, <95th percentile for age, sex and height, or <140/90 mm Hg for subjects aged 18 y or older (30). Other exclusion criteria included any chronic illness, medication use, or a positive pregnancy test.

Protocol. Weight, height, and BP were obtained at a screening visit before initiating the study protocol. The SIPN protocol has been previously described in detail (25–27). Briefly, subjects were placed on a controlled sodium and potassium diet for 3 d before testing. To do this, we met with the subjects before the week of their scheduled testing and planned each meal for each day, based on an average sodium and potassium intake of 4000 ± 200 mg/d and 2600 ± 200 mg/d, respectively. Girls were not tested while on their menses, and were tested on the week after the completion of menstrual flow to ensure all girls were tested in the same phase of their menstrual cycle. During the test week the meals were packed into coolers each day and the subjects or parents picked up the coolers from the Georgia Prevention Institute. Overnight urine collection was used to estimate the subject's compliance with the sodium-controlled diet. On the fourth day the subjects performed the stress test, after having a breakfast provided. They were seated in a comfortable chair for the entire protocol, and ambulation was only allowed for collection of urine samples. The subjects emptied their bladders at the beginning of the baseline period. The protocol included a baseline period of 1 h (hour 1) during which the subjects watched a movie. This was followed by a 1-h stress period (hour 2) during which a competitive video game task (Snowboarding, Sony Corp., Foster City, CA) was played for a monetary reward between two subjects. Finally, there was a 1-h poststress recovery period (hour 3) that was the same as the baseline period. Urine samples were obtained hourly, and hour 1 was considered as baseline $U_{Na}V$ to insure that the subjects had similar urinary flow rates. Hour 2 and hour 3 were considered stress $U_{Na}V$ and recovery $U_{Na}V$, respectively. BP was measured by a Dinamap monitor (Dinamap Compact Monitor, Tampa, FL) at an interval of 15 min. The individual BP values during each hour were averaged to obtain baseline BP (hour 1), stress BP (hour 2), and recovery BP (hour 3). The subjects were required to drink 250 mL of water every hour to insure that they remained hydrated and provided adequate urine samples.

Human urinary prostaticin measurement. Urinary levels of immunoreactive human prostaticin were determined by enzyme-linked immunosorbent assay (ELISA) prepared with the previously described antibody to human

prostaticin (31). Microtiter plates (96-well) were coated with anti-prostaticin IgG (IgG) ($1 \mu\text{g/mL}$, $100 \mu\text{L}$ per well) overnight at 4°C . Purified prostaticin standards (0.16 – 10 ng) or samples were added to individual wells in a total volume of $100 \mu\text{L}$ of PBS containing 0.05% Tween 20 and 0.5% gelatin (dilution buffer) and incubated at 37°C for 90 min. Biotin-labeled anti-human prostaticin IgG was added in each well at a concentration of $1 \mu\text{g/mL}$ in a total of $100 \mu\text{L}$ and incubated at 37°C for 60 min. Peroxidase-avidin at a concentration of $1 \mu\text{g/mL}$ in a total volume of $100 \mu\text{L}$ was added and incubated at 37°C for 30 min. The color reaction was performed by adding $100 \mu\text{L}$ of freshly prepared substrate solution [0.03% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 0.03% H_2O_2 in 0.1 M sodium citrate ($\text{pH } 4.3$)] to each well and incubating the mixture at room temperature for 30 min. The plates were read at 405 nm with a plate reader (Titertek Instruments, Huntsville, AL).

Aldosterone, electrolyte, and creatinine measurements. Aldosterone levels were determined using a competitive enzyme RIA kit (Diagnostic Systems Laboratories, Webster, TX). The sensitivity of the assay was 12 pg/mL. Intra-assay coefficient of variation was 7.1% ; and inter-assay coefficient of variation was 13.2% .

Electrolytes and creatinine were analyzed by the ion-selective electrode technique using a NOVA 16 Analyzer (NOVA Biomedical, Waltham, MA). The NOVA has an intra-assay coefficient of variation of less than 3% and an inter-assay coefficient of 4% .

Statistical analyses. The general characteristics of subjects were presented as mean $\pm$ SD. The variations of clinical phenotypes among baseline (hour 1), stress (hour 2), and recovery (hour 3) were analyzed by repeated measures ANOVA, with adjustment of age, sex, and body mass index (BMI). The correlations between urinary prostaticin concentration and urinary prostaticin concentration normalized by urinary creatinine, and $U_{Na}V$ or plasma aldosterone were tested by simple bivariate correlation analyses, or partial correlation analyses to adjust for potential confounders such as age, sex, and BMI. Log-transformation was performed to obtain approximation of normal distribution when necessary. A value of $p < 0.05$ was deemed statistically significant. The statistical analyses were performed with STATA 8.0 (StataCorp, College Station, TX).

RESULTS

The subject characteristics are presented in Table 1. Subjects were normotensive black adolescents aged 17.0 ± 1.2 y. The average height of the subjects was 170.2 ± 8.7 cm, and the average BMI was 25.3 ± 5.4 kg/m². The values of plasma creatinine and plasma aldosterone were within normal ranges. Urinary prostaticin was detectable in every subject, regardless of sex.

As shown in Table 1, the increment of systolic BP (SBP) and diastolic BP (DBP) from baseline (hour 1) to stress (hour

Table 1. General clinical characteristics

	Baseline	Stress	Recovery	P_{overall}	$P_{\text{b-s}}$	$P_{\text{s-r}}$
Age, y ($n = 102$)	17.0 ± 1.2	—	—	—	—	—
Male/female	45/57	—	—	—	—	—
Height, cm	170.2 ± 8.7	—	—	—	—	—
BMI, kg/m ²	25.3 ± 5.4	—	—	—	—	—
SBP, mm Hg	110 ± 12	115 ± 12	109 ± 11	<0.001	<0.001	<0.001
DBP, mm Hg	60 ± 6	64 ± 6	60 ± 6	<0.001	<0.001	<0.001
Plasma sodium, mmol/L*	136.7 ± 5.6	135.8 ± 6.0	136.2 ± 5.1	0.441	0.205	0.631
Plasma potassium, mmol/L*	4.4 ± 0.3	4.3 ± 0.4	4.3 ± 0.3	0.911	0.888	0.775
Plasma creatinine, mg/dL*	1.0 ± 0.4	0.9 ± 0.4	0.9 ± 0.4	0.038	0.046	0.685
Plasma aldosterone, pg/mL*	115.1 ± 61.0	99.9 ± 57.1	101.5 ± 55.2	<0.001	<0.001	0.521
Urinary creatinine, mg/dL*	114.8 ± 95.0	65.0 ± 49.4	53.4 ± 46.5	<0.001	<0.001	<0.001
Urinary prostaticin concentration, ng/mL	38.4 ± 32.7	17.2 ± 16.0	12.1 ± 16.2	<0.001	<0.001	<0.001
Urinary prostaticin excretion, ng/mg†	28 ± 11	26 ± 11	23 ± 10	0.005	0.038	0.014
$U_{Na}V$, mEq/h*	7.0 ± 4.1	12.9 ± 8.5	9.9 ± 6.8	<0.001	<0.001	0.041

* Analyses were performed based on log-transformed data.

† Urinary prostaticin excretion was calculated as urinary prostaticin concentration divided by urinary creatinine concentration.

Values = means $\pm$ SD. The repeated ANOVA analyses were applied to analyze the overall and subgroup differences in variables across baseline (hour 1), stress (hour 2), and recovery (hour 3), with adjustment of age, sex, and BMI. $P_{\text{b-s}}$, baseline to stress; $P_{\text{s-r}}$, stress to recovery.

Figure 1. Correlations between urinary prostatic normalized by creatinine and $U_{Na}V$ during stress. There were significant and negative correlations between urinary prostatic excretion (urinary prostatic concentration normalized by urinary creatinine concentration) and $U_{Na}V$ during stress ($r = -0.43$, $p = 0.0001$) (adjusted for age, sex, and body mass index).

2) was ~ 4 to 5 mm Hg, and both SBP and DBP dropped significantly during recovery (hour 3) compared with stress ($P_s < 0.001$). Similarly, $U_{Na}V$ was increased almost 2-fold from baseline (7.0 ± 4.1 mEq/h) to stress (12.9 ± 8.5 mEq/h) ($p < 0.001$), and declined during recovery (9.9 ± 6.8 mEq/h) compared with stress ($p = 0.04$). Conversely, urinary prostatic concentration had more than a 2-fold reduction from baseline (38.4 ± 32.7 ng/mL) to stress (17.2 ± 16.0 ng/mL), and continuously declined during recovery (12.1 ± 16.2 ng/mL) compared with stress ($p < 0.001$). As shown in Figure 1, urinary prostatic was inversely correlated with $U_{Na}V$ during stress ($r = -0.43$, $p = 0.0001$), even after being normalized by urinary creatinine. Urinary prostatic did not correlate with $U_{Na}V$ at baseline ($r = 0.06$, $p = 0.60$) or $U_{Na}V$ during recovery ($r = 0.07$, $p = 0.3$).

Plasma levels of aldosterone were significantly reduced during stress (99.9 ± 57.1 pg/mL) compared with baseline (115.1 ± 61.0 pg/mL) and remained lower during recovery (101.5 ± 55.2 pg/mL) ($p < 0.001$). We did not find any significant correlations between aldosterone, and $U_{Na}V$ or prostatic at baseline, stress, or recovery.

DISCUSSION

For sodium balance to be maintained in the face of increased arterial pressure, there must be a shift of renal pressure natriuresis to higher BP (1,32). The laboratory-based pressure natriuresis model (SIPN) exhibited a higher rate of $U_{Na}V$ when BP was elevated, which is a normal natriuretic response in healthy normotensive adolescents. We undertook this study to test for the relationship between urinary prostatic and the natriuretic process in response to stress-induced BP elevation, after controlling dietary sodium and potassium intake. Our data demonstrated that there was a decrease in urinary prostatic concentration from baseline to stress, and from stress to recovery. In particular, from baseline to stress, urinary prostatic concentration decreased 2-fold, whereas $U_{Na}V$ increased almost 2-fold. Furthermore, prostatic was inversely correlated with $U_{Na}V$ during stress, that is, lower prostatic was associated with higher $U_{Na}V$. These findings suggest that prostatic

could be involved in renal sodium reabsorption, and thus the renal-body fluid control of arterial pressure.

Discovered more than a decade ago, prostatic is one of the only two known mammalian serine proteases that are attached to the plasma membrane *via* a GPI anchor (10,33,34). It has been predominantly studied in the pathologic development of tumors and cancer such as prostate, breast, and ovarian cancer (10,31,35). Prostatic has recently attracted substantial attention as a potential mechanism underlying sodium and volume homeostasis and subsequent BP regulation, although the production, secretion, and clearance of distal tubular prostatic and urinary prostatic are largely unknown. It is believed that prostatic has channel-activating properties *via* the proteolytic cleavage of γ ENaC (16–18). Indeed, at the posttranslational level, proteolytic processing is thought to be important if not essential to ENaC activation.

Conversely, aldosterone regulates sodium balance, extracellular fluid volume, and BP (2,36,37). Aldosterone increases the rate of tubular sodium reabsorption across epithelia at the distal nephron by enhancing ENaC activity. Previously, Narikiyo *et al.* showed that urinary prostatic secretion was substantially increased in three patients with primary aldosteronism, and that adrenalectomy significantly reduced urinary prostatic secretion (19). In an independent study, Olivieri *et al.* demonstrated that spironolactone (100 mg), the traditional aldosterone receptor inhibitor, decreased urinary prostatic in five normotensive white adults in whom the renin/aldosterone axis was activated by low sodium intake (defined by urinary sodium < 150 mmol/d in 24-h urine), but it was ineffective in five subjects with high sodium intake (defined by urinary sodium > 150 mmol/d in 24-h urine) (38). Of note, urinary prostatic was evaluated by SDS–PAGE 2D gels, and Western blotting, which are generally considered to be less accurate and robust at the population level compared with the ELISA technique used in this study. We placed our subjects on a sodium- and potassium-controlled diet, yet failed to identify a significant correlation between aldosterone and prostatic at baseline, during stress, or recovery. Wang *et al.* evaluated the effects of adenovirus-mediated gene transfer of human prostatic on BP regulation and sodium reabsorption in Wistar rats. Elevated plasma aldosterone levels were detected 3 d after gene transfer and before the development of hypertension, indicating that stimulation of mineralocorticoid production was a primary target of prostatic (20). Alternatively, prostatic might modulate the pressure natriuresis relation, independent of aldosterone.

This study has some limitations. First, plasma and urine samples were collected and assayed only hourly throughout the SIPN protocol. Sample collections at more time points may enable us to better reveal the dynamic relationship between prostatic and $U_{Na}V$. Second, the basal levels of urinary prostatic in our subjects were not examined because we did not collect urine samples before the start of the sodium-controlled diet. Decreased dietary sodium intake might exaggerate the role of prostatic *via* the activation of aldosterone. Conversely, in our SIPN model, aldosterone was suppressed in response to stress, which might mask the true relationship between aldosterone and prostatic under stress. The dynamic

relationship among urinary prostaticin, salt intake, and aldosterone in normotensive adolescents needs to be studied using a salt sensitivity test, *e.g.* low salt diet (50 mmol/d) for 5 d followed by high salt (150 mmol/d) supplementation for 10 d (39,40). Third, as discussed above, because plasma aldosterone was only measured hourly, we cannot exclude the role of aldosterone in the pressure natriuresis relation over time in the SIPN model, even though no significant correlations between aldosterone and $U_{Na}V$ were found across baseline, stress, and recovery.

Nonetheless, there have been only few studies investigating the role of prostaticin in sodium balance in humans, although prostaticin expressed in the distal nephron has been suggested to be an ENaC regulatory mechanism *in vitro* and in animals since the late 1990s. Our data are the first to indicate that prostaticin is likely involved in pressure natriuresis at the population level, specifically in black adolescents. Clinically, considering that urine is easily collected and represents a medium with a relatively low number of interfering proteins, urinary prostaticin may be used as a possible biomarker for renal pressure and natriuresis. Our data in black adolescents warrant replication in other populations including white adolescents, normotensive adults, and patients with essential hypertension. Finally, although the cross-sectional correlation between prostaticin and stress-induced $U_{Na}V$ was recognized, a longitudinal study with repeated measures over time is under way to elucidate the contribution of prostaticin to pressure natriuresis and BP further in youth.

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