Lack of Validation of a Same-Day Outpatient Protocol for Determination of Salt Sensitivity of Blood Pressure

JoAnn Nichols, Fernando Eliovich, Cheryl L. Laffer

Abstract—Salt sensitivity of blood pressure has been studied in humans with a 48-hour inpatient protocol of salt loading and depletion or with longer outpatient protocols using high- and low-salt diets. Results have been reproducible, but both methods are laborious and costly. A 6-hour protocol of intravenous salt loading and furosemide has been reported but never validated. We studied 14 normal volunteers (39±2 years old; 86% women and 21% black) with the inpatient and 6-hour protocols, separated by 30 days. Four subjects (29%) were salt sensitive in the inpatient protocol. They had higher systolic blood pressure, higher body mass index, and somewhat lower plasma renin activity than salt-resistant subjects. Baseline systolic blood pressure before both protocols was highly reproducible (r=0.90; P<0.0001; limits of agreement: +6.2 to −8.0 mm Hg), whereas the response to salt depletion was not (r=0.09). Three salt-sensitive and 4 salt-resistant subjects were misclassified by the short protocol. Three-hour natriuresis by furosemide in the short protocol (344±15 mmol) was not different from the 12-hour natriuresis in the inpatient protocol (357±19). However, stimulation of plasma renin activity and aldosterone was significantly less in the short (+0.10±0.07 ng/AL/sec and −61±44 pmol/L) than in the inpatient protocol (+1.80±0.60 ng/AL/sec and +256±88 pmol/L; P<0.03 for both). Activation of hormonal changes that regulate depressor responses to salt depletion may not have occurred with the 3-hour natriuresis of the short protocol. This methodology cannot be used to study salt sensitivity of blood pressure, because the phenotype, mechanisms, and prognosis of the latter have been defined with the inpatient protocol. (Hypertension. 2012;59:00-00.)

Key Words: blood pressure monitoring • sodium • salt intake • risk factors • renin • aldosterone • salt sensitivity

Research on the relationship between salt balance and blood pressure (BP) has always been of interest to cardiorenal physiologists. However, unraveling the mechanisms of salt sensitivity of BP (SSSBP) became more important after the demonstration that the salt-sensitive (SS) phenotype has prognostic implications in humans.1 Studies in animal models were facilitated by dichotomization of the phenotype, that is, creation of pure SS and salt-resistant (SR) strains by inbreeding. In contrast, BP responses to salt loading and salt depletion in humans are normally distributed, not dichotomous.2 Therefore, classification of subjects into SS and SR groups requires selection of arbitrary cutoffs for the magnitude of BP responses to changes in salt balance, achieved with strictly controlled experimental protocols.

Many investigators of SSBP have used minor variations of the acute inpatient protocol devised at Indiana University (intravenous saline plus high-salt diet on the first day, followed by furosemide and low-salt diet on the second).2 Others have used chronic outpatient dietary manipulation of salt intake lasting several weeks. Results with these 2 methods are fairly reproducible.3,4 However, both are cumbersome and expensive. Although the outpatient approach avoids the cost of hospitalization, it is subject to patient dietary compliance.

Recently, a same-day protocol of sodium loading and depletion performed over the course of 6 hours has been reported.5 Although the advantages of such a protocol (easier to carry out and less expensive) are obvious, it has never been validated against the established ones.

Fourteen normal subjects who participated in our ongoing studies about the mechanisms of SSBP (conducted with the inpatient Indiana University protocol) were recruited to return 1 month later to undergo the short, same-day protocol in the clinic. We assessed whether classification of subjects into SS and SR groups was reproducible with these 2 methods.

Methods

Normotensive subjects were recruited into an institutional review board–approved protocol for the study of mechanisms of SSBP, and all gave informed consent. Fourteen of them completed both the inpatient and outpatient protocols, and their data are reported here.

We have described the inpatient protocol in detail in previous publications.6 Briefly, after obtaining demographic information and routine laboratory data, subjects were admitted to the hospital for an overnight stay, followed by salt loading on the first day of study (160

Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.111.185835
mEq of NaCl diet plus 2 L of normal saline infused from 8:00 AM to 12:00 PM) and salt depletion on the second (10 mEq of NaCl diet plus three 40-mg doses of oral furosemide). BP was recorded throughout the study with an ambulatory, automated, noninvasive oscillometric device (SpaceLabs 90207), every 15 minutes from 6:00 AM to 10:00 PM and every 30 minutes overnight. Average systolic BPs (SBPs) from 12:00 PM (end of saline on day 1 or second dose of furosemide on day 2) to 10:00 PM (bedtime) were used for classification of subjects into SS or SR groups, using as a cutoff a fall of 10 mm Hg from days 1 to 2.

Subjects brought a 24-hour urine specimen collected the day before study. Additional urine collections were obtained separately for the salt-loading day and for the initial (furosemide diuresis) and final (salt-depleted state) 12 hours of the second day. Urine chemistries, including creatinine and sodium, were measured in fresh samples. Blood samples for routine tests, plasma renin activity (PRA), aldosterone, and catecholamines (epinephrine, norepinephrine, and dopamine) were obtained at baseline (before saline infusion at 8:00 AM of the first day) and at the end of the salt-loading and salt-depletion days. PRA and aldosterone were measured by radioimmunoassay and plasma catecholamines by radioenzymatic assay.

The subjects returned 30 days later to undergo a same-day, 6-hour protocol similar to the one published previously by others. This was conducted in the clinic setting, with the patients comfortably seated in reclining chairs. Salt loading was achieved with 2 L of normal saline infused over 3 hours. This was immediately followed by salt depletion produced by 40 mg of IV furosemide. BP was monitored throughout the protocol with the SpaceLabs 90207 device, as above. The averages of all of the consecutive readings obtained over the last 15 minutes of saline infusion and over the 15 minutes before the third hour after the administration of IV furosemide were used to classify the patients as responders (ie, apparently SS) or nonresponders (ie, apparently SR). The cutoff used for this classification (a fall of 7 mm Hg in SBP between salt loading and salt depletion) was arbitrarily chosen by inspecting the distribution of BP responses to salt depletion in all of the participants and after determining that choice of any other arbitrary cutoff would not have changed the results.

Subjects again brought a 24-hour urine specimen collected the day before study. Additional urine collections were obtained separately for the salt-loading and salt-depletion 3-hour periods. Blood samples for routine tests, PRA, aldosterone, and catecholamines (epinephrine, norepinephrine, and dopamine) were obtained at baseline and at the end of the study.

Data are presented as mean ± SEM. Comparisons between SS and SR subjects and between data in the inpatient and outpatient protocols were made with unpaired t tests. Changes in variables between periods of each protocol were analyzed with paired t tests. Correlation coefficients were calculated with the Pearson method. Limits of agreement were obtained by plotting the individual average value for a parameter obtained with both protocols, versus the change between the values for the parameter in the 2 protocols. These tests and single linear regression analyses were performed with JMP software (version 3.0.2, SAS Institute). A P < 0.05 was used to reject the null hypothesis.

Results

The 14 volunteers were 39 ± 2 years old, 86% women, and 21% black, with systolic/diastolic BPs 116 ± 2/74 ± 2 mm Hg. Four of them (29%) were classified as SS with the inpatient protocol. Demographic and clinical characteristics of SS and SR subjects are shown in the Table. SS had significantly higher SBP and body mass index than SR subjects. Although their PRA was lower than that of SR subjects, this difference did not reach statistical significance. Creatinine clearance and urinary sodium excretion (on ad libitum diet) were not different between groups.

Table. Demographic and Clinical Characteristics of Normotensive SS and SR Subjects Classified With the Inpatient Protocol

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SS (n=4)</th>
<th>P</th>
<th>SR (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>39±2</td>
<td>NS</td>
<td>38±3</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>3/1</td>
<td></td>
<td>9/1</td>
</tr>
<tr>
<td>Race, black/white</td>
<td>2/2</td>
<td></td>
<td>1/9</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>37±7</td>
<td>&lt;0.05</td>
<td>28±2</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>125±4</td>
<td>&lt;0.01</td>
<td>112±2</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>79±5</td>
<td>NS</td>
<td>71±2</td>
</tr>
<tr>
<td>ClCr, ml/min/1.73 m²</td>
<td>139±13</td>
<td>NS</td>
<td>120±6</td>
</tr>
<tr>
<td>UNaV, μmol/mg of creatinine</td>
<td>105±14</td>
<td>NS</td>
<td>104±11</td>
</tr>
<tr>
<td>PRA, ngAI/L/s</td>
<td>0.18±0.10</td>
<td>NS</td>
<td>0.30±0.12</td>
</tr>
<tr>
<td>Aldosterone, pmol/L</td>
<td>222±65</td>
<td>NS</td>
<td>306±71</td>
</tr>
<tr>
<td>Catecholamines, nmol/L</td>
<td>1.5±0.2</td>
<td>NS</td>
<td>2.3±0.6</td>
</tr>
</tbody>
</table>

SSBP indicates salt-sensitivity of blood pressure; SS, salt-sensitive; SR, salt-resistant; BMI, body mass index; SBP, baseline systolic blood pressure; DBP, baseline diastolic blood pressure; ClCr, baseline creatinine clearance; UNaV, urinary sodium excretion before admission; PRA, baseline plasma renin activity; NS, not significant.

Individual baseline SBPs were highly reproducible at the onset of both protocols, despite the 30-day interval. The left panel of Figure 1 shows that the correlation coefficient for this relationship was highly significant, with a value of 0.90. Furthermore, the regression of outpatient on inpatient baseline SBPs (outpatient: 14.2 ± 10.9 mm Hg; inpatient: 6.17 ± 0.94 mm Hg) exhibited an intercept not statistically different from 0 and a slope not statistically different from the line of identity. The right panel of Figure 1 shows that the limits of agreement between the baseline SBPs of both protocols spanned an interval of 14.2 mm Hg, that is, a 12.3% maximum variability from one protocol to the other.

In contrast, responses to salt depletion in both protocols were completely unrelated. Figure 2 shows the changes in SBP from the salt-loading to the salt-depletion day of the inpatient protocol on the abscissa and those from the end of the 3-hour salt loading period to the third hour after IV furosemide on the ordinate. The vertical dotted line indicates the cutoff for definition of SSBP in the inpatient protocol (−10 mm Hg), whereas the horizontal one indicates the cutoff for responsiveness to salt depletion (ie, apparent SSBP) in the outpatient protocol (−7 mm Hg). Of the 4 subjects classified as SS in the inpatient protocol, only 1 was a responder to salt depletion in the outpatient protocol. That is, 3 SS subjects in the inpatient protocol were misclassified as apparently SR with the short outpatient protocol. Moreover, 4 subjects who were SR in the inpatient protocol were responders to salt depletion in the outpatient protocol, that is, misclassified as apparently SS. Overall, there was no correlation between the SBP responses to salt depletion by both protocols (r = 0.09), and the limits of agreement (data not shown) spanned an interval of 29 mm Hg, that is, 872% variability from one protocol to the other. Finally, when comparing subgroups of subjects by their apparent SSBP in the outpatient protocol (analogous to the comparison in the
Table for SS and SR in the inpatient protocol, the phenotypic characteristics observed in SS hypertension were absent in the outpatient responders to salt depletion. Hence, their body mass index (30.6±6.1 kg/m²) and SBP (117±5 mm Hg) were not higher than (30.3±2.2 kg/m² and 114±2 mm Hg, respectively) and their PRA (0.20±0.13 ngA1/L/sec) was not different from those of nonresponders (0.20±0.07 ngA1/L/sec).

Total urinary sodium excretion over the 12 hours of oral administration of furosemide in the inpatient protocol (357±19 mmol) was not significantly different from that achieved by IV furosemide over the 3 hours of the outpatient protocol (344±15 mmol). Despite this, responses of components of the renin-angiotensin-aldosterone system were different between protocols. The left and middle panels of Figure 3 show that, compared with baseline (before salt loading), PRA and aldosterone were significantly stimulated after salt depletion in the inpatient protocol (left open bars: +1.80±0.60 ngA1/L/sec and +256±88 pmol/L, respectively) but not in the outpatient protocol (right shaded bars: +0.10±0.07 ngA1/L/sec and −61±44 pmol/L). The differences between protocols were significant (ΔPRA −1.70±0.60 ngA1/L/sec, P<0.02, and Δaldosterone −318±120 pmol/L, P<0.03). In contrast, the right panel shows that mild stimulation of plasma catecholamines was not different between protocols.

**Figure 1.** The left panel depicts the relationship between baseline systolic blood pressures (SBPs) in the inpatient and outpatient protocols. For a discussion of the single linear regression between these 2 variables, see text. The right panel shows the limits of agreement for baseline SBP between the 2 protocols. The dotted line indicates that the average ΔSBP was 0.9-mm Hg less in the outpatient than in the inpatient protocol. The shaded area (limits of agreement) is defined by the average ±1.96 SDs of ΔSBP, spanning the interval +6.2 to −8.0 mm Hg. This interval (14.2 mm Hg) is equivalent to a maximum variability of 12.3% in SBP between protocols.

**Figure 2.** Systolic blood pressure (BP; SBP) responses to salt depletion. On the abscissa, those of the inpatient protocol, calculated as SBP of the salt depletion minus that of the salt loading day. On the ordinate, those of the outpatient protocol, calculated as SBP after 3 hours of furosemide minus that after 3 hours of saline infusion. The vertical dotted line indicates the cutoff for definition of salt sensitivity of BP (SSBP) in the inpatient protocol, whereas the horizontal dotted line indicates the cutoff for apparent SSBP in the outpatient protocol. Black circles depict subjects who were classified as salt-resistant (SR) by both protocols. White circles depict subjects classified as salt-sensitive (SS) by the inpatient protocol who were apparently SR in the outpatient protocol. Conversely, shaded squares depict subjects classified as SR by the inpatient protocol who were apparently SS in the outpatient protocol. The shaded circle was the only subject classified as SS by both protocols. There was no overall relationship between SBP responses to salt depletion by both protocols (r=0.09; not significant).

**Discussion**

SSBP is the pathophysiologic characteristic by which maintenance of salt balance becomes dependent on changes in BP that parallel salt intake, that is, the development of pressure-induced natriuresis and hypertension during salt loading or of depressor responses to salt depletion. This is different from normal physiology, in which equilibrium between the activity of natriuretic and antinatriuretic systems maintains salt balance independent of BP. Research on the abnormal mechanisms by which maintenance of salt balance becomes BP dependent has been facilitated by dichotomization of rodent strains into pure SS and SR phenotypes via selective inbreeding for many generations (eg, Dahl-SS and Dahl-SR rats and other strains). This has led to the description of multiple abnormalities in natriuretic and antinatriuretic systems, oxidative stress, and gene polymorphisms in SS strains. However, the ultimate cause of SSBP remains elusive, because many of these abnormalities were found in intersecting systems, precluding a definitive delineation of its primary cause.

Translation of this research to humans is even more difficult, because SSBP in normotensive or hypertensive subjects is not a dichotomous phenotype but a continuous, normally distributed characteristic instead. Therefore, classification of humans into SS and SR requires selection of arbitrary cutoffs in the BP responses to interventions involv-
ing salt loading or salt depletion. In our research on the roles of endothelin, oxidative stress, and 20-hydroxyeicosatetraenoic acid in SSBP of humans, we have used a minor modification (use of continuous monitoring of BP) of the 48-hour inpatient protocol of acute saline loading and furosemide-induced depletion devised at Indiana University decades ago.

Despite its arbitrariness, this protocol has unraveled common clinical characteristics associated with the SS phenotype when used by many different investigators (eg, increased prevalence in blacks, obese subjects, and elderly; exaggerated renal dysfunction and left ventricular hypertrophy; and suppressed activity of the renin-angiotensin system without or after salt loading). Some of these characteristics are also observed in pure SS rodent strains, indicating that the protocol is able to consistently identify subsets of SS normotensive and hypertensive subjects. Most importantly, long-term follow-up of a large number of subjects who underwent classification of SSBP using this protocol has led to the demonstration that the SS phenotype is a risk factor for cardiovascular mortality, independent of the level of BP. This has provided further impetus for research in humans, because unraveling the mechanisms of SSBP might ultimately lead to therapies that correct the SS phenotype and reduce cardiovascular morbidity and mortality.

Other investigators have chosen to study BP responses to changes in dietary salt intake in the outpatient setting. There has been large variation in the design of these protocols, involving different duration and order of administration of the high-salt and low-salt diets, and different magnitudes of dietary salt loading and depletion. However, it has been shown that results of dietary protocols reproduced in fair manner those obtained with the inpatient protocol above, provided that the contents of the high- and low-salt diets are 200 to 220 and 15 to 20 mmol of sodium per day, respectively, and that the duration of each diet is 1 week.

Both methodological approaches are laborious and costly. The inpatient protocol requires a clinical research center or defraying the cost of a 3-night admission to the hospital, a metabolic kitchen, and intensive involvement of research personnel. Carefully conducted outpatient protocols require provision of prepared meals for prolonged periods and are dependent on subjects’ dietary compliance. A recent publication on increasing prevalence of SSBP after menopause used a much simpler, shorter method (3 hours of salt loading followed by 3 hours of salt depletion) to classify their patients into SS and SR subgroups. The authors acknowledged, however, that their method had not been validated against traditionally accepted ones. In the expectation that we could show reproducibility between the inpatient and short protocols, for future use of the latter in our studies, we performed both protocols in normal volunteers who participate in our ongoing studies on the mechanisms of SSBP.

Using the inpatient protocol, the prevalence of SSBP in our volunteers was 29%, similar to that described previously for normotensive subjects. Also, SS subjects were more obese and had higher BP and somewhat lower PRA than the SR subjects, that is, they shared some classic characteristics of the SS phenotype described in hypertensive patients. Unfortunately, BP responses to salt depletion 30 days later, in the short protocol, were completely unrelated to those of the inpatient protocol. Only 50% of subjects were classified in the same subgroup (6 SR and 1 SS) by both protocols, whereas the other 50% were misclassified by the short protocol (4 SR as SS and 3 SS as SR). Furthermore, grouping of subjects by the results of the short protocol did not show the characteristics of the SS phenotype in the apparently SS patients, as the classification by the inpatient protocol did. This result of ours is different from that obtained in the menopause study, in which patients classified as SS with the short protocol had higher waist:hip and waist:thigh ratios than their SR counterparts.

We do not have an explanation for the lack of reproducibility of BP responses to salt depletion in the short protocol. Although our patients were younger (39±2 years old) than those in the menopause study (47±4 years old), prevalence of
SSBP was higher in our study (29% versus 23%), indicating that the SS phenotype had reached full expression in both populations. Moreover, baseline SBPs in both protocols were highly correlated, with narrow limits of agreement. Hence, stability of BP under ad libitum dietary conditions over a 30-day interval is unlikely to have played a role in the results. This is consistent with observations by others that BP responses to salt depletion are reproducible over time, when studied repeatedly with the same protocol.

Moreover, the magnitude of sodium depletion was very similar in both protocols. Total furosemide-induced natriuresis was almost equal when this drug was given in 3 oral doses of 40 mg in the inpatient protocol or as 1 IV 40-mg dose in the short protocol. Therefore, it was somewhat surprising that stimulation of the renin-angiotensin-aldosterone system was achieved by salt depletion in the inpatient but not in the short protocol.

A basic assumption in all of the research on SSBP is that “clamping” of natriuretic or antinatriuretic substances (ie, their inability to normally respond with physiological stimulation or inhibition to changes in salt balance) underlies the dependency of natriuresis on BP that is observed in the SS phenotype. For example, clamping of the renin-angiotensin system at a low level of activity by angiotensin-converting enzyme inhibitors or at a high level by angiotensin infusion induces SSBP in animals. It is, therefore, conceivable that a temporal factor was involved (ie, a longer period of lower perfusion pressure or of urinary sodium depletion in the inpatient protocol) determining lesser renin stimulation by equipotent natriuresis in the shorter outpatient protocol. Hence, differences in responsiveness of the renin-angiotensin-aldosterone system between SS and SR patients might not have been fully expressed in the latter, and their impact on BP responses to salt depletion was obscured. The same speculation about a role for temporal factors could be applied to other natriuretic or antinatriuretic systems that we did not study in our subjects. That is, the problem of the short protocol may be precisely its short duration, with inability to permit full expression of adaptive differences to changes in salt balance between SS and SR subjects.

Regardless of the reasons for the different BP responses to salt depletion with both protocols, our study clearly demonstrates that the short protocol cannot be used for classification of subjects into SS and SR phenotypes. Conclusions reached with this protocol, regarding any clinical or mechanistic observations, cannot be extrapolated to the mechanisms or prognostic implications described for the SS phenotype by use of traditional methods, that is, the inpatient protocol or validated outpatient dietary protocols that may be as short as a few days.

Perspectives
Our study addresses a methodological issue that is important for research on the mechanisms determining SSBP in humans, which is known to be a cardiovascular risk factor independent of BP. Research on SSBP is relevant because it may lead to therapeutic approaches for the SS phenotype, above and beyond treatment of hypertension itself, with consequent improvement in prognosis. We unequivocally show that, until an easier but validated methodology for classification of humans into SS and SR subgroups is developed, investigators must continue using traditionally established techniques with proven reproducibility, no matter how laborious. We provide a word of caution regarding the use of seemingly easier methods without proper validation. Phenotype classification must reproduce that of the established methods if the results of a new technique are to be incorporated into our current body of knowledge in the field, because everything we know about clinical characteristics, mechanisms, and prognostic implications of the SS phenotype has been learned with the traditional validated protocols.

Sources of Funding
These studies were supported by a grant from the Scott, Sherwood, and Brindley Foundation and by funds from the Department of Medicine, Scott and White Healthcare, Texas A&M Health Science Center College of Medicine.

Disclosures
None.

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Hypertension. published online December 19, 2011;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2011/12/19/HYPERTENSIONAHA.111.185835

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