Partial baroreceptor dysfunction and low plasma nitric oxide bioavailability as determinants of salt-sensitive hypertension: a reverse translational rat study

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Abstract

This study determined whether clinical salt-sensitive hypertension (cSSHT) results from the interaction between partial arterial baroreceptor impairment and a high-sodium (HNa) diet. In three series (S-I, S-II, S-III), mean arterial pressure (MAP) of conscious male Wistar ChR003 rats was measured once before (pdMAP) and twice after either sham (SHM) or bilateral aortic denervation (AD), following 7 days on a low-sodium (LNa) diet (LNaMAP) and then 21 days on a HNa diet (HNaMAP). The roles of plasma nitric oxide bioavailability (pNOB), renal medullary superoxide anion production (RMSAP), and mRNA expression of NAD(P)H oxidase and superoxide dismutase were also assessed. In SHM (n=11) and AD (n=15) groups of S-I, LNaMAP–pdMAP was 10.5±2.1 vs 23±2.1 mmHg (P<0.001), and the salt-sensitivity index (SSi; HNaMAP–LNaMAP) was 6.0±1.9 vs 12.7±1.9 mmHg (P=0.03), respectively. In the SHM group, all rats were normotensive, and 36% were salt sensitive (SSi>10 mmHg), whereas in the AD group ~50% showed cSSHT. A 45% reduction in pNOB (P#0.004) was observed in both groups in dietary transit. RMSAP increased in the AD group on both diets but more so on the HNa diet (S-II, P<0.03) than on the LNa diet (S-III, P<0.04). MAP modeling in rats without a renal hypertensive genotype indicated that the AD*HNa diet interaction (P=0.008) increases the likelihood of developing cSSHT. Translationally, these findings help to explain why subjects with clinical salt-sensitive normotension may transition to cSSHT.

Key words: Salt-sensitive hypertension; Aortic barodenervation; Nitric oxide bioavailability

Introduction

Salt sensitivity is a condition in susceptible normotensive or hypertensive individuals in which a significant and sustained rise in mean arterial pressure (MAP) accompanies chronic high sodium chloride (salt) intake (1). Clinically, the difference in MAP between the ends of salt depletion and repletion regimes (acute or chronic), or vice versa, is called the salt-sensitivity index (SSI). This is a normally distributed, inherited trait used to classify an individual as salt sensitive (SS) if the value is >10 mmHg, as indeterminate (IND) if the value is >5<10 mmHg, or as salt resistant (SR) if the value is ≤5 mmHg (2). In addition, SS subjects exhibit a characteristically blunted slope in pressure-natriuresis or in pressure-Na diet relationships, whereas SR subjects do not (3). Experimentally, however, no defined protocol exists for identifying the SS condition as clinically defined. Thus, animals that show any increase in MAP induced by high salt intake, especially if MAP reaches the hypertensive range, are considered to have ”experimental” SS hypertension (eSSHT). To our knowledge, the “clinical” concept of SS normotension (cSSNT) does not exist in the experimental field.

Studies of renal cross-transplantation between Dahl SS and Dahl SR rats performed by Dahl et al. (4), Dahl and Heine (5), and Morgan et al. (6), using both normal (0.3%, NNa) and high (8.0%, HNa) sodium diets, allowed them to arrive at two conclusions that others have come to endorse (7,8). They concluded that eSSHT in Dahl SS rats is determined by the hypertensive genotype of the
kidney, which reduces the renal sodium excretory capacity (4-6), and by the systemic genotype of the rat, which determines how systemic factors (almost exclusively extrarenal) respond to a HNa diet (6). These systemic factors would further reduce the renal sodium-excretory capacity of the kidney, which would be overcome only by further increasing MAP (7). To date, research has implicated several differential systemic factors in Dahl SS and Dahl SR rats. Among them are adrenal steroidogenesis (9), sympathetic nerve activity (7,10), and endothelial function (11,12).

In Dahl SS rats on a low salt diet (0.04%, LNa) and, therefore, in a prehypertensive state, Gordon and Mark (10,13) demonstrated a partial arterial baroreceptor impairment (vs Dahl SR rats on a LNa diet) that translated into a diminution of both the heart rate (HR) and the neurosympathetic-mediated hindlimb vascular resistance baroreflex sensitivities, without altering the baseline MAP. They proposed that this partial impairment, the most common form of baroreceptor impairment (14), might amplify the effects of the HNa diet by failing to adequately buffer increases in arterial pressure (13). However, to our knowledge, this interaction has not been explored. If such an interaction exists, partial baroreceptor impairment might be one of the phenotypic elements of the SS systemic genotype (its baroreceptor element). On the other hand, it has been documented in Dahl SS rats (vs Dahl SR rats) that a HNa diet induces endothelial dysfunction, as manifested by increased vascular superoxide anion production (12), with reduction in both the plasma levels of nitrogen oxides (11) - an index of nitric oxide (NO) bioavailability - and in the endothelium-dependent relaxation to acetylcholine (12). If such interaction between endothelium and salt exists, endothelial dysfunction might constitute another of the phenotypic elements of the SS systemic genotype (its endothelial element).

The present study, performed in primarily normotensive rats, had two objectives: 1) to test the effects of sequential exposure, first to a LNa diet for 7 days and then to a HNa diet for 21 days, in rats with and without partial arterial baroreceptor impairment (to simulate the prehypertensive SS baroreceptor systemic phenotype) on SSI and on MAP after 21 days on a HNa diet, and 2) to study the roles of plasma NO bioavailability (pNOB), renal medullary superoxide anion production, and renal mRNA expression of NAD(P)H oxidase and superoxide dismutase (SOD) in such responses. We hypothesized that clinical salt-sensitive hypertension (cSSHT) should develop in all rats on a HNa diet with partial arterial baroreceptor impairment, if in effect an interaction between them exists.

**Material and Methods**

**Series I, II, and III**

Male rats bred from the Wistar Charles River strain 003 (ChR003; Charles River, USA) were used in three series (S-I, S-II, and S-III). The effects of sequential exposure, first to a LNa diet for 7 days and then to a HNa diet for 21 days, in rats with and without chronic bilateral aortic denervation (partial barodenervation), on MAP, SSI, and pNOB were tested in S-I, and on baseline and NADH-stimulated renal medullary superoxide anion production (RMSAP) and renal mRNA expression of NAD(P)H oxidase and SOD at the end of a HNa diet in S-II. S-III tested the effects of a LNa diet for 7 days in rats with real or sham aortic denervation at baseline and NADH-stimulated RMSAP. All experimental procedures were conducted in accordance with the Mexican Guidelines for the Care and Use of Experimental Animals (NOM-062-200-1999).

**General protocol**

Weaned rats (70 to 90 g) housed individually at the animal care facility (with an automated 12:12-h light-dark cycle, temperature, and humidity control) were weighed daily and had free access to a NNa diet (LabDiet 5P14, 0.4% Na, PMI Nutrition International, Inc., USA) and 0.9% isotonic saline as drinking water, to produce conditions for the expression or not of a renal hypertensive genotype. When rats reached 200-235 g body weight, MAP was measured when the rats were in a conscious state before aortic denervation [predenervation MAP (pdMAP)]. Next, rats were randomly assigned to sham (SHM) or real aortic denervated (AD) groups. After barodenervation, they were returned to the animal care facility, having free access to the NNa diet plus filtered tap water until recovering their predenervation weight. Then, rats were exposed freely and in succession to a LNa diet (0.04% Na, TD90228 diet, Harlan Teklad, USA) for 7 days, followed by a HNa diet (8% Na, TD92012 diet, Harlan Teklad) for 21 days. MAP was measured again in the conscious state at the end of both LNa and HNa dietary regimes in S-I and S-II (3 occasions in total: pdMAP, LNaMAP, and HNaMAP) and at the end of the LNa dietary regime in S-III (2 occasions in total: pdMAP and LNaMAP).

**Predenervation measurement of MAP**

A catheter was implanted into the tail artery using aseptic techniques while the rat was under short-duration ether anesthesia. Once conscious, rats received 570 mg/kg metamizole sodium (PISA, Mexico) sc and were placed in a very well-ventilated, semirestrictive (permitting back and forth movement), comfortable, low-stress rat holder (i.e., not a Lucite restrainer or Perspex tube). After a 4-h stabilization period, MAP and HR were recorded continuously for 60 min. Then, rats received 90 mg/kg sodium ceftriaxone (Amcef, AMSA, Mexico) ia and were subjected to sham or real aortic denervation.

**Aortic barodenervation procedure**

Using aseptic techniques and after administering ip ketamine (33.3 mg/kg; Anesket, PISA) and xylazine...
(6.7 mg/kg; Rompun, Bayer, Mexico) anesthesia, atropinized (0.05 mg/kg ip; Atropisa, PiSA) rats were subjected to either sham or real chronic bilateral aortic barodenervation using Krieger’s technique (15) slightly modified by us (16). Sham or real barodenervations lasted 30-40 min. After tail and neck incisions were closed, rats received 570 mg/kg metamizole sodium sc and were warmed until they became conscious. Over the subsequent 3 postsurgical days, rats received 140 mg/kg sodium ceftriaxone im.

**Postdenervation measurement of MAP**

Twenty-four hours before measuring LNaMAP and HNaMAP, under aseptic conditions and with the rat under ether anesthesia, catheters were inserted into either the right femoral artery to record LNaMAP or the left femoral artery and vein to record HNaMAP and drug administration. Catheters exited the interscapular region and passed through a lightweight flexible spring to a 360° rotary dispositive mounted above the cage. Then, 320,000 IU/kg penicillin G benzathine (Pendiben L-A, PiSA) im and 570 mg/kg metamizole sodium sc were administered. The next day, the rat was placed in the holder, the flexible spring was removed, and the arterial catheter was connected to a P23 XL transducer (Gould, USA). After a 30-min stabilization period beginning at 7:00 am, MAP and HR were recorded continuously for 60 min. In S-I and S-II, after measuring LNaMAP, 1.5 mL arterial blood was collected in sterile and ice-cooled tubes without replacement. After removing the catheter(s) and over the subsequent 3 postsurgical days, rats received 80,000 IU/day penicillin G benzathine im.

After measurement of HNaMAP in S-I and S-II and LNaMAP in S-III, 1.5 mL arterial blood was collected again and replaced with a 750-μL bolus of 6% hydroxy-starch solution (Hestar, PiSA) administered slowly iv. After a 30-min stabilization period, a subprotocol was performed to evaluate the effectiveness of aortic denervation. This consisted of recording MAP and HR for 1 min before (control) and 4 min after administering three 100-μL boluses of phenylephrine at 10-min intervals [PHE; 10 μg/kg iv; P-6126, Sigma-Aldrich Quimica (S-AQ), Mexico] and three 100-μL boluses of sodium nitroprusside at 6-min intervals (SNP; 5 μg/kg iv; S-0501, S-AQ).

**MAP recording settings**

Blood pressure was recorded by coupling the P23 XL transducer to a 79D Grass polygraph (Grass Instrument Co., USA) via Grass 7P1-7DA amplifiers. The pulsatile waveform signal was led via J1-J2 to another Grass 7DA amplifier to obtain the MAP value by electronic attenuation (0.1) and to a Grass 7P4 tachograph to record the instantaneous HR. Both signals (MAP and HR) were digitized at 2 Hz using a Data Translation DT 2801 analog-digital board with the HP-Vee software (Hewlett-Packard Co., USA). The difference between LNaMAP and pdMAP was calculated for each animal. MAP lability was measured as the standard deviation of the MAP recordings made of each conscious rat (7200 samples) over 1 h after either 7 days on the LNa diet (S-III) or 21 days on the HNa diet (S-I and S-II). The SSI was calculated as the difference between HNaMAP and LNaMAP.

**Effectiveness of aortic barodenervation**

The effectiveness of aortic barodenervation was evaluated in the conscious state using the transformed general sensitivity index (TGSI) previously described and validated by us (16). This index allows us to differentiate, on an individual basis, among rats with sham, aortic, or sino-aortic barodenervation: 

$$
\text{TGSI} = \frac{\{(H_{\text{SNP}} – H_{\text{PHE}})/d\text{MAP}_{\text{SNP}} – d\text{MAP}_{\text{PHE}}\}^{0.04597}}{1 – 0.04597}
$$

TGSI ranges previously established (16) were as follows: for sham barodenervation, 10.09 to 11.46; for aortic barodenervation, 8.40 to 9.94, and for sino-aortic denervation, 7.68 to 8.24.

**Termination of the experiment**

At the end of the experiment, all rats in the three series were anesthetized with 50 mg/kg ip sodium pentobarbitone (Pentovet, Norvet, Mexico). Using aseptic techniques, a retrograde PE50 catheter (Intramedic, Clay Adams, USA) was inserted into the abdominal aorta near the iliac bifurcation and advanced to just below the left renal artery. This catheter was connected to a cold pack-wrapped 20-mL syringe containing ice-cold PBS, pH 7.4, which was placed in an infusion pump (model 901, Harvard Apparatus Co., USA). Then, in rapid sequence, the infusion pump was turned on (at a 2.5 mL/min infusion rate to hold a perfusion pressure at ~110 mmHg); first, the left renal vein was transected, then the right renal vein was transected, and the proximal (caval) ends of the veins were knotted. Next, the mesenteric artery and upper aortic artery were knotted so that perfusion continued until the kidneys blanched (~15 min). Both kidneys were removed, placed in separate Petri boxes with ice-cold PBS, decapsulated, and sectioned longitudinally (frontal plane) in three slices. The left and right renal medullas were excised from the middle slices.

**Measurement of pNOB**

pNOB was measured as total plasma nitrogen oxide concentration ([NO$_2$]p = [NO$_2$] + [NO$_3$]p) using Griess’s colorimetric reaction as described by Miranda et al. (17) but adapted by us for a microtube and 540 nm double-beam spectrophotometer assay (DMS-70 UV-Vis; Varian, Australia). The change in plasma NO bioavailability (ΔpNOB) was calculated as LNa[NO$_2$]p–HNa[NO$_3$]p.

**Measurement of RMSAP**

Baseline and NADH-stimulated RMSAP were measured in S-II and S-III, as described by Guzik and Channon (18). About 100 mg renal medulla in 2 mL ice-cold PBS lysis buffer [final concentrations: 1% NP40
(11332473001, Roche, Mexico). 0.5% sodium deoxycholate (D-6750, S-AQ), 0.1% SDS (1610300, BioRad, USA), 1 mM PMSF (P-7626, S-AQ), 10 U/mL aprotinin (A-1153, S-AQ), and 10 mM sodium orthovanadate (B-49203, S-AQ) was homogenized and centrifuged at 4°C at 14,835 g for 15 min (model RMC14, Sorvall, USA), and then the supernatant was cooled on ice. Next, under dark conditions, clean vials were counted (5 min) in a liquid scintillation counter (RackBeta, model 1209, Pharmacia Wallac Oy, Finland) in out-of-coincidence mode to obtain background (BKG) counts. To measure NADH-stimulated RMSAP, we counted 950 μL of a mixture [4500 μL lysis buffer plus 250 μL 10 μM lucigenin (B49203, S-AQ)] and obtained the baseline (BL) count. Then, 50 μL supernatant, 20 μL 100 μM NADH (N8129, S-AQ), and finally 20 μL 400 U/mL SOD (S7571, S-AQ) were added in succession and counted each time. The same procedure was used to measure the BL RMSAP but without adding NADH. All measurements were done in duplicate. Homogenate protein concentration was measured by Bradford’s method at 595 nm. For each replicate, the integral of the counts per minute (cpm) for the last 4.5 min was obtained for BKG, BL, NADH, and SOD, and the duplicates were averaged. From the last three integrals, BKG was subtracted to obtain ΔBL, ΔNADH, and ΔSOD. We calculated RMSAP as follows: BL = ΔBL–ΔSOD/mg protein, and NADH-stimulated = (ΔNADH–ΔSOD)/(ΔBL–ΔSOD)/mg protein.

Semiquantitative reverse-transcription PCR (RT-PCR)
Renal medulla was homogenized in a mixture of phenol and guanidine isothiocyanate (TRIzol, 15596-026, Invitrogen, USA) using a Polytron PT 1200 (Kinematica, Switzerland) and stored at –80°C until the processing of total RNA extraction. RT reactions were performed using 2 μg total RNA and a Superscript II Reverse Transcriptase kit in a 20-μL mixture reaction. PCR was performed using the PTC-100 Programmable Thermal Controller system (MJ Research, USA) with a Platinum Taq DNA polymerase kit (10966, Invitrogen) using 1 μg cDNA containing 0.2 mM of each dNTP (18427, Invitrogen) in a 25-μL mixture reaction. The reaction was incubated at 95°C for 5 min, and 35 cycles of the following sequential steps were performed: 94°C for 30 s, 58°C for 40 s, and 72°C for 90 s. The β-actin control was processed under the same conditions but for only 25 cycles. The number of cycles for each cDNA was previously determined by an amplification curve. The rat sense and antisense primer sequences shown in Table 1 were designed using the gene-specific sequence deposited in the GenBank. Ten-microliter aliquots were electrophoresed on a 2.5% agarose gel and visualized with the Gel Logic 100 Imaging System (Kodak, USA) using ethidium bromide. The densitometric band values for gp91phox, p22phox, intra-cytoplasmic (IC)-SOD, and β-actin were obtained using the Image J software V 1.43 and were reported as relative band intensities.

Experimental design and statistical analysis
The three series were designed as cross-sectional studies because, even though in S-I and S-II the MAP and pNOB were measured at least two times in each rat (longitudinal subprotocols), the main response variables in such series were differences (SSI or ΔpNOB). MAP data in S-I were first analyzed (95% CI) by linear mixed-effects models (19) to evaluate the denervation+diet interaction and by ANCOVA to evaluate the effects of pdMAP and group on LNaMAP. Thereafter, SSI and pNaMAP data in S-I were modeled by multiple regression (linear fixed models, 95% CI) using re-parametrized variables. The residuals of the models were tested for normality (Shapiro-Wilk test) and homoscedasticity (Brown-Forsythe test) (20). When necessary, the data were subjected to a Box-Cox transformation to adhere to the assumptions of parametric statistics. The best models were chosen on the basis of the highest adjusted r² (determination coefficient = explained variation), the lowest Akaike information criterion (21), the likelihood ratios, and the principle of parsimony. Post hoc multiple comparisons were performed by the Tukey test. MAP data in S-II were analyzed by multivariate analysis of variance (MANOVA) with repeated measures for the diet factors. In the three series, as necessary, classical parametric tests for nonindependent and independent samples were used. All values are reported as means±SE, and data were analyzed using the JMP V.7.01 software (SAS Institute, USA) and the R V 2.14.1 software.

Results

Series I

Body weight time course in SHM and AD groups. Only 26 of 36 sham or real aortic denervated rats showed a satisfactory body weight evolution and fulfilled the TGSI barodenervation criteria (SHM group, n=11 and AD group, n=15). Despite randomization, predenervation body weight was modestly (~7 g) but significantly greater (P<0.02, unpaired t-test) in the AD group than in the SHM group (Table 2). The AD group showed a greater decrease in body weight (~17 g more, P=0.001, unpaired t-test) requiring a longer recovery time than the SHM group to reach its predenervation body weight (~3 days more, P=0.001, unpaired t-test). However, at the end of the LNad diet and the HNa diet, both groups had similar body weights.

Pre- and postdenervation MAP time courses in SHM and AD groups. Table 3 shows the pdMAP, LNadMAP, and HNaMAP recorded in each group. Since preliminary experiments in SHM and AD rats showed stable urinary sodium excretions of ~0 and ~30 mEq/day with the LNad and HNa diets, respectively, LNadMAP and HNaMAP are plotted in Figure 1 as pressure-sodium diet relationships. There was no difference in pdMAP between groups, and
The TGSI value showed a statistically significant difference ($P=0.001$, unpaired $t$-test) between groups. MAP during the LNa diet increased above the pdMAP value in both groups, but more so in the AD group (23±2.1 mmHg) than in the SHM group (10.5±2.1 mmHg). The difference between LNaMAP and pdMAP was 12.5 mmHg greater ($P=0.001$, unpaired $t$-test) in the AD group than in the SHM group (Figure 1). The modeling of LNaMAP by ANCOVA indicated that 50% of the variation in LNaMAP ($r^2=0.4987$, ANOVA $P=0.001$) could be explained by the pdMAP and group predictor variables, without any interaction between them. Moreover, after 21 days on the HNa diet, MAP increased relative to its corresponding LNaMAP, but to a lesser extent in the SHM group (SSi: 6.0±1.9 mmHg; an SSi of the IND subtype; see later) than in the AD group (12.7±1.9 mmHg; an SSi of the SS subtype; see later). The difference between SSi values was statistically significant ($P=0.03$, unpaired $t$-test) as were the HNaMAP values (SHM: 118.8±1.9 vs AD: 135.0±2.1 mmHg, $P=0.001$, unpaired $t$-test). The modeling of these MAP data by linear mixed models ($r^2=0.6791$) showed effects of pdMAP ($P=0.002$, chi-square test), denervation ($P=0.001$, chi-square test), diet ($P=0.001$, chi-square test), and a clear denervation*diet interaction ($P=0.008$, chi-square test).

**SSi, frequency of SSi subtypes, and hypertension in SHM and AD groups.** The SSi in each group fitted a normal distribution (Shapiro-Wilk test), and each rat was stratified to any of the three SSi subtypes described in humans (2). Given that the standard deviation of HNaMAP in the SHM group ($n=11$) was 6.30 mmHg, 95.4% of the sample values fell between 106.2 and 131.3 mmHg ($±2$ standard deviations). Accordingly, a cut-off value of ≥130 mmHg was chosen to indicate the presence of hypertension under our experimental conditions (e.g., substrain used, age of the rats, or use of the low-stress rat holder). Figure 2 shows the frequencies of SSi subtypes in both groups. In the SHM group, most of the rats (64%) showed either an SR or IND subtype, but, unexpectedly, 36% showed an SS subtype. In this group, 50% of SR rats decreased their MAP on the HNa diet (Figure 2, inverted arrows). In contrast, in the AD group only 60% of the rats showed an SS subtype and 67% were hypertensive ($>130$ mmHg). The hypertensive rats in this group represented 77% of the SS subtype rats, 50% of the IND subtype rats, and 50% of the SR subtype rats. Globally, 50% of the rats in the AD group developed cSSHT (SSi $>10$ mmHg plus HNaMAP $>130$ mmHg). Moreover, the HNa diet increased HNaMAP in all rats of the AD group, regardless of their SSi subtype. Finally, we found a modest yet significant association between SSi and HNaMAP ($r=0.7088$, $P=0.001$, Pearson).

**Total plasma nitrogen oxide concentration ([NO$_x$]p) on LNa and HNa diets.** Independent of the group, we found that [NO$_x$]p levels fell significantly (45%, $P<0.004$, paired $t$-test) from the LNa to HNa diet (Figure 3). Thus, in both groups, the TGSI value showed a statistically significant difference ($P=0.001$, unpaired $t$-test) between groups.

### Table 1. Oligonucleotide sequences for forward (F) and reverse (R) primers (5’-3’).

| Gene  | Sequence | Location of product (bp) | GenBank accession No. | Product length (bp) |
|-------|----------|--------------------------|-----------------------|---------------------|
| gp91phox | F 5’-AAAGGAGTGCACCCAGTACAAAGT-3’ | 762-784 | AF298656 | 78 |
|        | R 5’TACAGGAACATGGGACACCACATAT-3’ | 839-817 | | |
| p22phox | F 5’-AAGTACCATCCAGTGAGTCCAGG-3’ | 259-277 | U18729 | 68 |
|        | R 5’-TGAGGCCACCCGGGACCCGGG-3’ | 326-310 | | |
| IC-SOD | F 5’TGGTGCCAATGAGATCTGTTCTG-3’ | 384-406 | NM-017050 | 84 |
|        | R 5’TGGTGCCAATGAGATCTGTTCTGCTG-3’ | 467-447 | | |
| β-actin | F 5’TGGTGCCAATGAGATCTGTTCTGCTG-3’ | 926-951 | M-031144 | 285 |
|        | R 5’-CCAAAGGACCAATGAGATCTGTTCTGCTG-3’ | 1210-985 | | |

* gp91phox and p22phox are subunits of NAD(P)H oxidase; IC-SOD: intra-cytoplasmic superoxide dismutase.

### Table 2. Evolution of body weight in SHM and AD groups of series I.

| Group | pbw (g) | min bw (g) | Δbw (g) | RT (days) | 7 days LNa bw (g) | 21 days HNa bw (g) |
|-------|---------|------------|---------|-----------|------------------|-------------------|
| SHM ($n=11$) | 207 ± 1.9 | 196 ± 2.1 | 11 ± 1.0 | 3 ± 0.2 | 242 ± 4.6 | 348 ± 10.0 |
| AD ($n=15$) | 215 ± 2.3* | 186 ± 2.5* | 28 ± 1.7** | 6 ± 0.4** | 255 ± 6.0 | 337 ± 9.0 |

Data are reported as means±SE. pbw: predenervation body weight; min bw: minimum body weight reached by the rats after denervation; Δbw: change in body weight after denervation (pbw minus min bw); RT: recovery time (days after denervation for recovering its pbw); 7 days LNa bw: body weight after 7 days on low-sodium diet; 21 days HNa bw: body weight after 21 days on high-sodium diet; SHM: sham chronic aortic bilateral denervation group; AD: renal chronic aortic bilateral denervation group. *$P=0.02$, **$P=0.001$ vs SHM group (unpaired $t$-test).
groups, the difference in [NO\textsubscript{2}] between LNa and HNa diets (Δ[NO\textsubscript{2}]p) was positive and similar, indicating that the two groups had similar reductions in plasma NO bioavailability (positive ΔpNOB). The ΔpNOB fitted a normal distribution in both groups (Shapiro-Wilk test) but did not show any association with SSi (r=0.0031, P=0.99, Pearson) or HNaMAP (r=0.031, P=0.87, Pearson).

**Modeling of SSi and HNaMAP.** When TGSI was taken as an index of the extent of barodenervation (16) and the ΔpNOB was taken as an index of the change in sodium diet, the modeling of SSi (by multiple regression) indicated that ~60% of the variation in SSi (r\textsuperscript{2}=0.5730; ANOVA P=0.003) could be explained by the predictor variables TGSI (β=−4.357; P=0.003; power=92%), ΔpNOB (β=−0.2986; P=0.62; power=8%), and the TGSI*ΔpNOB interaction (β=1.6297; P=0.02; power=70%). In contrast, the reduction of this model (SSi) leaving only TGSI as the predictor variable, reduced the r\textsuperscript{2} to 0.2368 (ANOVA P=0.02; power 74%). Moreover, the modeling of HNaMAP (by multiple regression) indicated that ~99% of the variation in HNaMAP (r\textsuperscript{2}=0.9999; ANOVA P=0.001) could be explained by the predictor variables LNaMAP (β=1.000; P<0.001; power = 100%) and the SSi (β=1.000; P<0.001; power = 100%), with no interaction between them.

**Renal medullary mRNA expression of IC-SOD and NAD(P)H oxidase subunits.** After 21 days on a HNa diet (Figure 4), there were no differences between groups in the renal medullary mRNA expression of the two NAD(P)H oxidase subunits studied, and that of IC-SOD was 30% lower in the AD group than the SHM group, although the latter fell short of statistical significance (P=0.06, unpaired t-test).

**Series II**

**RMSAP at the end of the HNa diet.** Six of 8 rats in the SHM group and all 8 rats in the AD group fulfilled TGSI criteria. The behavior of TGSI, pMAP, LNaMAP, LNaMAP−pMAP, and HNaMAP in S-II was similar to that shown for those variables in S-I, except that the SSi difference between groups fell short of statistical significance, likely due to the elimination of two SHM experiments (SHM=7.4±2.8 vs AD=12.8±3.4 mmHg). At the end of the HNa diet, there was no difference in baseline RMSAP (~215,000 cpm) between the groups. The NADH-stimulated RMSAP in the SHM group with respect to its baseline RMSAP exhibited a near 4-fold increase (697,075±120,208 cpm/mg protein, P<0.006, paired t-test), whereas the AD group exhibited a corresponding near 9-fold increase (1,908,498±490,274 cpm/mg protein, P<0.004, paired t-test). NADH-stimulated RMSAP was 180% greater in the AD group than in the SHM group (P<0.03, unpaired t-test, right bars in Figure 5).

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**Table 3.** Pre- and postdenervation mean arterial pressure, MAP lability, salt-sensitivity index, and transformed general sensitivity index, in SHM and AD groups of series I.

| Group      | pdMAP (mmHg) | LNaMAP (mmHg) | HNaMAP (mmHg) | SD of MAP (mmHg) | SSi (mmHg) | TGSI (w/o units) |
|------------|--------------|---------------|---------------|------------------|------------|------------------|
| SHM (n=11) | 102.3 ± 2.4  | 112.8 ± 1.5\textsuperscript{+} | 118.8 ± 1.9\textsuperscript{+} | 2.5 ± 0.2 | 6.0 ± 1.9 | 10.9 ± 0.1 |
| AD (n=15)  | 99.3 ± 2.1  | 122.3 ± 1.7\textsuperscript{+b} | 135.0 ± 2.1\textsuperscript{+b} | 5.4 ± 0.4 | 12.7 ± 1.9 | 9.3 ± 0.2\textsuperscript{b} |

Data are reported as means±SE. MAP: mean arterial pressure; pdMAP: predenervation MAP; LNaMAP: postdenervation MAP after 7 days on low-sodium diet; LNaMAP−pMAP: LNaMAP minus pdMAP; HNaMAP: postdenervation MAP after 21 days on high-sodium diet; SD of MAP: standard deviation of MAP as index of MAP lability; SSi: salt-sensitivity index; TGSI: transformed general sensitivity index (adimensional, validated index of effectiveness of barodenervation); SHM: sham chronic aortic bilateral denervated group; AD: real chronic aortic bilateral denervated group; "P=0.001 vs pdMAP (paired t-test); "P=0.005, *P=0.001 vs LNaMAP (paired t-test); "P=0.03, "P=0.001 vs SHM group (unpaired t-test).

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**Figure 1.** Pressure-sodium diet relationship constructed from the postdenervation mean arterial pressure (MAP) measured after 7 days on the low-sodium diet (LNaMAP) and after 21 days on the high-sodium diet (HNaMAP) in each group of Series I. The salt-sensitivity index (SSi) for each group is indicated, as is the MAP threshold (gray dashed line = 130 mmHg) for hypertension (see Results). SHM: sham group; AD: aortic denervated group; Den: denervation. Data are reported as means±SE. \textsuperscript{+}P=0.005, \textsuperscript{+}P=0.001 vs LNaMAP (paired t-test); \textsuperscript{b}P=0.03, \textsuperscript{b}P=0.001 vs SHM group (unpaired t-test).
Series III

RMSAP at the end of the LNa diet. Six of 8 rats in the SHM group and all 8 rats in the AD group fulfilled TGSi criteria. The behavior of TGSi, pdMAP, LNaMAP, and LNaMAP-pdMAP in S-III was similar to that shown for those variables in S-I and S-II. The SSi was not calculated in this series because the experiments ended after 7 days on the LNa diet, and at that time, there was no difference between the groups in baseline RMSAP (∼270,000 cpm). In the SHM group, the NADH-stimulated RMSAP (296,397 ± 144,430 cpm/mg protein, not significant, paired t-test) was the same as its corresponding baseline RMSAP, whereas in the AD group this variable was different from its baseline RMSAP (887,520 ± 254,074 cpm/mg protein, P<0.02, paired t-test). The NADH-stimulated RMSAP was ∼200% greater (P<0.04, unpaired t-test, left bars in Figure 5) in the AD group than in the SHM group. Since the study was not primarily designed to evaluate differences in baseline or NADH-stimulated RMSAP between a HNa diet (S-II) and a LNa diet (S-III) in either the SHM or AD group, the interseries RMSAP data are not strictly comparable. This limitation notwithstanding, the NADH-stimulated RMSAP (Figure 5) was significantly greater (P<0.03, unpaired t-test) in S-II, after 7 days on the LNa diet plus 21 days on the HNa diet (right bars), than in S-III, after only 7 days on the LNa diet (left bars).

Discussion

The principal findings of this study performed in male Wistar rats bred from the ChR003 substrain were the following: 1) the SS subtype of the SSi was present in 36% of the rats in the SHM group and in 60% of the rats in the AD group; 2) independent of the group and SSi subtype, the change from a LNa to a HNa diet significantly reduced plasma NO bioavailability (positive ΔpNOB); 3) under a LNa diet, chronic bilateral aortic denervation induced a significant rightward shift of MAP; 4) the interaction between chronic bilateral aortic denervation
and the HNa diet induced a significant increase in SSi; 5) together, these last two events induced cSSHT in ~50% of the rats in the AD group; 6) under either a LNa diet or a HNa diet, aortic denervation increased renal medullary NADH-stimulated superoxide anion production, although apparently the increase was greater in extent under a HNa diet; 7) after 21 days on the HNa diet, renal medullary expression of mRNA of the IC-SOD and of the gp91phox, and p22phox NAD(P)H oxidase subunits was not different between groups.

To find a HNaMAP of 119±1.9 mmHg in 11- to 12-week-old rats in the SHM group (350 g body weight) after two periods of salt loading (postweaning and post-sham denervation), besides being similar to the MAP measured over 2 h by Franchini and Krieger (22) and Franchini et al. (23) in tethered, freely moving Wistar rats of 300 g (114±2 mmHg) and 250 g (118±4 mmHg) body weight, respectively, on a normal sodium diet, indicates that rats of the Wistar ChR003 substrain used in the present study do not carry a primary renal hypertensive genotype. Since rats are more resistant to salt than humans (24), a change of at least 10 mmHg in SSi was imposed as a criterion to classify rats of the SS subtype. Thus, through a longitudinal, inductive and diagnostic subprotocol, it was found that the SSi fitted a normal distribution, and the frequency of the subtypes of SSi (SR, IND, or SS) within each group was documented (Figure 2). Unexpectedly, in the SHM group, 36% of the rats were of the SS subtype, although all rats were normotensive. This demonstrates experimentally for the first time that the cSSNT (SSi > 10 mmHg plus MAP > 130 mmHg) phenotype exists. Also unexpectedly, in the AD group only 60% of the rats were of the SS subtype and 67% of the rats developed hypertension, such that only 50% of the rats showed cSSHT (SSi > 10 mmHg plus MAP > 130 mmHg). Although the sample size was not large (n=26 rats), these SS frequencies are quite similar to those described in normotensive (25%) and hypertensive (50%) human subpopulations (2), which suggests that the present model in this rat substrain might recreate the salt sensitivity of humans. These results also suggest that SS, as clinically defined (SSi > 10 mmHg), could be an epiphenomenon of hypertension. Whether the cSSNT phenotype exists in other outbred or inbred rat strains or substrains (e.g., Dahl SR rats) is unknown, but this could be determined by performing a subprotocol similar to the one described here.

To simulate the prehypertensive SS baroreceptor systemic phenotype of partial baroreceptor impairment (10,13), a chronic bilateral aortic denervation was performed instead of a chronic bilateral carotid sinus denervation, because the destruction of chemoreceptors during the carotid sinus denervation (22,23) in a species like the rat, which lacks functional aortic body chemoreceptors (25), causes chronic hypoventilation with consequent chronic...
SHM group

LNa diet (unpaired A.S. Rodrı́quez-Pérez et al. vs NADH-stimulated renal medullary superoxide anion production was not different from the maximal value of 15 mmHg observed in another group of rats evaluated 1 day after aortic denervation. However, no difference in absolute MAP (104 ± 2 mmHg) was reported by Van Vliet et al. (26) in telemetry data from freely moving, unstressed 6-week post-aortic denervated Long-Evans rats on a NNa diet when compared with a 6-week sham group (102 ± 1 mmHg). Similarly, previous unpublished longitudinal observations of our laboratory indicate that the MAP in conscious, aortic-denervated Wistar ChrR003 rats fed a NNa diet the first 10 postdenervation days and then a LNa diet for the following 21 days (116 ± 2.3 mmHg) was not different from the MAP in the same rats (n = 10) measured before aortic denervation (113.6 ± 1.8 mmHg). Although direct evidence from continuous longitudinal studies is lacking, all these studies strongly suggest that, in the conscious aortic-denervated rat, after an early and maximum nonhypertensive MAP increase, MAP decreases gradually toward its predenervation value, with this process being apparently faster with a LNa diet (at most 4 weeks) than with a NNa diet (at most 6 weeks). For both diets, after 15 days of aortic denervation, the MAP is shifted to the right. It must be noted that Kline et al. (27) reported in tethered Wistar rats on a NNa diet that chronic renal denervation reversed the increase in MAP observed 19 days after aortic denervation, although that study lacked aortic denervation criteria. This limitation notwithstanding, such a study suggests that the observed rightward shift of LNaMAP in our AD group might be related to a rise in either baseline efferent or baseline afferent renal sympathetic nerve activity (RSNA) or in both. Finally, the modeling of HNaMAP explained almost entirely its variation and included as predictors variables to not only LNaMAP, as expected, but also to SSi. Thus, HNaMAP in the AD group is a mixed phenomenon composed of the already discussed rightward shift of LNaMAP plus the amplification of SSi (vs the SHM group) induced by the interaction of both aortic denervation and the change from a LNa diet to a HNa diet. All of this explains why this group showed a greater rightward shift of MAP on the HNa diet than on the LNa diet (and, therefore, an SSi ~7 mmHg greater than the SHM group), with greater frequency of the SS subtype of SSI and cSSHT.

Comparison of this new model of cSSHT with the total arterial barodenervation model, comprised of sino-aortic denervation plus salt, which is the classical model of induced eSSHT, is almost inescapable. In 2-week post-sino-aortic denervated and telemeterized, freely moving Sprague-Dawley rats, Osborn and Hornfeldt (28) measured MAP while the dietary sodium regime was changed from a NNa diet for 1 week to a progressive HNa diet (first 4% and then 8%) during the next 6 weeks. Using the group’s MAP lability as the criterion of barodenervation, they found no between-group differences in MAP during the NNa diet. However, during the 4% and 8% HNa diets, MAP increased progressively more in the sino-aortic denervated group than in the sham group, so that,

systemic hypoxia, which in turn has the chronic effect of lowering blood pressure (23). In addition, the pdMAP was measured, and a validated individualized criterion of aortic barodenervation (TGS) (16) was used in each rat. The general modeling of MAP data by mixed models showed that MAP differences between groups could be explained by the pdMAP, the effect of partial arterial barodenervation, the transition from a LNa to a HNa diet, and the interaction between partial arterial barodenervation and diet.

We showed that the AD group, after about 13 postdenervation days (including 7 days on the LNa diet) had a greater rightward shift in both absolute MAP (LNaMAP) and relative MAP (LNaMAP–pdMAP) than the SHM group, all absolute values being within the normotensive range (<130 mmHg). The difference in absolute MAP (LNaMAP) between groups was ~10 mmHg, which was the same difference between groups reported by Franchini and Krieger (22) in tethered, freely moving, 20-day-post-aortic denervated Wistar rats on a normal sodium diet. Given that the modeling of LNaMAP data indicated that both the covariate pdMAP and group factor were their predictor variables, a number of factors might contribute to the observed absolute and relative changes in LNaMAP in our two groups: saline consumption from weaning to predenervation, the postdenervation recovery time, and the 7 days on the LNa diet. However, in the AD group it is also likely that chronic aortic denervation contributed significantly to the changes observed in LNaMAP.

The next question was: How durable is this LNaMAP rightward shift when AD rats consume a LNa diet? In the above-mentioned study by Franchini and Krieger (22), the ~10 mmHg MAP increase observed 20 days postdenervation was not different from the maximal value of 15 mmHg observed in another group of rats evaluated 1

Figure 5. NADH-stimulated renal medullary superoxide anion production in SHM and AD groups of Series III (after only 7 days on the LNa diet, left bars) and of Series II (after 7 days on the LNa plus 21 days on the HNa diet, right bars). SHM: sham group; AD: aortic denervated group; n: number of experiments. Data are reported as means ± SE. *P = 0.04, **P = 0.03 vs SHM group (unpaired t-tests); tP 0.03 vs LNa diet (unpaired t-test).
from 24-h averages, they found that at the end of the 8% 
HNa dietary regime, MAP had increased (vs the NNa diet) 
by 4 ± 1 mmHg in the sham group and 15 ± 2 mmHg in 
the sino-aortic denervated group (these values were not 
statistically compared in the article); the latter group 
achieved a maximal MAP lower than 130 mmHg. Even 
though Osborn and Hornfeldt (28) did not explicitly 
postulate the existence of an denervation−diet interaction, 
their data (HNaMAP−NNaMAP) strongly support its 
existence even under the very unusual stress-free 
conditions. The authors concluded that arterial baro-
receptors play a major role in long-term control of MAP 
under the conditions of a HNa diet. The present study 
performed in rats under low-stress conditions (a more 
common condition) also demonstrates the existence of 
such an interaction, but in a model including partial 
baroreceptor impairment, a more common form of 
baroreceptor impairment than total sino-aortic denerva-
tion, which suggests that the interaction is independent of 
the stress conditions. On the other hand, in contrast to 
aortic denervation, sino-aortic denervation, as usually 
performed, also destroys carotid chemoreceptors and 
results in hypoventilation, which by itself induces chronic 
hypoxia with its consequent chronic lowering of blood 
pressure (22,23). This last fact alone could explain why 
Osborn and Hornfeldt (28) did not find differences 
between groups in MAP on an NNa diet and why the 
 maximal sino-aortic denervation group’s MAP levels did 
not exceed 130 mmHg. In other words, our AD model 
likely allowed us to observe the LNaMAP differences 
by avoiding the confounding hypotensive effects of sino-
aortic denervation-induced hypoxia. Alternatively, these 
latest changes in our AD model might be, in addition, the 
result of both the interaction between AD and the use of a 
low-stress rat holder (LNaMAP differences) and the 
interactions among AD, the use of a low-stress rat holder, 
and a HNa diet (HNaMAP differences). Identifying which 
is the case is a task left to those research groups with 
available telemetric systems.

In the present study, the HNa diet reduced pNOB 
compared to the LNa diet (positive ΔpNOB). This pattern 
was the opposite of that reported by Shultz and Tolins (29) 
negative ΔNOB) in intact Sprague-Dawley rats. Thus, 
there seem to be different HNa diet−responsive endothelial 
systemic genotypes among rat strains, with our rat 
substrain showing some degree of endothelial salt sensi-
tivity (positive ΔpNOB), although this phenotype would not 
be exclusively extrarenal because kidneys also have blood 
vessels. Moreover, the SHM and AD groups had the same 
positive ΔpNOB (~45%) pattern, indicating that this change 
was not under aortic baroreflex control. Although the 
present work was not designed to study the mechanisms 
of a positive ΔpNOB, it is possible that this may be mediated 
by salt-induced endogenous inhibition of endothelial nitric 
oxide synthase and/or by increased availability of vascular 
superoxide due to increased production and/or to reduced 
degradation (30). Furthermore, because both ΔpNOB and 
SSi were affected by the dietary change, SSi was 
remodeled in terms of ΔpNOB. Interestingly, this modeling 
indicated that ~60% of its variation could be better 
explained by TGSI (which per se was negative and 
significantly associated with SSi), by the change in diet 
judged by ΔpNOB (which per se was not associated with 
SSi), and by the interaction between both (which per se was 
positive and significantly associated with SSi). These 
associations suggest that a positive ΔpNOB in response to 
a HNa diet may be a necessary, but not sufficient factor 
for the expression of SSi. Moreover, the lack of association 
between ΔpNOB and SSi, coupled with the fact that only 
36% and 60% of the rats in the SHM and AD groups, 
respectively, showed an SS subtype, indicates that a 
positive ΔpNOB is not synonymous with the specific 
existence of the SS subtype. Furthermore, we found no 
association between ΔpNOB and HNaMAP, notwithstanding 
the significant (although smaller than expected) 
association between SSi and HNaMAP (r = 0.7089). Even 
though, experimentally, Bayorh et al. (11) documented that 
a positive ΔpNOB was found in Dahl SS (hypertensive) rats 
but not in Dahl SR (normotensive) rats, such results are 
difficult to interpret, because these authors did not evaluate 
the SSi, as we have done here. Therefore, it is unknown 
whether all Dahl SS rats have a clinical SS subtype or 
whether all Dahl SR rats have a clinical SR subtype. 
Evidently, the criterion used to define Dahl rats as SS or SR 
was based on whether or not the rats consistently 
developed hypertension on a HNa diet; however, our data 
show that hypertension on a HNa diet can coexist without a 
clinical SS subtype. In contrast, our results are consistent 
with clinical studies of Campese et al. (31) and Bragulat et 
al. (32), who showed a similar and positive ΔpNOB in SS 
and SR hypertensive Afro-American and Spanish patients, 
respectively, all of whom were evaluated for SSi. However, 
our results are not consistent with the clinical results of 
Fang et al. (33), who found a positive ΔpNOB only in SS 
but not in SR normotensive Chinese individuals, all of 
whom were evaluated for SSi. We do not currently have 
an explanation for these differences, although racial differ-
cences could play a role. What is clear is that a thorough 
investigation of SSi - at both experimental and clinical 
levels, in normotensive and hypertensive subjects of 
diverse strains, origins, lineages, and races - and its 
association with pNOB are mandatory.

Given that the renal medulla is critical for regulating 
blood pressure (34), and knowing the role played by free 
radicals in modulating both renal medullary blood flow 
(RMBF) and tubular functions (35), we first explored the 
effects of sequential exposure to a LNa (0.04% Na, for 7 
days) diet followed by a HNa (8% Na, for 21 days) diet 
regime (S-II) in our model, and then the effects of isolated 
exposure to a LNa (0.04% Na) diet for 7 days (S-III) on 
RMSAP. We found a greater increase in NADH-stimulated
RMSAP in the AD group than in the SHM group in both the LNa diet (S-III) and the HNa diet (S-I), although apparently the difference was greater during the HNa diet than the LNa diet. This contrasts with a recent study by Johns et al. (36), who found no difference in NADH-stimulated RMSAP in sham Wistar rats subjected to normal (0.3%) and high (3%) sodium diets. Thus, the difference in findings in the sham rats evaluated in these studies could be explained by the range of diets used (7.97 vs 2.7%, respectively). Moreover, our findings strongly suggest that NADH-stimulated RMSAP might be under the inhibitory control of aortic baroreceptors, suggesting again that this response could be mediated by an increase in baseline efferent RSNA. Our findings also suggest that this local RMSAP is increased by a HNa diet per se, and even more so when aortic denervation and a HNa diet are conjoined. This scenario, then, might constitute the functional background of the denervation*diet interaction indicated by the analysis. In that sense, Mattsson et al. (37) reported that reductions in RMBF are capable of inducing chronic sodium retention and chronic increase of MAP in rats. Years later, Zou et al. (38) demonstrated in rats that renal medullary superoxide anions exert tonic renal medullary vasoconstrictive and antinatriuretic actions. Moreover, Eppel et al. (39) reported that, in anesthetized rabbits, stimulation of the renal nerves decreases RMBF in a frequency-dependent manner, and that the systemic inhibition of NO by L-NG-nitroarginine (represented in the present study by a positive $p_{NOB}$) reduced baseline RMBF and revealed the RMBF-reducing effect of low-frequency renal nerve stimulation. It is possible, therefore, that aortic denervation, by increasing baseline efferent RSNA, might directly reduce RMBF - especially if there is concurrently a positive $\Delta p_{NOB}$ - but also indirectly by raising RMSAP, and even more so under a HNa diet.

Whatever the mechanism involved in the differential rise of RMSAP in the AD group not seem to affect, in S-I (Figure 4), the renal medullary mRNA expression of NAD(P)H oxidase subunits gp91phox or p22phox, although it tended to decrease the mRNA expression of IC-SOD in this group, suggesting that this mechanism first affects the mRNA expression of antioxidant enzymes and that it does not use AngII-dependent mRNA expression of the p22phox NAD(P)H oxidase subunit pathway for that purpose (40). However, we cannot rule out the possibility that such a mechanism could affect, at the translational or posttranslational level, the activities of these enzymes.

In summary, in a process of reverse translation, the present study systematized in male Wistar CHR003 rats a longitudinal, inductive and diagnostic subprotocol (7 days on a LNa diet plus 21 days on a HNa diet) for obtaining to an experimental level the SSI, which is the most widely used clinical SS diagnostic tool. All SSI subtypes were found in both the SHM group (normotensive) and the AD group (mostly hypertensive), and the condition of cSSNT has been described for the first time in the intact rat, raising the possibility that SS is an epiphenomenon of hypertension. On the other hand, independently of aortic denervation status, the rats showed a 45% reduction in plasma NO bioavailability (positive $\Delta p_{NOB}$) when they changed from a LNa diet to a HNa diet, which was interpreted as indicating that this rat substrain shows some degree of endothelial salt sensitivity, and suggests that this sensitivity may be one of the constitutive elements of the SS systemic phenotype (the endothelial element). Moreover, the analysis indicated that, in primarily normotensive rats without a renal hypertensive genotype but under low-stress conditions (a more common condition than no-stress and induced here by using a rat holder), aortic denervation increased LNaMAP, but the interaction of aortic denervation with a HNa diet increased the likelihood of developing cSSHT, which supports the idea that partial baroreceptor impairment is one of the constitutive elements of the SS systemic phenotype (the baroreceptor element). Furthermore, it is reported for the first time that, while aortic denervation and a HNa diet each alone increased NADH-stimulated RMSAP, together they might cause an even higher increase in RMSAP. The main translational implication of these findings is that they help to explain why subjects with cSSNT may transition to cSSHT.

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