High Salt Up-Regulates Ca\textsuperscript{2+}-Sensing Receptor Expression and Ca\textsuperscript{2+}-Induced Relaxation of Contracted Mesenteric Arteries from Dahl Salt-Sensitive Rats

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Running Title: High Salt Diet Up-regulates CaSR Expression and Signaling.

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Abbreviations: BK, Ca\(^{2+}\)-activated big potassium (K\(^{+}\)) channel; CaSR, Ca\(^{2+}\)-sensing receptor; eNOS, endothelial Nitric Oxide (NO) Synthase; [Ca\(^{2+}\)]\(_e\), extracellular Ca\(^{2+}\) concentration; IBTx, iberiotoxin; L-NAME, N\(^{G}\)-Nitro-L-arginine methylester; 7-NI, 7-Nitroindazole; MAP, mean arterial pressure; PE, phenylephrine; PSS, physiological salt solution; SR, salt-resistant; SS, salt-sensitive.
Abstract

High Ca\textsuperscript{2+} lowers BP in hypertension but the mechanism is not clear. The missing link may be the perivascular sensory nerve CaSR that mediates a vasodilator system following activation by interstitial Ca\textsuperscript{2+}. Our results show that high salt increased CaSR expression in mesenteric arteries as well as Ca\textsuperscript{2+} relaxation of contracted mesenteric arteries from salt-sensitive (SS) rats. The CaSR was expressed as a doublet (≈ 120 - 150 kDa) in arteries from animals fed a high salt diet for 1-4 weeks. The higher molecular weight, glycosylated protein increased in arteries from SS animals, however, expression of the low molecular weight, high mannose protein decreased over four weeks of feeding the diet. In tissues from salt-resistant (SR) rats, the diet decreased CaSR expression after 4 weeks. Ca\textsuperscript{2+} relaxation of mesenteric arteries, under phenylephrine tone, increased in SS rats but decreased in arteries from SR fed the high salt diet. BK channels have a major role in Ca\textsuperscript{2+} relaxation of arteries in SR than SS rats. The data suggest that high salt epigenetically regulates the receptor at the translational level \textit{in vivo}, and the in vitro effect of Ca\textsuperscript{2+} is on receptor trafficking and signaling. In conclusion, up-regulated expression of the CaSR in salt sensitivity increased receptor-mediated vascular relaxation. These findings show that CaSR signaling may compensate for changes in the vasculature in salt-sensitive hypertension.
Significance Statement

The perivascular sensory nerve Ca\textsuperscript{2+}-sensing receptor mediates Ca\textsuperscript{2+} relaxation of isolated mesenteric arteries under tension. This receptor may, therefore, play a significant role in relaxation of resistance arteries tone \textit{in vivo} thus, explaining the BP lowering effect of dietary Ca\textsuperscript{2+}. The present studies describe the effect of high salt-induced upregulation of the CaSR in SS rats and the roles played by BK channels and nitric oxide in Ca\textsuperscript{2+}-responses.
INTRODUCTION

The CaSR provides the primary pathway for Ca\(^{2+}\)-mediated regulation of the release of parathyroid hormone (Brown and MacLeod, 2001). However, the CaSR is also involved in the fine tuning of serum Ca\(^{2+}\) levels and Ca\(^{2+}\) excretion (Kos et al., 2003). The mechanisms underlying these latter effects are not clear, therefore, the role of the CaSR in other tissues needs to be characterized. Studies on the role of the receptor in cardiovascular function, particularly in salt-sensitive hypertension is lacking. We previously showed that eNOS knockout up-regulates expression of the CaSR in mesenteric arteries in mice (Awumey et al., 2013). Human and animal studies indicate that high calcium lowers BP in hypertension (Hatton and McCarron, 1994), and high calcium (1.5 g/day) intake supplemented with fruits, vegetables and low fat milk lowered BP in hypertension compared with fruits and vegetables only diets (Appel et al., 1997; Akita et al., 2003; Appel et al., 2006). Findings by Bukoski et al. (Bukoski, 2001; Bukoski et al., 2001) support the notion that dietary calcium lowers BP by increasing interstitial fluid Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{ISF}\)) and activation of CaSR signaling in perivascular sensory nerves. Increased [Ca\(^{2+}\)]\(_{ISF}\) up-regulates CaSR signaling in mesenteric arteries resulting in activation of the cannabinoid system and vascular relaxation. A previous study from this laboratory (Awumey et al., 2008) led us to propose that activation of the CaSR ultimately activates release of arachidonic acid (AA) and the breakdown of diacylglycerol to 2-arachidoglycerol (2-AG), an endocannabinoid. Both AA and 2-AG are metabolized by cytochrome P450 (CYP) to vasodilators such as epoxyeicosatrienoic acid (EET) and glycerated epoxyeicosatrienoic acid (GEET), respectively. These metabolites stimulate smooth
muscle Ca\(^{2+}\)-activated K\(^+\) (BK) channels and hyperpolarize the cell leading to relaxation. CYP2J2, a CYP epoxygenase isoform, catalyzes the oxidative metabolism of AA to EETs (Zeldin, 2001). Renal expression of the enzyme and EET biosynthesis are reduced in animal models of hypertension (Capdevila et al., 2007). We have also shown that synthetic 2-AG and GEETs induce relaxation of isolated, and pre-contracted mesenteric arteries from Wistar rats (Awumey et al., 2008).

Hypertension is a leading cause of vascular and renal morbidity that leads to mortality world-wide, and the incidence of the disease is high in the US. Salt-sensitive hypertension is particularly widespread in the aging population and more prevalent in the US population of African descent than whites because of the tendency of the former group to retain sodium in their kidneys (Lindhorst et al., 2007). The disease is associated with vasoconstriction, endothelial as well as renal dysfunction and treatment is difficult because the mechanisms are not fully understood. Understanding the mechanism of CaSR signaling in normal physiology and hypertension is, therefore, essential to evaluating its potential as a therapeutic target. Studies by our group have shown that a high salt diet reduced renal [Ca\(^{2+}\)]\(_{ISF}\) and increase SBP in SS rats (Palmer et al., 2003; Eley et al., 2008), and the effects of dietary Ca\(^{2+}\) in human studies show that diets high in fruits and vegetables, supplemented with low fat milk, reduce BP (Appel et al., 1997; Sacks et al., 1999; Conlin et al., 2000; Obarzanek et al., 2001; Sacks et al., 2001; Vollmer et al., 2001a; Vollmer et al., 2001b; Akita et al., 2003; Nowson et al., 2004; Nowson et al., 2005; Appel et al., 2006). However, there is no consensus on the BP-lowering effect of dietary Ca\(^{2+}\), but studies using animal models of hypertension have shown that high Ca\(^{2+}\) intake lowers resistance artery tone and BP.
(Kageyama and Bravo, 1987; Hatton et al., 1989; Muntzel et al., 1989; Hatton and McCarron, 1994; Appel et al., 1997; Sacks et al., 1999; Akita et al., 2003; Nowson et al., 2004; Nowson et al., 2005; Appel et al., 2006; Koobi et al., 2006). In the present study, we used the well-established model of low renin, salt-sensitive hypertensive Dahl rats to evaluate the hypothesis that high salt modulates CaSR expression and vascular signaling in salt-sensitivity.
MATERIALS AND METHODS

Male salt-resistant (SR) and SS rats (8 weeks old) were obtained from Envigo RMS, Inc. (Indianapolis, IN). An 8% NaCl diet (TD.92012) was from Envigo Tekland Diets (Madison, WI). Tween-20 solution (10%), Sodium Dodecyl Sulfate (SDS; 10%), 30% normal acrylamide, Tris–HCl Buffer (1.5 M, pH 8.8, Tris–HCl 0.5 M, pH 6.8), 10% Ammonium persulfate, Immuno-Blot PVDF membranes and TEMED (N,N,N,N-Tetramethylene-diamine) were bought from Bio-Rad Laboratories, Inc. (Hercules, CA). Tris-Glycine (with 0.1% SDS), Tris-Glycine Buffer (TG 10X), Tris-Buffered Saline (TBS, 10X; pH 7.4), polyclonal CaSR antibody (PA1-37213, raised against a synthetic peptide corresponding to the N-terminus of the rat CaSR) and methanol were from ThermoFisher Scientific, Inc. (Pittsburgh, PA). Electrophoresis sample buffer (2X), and rabbit polyclonal GAPDH (FL-335) (sc-25778, raised against amino acids 1-335, being the full length GAPDH) of human origin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Iberiotoxin (IBTx), a BK channel inhibitor was from Sigma (St. Louis, MO). All other chemicals used in the present studies were of the purest grades commercially available.

Animals and BP Analysis

The protocol for the use of SR and SS rats was approved by the Institutional Animal Care and Use Committee of North Carolina Central University. Animals were kept in the Animal Research Center at our Institute for one week to allow them to acclimatize before use. Some animals were used for baseline determinations of CaSR expression and Ca$^{2+}$ relaxation studies before initiation of diet treatment. The remaining study
animals were randomly divided into 3 groups each as follows: Group 1 (8% NaCl for 1 week); Group 2 (8% NaCl for 2 weeks); Group 3 (8% NaCl for 4 weeks). All animals were allowed free access to food and water. Pulses (mV) and systolic blood pressures, SBP (mm Hg) were determined weekly for 4 weeks by an indirect computerized (PowerLab), non-invasive BP (NIBPS) tail-cuff method (ADInstruments, CO). Animals were trained, habituated to the protocol and carefully handled to give reproducible data. The cuff is positioned proximally at the end of the tail to occlude blood flow that is measurable in the caudal artery.

**Extraction of Proteins and Analysis by Western Blotting**

Mesenteric arteries were isolated from animals under isoflurane anesthesia for protein extraction and wire myography, as previously described (Awumey et al., 2013; Odutola et al., 2019). Briefly, total proteins were extracted from tissues by homogenization in tris-HCl (pH. 5) buffer and centrifuged. Proteins were separated on 8% SDS-PAGE gels and transferred onto PVDF membranes for blotting with non-fat milk followed by incubation with polyclonal CaSR antibodies overnight. Protein bands were visualized using the chemiluminescence (ECL™ method). GAPDH was used as the loading control and protein bands were then analyzed by densitometry.

**Mesenteric Artery Isolation and Ca^{2+} Relaxation Studies**

Mesenteric arteries (≈ 200-300 µm internal diameter), isolated from anesthetized animals (at baseline, and on diets for 2 or 4 weeks), were analyzed for Ca^{2+}-induced relaxation of PE-contracted artery segments by wire myography as previously described (Awumey et al., 2008; Awumey et al., 2013; Odutola et al., 2019). Briefly, dissected mesenteric artery segments (2 mm long) were mounted in a small vessel Mulvany-
Halpern 510A Auto Dual Wire Myograph and maintained in PSS with 100 µM ascorbic acid (antioxidant) and aerated with a mixture of 95% air/5% CO₂ at 37 °C for 30 min. Arteries were stretched automatically and “normalized” using parameters defined for the myograph (Target trans-mural pressure = 13.3 kPa (100 mm Hg); Time = 60 sec; Normalization Factor (IC₁/IC₁₀₀ Ratio) = 0.9) (Angus and Wright, 2000; Lay et al., 2000; Wright and Angus, 2000). Mounted vessels were then challenged with 5 µM phenylephrine (PE) to obtain reproducible contractions. Active force (AF) development of ≥ 10 mN in arteries were considered optimum for the experiments to proceed. Ca²⁺ was added, cumulatively to vessels pre-contracted to ≈ 90% of maximal tone. When IBTx, L-NAME and 7-NI were used, tissues were pre-incubated with the compounds in the myograph chamber for 10 min and were present during relaxation assays. All studies were conducted with endothelium-replete mesenteric arteries.

**Statistical Analysis**

Data analysis was carried out by One-Way Repeated Measures ANOVA using the SigmaPlot 14.0 computer software program from SYSTAT (Port Richmond, CA). Values plotted are means (± SE). Differences between groups were determined by multiple comparisons using the Holm-Sidak method and were considered significant at p < 0.05. Statistically significant results were obtained with 4-6 animals, based on power analysis of estimates of the expected variance. Power of performed test with α = 0.0500:1.000.
RESULTS

Our data show that an 8% NaCl diet increased SBPs in SS rats from week 1-4 as expected but in SR rats, increased BPs were recorded only in weeks 3 and 4. The high salt diet also upregulated CaSR levels in mesenteric arteries from SS animals. Feeding of the high salt diet to SR for 4 weeks reduced CaSR expression. Determination of Ca\textsuperscript{2+}-induced relaxation of isolated, PE-contracted mesenteric arteries showed that the high salt diet increased relaxation of vessels from SS but decreased it in those from SR rats. BK inhibition reduced Ca\textsuperscript{2+} relaxation of PE-contracted mesenteric arteries from SR and SS fed the low salt diet. The detailed results are presented below.

**Effect of an 8% NaCl Diet on BP in SR and SS rats**

Fig. 1 shows body weights of animals at baseline and after 4 weeks on the high salt diet. Body weights increased in SR and SS rats, compared to baselines, but the increases were much higher in SS. Fig. 2 shows the effect of the high salt diet on SBP in SS animals. The caudal artery pulses and cuff pressure recordings in SS rats on the high salt diet for 1 and 2 weeks were determined by a non-invasive BP measurement by the tail-cuff method. Rapid pulses were recorded in SS rats on high salt diet during cuff inflation compared to baseline (i.e., before the start of diet treatment). Fig. 3 shows analysis of SBPs in SR and SS animals over the 4-week feeding period. SBPs significantly increased \((p < 0.05)\) in SS rats fed the high salt diet for 2-4 weeks compared to SR rats on the same diet. In SR rats, BPs were significantly higher \((p < 0.05)\) after 4 weeks on the high salt diet.
Western Blot Analysis of the Effect of an 8% NaCl Diet on CaSR Levels in Mesenteric Arteries from SR and SS rats.

Protein extracts from rats, fed the high salt diet, were analyzed by Western blotting to determine the effect of high salt on expression of the CaSR in mesenteric arteries. Fig. 4 shows that expression of the CaSR doublet (≈ 120 and 150 kDa) was significantly increased ($p < 0.05$; ANOVA) in mesenteric arteries from SS rats fed the high salt diet compared to SR rats on the same diet. The bar charts are densitometry analysis of the expression of the mature CaSR from SR and SS rats fed the high salt diet for 1-4 weeks. The bar charts show densitometry analysis of the expression of the immature and mature CaSR. There was no difference in the expression of the mature receptor in SR animals on the high salt diet for 1-2 weeks compared to baseline, but the level decreased significantly ($p < 0.05$) after 4 weeks. Expression of the immature receptor increased from baseline to 2 weeks but not detected after 4 weeks. In SS, the high salt diet decreased expression of the immature, low molecular weight (≈ 120 kDa) protein in mesenteric arteries from over the 4-week feeding period. In SS, the high salt significantly increased ($p < 0.05$; ANOVA) expression of the 150 kDa CaSR protein in mesenteric.

Effect of an 8% NaCl diet on “Normalized” Tension, Phenylephrine Tension and $Ca^{2+}$-induced Relaxation of Mesenteric Arteries from SR and SS rats.

Fig. 5 shows “Normalizations” and tensions in mounted artery segments from animals, fed the control or high salt diets for 4 weeks. Artery segments were mounted in a wire myograph chamber and stretched automatically to achieve a lumen diameter that approximates the vessel diameter in vivo. During mechanical studies of isolated
vessels, setting consistent baseline condition is necessary to obtain reproducible results. “Normalization” is the process of standardizing the baseline experimental conditions for vessel function measurements assessed by myography. The slope of “Normalization” curves for SR on control and high salt diets were different but the resting tensions (RTs) were similar. The difference between the mean RTs in arteries were not significant Fig. 6 shows normalized tensions and tensions developed in mounted artery segments in response to 5 µM PE before applications of Ca\(^{2+}\). PE tensions increased in SS arteries in weeks 2 and 4 but decreased in arteries from SR after 4 weeks on the high salt diet. Tension changes in tissues from SR and SS animals after PE applications followed by relaxations due to cumulative additions of Ca\(^{2+}\) are shown in Fig. 7, and Fig. 8. The high salt diet reduced Ca\(^{2+}\) relaxation in arteries from SR but did not change the responses in tissues from SS. The [Ca\(^{2+}\)]e-response curves in arteries from SR and SS animals are shown in Fig. 9. Relaxation of arteries from SS animals on the high salt diet for 2-4 weeks were shifted to the left compared to those from SR. EC\(_{50}\) values for Ca\(^{2+}\) relaxations were extrapolated fitted data. The high salt diet treatment significantly decreased the EC\(_{50}\) value (Insets). Fig. 10 shows “normalization” and force tracings in an artery segment from an SS rat on control diet. The effect of the BK channel inhibitor, IBTx on Ca\(^{2+}\)-induced relaxation is shown. Fig. 11 shows that inhibition of BK channels increased the EC\(_{50}\) values for Ca\(^{2+}\) relaxation of PE-contracted arteries from both SR and SS rats on the control diet. The effect of IBTx in arteries from SR was much more pronounced than those from SS. The Ca\(^{2+}\)-induced relaxation curves of PE-contracted arteries were shifted to the right by IBTx.
**DISCUSSION**

CaSR-mediated relaxation of isolated, phenylephrine-contracted mesenteric arteries (Bukoski et al., 1997; Wang and Bukoski, 1998) may play a role in blood pressure regulation (Bukoski, 1998). Earlier studies from our laboratory show that Ca\(^{2+}\)-activated K\(^+\) (BK) channels and cytochrome P450 metabolites of 2-AG play a role in this process (Awumey et al., 2008). The current data revealed novel findings showing up-regulation of CaSR expression and CaSR-mediated vascular relaxation in SS hypertension and suggest a compensatory mechanism to counteract the effect of high salt. High salt increased systolic blood pressure in SS rats, as expected, using the tail-cuff method, which gives an estimate of the SBP (within 5% of direct BP) derived from the caudal artery pressure. The data show that high salt diet affects both pulse and SBP.

The detection of the CaSR doublet in mesenteric arteries from SR and SS rats may be due to differential expression of the immature and mature forms of the receptor (Bai et al., 1996; Pearce et al., 1996; Bai et al., 1998; Wang et al., 2003; Bouschet et al., 2005; Awumey et al., 2007; Cavanaugh et al., 2010; Stepanchick and Breitwieser, 2010; Awumey et al., 2013; Grant et al., 2015). Western analysis of CaSR expression produces three different protein bands between 100 and 160 kDa that represent different monomeric forms of the receptor (Bai et al., 1996). The low molecular weight band of 120 kDa (with high mannose content) is the non-glycosylated form of the receptor that is expressed at a lower level than the other forms and is known to be restricted to the endoplasmic reticulum (Huang and Miller, 2007). The high molecular weight (150 kDa) band is the mature, glycosylated form of the receptor that is
expressed on the cell surface (Bai et al., 1996). The extracellular domain of the CaSR has several glycosylation sites (Brown and MacLeod, 2001). The current data suggest that the high salt diet increased the post-translational modification of the receptor. An interesting new finding in mesenteric arteries is the observation that expression of the mature CaSR increased in tissues from SS animals on the high salt diet whilst the levels of the immature receptors decreased. Presumably, the high salt diet up-regulated maturation of the receptor to counteract the effect of the high salt. Our earlier studies show that endothelium-denudation only reduced Ca$^{2+}$ responses by about 20% in mesenteric arteries from Wistar rats indicating that Ca$^{2+}$ relaxation is largely independent of the endothelium (Awumey et al., 2008). In mice, eNOS knockout increased CaSR expression in mesenteric arteries, and NOS inhibition reduced Ca$^{2+}$ response maxima marginally, without affecting the EC$_{50}$, compared to control C57 BL/6 and nNOS$^{-/-}$ mice (Awumey et al., 2013). BK channel inhibition reduced the Ca$^{2+}$ relaxation responses in mesenteric arteries from mice and rats, showing a significant involvement of these channels. Thus, in two animal models of hypertension- eNOS$^{-/-}$ and SS rats fed a high salt diet, CaSR expression is up-regulated suggesting a physiological role for the receptor in these conditions. Common variations in the CaSR gene are associated with BP in young African Americans (Jung et al., 2009) suggesting a role for the receptor in BP regulation. Increased expression of the mature form of the CaSR may suggest that there should be a reduction in BP, but the present findings show no reduction in BP in salt-sensitive animals on the high salt diet. This may be because Ca$^{2+}$ relaxation of mesenteric arteries occurs only after vessel contraction and contribution from this may not be enough to overcome the increased pressure due to
the diet. Phenylephrine has an indirect effect on endothelium-derived vasodilators in small mesenteric arteries (Dora et al., 2000; Liu et al., 2006). Thus, the CaSR signaling pathway that leads to relaxation is activated under conditions of increased vascular tone and may serve only a compensatory role. The effect of increased expression of the receptor on BP may, therefore, be proven only after in vivo activation of the receptor or by using animals in which the receptor has been knocked out. It is also possible that the role of the receptor may be more important in other tissues, such as kidneys, which play a much larger role in BP regulation. We will explore these possibilities in future studies to show any linkage between increased receptor expression and BP changes.

During the mechanical study of isolated vessels, the initial passive conditions at which vessels are set presents a major source of error, therefore consistency from sample to sample is needed. “Normalization” of mounted vessel segments standardizes baseline conditions for direct comparisons and optimizes vessel responses since sensitivity to agonists and active responses are dependent on stretch. The data suggest that high salt induced an increase in passive stiffness but reduced active force development and Ca\(^{2+}\) relaxation in arteries from salt-resistant rats. This corresponds to reduced expression of the CaSR in mesenteric arteries after 4 weeks on the high salt diet. Active forces increased in arteries from salt-sensitive rats on the high salt diet for 2-4 weeks and decreased in tissues from SR after 4 weeks showing an effect of the high salt on the in vivo translation of the CaSR and possibly its downstream signaling complements, thus affecting the contractile mechanism. The high salt diet did not change relaxation responses to Ca\(^{2+}\) in arteries from SS rats, but responses were reduced in arteries from salt-resistant rats showing normal active force development in
SS and Ca$^{2+}$ relaxation in salt-sensitivity. The exposure of arteries to Ca$^{2+}$ was carried out *in vitro*, after mounting of segments in myograph chambers and contracting them with phenylephrine, therefore the effect is on receptor trafficking and signaling.

The effect of IBTx on Ca$^{2+}$ relaxation responses indicates the involvement of BK channels as we observed in earlier studies (Awumey *et al.*, 2008; Awumey *et al.*, 2013). Using Wistar rats, we consistently demonstrated that CaSR signaling, and relaxation of mesenteric arteries involves the activation of BK channels, and this is confirmed in arteries from Dahl salt rats. Apparently, CaSR upregulation also increase BK-mediated vasodilation. Our group has shown, in a previous study, that tretraethylammonium and iberiotoxin (BK inhibitors) blocked Ca$^{2+}$ relaxation of isolated, PE-contracted rat mesenteric arteries but apamin and charybdotoxin (small K$_{Ca}$ inhibitor), glinbenclamide (K$_{ATP}$ inhibitor) and 4-aminopyridine (voltage-dependent K$^{+}$ channel inhibitor) had no effect ((Ishioka and Bukoski, 1999). BK channels are expressed in vascular smooth muscle cells and regulate contractile function in hypertension (Cox, 2002; Goto *et al.*, 2012; Zhang *et al.*, 2018). In the presence of Ca$^{2+}$, inhibitors of BK channels produce spontaneous contractions with rhythmic oscillations in basilar arteries from spontaneous hypertensive rats (Kamouchi *et al.*, 2002). Thus, BK channels may play an important role in regulating the resting tone because removal Ca$^{2+}$ abolished this rhythmic contraction. In the present studies arteries were pre-contracted with phenylephrine, therefore, this pathway may not be significant. Our data also suggest that, in SR rats, reduced Ca$^{2+}$ sensitivity may be due to direct alteration of CaSR expression or regulation, reduction in signaling efficacy and/or expression of downstream effectors required for contraction. Expression of CaSR after 4 weeks on the high salt diet could
be due to downregulation of mRNA and/or translation, and Ca\textsuperscript{2+}-induced clearance of existing receptors. However, immature CaSR levels appear to increase over the first two weeks and decreased after the 4 weeks suggesting mRNA downregulation. We have developed CaSR knockouts on the SS (Odutola et al., 2019) and SR backgrounds to explore these mechanisms in future studies. In the present study, inhibition of NOS by L-NAME enhanced Ca\textsuperscript{2+\textsubscript{e}} relaxation of mesenteric arteries from SS rats, but the nNOS selective 7-NI had no effect in SR and SS. The data show that eNOS-derived NO inhibits CaSR signaling in mesenteric arteries from SS rats and presents a new dynamic in CaSR-mediated vascular relaxation that requires further studies. Our findings are novel and suggest that up-regulation of CaSR expression in mesenteric arteries, and CaSR-mediated vasodilation in hypertension serve a compensatory role to counteract the effects of high salt.

In conclusion, salt sensitivity is a principal factor in the development of hypertension and our current findings show that CaSR signaling constitute a novel and important physiological pathway that may be a target for managing the disease.
Authorship Contributions

Participated in Research Design: Awumey.

Conducted Experiments: Williams, Bridges.

Performed Data analysis: Bridges, Awumey.

Wrote or contributed to the writing of manuscript: Awumey.
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**Footnotes**

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LEGENDS FOR FIGURES

Figure 1. Effect of an 8% NaCl diet on body weights of SR and SS rats. Body weights of animals fed the high salt diet for 4 weeks. Data plotted are means (± SEM) of determinations in 5 animals as shown. *Significantly different from baseline values; #Significantly different from SR.

Figure 2. Effect of an 8% NaCl diet on BP in SS rats. Animals were fed the high salt diet for 1-2 weeks and SBPs determined by a computerized tail-cuff method. A. Baseline pulse and SBP recordings in an SS rat before initiation of diet treatment, and after feeding of the 8% NaCl diet for, B. 1 week or C. 2 weeks. Cuff inflations and deflations are shown on the tracings.

Figure 3. Analysis of the effects of an 8% NaCl diet on BP in SR and SS rats. A. Baseline pulse and SBPs, B. 8% NaCl diet for 1 week; C. 8% NaCl diet for 2 weeks in SS animals. SBPs were analyzed after feeding of the high salt diet for 1-4 weeks to SR and SS animals. Data are means (± SEM) of determinations in 5 animals from each group. *Significantly different from baseline and SR; **Significantly different from SR baseline.

Figure 4. Effect of an 8% NaCl diet on expression of the CaSR in mesenteric arteries from SR and SS rats. Animals were fed the high salt diet for over 4 weeks and protein extracts from mesenteric arteries were analyzed by Western blotting with a polyclonal CaSR antibody (PA1-37213) and compared to baseline expressions. A.
Western blot analysis of protein extracts from mesenteric arteries from SR and SS rats. 

B. Bar charts showing densitometry analysis of the high molecular weight CaSR band normalized to polyclonal GAPDH antibody (FL-335) as the loading standard; i. Analysis of protein extracts of mesenteric arteries from SR showing expression of the high molecular weight, glycosylated, mature CaSR. ii. Analysis of protein extracts of mesenteric arteries from SS showing expression of the high molecular, glycosylated, mature and the low molecular weight, high mannose, immature CaSR. Data are means (± SEM) of 4 animals. *Significantly different from baseline.

Figure 5. Effect of an 8% NaCl diet on “normalization” of mounted mesenteric arteries from SR and SS rats. Segments (2 mm) of isolated mesenteric arteries were mounted in a wire myograph chamber in PSS medium (with 1 mM Ca$^{2+}$ and 100 µM ascorbic acid), equilibrated at 37 °C with aeration (95% air/5% CO$_2$) and “Normalized” by stepwise increases in passive force (in the absence of smooth muscle activation) until a resting tension (RT) is achieved. “Normalization” of mounted vessels from, A. SR; and B. SS, rats at baselines (on a 0.45% NaCl diet) or on 8% NaCl diet for 4 weeks.

Figure 6. Effect of an 8% NaCl diet on normalized and PE tensions in mesenteric arteries. Normalized tensions and tensions generated by addition of 5 µM PE (active force excluding passive force) were determined in mounted vessels from SR and SS animals at baseline or on 8% NaCl diet for 4 weeks are shown. A. Normalized tensions;
B. PE tensions. *Significantly different from SR; **Significantly different from SR baseline.

Figure 7. Ca\(^{2+}\) relaxation of isolated, PE-contracted mesenteric arteries from SR rats. Artery segments (2 mm) were mounted in PSS medium (with 1 mM Ca\(^{2+}\) and 100 µM ascorbic acid) in a wire myograph chamber, equilibrated at 37 °C with aeration (95% air/5% CO\(_2\)) and “normalized” as in Fig. 6. Ca\(^{2+}\) relaxation of PE-contracted mesenteric arteries from SR rats on, A. 0.45% NaCl diet (baseline) or B. 8% NaCl diet for 4 weeks are shown. The tracings show relaxations following application of cumulative concentrations of Ca\(^{2+}\).

Figure 8. Effect of an 8% NaCl diet on Ca\(^{2+}\) relaxation of isolated, PE-contracted mesenteric arteries from SS rats. Artery segments (2 mm) were mounted in PSS medium (with 1 mM Ca\(^{2+}\) and 100 µM ascorbic acid) in a wire myograph chamber, equilibrated at 37 °C with aeration (95% air/5% CO\(_2\)) and “normalized” as in Fig. 6. Ca\(^{2+}\) relaxation of PE-contracted mesenteric arteries from SS rats on; A. 0.45% NaCl diet (baseline) or B. 8% NaCl diet for 4 weeks are shown. The tracings show relaxations following application of cumulative concentrations of Ca\(^{2+}\).

Figure 9. Analysis of [Ca\(^{2+}\)]-responses. EC\(_{50}\) values were derived from force tracings in mesenteric arteries from SR and SS animals on; A. 0.45% NaCl diet (baseline), B. 8% NaCl diet for 2 weeks, or C. 8% NaCl diet for 4 weeks. Data shown were fitted to four parameter sigmoid curves (SigmaPlot 14.0) to obtain EC\(_{50}\) values for comparison.
Inset are the EC$_{50}$ values derived from the fitted curves and presented as bar charts with standard errors of the estimates. *Significantly different from SR.

**Figure 10. Effect of Inhibition of Ca$^{2+}$-activated K$^+$ (BK) channels on force tracings in an Artery segment from an SS rat.** Force tracings showing “normalization” followed by PE tensions, and Ca$^{2+}$ relaxations of artery segment from an SS rat at baseline.

**Figure 11. Inhibition of BK and NOS in mesenteric arteries on [Ca$^{2+}$]$_e$-responses.** Response curves showing the effects of IBTx and L-NAME on relaxation of normalized and pre-contracted mesenteric arteries from SR at baseline. [Ca$^{2+}$]$_e$-response curves showing the effects of IBTx and L-NAME on relaxation of normalized and pre-contracted mesenteric arteries from SR (A) and SS (B) at baseline. Four parameter sigmoid curves were fitted to the data to obtain EC$_{50}$ values for comparison. Inset are bar charts showing EC$_{50}$ values determined from the fitted curves with standard errors of the estimates. *Significantly different from Controls; **Significantly different from SS control.
Analysis of Effect of a 8% NaCl Diet on Systolic BP in SS Rats.

A. Baseline
B. 8% NaCl (1 Week)
C. 8% NaCl Diet (2 Weeks)

Figure 2
Figure 3

C. Analysis of Blood Pressure

SBP (mm Hg)

Baseline

Weeks on 8% NaCl Diet

SR

SS

n = 5

* Significant difference

** Highly significant difference
A. Western Blot

Weeks: 0 1 2 4

B. Densitometry

i. SR CaSR

ii. SS CaSR Doublet

Figure 4
Figure 5

Normalization of Mesenteric Artery Segments

A. SR
- 0.45% NaCl
- 8% NaCl

B. SS
- 0.45% NaCl
- 8% NaCl

Stepwise increase in passive force

Tension (mN)

Time (min)
Figure 6
Figure 7

Force tracings in isolated artery segments from SR rats

A. Baseline (0.45% NaCl)

B. 8% NaCl Diet (4 Weeks)
Figure 8

Force tracings in isolated artery segments from SS rats

A. Baseline (0.45% NaCl)

B. 8% NaCl Diet (4 Weeks)
Figure 9

A. Baseline

B. 8% NaCl Diet (2 Weeks)

C. 8% NaCl Diet (4 Weeks)
Figure 10

Effect of IBTx on force tracings in artery segment from an 55 rat on 0.45% NaCl diet
Figure 11