Clinica Chimica Acta 421 (2013) 236–242

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

A linear relationship between the ex-vivo sodium mediated expression of two sodium regulatory pathways as a surrogate marker of salt sensitivity of blood pressure in exfoliated human renal proximal tubule cells: The virtual renal biopsy

John J. Gildea [a], Dylan T. Lahiff [a], Robert E. Van Sciver [a], Ryan S. Weiss [a], Neema Shah [a], Helen E. McGrath [a], Cynthia D. Schoeffel [a], Pedro A. Jose [b], Robert M. Carey [a], Robin A. Felder [a,*]

[a] University of Virginia, Charlottesville, VA, United States
[b] University of Maryland, Baltimore, MD, United States

ARTICLE INFO

Article history:
Received 28 August 2012
Received in revised form 14 January 2013
Accepted 21 February 2013
Available online 27 February 2013

Keywords:
Salt sensitivity
Cell-based assay
Renal cells
Urinalysis
Sodium diet
Personalized medicine

ABSTRACT

Background: Salt sensitivity (SS) of blood pressure (BP) affects 25% of adults, shares comorbidity with hypertension, and has no convenient diagnostic test. We tested the hypothesis that urine-derived exfoliated renal proximal tubule cells (RPTCs) could diagnose the degree of an individual's SS of BP.

Methods: Subjects were selected who had their SS of BP determined 5 y prior to this study (salt-sensitive: ≥7 mm Hg increase in mean arterial pressure (MAP) following transition from a random weekly diet of low (10 mmol/day) to high (300 mmol/day) sodium (Na^+) intake, N = 4; inverse salt-sensitive (ISS): ≥7 mm Hg increase in MAP transitioning from a high to low Na^+ diet, N = 3, and salt-resistant (SR): <7 mm Hg change in MAP transitioned on either diet, N = 5). RPTC responses to 2 independent Na^+ transport pathways were measured.

Results: There was a negative correlation between the degree of SS and dopamine-1 receptor (D_1R) plasma membrane recruitment ($y = -0.0107x + 0.68$ relative fluorescent units (RFU), $R^2 = 0.88$, $N = 12$, $P < 0.0001$) and angiotensin II-stimulated intracellular Ca^{++} ($y = -0.0016x + 0.0336$, $R^2 = 0.7112$, $P < 0.001$, $N = 10$) concentration over baseline.

Conclusions: Isolating RPTCs from urine provides a personalized cell-based diagnostic test of SS index that offers advantages over a 2-week controlled diet with respect to cost and patient compliance. Furthermore, the linear relationship between the change in MAP and response to 2 Na^+ regulatory pathways suggests that an individual's RPTC response to intracellular Na^+ is personalized and predictive.

1. Introduction

The long-term effect of high dietary sodium (Na^+) on blood pressure (BP) and overall health is still under considerable debate [1]. While it is well documented that a high Na^+ diet may lead to increased BP and cardiovascular risk in some individuals, there is also a segment of the population that demonstrates an increase in BP and cardiovascular risk on a low Na^+ diet [1,2]. Indeed, the relationship between mortality, end-stage renal disease, or hypertension and the amount of Na^+ that is consumed in the diet is "J" shaped, indicating that there is cardiovascular risk associated with high or low Na^+ intake in some individuals [3–5]. Thus, there is a significant need for definitive diagnostic tools in order to stratify test subjects according to their ability to excrete a salt load so that health outcomes can be properly predicted [6].

Cell-based assays are allowing the diagnosis, prediction of clinical outcomes, and response to therapy in an increasing number of diseases including cancer, organ rejection, and diabetes [7]. Exfoliated organ cells have the potential to supplement current biomarker-based analyses since cell-based assays can measure aberrations in cell activity that may be present in complex chronic diseases. The kidneys play a major role in regulating BP as they regulate water and electrolyte homeostasis [6,8–10]. Excessive renal Na^+ reabsorption, especially in the renal proximal tubule and thick ascending limb of Henle, leads to essential hypertension [11,12]. Salt sensitivity (SS) of BP is related to but distinct from hypertension in that there is an abnormal increase in BP following high Na^+ intake even in subjects with normal or optimal BP. SS, even if the increase in blood pressure does not fulfill the definition of hypertension, leads to the same negative health outcomes as subjects with hypertension [13], and is a major public health challenge that affects 25% of middle-aged Americans, with a high prevalence in African-Americans.

* Corresponding author at: University of Virginia, Pathology, 450 Ray C Hunt Dr., Aurbach Building, Room 2312, PO Box 801400, Charlottesville, VA 22903, United States. Tel.: +1 434 924 5151; fax: +1 434 924 5718.
E-mail address: raf7k@virginia.edu (R.A. Felder).

0009-8981/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2013.02.021

Despite its high prevalence and significant morbidity and mortality, the diagnosis of SS can only be determined using an extensive dietary salt loading and salt depletion study performed in an inpatient setting (i.e., clinical research center). Furthermore, the results obtained with a simpler and more rapid protocol [13] correlate poorly with the more generally accepted 2-week controlled Na^+ diet protocol [14–17].

Target organ damage (e.g., kidney failure, stroke, and heart disease) resulting from hypertension and SS could be a result of the higher BP required to excrete the same amount of Na^+ [3–5]. An important mechanism in the increase in Na^+ excretion following a salt load is the inhibition of renal Na^+ reabsorption via dopamine and dopamine 1-like receptors (D_1R/D_5R) which regulate >50% of total renal Na^+ transport [18]. Defects in dopamine-mediated natriuresis have been directly implicated in the etiology of hypertension [19] that impairs D_1R/D_5R function, most evident in the renal proximal tubule (RPT) and RPT cells (RPTCs) [20].

Dopamine receptor activation or an increase in intracellular Na^+ has been shown to recruit the predominantly cytoplasmic D_1R to the plasma membrane [21]. The recruitment of D_1R to the plasma membrane is important in the D_1R/D_5R-mediated decrease in Na^+ transport in RPTCs [21]. Defects in D_1R action in RPTCs, including D_1R recruitment to the plasma membrane in the RPTCs, have been shown in human essential hypertension [19,20,22]. An abnormal interaction between renal D_1R and angiotensin type 1 receptor (AT_1R) in RPTCs [23–25] in the renal renin–angiotensin system may also lead to Na^+ retention in hypertension, including salt-sensitive hypertension [26,27]. There is an aberrant interaction between D_1R and AT_1R in RPTCs from humans with essential hypertension [28].

Since SS involves aberrant regulation of Na^+ transport in the RPT, we hypothesized that the use of living RPTCs isolated from urine (i.e., a virtual renal biopsy) could serve as a convenient method to assess RPT physiology almost instantaneously. Studies using living RPTCs isolated from urine have provided important clues about the etiology of disease [7,29–32]. We hypothesized that the physiological consequences of stimulation of D_1R and AT_1R could be recapitulated in freshly isolated RPTCs. Specifically, we tested the hypothesis that the intracellular Na^+-induced recruitment of the D_1R from cytosol to the plasma membrane and an increase in intracellular Ca^{++} with angiotensin II may be associated with the salt-sensitive phenotype. We further hypothesized that the degree of SS (i.e., SS index) is proportional to the response to D_1R and AT_1R stimulation.

2. Methods

2.1. Subjects

Our study was performed on 12 subjects selected from a pool of subjects that had been characterized 1 to 5 y previously for their SS index using a 2-week randomized controlled diet protocol [33]. Appropriate power calculations were performed for within group and between group comparisons at a 2-tail $P \leq 0.05$ level of significance. During their initial characterization for SS index, subjects were placed on an isocaloric constant diet containing 1 gram protein/kg body weight/day and either 300 mmol/day Na^+ (high Na^+) or 10 mmol/day Na^+ (low Na^+), and 60 mmol/day potassium (K^+) for 7 days (on each diet) in a randomized alternating protocol. At the time of study, the isocaloric and protein controlled diet was used to eliminate effects of dietary protein variations on renal dopamine formation. Body weight was recorded, and heart rate and arterial blood pressure were measured in the sitting position (at least 5 min rest) with an automated BP device (DINAMAP ProCare, Critikon) which has been validated according to the International Protocol in an adult population. Mean arterial pressure (MAP) was calculated as MAP = diastolic pressure (DP) + [0.33 + (heart rate (HR) × 0.0012)] × [systolic pressure (SP)]. Twenty-four hour urine collections were analyzed daily for Na^+, K^+ and creatinine to determine the state of Na^+ balance. Exclusion criteria included a history of accelerated or malignant hypertension, contraindications to discontinuing antihypertensive therapy, or renal dysfunction as evidenced by a serum creatinine >1.5 mg/dl or proteinuria >300 mg/day [34]. Subjects which had previously experienced a myocardial infarction, stroke or transient ischemic episode, congestive heart failure, severe small vessel disease, or concurrent pregnancy were also excluded as previously described [33].

One to five years after their initial characterization, subjects were randomly selected from each pool of salt-sensitive, salt-resistant (SR), and inverse salt-sensitive (ISS) and then reenrolled in this follow-up study. Morning to mid-day voided urines were collected by normotensive subjects and brought to the laboratory for viable cell isolation and testing. There was no significant correlation between each subject's SS index and urine Na^+, K^+ or osmolarity indicating that the measured RPTC responses were not related to the amount of electrolytes in the urine.

2.2. Renal cell isolation

There is slow normal turnover of cells in normal healthy individuals which results in shedding of renal tubule cells [35]. The isolation and culture of RPTCs have been reported in the literature from rats and humans [7,29–32,36]. RPTCs were isolated to approximately 98% purity with a yield of 150–200 cells/void (approximately 120 ml). In order to validate that cells that were isolated were RPTCS, we used 2 independent isolation methods, as well as antibodies and lectins to identify markers on their outer membrane (vide infra).

Briefly, our method of isolating RPTCs from urine involved removing casts, cuboidal epithelial cells, and other contaminants using the following multi-step purification procedures. This procedure allowed >95% recovery of exfoliated RPTCs as determined from an internal control consisting of cultured RPTCs bioengineered to express tandem tomato fluorescent protein. The entire voided urine sample from a well hydrated individual (100 ml minimum) was centrifuged at 800 ×*g* for 5 min and allowed to decelerate without a brake in order not to disturb the pellet. The pellet was carefully aspirated with a 10 ml volume of urine and placed into a 15 ml tube. The pellet was washed 3 times in 10 ml PBS^{++} (PBS with Ca^{++} and Mg^{++}, pH 7.4) and centrifuged to produce a new pellet following each wash. The final pellet was collected, filtered through a 40 μm filter, and placed into a 1.5 ml microfuge tube. RPTCs were isolated using 2 different methods with similar results. In the first method, 2 μg biotinylated BSA in 200 μl of serum-free media were added to the urine pellet for 30 min at 4 °C, followed by 25 μl Cellection Biotin Binder Kit, according to manufacturer's instructions (Invitrogen). This method utilizes the high affinity binding of cubulin/megalin complex for albumin and expressed in RPT but not in other nephron segments [37]. In the second method, we added 2 μg biotinylated CD13 monoclonal antibody in 200 μl of PBS^{++} (with 0.1% BSA and 2 mmol/l EDTA) to the urine pellet for 30 min at 4 °C. This was followed by the addition of 25 μl of Anti-Biotin Microbeads (Miltenyi Biotec), following manufacturer's instructions. Mixing with magnetic particles was performed for 30 min at 4 °C with end-over-end rotation, at 12 rotations/min. The supernatant containing the suspended cells was collected and centrifuged in a separate 1.5 ml microfuge tube at 200 ×*g* for 5 min. The isolated cells were RPTCs, as determined by gamma glutamyl transpeptidase (GGT) [38], aminopeptidase N (CD13) [39,40], and *Lotus tetragonolobus* agglutinin (LTA) [41] staining. In some experiments, for controls, we used cultured human RPTCs isolated from kidney tissue as previously published [42]. Na^+ hydrogen exchanger 3 (NHE3) is expressed in the renal proximal tubule and thick ascending limb; Na^+ potassium-2 chloride cotransporter (NKCC2) is expressed only in the thick ascending limb; Na^+ chloride cotransporter (NCC) is only expressed in the distal convoluted tubule while epithelial Na^+ channel (ENaC) is expressed only in late connecting tubule and collecting duct [43]. Therefore, the presence of NHE3 but not NKCC2, NCC, or ENaC indicates the presence of RPTCs