Dietary sodium modulates the interaction between efferent renal sympathetic nerve activity and afferent renal nerve activity: role of endothelin

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Running head: Sodium and endothelin modulates afferent renal nerve activity

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ABSTRACT

Increasing efferent renal sympathetic nerve activity (ERSNA) increases afferent renal nerve activity (ARNA) which in turn decreases ERSNA via activation of the renorenal reflexes in the overall goal of maintaining low ERSNA. We now examined whether the ERSNA-induced increases in ARNA are modulated by dietary sodium and the role of endothelin (ET). The ARNA response to reflex increases in ERSNA was enhanced in high (HNa) vs. low sodium (LNa) diet rats, 7560±1470 vs. 900±390%⋅sec. The norepinephrine (NE) concentration required to increase PGE₂ and substance P release from isolated renal pelvises was 10 pM in HNa and 6250 pM in LNa diet rats. In HNa diet pelvises 10 pM NE increased PGE₂ release from 67±6 to 150±13 pg/min and substance P release from 6.7±0.8 to 12.3±1.8 pg/min. In LNa diet pelvises 6250 pM NE increased PGE₂ release from 64±5 to 129±22 pg/min and substance P release from 4.5±0.4 to 6.6±0.7 pg/min. In the renal pelvic wall, ETB-R are present on unmyelinated Schwann cells close to the afferent nerves and ETA-R on smooth muscle cells. ETA-receptors (R) protein expression in the renal pelvic wall is increased in LNa diet. In HNa diet, renal pelvic administration of the ETB-R antagonist BQ788 reduced ERSNA-induced increases in ARNA and NE-induced release of PGE₂ and substance P. In LNa diet, the ETA-R antagonist BQ123 enhanced ERSNA-induced increases in ARNA and NE-induced release of substance P without altering PGE₂ release. Conclusion: Activation of ETB-R and ETA-R contributes to the enhanced and suppressed interaction between ERSNA and ARNA in conditions of HNa and LNa diet, respectively, suggesting a role for ET in the renal control of ERSNA that is dependent on dietary sodium.
INTRODUCTION

The majority of the afferent renal nerve fibers are localized in the renal pelvic wall (28,29,37) where many of these nerve fibers are in close contact with sympathetic nerves (29). The close contact between the renal afferent and sympathetic nerves provides anatomical support for a functional interaction between efferent renal sympathetic nerve activity (ERSNA) and afferent renal nerve activity (ARNA). Indeed, our previous studies have provided evidence for such an interaction, whereby increases in ERSNA increase ARNA (29). The interaction between ERSNA and ARNA is modulated by norepinephrine (NE) which increases and decreases the activation of the renal sensory nerves by stimulating $\alpha_1$- and $\alpha_2$-adrenoceptors, respectively, located on the renal pelvic sensory nerve fibers. Not only is there an interaction between ERSNA and ARNA, but there is also a reciprocal interaction between ARNA and ERSNA. Increases in ARNA decrease ERSNA causing a natriuresis, i.e., a renorenal reflex response (33). Thus, ERSNA-induced increases in ARNA would exert a powerful negative feedback control of ERSNA via activation of the renorenal reflexes in the overall goal of maintaining low ERSNA to prevent renal sodium retention.

We reasoned that the ERSNA-ARNA interaction would play a significant role in the renal control of body water and sodium balance if the interaction was modulated by dietary sodium. In conditions of low sodium (LNa) dietary intake, suppression of the ERSNA-ARNA interaction would increase ERSNA via impairment of the renorenal reflex mechanism leading to sodium retention. In contrast, in conditions of high sodium (HNa) dietary intake, enhancement of the ERSNA-ARNA interaction would increase the inhibitory renorenal reflex control of ERSNA
resulting in suppression of ERSNA to prevent/limit sodium retention. This hypothesis derives support from our studies in afferent renal denervated rats fed HNa diet which are characterized by increased responsiveness of ERSNA to various sympathetic stimuli and increased arterial pressure (23,31). Furthermore, the responsiveness of the renal mechanosensory nerves to increases in renal pelvic pressure is enhanced in rats fed HNa diet and suppressed in LNa diet rats (25).

Because our studies showed that the interaction between ERSNA and ARNA was enhanced by HNa diet and suppressed by LNa diet, we examined the possible mechanisms involved. We focused on endothelin (ET). ET-1 exerts its effects by activating two G-protein coupled receptors, ETA-R and ETB-R (17). The responses to ET vary with the cell type/organ. The major vascular effects of ETA-R activation are vasoconstriction. The results of activation of ETB-R are more diverse and include vasoconstriction, vasodilation, diuresis, and natriuresis (47). In addition, our previous studies suggested a role for ET-1 in the activation of renal mechanosensory nerves, the nature of which is dependent on dietary sodium intake (24). Activation of renal pelvic ETB-R contributes to the enhanced ARNA responsiveness to renal mechanosensory nerve activation in conditions of HNa diet. In contrast, activation of ETA-R contributes to the suppressed ARNA responsiveness of the renal mechanosensory nerves in conditions of LNa diet. We therefore examined whether activation of ETA-R and/or ETB-R contributed to the dietary sodium-induced modulation of the interaction between ERSNA and ARNA.

The in vivo studies were complemented by in vitro studies in the isolated renal pelvic wall from HNa and LNa diet rats. Because this preparation is by design sympathetically denervated, any effects of dietary sodium on NE-induced release of PGE2 and substance P in the
absence or presence of ET-R antagonists would be related to modulation of a postsynaptic mechanism(s) at the sensory nerve endings.

Furthermore, we examined whether altered gene and/or protein expression of ETA-R and/or ETB-R contributes to the differential effects of ET-1 on the responsiveness of the afferent renal nerves in conditions of HNa and LNa diet. We also carried out immunohistochemical studies to determine the cellular distribution of ETA-R and ETB-R in the renal pelvic wall and in T9-L1 dorsal root ganglia (DRGs) which contain the majority of the cell bodies of the afferent renal nerves (3,6).

METHODS

The study was performed on male Sprague-Dawley rats weighing 197-420 g (mean 291±3 g). Two weeks before the study, rats were placed on either sodium (Na⁺)-deficient pellets (ICN, Na⁺=1.6 meq/kg) with tap water drinking fluid (LNa diet, n=84), normal Na⁺ pellets (Teklad, Na⁺=163 meq/kg) with tap water drinking fluid (normal sodium (NNa) diet, n=47) or normal Na⁺ pellets with 0.9% NaCl drinking fluid (HNa diet, n=65) (25).

The experimental protocols were approved by the Institutional Animal Care and Use Committee and performed according to the “Guide for the Care and Use of Laboratory Animals” from the National Institutes of Health.

Anesthesia was induced with pentobarbital sodium (0.2 mmol/kg i.p. Abbott Laboratories).

Gene Expression

RNA was isolated from renal pelvises and T9-L1 DRG dissected from anaesthetized rats fed HNa (n=8) and LNa diet (n=8) using the RNAqueous™-4PCR kit (Ambion, Huntingdon,
UK). Concentration and purity of RNA were determined spectrophotometrically using absorbance at 260 nm and the $A_{260/280}$ ratio, respectively. No relevant contamination with proteins was present in the samples as shown by an $A_{260/280}$ ratio higher than 1.9. Reverse transcription was performed with approximately 150 ng RNA per reaction and random hexamer primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Expression levels were determined by TaqMan real-time PCR performed with ready to use TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) using cDNA corresponding to 3 ng RNA as template and the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). Design of synthetic oligonucleotide primers and fluorescent probes for ETA-R, ETB-R and porphobilinogen deaminase (PBGD) were identical to those used previously (48). Furthermore, we included tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz) using the following primers and probe: forward primer: CATCTGCAACGACGTACTGTCTCT; probe: ACTACTACCGCTACTTGGCTGAGGTTGCTG reverse primer: GACTGGTCCACAATCTTTTTCTTG. The forward primer spans nucleotides 337 through 360, the probe nucleotides 432 through 461 and the reverse primer nucleotides 472 through 495. The PCR included a 10 min activation of the DNA polymerase at 95°C, 40 cycles at 95°C for 15s for denaturation, and 1 min at 60°C for annealing and extension. PCRs were performed in triplicate. In each experimental plate a no-template control was included to check for any contamination affecting the PCR.

The fluorescence raw data from kinetic PCR were analysed using Data Analysis for Real-Time PCR [DART-PCR Version 1.0; (43) http://directory.gene-quantification.info/] which allows comparison of DNA amplification efficiency among experimental groups as a
prerequisite for quantitative analysis of PCR data. The relative content of mRNA was determined using the geometric mean of the content of PBGD and Ywhaz as endogenous references for normalization.

**Western Blot Analysis**

Western blot analysis was performed as previously described (49). In short, homogenized renal pelvic and DRG tissues from 8 HNa and 8 LNa diet rats were centrifuged at 100,000 g for 1 h at 4°C. The pellets (membrane fractions) were resuspended and stored at -70°C until western blot analysis. Total protein concentration was determined by the Biuret reaction (Biorad, München, Germany) using bovine serum albumin (BSA) as a standard. 25-50 µg protein of DRG tissue and 100 µg protein of pelvic wall tissue were electrophoretically separated on a 10% SDS polyacrylamide gel with a 5% stacking gel and transferred to nitrocellulose (0.2 µm, Schleicher&Schuell/BioSciences GmbH, Dassel, Germany). The membrane fractions were blocked with nonfat milkpowder and incubated for 18 hrs at 4°C with antiserum against ETB-R or ETA-R (rabbit; 1:200, Alomone Labs, Jerusalem, Israel) followed by incubation with peroxidase-labeled goat anti-rabbit (1:10,000, Biorad, München, Germany). Specificity of the antisera was verified by incubating the antisera against the ET-R with excess immunizing peptide. To ascertain adequate amount of protein, renal pelvic and DRG tissues from 2 rats were combined for the western blot analysis resulting in N=4 from each group of 8 rats.

Immunoreactive bands were detected using enhanced chemiluminescence kit (ECL Plus, Amersham Pharmacia Biotech, Inc). After development, the films were scanned and the bands quantified by densitometry (Gelscan Standard V5.02, BioSciTec). Actin was used as loading control. The data represent the average of 3-5 assays per tissue and rat.

**Immunohistochemistry**
The immunohistochemical procedures for kidney and DRG tissue have been previously described in detail (28,29). In brief, anesthetized male Sprague Dawley rats were transcardially perfused with calcium-free Tyrode solution followed by phosphate-buffer, the kidneys dissected out and stored in 10% sucrose at 4°C. 14 µm thick sections were cut on a cryostat, thaw-mounted onto gelatin-coated slides and fixed in ice-cold acetone. All primary antibodies were incubated for 24 hrs at 4°C.

**ETB-R.** The sections were incubated with antiserum against glial fibrillary acidic protein (GFAP, mouse; 1:1,000, Neuromics, Edina, MN), a marker for satellite cells in DRG and unmyelinated ensheathing Schwann cells in the peripheral nervous system (16), or calcitonin gene-related peptide (CGRP, mouse; 1:8,000; Drs JH Walsh and HC Wong), a marker for sensory nerves. Immunoreactivity was visualized using the tyramide signal amplification system (TSA-Plus: PerkinElmer Life and Analytical Sciences Inc., Waltham, MA). After completion of the protocol for TSA for detection of GFAP or CGRP, the tissue sections were incubated with antiserum for ETB-R (rabbit; 1:100, Alomone Labs) and processed by the indirect immunofluorescence technique. In double labeling studies with antisera against ETB-R and α-smooth muscle actin (αSMA, mouse; 1:400, Sigma-Aldrich, St Louis, MO), a mixture of the two primary antisera was applied to the tissue sections and processed by the indirect immunofluorescence technique.

**ETA-R.** The sections were incubated with antiserum against ETA-R (rabbit; 1:10,000, Alomone Labs). Immunoreactivity was visualized using TSA-Plus. After completion of the protocol for TSA for detection of ETA-R, antiserum for CGRP (mouse; 1:400) or αSMA was applied and the tissue sections processed by the indirect immunofluorescence technique.
The specificity of the antisera for ETB-R and ETA-R was tested by preincubation of the primary antisera with an excess amount of the fusion protein used as immunogen, the concentration of the immunogen being 100 µg/ml.

The tissue sections were examined using a Nikon Eclipse E600 fluorescence microscope (Tokyo, Japan) equipped with epifluorescence with the appropriate filter combinations. Photographs were taken with a Hamamatsu ORCA-ER C4762-80 digital camera (Hamamatsu City, Japan) using Hamatsu photonics Wasabi software. For confocal analysis a Radiance Plus confocal laser scanning system (Bio-Rad, Hemel Hemstead, UK) installed on a Nikon Eclipse E600 fluorescence microscope was used. Digital images from the microscopy were optimized for image resolution brightness and contrast and color images were merged using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA).

**In vivo Studies**

After induction of anesthesia (see above), an intravenous infusion of pentobarbital sodium (0.04 mmol·kg\(^{-1}\)·hr\(^{-1}\)) at 50 µl/min into the femoral vein was started and maintained throughout the course of the experiment. Arterial pressure was recorded from a catheter in the femoral artery. The left renal pelvis was perfused with vehicle or various perfusates, described below, throughout the experiment at 20 µl/min via a PE-10 catheter placed inside a PE-60 catheter located in the left ureter. A PE-10 catheter was placed in the right ureter for collection of urine. ERSNA and ARNA were recorded from the central and peripheral portions, respectively, of the cut ends of two adjacent left renal nerve branches which were placed on bipolar silver wire electrodes. ERSNA and ARNA were integrated over 1-second intervals, the unit of measure being microvolts per second per 1 second. All data were collected at 500 Hz and averaged over 2 seconds. Postmortem renal nerve activity, assessed by crushing the renal nerve bundles central or
peripheral to the recording electrode, was subtracted from all values of ERSNA and ARNA, respectively. Renal nerve activity was expressed in percentage of its baseline value during the control period (21-33).

**Stimulation of Renal Sensory Nerves:** Renal sensory nerves were stimulated by reflex mediated increases in ERSNA which were produced by placing the rat’s tail in warm water (29). The threshold for activation of thermal cutaneous stimulation was 49°C in rats fed NNa and LNa diet and 47°C in rats fed HNa diet. Because pilot studies showed poor reproducibility of the ERSNA responses to thermal cutaneous stimulation at 49°C in rats fed HNa diet, subsequent studies used a water temperature of 47°C in these rats.

Because our previous studies have shown that increases in ERSNA modulate ARNA by a release of NE, we also examined whether dietary sodium modulated the ARNA responses to renal pelvic perfusion with NE. The renal pelvis was perfused with NE at 2 and 10 pM in rats fed HNa diet (N=11) and NNa diet (N=8) and 10, 50 and 250 pM in rats fed LNa diet (N=11).

In all experimental groups, a 10-min control and a 10-min recovery period bracketed the experimental period.

**High sodium diet:** Effects of an ETB-R antagonist on the ARNA responses to reflex increases in ERSNA. The experiments performed in HNa diet rats were divided into two parts. During each part, the rat’s tail was placed in 47°C water during two 3-min experimental periods. Following each experimental period, the rat’s tail was immediately placed in room temperature water to quickly terminate the heat stimulation. Twenty minutes after the end of the first part, the renal pelvic perfusate was switched from vehicle to the ETB-R antagonist BQ788 (13) at 1 µM (n=14). Ten minutes later, the control, experimental and recovery periods were repeated. Time
control experiments (N=7) were performed similarly except the renal pelvis was perfused with vehicle throughout the experiment.

**Low sodium diet**: Effects of an ETA-R antagonist on the ARNA responses to reflex increases in ERSNA. The experiments performed in LNa diet rats used a similar protocol as above except the rat’s tail was placed in 49°C water. The ETA-R antagonist BQ123 (12) at 5 µM was administered during the second part of the experiment (N=14). Time control experiments (N=8) were performed similarly except the renal pelvis was perfused with vehicle throughout the experiment.

**Normal sodium diet**: Effects of an ETB-R or ETA-R antagonist on the ARNA responses to reflex increases in ERSNA. The experiments performed in NNa diet rats used a protocol similar to that above. BQ788 and BQ123 were administered during the second part of the experiments in two separate groups of rats, each N=10.

**In vitro Studies**

**Stimulation of renal sensory nerves in an isolated renal pelvic wall preparation**: Because ET-1 and ET-3 have been shown to modulate the release of NE by presynaptic mechanisms (39,41,52), further studies were undertaken in an isolated renal pelvic wall preparation to examine whether the effects of the ET-R antagonists on renal sensory nerve activation involved a mechanism(s) at the peripheral sensory nerve endings; the isolated renal pelvic preparation by design being sympathetically denervated. NE was added to the incubation bath containing isolated renal pelvises from rats fed HNa, NNa or LNa diet and the release of substance P and PGE₂ were measured.

The isolated renal pelvic wall preparation has previously been described in detail (21-30). In brief, following anesthesia renal pelvises dissected from the kidneys were placed in wells
containing 400 µl HEPES buffer maintained at 37°C. Each well contained the pelvic wall from one kidney. Throughout the experiment, the incubation medium was replaced with fresh HEPES every 5 min. The incubation medium was collected in siliconized vials and stored at -80°C for later analysis of substance P and PGE₂. After a 2-hrs’ equilibration period, the experiment started with four 5-min control periods followed by one 5-min experimental period and four 5-min recovery periods. NE was added to the incubation bath to both pelvises during the experimental periods.

In our initial studies, we examined whether dietary sodium modulated the release of substance P and PGE₂ produced by NE at 250 pM, the threshold concentration required to activate renal sensory nerves in isolated renal pelvises from NNA diet rats (29).

**High sodium diet: Effects of an ETB-R antagonist on the NE-induced release of substance P and PGE₂.** Initial experiments were performed to determine the threshold concentration of NE required to increase the release of substance P. Both pelvises were incubated in HEPES buffer as described above. During the experimental period, one pelvis was exposed to 2 pM NE and the other to 10 pM NE (N=9). In subsequent experiments examining the effects of BQ788 (N=12), both pelvises were exposed to 10 pM NE during the experimental period. In these experiments, one pelvis was incubated in HEPES buffer and the other pelvis in HEPES buffer containing 1 µM BQ788 throughout the control, experimental and recovery periods. Renal pelvic release of substance P and PGE₂ into the incubation bath was measured throughout the experiment.

**Low sodium diet: Effects of an ETA-R antagonist on the NE-induced release of substance P and PGE₂.** One pelvis was incubated in HEPES buffer and the other pelvis in HEPES buffer containing 1 µM BQ123 throughout the control, experimental and recovery periods. During the
experimental period, both pelvises were exposed to NE at 1250 pM (N=12) or 6250 pM (N=10). Because our previous studies in NNa diet rats showed that PGE$_2$ facilitated the NE-mediated release of substance P (29), further studies were performed to examine whether addition of PGE$_2$ at a subthreshold concentration for substance P release, 0.03 µM (25), would alter the substance P release by 50 pM NE, in the absence or presence of 1 µM BQ123.

Normal sodium diet; Effects of an ETB-R or ETA-R antagonist on the NE-induced release of substance P. One pelvis was incubated in HEPES buffer and the other pelvis in HEPES buffer containing 1 µM BQ123 (N=6) or 1 µM BQ788 (N=8) throughout the control, experimental and recovery periods. During the experimental period, both pelvises were exposed to 250 pM NE.

Renal Pelvic Contractions - Myography. Because our immunohistochemical studies suggested the presence of ETA-R on smooth muscle cells in the renal pelvic wall, we examined whether ET-1 induced pelvic wall contractions and if so, whether pretreatment with BQ123 modified the ET-1 induced contractions. Renal pelvises from 5 NNa diet rats were mounted in a small vessel wire myograph (model 410A, Danish Myotechnology, Aarhus, Denmark) on two stainless steel wires with diameters of 40 µm and stretched until rhythmic contractions were observed. Tissues were equilibrated for 45-60 min. The incubation medium (NaCl 118.5 mM, KCl 4.7 mM, Mg SO$_4$ 1.2 mM, KH$_2$PO$_4$ 1.2 mM, NaHCO$_3$ 25 mM, CaCl$_2$ 2.5 mM, Glucose 5.6 mM, pH 7.4. fluid) was replaced every 15 min with fresh solution. At steady state, ET-1 (1-300 nM) was added to the incubation bath in a cumulative manner at 6-min intervals with and without addition of 10 µM BQ123.

Drugs

Substance P antibody (IHC 7451) was acquired from Peninsula Laboratories (San Carlos, CA) and PGE$_2$ from Cayman Chemicals (Ann Arbor, MI). All other reagents/chemicals
were from Sigma Aldrich (St. Louis, MO) unless otherwise stated. NE was dissolved in 0.1% ascorbic acid in incubation buffer or 0.15 M NaCl. All other agents were dissolved in incubation buffer (in vitro studies) or 0.15 M NaCl (in vivo studies).

Analytical Procedures

Substance P and PGE$_2$ in the incubation medium was measured by ELISA, as previously described in detail (21-30).

Statistical Analysis

In vivo: Increases in ERSNA, ARNA, and mean arterial pressure (MAP) produced by placing the tail in warm water were evaluated by calculating the area under the curve of each parameter vs. time, with baseline being the average value of each control period. Likewise, the ARNA responses to NE were calculated as the area under the curve of ARNA vs. time. D’Agostino and Pearson omnibus normality tests were used to determine whether the data were normally distributed. Normally distributed data were analyzed using Student’s unpaired t-test, one or two-way analysis of variance for repeated measurements followed by t-test with Bonferroni correction for multiple comparisons. Data which were not normally distributed were analyzed using Wilcoxon signed rank test, Friedman 1-way analysis of variance with repeated measures or Kruskal Wallis one way analysis of variance, each followed by Dunn’s multiple comparison test (50). A significance level of 5% was chosen. Data in text and figures are expressed as means ± SE.

RESULTS

Gene and protein expression of ETB-R and ETA-R. In the renal pelvic wall, there was no difference in gene expression of ETA-R or ETB-R in fed HNa or LNa diet (Fig. 1). In T$_9$-L$_1$
DGR, gene expression of ETB-R was slightly higher in rats fed HNa diet than in rats fed LNa diet (P<0.05). Gene expression of ETA-R was similar in the two groups. In renal pelvic tissue, protein expression of ETA-R was lower from rats fed HNa diet than in rats fed LNa diet (P<0.05). Protein expression of ETB-R was similar in the two groups. In T9-L1 DRG, protein expression of ETA-R and ETB-R was similar in rats fed HNa and LNa diet.

**Immunohistochemistry**

*Localization of ETB-R and ETA-R in renal pelvic and DRG tissue.* Strong labeling with the antibodies against ETB-R and ETA-R was observed in the renal pelvic wall (Fig. 3a&b). However, the two antibodies labeled different structures. Whereas, the ETB-R antibody appeared to label nerve fibers (Fig. 3a), the ETA-R antibody appeared to label smooth muscle cells in the pelvic wall and adjacent blood vessels (Fig. 3b). To test this hypothesis we double labeled the sections with antibodies against the ET-R, αSMA and CGRP.

As shown in Fig. 4a-c &d-f, the ETB-R antibody labeled fiber-like structures running among smooth muscle fibers in the renal pelvic wall that were close to CGRP-immunoreactive (ir) fibers (Fig. 4d-f). Also, ETB-R-like immunoreactivity (LI) was seen within the uroepithelium. The close localization of the ETB-R-ir structures to the CGRP-ir fibers was further observed in nerve bundles in the renal pelvic area (Fig. 4g-i). Because these studies suggested that ETB-R were located on or close to CGRP-ir nerve fibers, DRG T9-L1 were labeled with the ETB-R antibody. The ETB-R antibody did not label cell bodies in the DRG but rather satellite cells surrounding the cell bodies as shown by the co-localization of ETB-R-ir and GFAP-ir (Fig. 5a-c). Because peripheral supporting cells, i.e., unmyelinated ensheathing Schwann cells, can be identified by the GFAP antibody (16), we also double-labeled renal tissue sections with antibodies against ETB-R and GFAP. As shown in Figs. 5d-f, the majority of the
ETB-ir structures were colocalized with GFAP-ir structures. This is further shown in nerve bundles in the pelvic area (Fig. 5g-i).

Double-labeling kidney and DRG T9-L1 tissue sections with antibodies against ETA-R, αSMA and/or CGRP showed that the ETA-R-LI colocalized with αSMA-ir cells (Fig. 6a-c) but not with CGRP-ir fibers in the pelvic wall (Fig. 6d-f). The CGRP-ir nerve fibers ran among the ETA-R-ir smooth muscle cells (Fig. 6f). Likewise in DRG T9-L1, the ETA-R antibody labeled smooth muscle cells in vessels (Fig. 6g-i) found among the CGRP-ir neural cell bodies (Fig. 6j-l). The presence of ETA-R on pelvic smooth muscle cells was functionally confirmed by the marked reduction produced by the ETA-R antagonist BQ123 of the ET-1-induced renal pelvic contractions (Please see S1 - online supplement).

Similarly to previously reported (54,58,59), the ETB-R antibody produced marked labeling of endothelial cells in the glomeruli, endothelial cells and some smooth muscle cells in blood vessels, including efferent and afferent arterioles, and inner medullary collecting duct cells. The ETA-R antibody labeled smooth muscle cells in blood vessels throughout the kidney including afferent and efferent arterioles and vasa recta (data not shown).

All ETB-R and ETA-R labeling in the kidney and DRG sections was blocked by adsorption with the peptide used as immunogen for the generation of the ETB-R and ETA-R antibody, respectively (Please see S2 - online supplement).

In Vivo

Effects of dietary sodium on the reflex responses to thermal cutaneous stimulation. To evaluate the effects of dietary sodium on the responses to thermal cutaneous stimulation, the first responses in the time control groups and the groups subsequently treated with either BQ788 or BQ123 were pooled in each diet group. As shown in Fig. 7 and Table 1, the increases in ERSNA,
ARN and MAP were suppressed in rats fed LNa diet compared to those in rats fed NNa or HNa diet (P<0.01). Thermal cutaneous stimulation increased contralateral urinary sodium excretion in all three groups; HNa diet, 45±8% from 1.5±0.2 µmol·min⁻¹·g⁻¹ (P<0.01), NNa diet, 32±9% from 1.1±0.2 µmol·min⁻¹·g⁻¹ (P<0.02) and LNa diet, 35±9% from 0.5±0.2 µmol·min⁻¹·g⁻¹ (P<0.01).

To examine whether the suppressed ARNA responses in LNa diet rats were related to the smaller increases in ERSNA produced by thermal cutaneous stimulation in these rats, we examined whether dietary sodium modulated the ARNA responses to NE administered directly into the renal pelvis. NE caused a dose dependent increase in ARNA in all rats. The threshold concentration required to increase ARNA was dependent on the Na diet, being 2 pM in HNa diet rats, 10 pM in NNa diet rats and 50 pM in LNa diet rats (Fig. 8). The ARNA responses to 2 pM NE in HNa diet rats were greater than those produced in NNa diet rats (P<0.01). Also, the ARNA responses to 10 pM NE in HNa diet rats were greater than those produced in NNa diet rats (P<0.05) and LNa diet rats (P<0.01). Contralateral urinary sodium excretion was increased by 2 and 10 pM NE in HNa diet rats, 24±8% from 1.3±0.3 µmol·min⁻¹·g⁻¹ (P<0.05) and 34±7% from 1.5±0.3 µmol·min⁻¹·g⁻¹ (P<0.01), respectively, and by 10 pM NE in NNa diet rats, 21±5% from 1.0±0.1 µmol·min⁻¹·g⁻¹ (P<0.05). Contralateral urinary sodium excretion was not altered by NE in LNa diet rats which may be related to the low baseline level, 0.11 µmol·min⁻¹·g⁻¹.

Importantly, MAP was not affected by renal pelvic administration of NE in any of the groups. Basal MAP was 106±4, 115±4 and 109±5 mmHg in rats fed HNa, NNa and LNa diet, respectively.

High sodium diet-in vivo: Effects of an ETB-R antagonist on the ARNA responses to reflex increases in ERSNA. The results depicted in Figures 7 and 8 suggested that the responsiveness of the renal sensory nerves to NE and reflex increases in ERSNA are modulated
by dietary sodium. Because of our previous studies (24), we hypothesized that blocking the renal pelvic ETB-R may modulate the ARNA responses to reflex increases in ERSNA. As shown in Fig. 9A, renal pelvic perfusion with BQ788 reduced the increases in ARNA produced by placing the rat’s tail in 47°C water (P<0.01) without affecting the increases in ERSNA or MAP, 4,700±650 vs. 3,700±450 mmHg⋅sec (NS).

In time control experiments the second ARNA and ERSNA responses to thermal cutaneous stimulation were greater than those produced by the first stimulation (P<0.05). No differences were seen in the two MAP responses (Table 2).

Low sodium diet-in vivo: Effects of an ETA-R antagonist on the ARNA responses to reflex increases in ERSNA. Because of the results of our previous studies (24), we tested the notion that activation of ETA-R might contribute to the suppressed ARNA response to reflex increases in ERSNA. Renal pelvic administration of BQ123 enhanced the ARNA response to thermal cutaneous stimulation to a value similar to that seen in vehicle treated HNa diet rats (Fig. 9A&B). BQ123 had no effect on the suppressed ERSNA and MAP responses, 2800±3390 vs. 2,900±470 mmHg⋅sec.

Repeated placement of the rat’s tail in 49°C water in the presence of vehicle (time control) resulted in reproducible increases in ERSNA, ARNA and MAP (Table 2).

Normal diet-in vivo: Effects of an ETB-R or ETA-R antagonist on the ARNA responses to reflex increases in ERSNA. In contrast to our findings in rats fed HNa or LNa diet, renal pelvic administration of BQ788 or BQ123 had no effect on the ARNA responses to thermal cutaneous stimulation in rats fed NNa diet (Fig. 10). Likewise, the increases in ERSNA, 9150±2610 and 8.400±1430%⋅sec and MAP, 3060±520 and 5,100±760 mmHg⋅sec, produced by thermal cutaneous stimulation in the two groups were unaltered by BQ788 and BQ123.
**In Vitro**

*Effects of dietary sodium on the norepinephrine-induced release of substance P and PGE₂.* Baseline substance P release from renal pelvises from HNa diet rats was greater than that from LNa diet rats (P<0.01) (Fig. 11). Adding 250 pM NE to the bath resulted in a reversible release of substance P from renal pelvises from HNa and NNa diet rats (P<0.01). The NE-mediated release of substance P from HNa diet pelvises was greater than that from NNa diet pelvises (P<0.01). The NE-mediated increases in substance P release were associated with increases in renal pelvic release of PGE₂. In contrast, 250 pM NE failed to increase PGE₂ and substance P release from renal pelvises from LNa diet rats.

*High sodium diet-in vitro: Effects of an ETB-R antagonist on the NE-induced release of substance P and PGE₂.* Next we examined whether lower concentrations of NE would increase substance P and PGE₂ in HNa diet rats. Whereas 2pM failed to increase substance P release, 8.1±1.3 to 9.4±1.6 pg/min, 10 pM NE resulted in a reversible increase in the release of substance P, from 8.1±1.5 to 14.7±2.8 pg/min and PGE₂, from 57±4 to 144±16 pg/min, both P<0.01. Subsequent studies showed that adding BQ788 to the incubation bath reduced both the increases in substance P and PGE₂ produced by 10 pM NE (Fig. 12A).

*Low sodium diet-in vitro: Effects of an ETA-R antagonist on the NE-induced release of substance P and PGE₂.* Because 250 pM NE failed to increase substance P release in rats fed LNa diet, we tested whether higher concentrations of NE would increase substance P release in the absence or presence of BQ123. Adding 1250 pM NE to the bath did not increase substance P release in the absence or presence of BQ123 in the bath (Table 3). Adding 6250 pM NE to the bath resulted in a small increase in substance P release which was enhanced by BQ123 in the bath (Fig. 12B). However, BQ123 had no effect on the NE mediated increase in PGE₂ release.
(Fig.12B). Adding 0.03 µM PGE$_2$ to the bath lowered the NE concentration required to increase substance P release from 6250 to 50 pM, but only in the presence of BQ123 (Table 4).

**Normal sodium diet-in vitro: Effects of an ETB-R and ETA-R antagonist on the NE-induced release of substance P and PGE$_2$.** Similar to our *in vivo* studies in NNa diet rats (Fig. 10), adding BQ788 or BQ123 had no effect on the release of substance P produced by NE from renal pelvises derived from rats fed normal sodium diet (Fig. 13).

**DISCUSSION**

Increases in ERSNA increase ARNA which in turn provides a powerful negative feedback control of ERSNA in the overall goal of maintaining low ERSNA to limit sodium retention (29, Fig.14). The present study shows that the ERSNA-ARNA interaction is modulated by dietary sodium. The increases in ARNA produced by reflex increases in ERSNA are enhanced by HNa diet and suppressed by LNa diet by a mechanism(s) involving PGE$_2$-dependent release of NE. Further, our data suggest a role for ET in the modulation of the ERSNA-ARNA interaction produced by dietary sodium. In the renal pelvic wall, ETA-R-LI was found on smooth cells and ETB-R-ir fibers close to sensory nerve fibers. The ARNA responses to reflex increases in ERSNA were suppressed by renal pelvic administration of an ETB-R antagonist in HNa diet and enhanced by an ETA-R antagonist in LNa diet rats. Our data suggest that activation of ETB-R on or close to the sensory nerves in the renal pelvic wall contributes to the enhanced interaction between ERSNA and ARNA in HNa diet rats. Conversely, activation of ETA-R on smooth muscle cells in the renal pelvic wall contributes to the reduced interaction between ERSNA and ARNA in LNa diet rats. The differential effect of ET-1 on ETB-R and ETA-R in
conditions of HNa and LNa diet may, at least in part, be related to increased ETA-R protein expression in LNa diet rats (Fig.14).

Dietary sodium modulates the interaction between ERSNA and ARNA. The interaction between ERSNA and ARNA involves renal pelvic release of NE which activates $\alpha_1$- and $\alpha_2$-adrenoceptors on renal sensory nerves leading to increases and decreases in renal PGE$_2$ synthesis/release, respectively (29). The current data show that the ERSNA-ARNA interaction is suppressed by LNa diet and enhanced by HNa diet. These data suggest an important physiological role for the ERSNA-ARNA interaction in renal control of water and sodium homeostasis. In HNa dietary condition, the enhancement of the ERSNA-ARNA interaction would increase the inhibitory renorenal reflex control of ERSNA, resulting in suppression of ERSNA to prevent/limit sodium retention. In LNa dietary condition, the reduced ERSNA-ARNA interaction would increase ERSNA via suppression of the renorenal reflex mechanism leading to sodium retention (Fig. 14).

The reduced ARNA response to thermal cutaneous stimulation was associated with smaller increases in ERSNA and MAP in LNa vs. HNa and NNa diet rats. However, it is unlikely that the reduced ARNA response to reflex increases in ERSNA was due to the reduced ERSNA response to thermal cutaneous stimulation in these rats. Whereas in HNa diet rats, the ARNA responses to thermal cutaneous stimulation were correlated with the magnitude of the ERSNA responses ($r^2=0.59$, $P<0.01$), they were not in LNa diet rats ($r^2=0.09$). Calculation of the expected increases in ARNA from the measured increases in ERSNA in LNa diet rats using the regression curve equation derived from the data in HNa diet rats shows larger ARNA responses compared to those actually achieved by thermal cutaneous stimulation in the LNa diet rats. These data suggest that the ARNA responses to thermal cutaneous stimulation in LNa diet rats are
controlled/suppressed by some mechanism(s) other than the increases in ERSNA. Further studies examining the effects of NE administered directly into the renal pelvis and to an isolated renal pelvic wall preparation from rats fed various sodium diets supported this hypothesis. The threshold concentration of NE required to increase ARNA and substance P release was lowest in HNa diet rats and highest in LNa diet rats.

The increases in ARNA produced by renal pelvic administration of NE were associated with increases in contralateral urinary sodium excretion. Because MAP was not affected by renal pelvic administration of NE, the contralateral natriuretic responses most likely were the result of activation of the renorenal reflex mechanism(33).

It is unlikely that the differential responsiveness of the renal sensory nerves in rats fed various sodium diets was related to basal urinary sodium concentration. The responsiveness of the renal sensory nerves is not affected by changes in urinary sodium concentrations between 10-900 mM (32). In the current study, urinary sodium concentrations varied from 39±6 mM in LNa diet rats (n=27), 107±16 mM in NNa diet rats (n=27) to 160±13 mM in HNa diet rats (n=32).

**ETB-R and ETA-R in renal pelvic and DRG tissue.** The similar ET-1 content in renal pelvic and T3-L1 DRG tissue in HNa and LNa diet rats (24) excluded the possibility that the differential effects of ET-1 in the activation of renal sensory nerves in HNa and LNa diet rats were related to different ET-1 expression. Our previous functional studies suggested that ET-R are localized in the renal pelvic wall tissue (24). Our current studies showed (1) that both ET-R are synthesized in renal pelvic and T3-L1 DRG tissue and (2) an increased expression of ETA-R in renal pelvic tissue from LNa diet rats, a finding similar to that in renal medullary tissue (19). The increased ETA-R expression may reflect increased endogenous angiotensin (ANG) II modulating the expression of these receptors (56).
ETB-R-ir structures are in close contact with CGRP-ir fibers and GFAP-ir structures among smooth muscle fibers in the renal pelvic wall. These data suggest that ETB-R are localized on or close to unmyelinated Schwann cells surrounding sensory nerve fibers in the renal pelvic wall. The presence of Schwann cells in the renal pelvic wall has previously been shown in close conjunction to sympathetic nerve fibers (5). The presence of ETB-R on Schwann cells close to renal pelvic sensory nerves is supported by colocalization of ETB-R-LI and GFAP-LI in satellite cells surrounding CGRP-ir cell bodies in T₀-L₁ DRGs, a finding similar to that by Pomonis et al. in lumbar DRGs (45). The exact mechanism(s) by which activation of ETB-R on peripheral Schwann cells may modulate ARNA remains unclear, but it is well known that glial cells can modulate neurotransmission by increasing intracellular Ca⁺⁺ in response to various neurotransmitters, including ET-1 (10,53). ETB-R are expressed on glial cells throughout the central nervous system (40) and on immortalized Schwann cells (55).

Our studies further showed co-labeling of ETA-R and αSMA in smooth muscle cells in the pelvic wall, and in small vessels in and adjacent to the renal pelvic wall, throughout the kidney and in T₀-L₁ DRG. CGRP-ir fibers were found among ETA-R-ir smooth muscle cells in the renal pelvic wall. The presence of ETA-R on renal pelvic smooth muscle cells was functionally supported by our studies showing abolition of the ET-1 induced renal pelvic contraction by the ETA-R antagonist BQ123. Our data in DRG are in an apparent conflict with those by Pomonis et al. (45) which showed ETA-R labeling in CGRP-ir neural cell bodies but no labeling of vascular smooth muscle in lumbar DRG. However our data are in agreement with numerous studies showing the presence of ETA-R in vascular smooth muscle in various central and peripheral organs, including brain (10), spinal cord (43), heart and kidney (e.g. 10). Even if the intense labeling of the smooth muscle cells would obscure a faint labeling of the sensory
nerves in our studies, our findings would suggest that the predominant structures labeled with the ETA-R antibody are the smooth muscle fibers in the renal pelvic wall and vasculature both in the kidney and DRG.

**Role of ETB-R and ETA-R in the dietary sodium induced modulation of the interaction between ERSNA and ARNA.** Although much attention has been placed on ET as a mediator of pain (e.g. 4,18), previous studies suggested that ET may also play a role in the maintenance of water and sodium homeostasis. Activation of ETB-R is known to cause a diuresis and natriuresis (47). The ETB-R deficient rat and the mouse with collecting duct-specific knockout of ET-1 develop salt sensitive hypertension (1,7,44), presumably to facilitate excretion of an increased sodium load. Moreover, our previous studies demonstrated a role for ET in the activation of renal mechanosensory nerves that was dependent on dietary sodium (24). The current results extend our previous findings and provide further evidence for ET as an important component of the spectrum of renal mechanisms involved in the renal control of water and sodium during changes in dietary sodium intake. The ERSNA-induced increases in ARNA were reduced by an ETB-R antagonist in HNa diet rats and enhanced by an ETA-R antagonist in LNa diet rats. The route of administration of the ET-R antagonists, i.e., directly into the renal pelvis, suggested that the effects of the ET-R antagonists on the ERSNA-ARNA interaction involved mechanisms at the peripheral sensory nerve endings in the renal pelvic area. This hypothesis was confirmed by the presence of ETA-R and ETB-R receptors close to sensory nerves in the renal pelvic wall and our studies in the isolated sympathetically denervated renal pelvic wall preparation. Adding an ETB-R antagonist to the incubation bath reduced the NE-induced release of PGE2 and substance P from HNa diet pelvises. Because the NE-release of substance P is dependent on intact PG syntheses (29), these data suggest that stimulation of ETB-R contributes to the enhanced
activation of renal sensory nerves by a PGE2-dependent mechanism. In contrast in renal pelvises from LNa diet rats, the ETA-R antagonist enhanced the NE-mediated release of substance P without affecting the release of PGE2, suggesting that the activation of ETA-R modulates renal sensory nerve activity by a mechanism downstream of PGE2 synthesis (Fig. 14).

The NE concentration required to activate renal sensory nerves in vitro was much lower than that in vivo. This was especially true for LNa dietary conditions. Renal COX-2 expression is regulated by dietary sodium intake. Renal medullary COX-2 expression is increased in HNa and decreased in LNa diet, whereas the reverse is true for renal cortical COX-2 expression (8,14,57). COX-2 is also present in the renal pelvic wall (27). Assuming that the expression of COX-2 is regulated by similar mechanisms in the renal pelvic wall and medulla, the impaired interaction between ERSNA and ARNA in LNa diet may in part be related to reduced renal pelvic wall COX-2 expression and increased activation of ETA-R. This hypothesis was tested and confirmed by our studies showing that adding an ETA-R antagonist and PGE2, at a subthreshold concentration for substance P release, to the incubation bath lowered the NE concentration required for substance P release from 6250 to 50 pM.

Possible mechanisms involved in the contribution of ET-1 to the modulation of interaction between ERSNA and ARNA produced by dietary sodium. There are numerous studies linking activation of ETB-R to increased PGE2 synthesis. Activation of ETB-R increases intracellular Ca++ via activation of phospholipase C (PLC) in isolated glomerular cells (46). This is of interest in view of numerous studies showing increases in intracellular Ca++ in glial cells in response to ET-1 (52) and that activation of protein kinase C (PKC) in the renal pelvic wall leads to induction of COX-2, increased PGE2 synthesis, activation of cAMP and substance P release (26,27,29). Furthermore, studies in astrocytes (34) and renal non-neural tissue (11) show that
ET-3 and ET-1 induce COX-2 expression, increase PGE_2 synthesis and activate cAMP (51), possibly by activating ETB-R (20). These previous studies together with our current findings suggest that in conditions of HNa intake, activation of ETB-R on Schwann cells enveloping sensory nerves in the renal pelvic wall contributes to the NE-mediated release of PGE_2, possibly involving activation of PLC/PKC, resulting in release of substance P and activation of the renal sensory nerves (Fig. 14).

The mechanisms involved in the ETA-R mediated suppression of the ERSNA-ARNA interaction are currently unknown. It is unlikely that the ETA-R mediated suppression of the responsiveness of renal sensory nerves is related to ETA-R induced renal pelvic wall contractions which most likely would increase rather than decrease ARNA. On the other hand, the presence of ETA-R on smooth muscle cells in small vessels in and adjacent to the renal pelvic wall may suggest that the ETA-R-induced modulation of renal sensory nerve activation is related to local vasoconstriction with ischemia leading to an increase in oxygen free radicals (35,38) which have been shown to impair carotid baroreceptor activity (36). The close anatomical relationship between ETA-R-ir smooth muscle cells and CGRP-ir fibers may support such an hypothesis. The lack of effect of BQ123 on the NE-induced release of PGE_2 suggests that activation of ETA-R suppresses the activation of renal sensory nerves by a mechanism(s) downstream of PGE_2 release (Fig. 14). These data together with our previous studies which showed that activation of ETA-R contributes to the ANG II induced suppression of the activation of renal mechanosensory nerves (21) may suggest that the ETA-R induced suppression of the renal sensory nerves involves inhibition of the PGE_2-mediated activation of adenylyl cyclase by a pertussis toxin sensitive mechanism (22).

**Perspectives**
In conditions of HNa dietary conditions, the enhancement of the interaction between ERSNA and ARNA would increase the inhibitory renorenal reflex control of ERSNA resulting in suppression of ERSNA to prevent/limit sodium retention. In contrast in conditions of LNa dietary intake, suppression of the interaction between ERSNA and ARNA would increase ERSNA via impairment of the renorenal reflex mechanism leading to sodium retention. The importance of this interaction in the control of water and sodium homeostasis and the regulation of arterial pressure has been shown in rats with disrupted afferent renal innervation. These rats are characterized by increased ERSNA responsiveness to general activation of the sympathetic nervous system eventually leading to renal sodium retention and increased arterial pressure in conditions of increased dietary sodium intake (23,31).

Our data showing that ET-1 may enhance or suppress the interaction between ERSNA and ARNA dependent on the dietary sodium intake suggest that ET-1 contributes importantly to the renal mechanisms involved in the control of ERSNA and maintenance of sodium balance. Thus an impairment of the interaction between ERSNA and ARNA may contribute to the salt sensitive hypertension in the ETB-R deficient rat (7,44). Interestingly, administration of an ETA-R antagonist reduces arterial pressure in these rats.
ACKNOWLEDGMENT

This work was supported by grants from the Department of Veterans Affairs, The National Institutes of Health, Heart, Lung and Blood Institute, RO1 HL66068, American Heart Association Heartland Affiliate Grant-In-Aid 0750046Z, the Alfried Krupp von Bohlen and Halbach Foundation Collegiate Program “Life Science – Medicine, Life and Health” Sciences at the Alfried Krupp Science College in Greifswald, Germany, Swedish Research Council (04X-2887) and Knut and Alice Wallenberg Foundation, Sweden.

We are grateful for generous supply of the CGRP antisera from the late Dr. J.H. Walsh and Dr. H.C. Wong, The Center for Ulcer Research and Education of the Veterans Affairs/University of California Gastroenteric Biology Center, Los Angeles (antibody/RIA Core Grant #DK41301).

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung and Blood Institute or the National Institutes of Health.
REFERENCES


40. **Nakagomi S, Kiryu-Seo S, Kiyama H.** Endothelin-converting enzymes and endothelin receptor B messenger RNAs are expressed in different neural cell species and these
messenger RNAs are coordinately induced in neurons and astrocytes respectively following nerve injury. *Neurosci* 101:441-449, 2000.


56. Xia Y, Karmazyn M. Obligatory role for endogenous endothelin in mediating the hypertrophic effects of phenylephrine and angiotensin II in neonatal rat ventricular


FIGURE LEGENDS

**Figure 1.** mRNA expression of ETB-R (left panel) and ETA-R (right panel) in T\(_9\)-L\(_1\)DRG and renal pelvic wall from rats fed HNa and LNa diet. mRNA of ET-R was normalized to the geometric mean of two housekeeping genes, PBGD and Ywhaz. *P<0.05 LNa vs HNa diet.

**Figure 2.** Protein expression of ETB-R (left panel) and ETA-R (right panel) in T\(_9\)-L\(_1\)DRG and renal pelvic wall from rats fed HNa and LNa diet. The ratio of ET-R/actin protein expression was normalized to the average value of the ratio in HNa diet rats. *P<0.05 LNa vs. HNa diet.

**Figure 3.** Immunofluorescence for ETB-R (a) in renal pelvic tissue shows nerve fiber-like structures (arrows) among smooth muscle cells. ETA-R-LI is present on smooth muscle cells in the renal pelvic wall and adjacent blood vessels (arrows, b).

**Figure 4.** Immunofluorescence double-labeling of renal pelvic tissue shows ETB-R-ir fiber-like structures among smooth muscle cells (a-c) close to CGRP-ir sensory nerve fibers (d-f). Likewise, ETB-R-ir and CGRP-ir fibers are overlapping in a nerve bundle close to the renal pelvic wall (g-i).

**Figure 5** Immunofluorescence double-labeling of T\(_{13}\)DRG shows colocalization of ETB-R-LI with GFAP-LI (arrows) surrounding neuronal cell bodies (a-c). Also, many ETB-R-ir fiber-like structures are colocalized with GFAP-ir structures the renal pelvic wall (d-f) and in a nerve bundle close to the renal pelvic wall (g-i)(arrows).

**Figure 6** Immunofluorescence double-labeling of renal pelvic tissue shows ETA-R-ir on smooth muscle cells (a-c). CGRP-ir sensory nerve fibers (arrows) are found among the ETA-R-ir smooth muscle cells (d-f). In T\(_{11}\)DRG (g-i) and T\(_{10}\)DRG (j-l) ETA-R-LI was found on smooth muscle cells in vessels distributed among CGRP-ir neuronal cell bodies (arrows).
**Figure 7 in vivo:** Effects of placing the rat’s tail in water at 47°C or 49°C (thermal cutaneous stimulation) on ERSNA and ARNA in rats fed HNa, NNa and LNa diet. **P<0.01 vs. baseline; † P<0.05, ‡ P<0.01, responses in LNa vs. NNa and HNa diet, respectively. Δ ERSNA, efferent renal sympathetic nerve activity response; Δ ARNA, afferent renal nerve activity response; AUC area under the curve of RNA vs time.

**Figure 8 in vivo:** Effects of renal pelvic administration of norepinephrine (NE) at increasing concentrations on ARNA in rats fed HNa, NNa and LNa diet. **P<0.01 vs. baseline; ‡ P<0.01, ARNA responses to 2 pM NE in rats fed HNa vs. NNa diet and ARNA responses to 10 pM NE in rats fed HNa vs. LNa diet. † P<0.05, ARNA responses to 10 pM NE in rats fed HNa vs. NNa diet. For abbreviations see legend for Figure 7.

**Figure 9 in vivo:** (A). Effects of renal pelvic administration of the ETB-R antagonist BQ788 on the ERSNA and ARNA responses to placing the rat’s tail in 47°C water in rats fed HNa diet. (B). Effects of renal pelvic administration of the ETA-R antagonist BQ123 on the ERSNA and ARNA responses to placing the rat’s tail in 49°C water in rats fed LNa diet. **P<0.01 vs baseline; ‡ P<0.01, ARNA responses to thermal cutaneous stimulation in the absence and presence of renal pelvic perfusion with BQ788 (A) and BQ123 (B). For abbreviations see legend for Figure 7.

**Figure 10 in vivo:** Effects of renal pelvic perfusion with BQ788 and BQ123 on the ARNA responses to placing the rat’s tail in 49°C water. **P<0.01 vs. baseline. For abbreviations see legend for Figure 7.

**Figure 11 in vitro:** Effects of 250 pM NE on the release of substance P (left panel) and PGE₂ (right panel) from isolated renal pelvic wall preparations from rats fed HNa, NNa and LNa diet. **P<0.01 vs. baseline; ‡ P<0.01, NE-induced release of substance P and PGE₂ from renal
pelvises derived from HNa and NNa diet rats vs. LNa diet rats; § P<0.01 NE-induced substance P release from renal pelvises from rats fed NNa vs HNa diet rats; † P<0.01, baseline substance P release from renal pelvises from LNa vs HNa diet rats. For abbreviations see legend for Figures 7&8.

**Figure 12 in vitro:** (A) Effects of BQ788 on the release of substance P and PGE2 produced by 10 pM NE in isolated renal pelvic wall preparations from rats fed HNa diet. (B) Effects of BQ123 on the release of substance P and PGE2 produced by 6250 pM NE in isolated renal pelvic wall preparations from rats fed LNa diet. **P<0.01, vs. baseline; † P<0.05, NE-induced PGE2 release in the presence of vehicle vs. BQ788; ‡ P<0.01 NE-induced substance P release in the presence of vehicle vs. BQ788 (A) and BQ123 (B). For abbreviations see legend for Figures 7&8.

**Figure 13 in vitro:** Effects of BQ788 and BQ123 on the release of substance P produced by NE, 250 pM (left panel) and 50 pM (right panel), respectively, in NNa diet rats. **P<0.01 vs baseline. For abbreviations see legend for Figures 7&8.

**Figure 14** Our studies suggest that ERSNA-induced increases in NE release lead to an increase in renal pelvic PGE2 synthesis/release. In conditions of HNa dietary intake, activation of ETB-R on or close to unmyelinated Schwann cells surrounding the sensory nerves in the renal pelvic wall facilitates/enhances the synthesis/release of PGE2 leading to enhanced release of substance P and increases in ARNA. The increased ARNA exerts a negative feedback control of ERSNA via activation of the renorenal reflexes in the overall goal of maintaining a low level of ERSNA during HNa dietary intake. In conditions of LNa dietary intake, increased expression of ETA-R on smooth muscle fibers close to the sensory nerves in the renal pelvic wall leads to ET-1-induced suppression of NE-induced release of substance P and ARNA by a mechanism(s)
downstream of PGE₂ synthesis. Suppression of ARNA leads to disinhibition of the renorenal reflex control of ERSNA which would contribute to compensatory sodium retention during LNa dietary intake For abbreviations see legend for Figures 7&8.
Table 1. Effects of thermal cutaneous stimulation on mean arterial pressure in rats fed high, normal and low sodium diet during vehicle administration.

<table>
<thead>
<tr>
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<th>High NaCl diet, n=20</th>
<th>Normal NaCl diet, n=20</th>
<th>Low NaCl diet, n=22</th>
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<tr>
<td>ΔMAP, mmHg·sec</td>
<td>4470±550**</td>
<td>4080±20**</td>
<td>2280±300**‡†</td>
</tr>
</tbody>
</table>

ΔMAP responses expressed as area under the curve of MAP vs time; ** P<0.01 vs baseline; † p<0.05 vs normal sodium diet, ‡ P<0.01 vs high sodium diet.
Table 2. Effects of repeated thermal cutaneous stimulation in rats fed high or low sodium diet in the presence of vehicle, time control

<table>
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<th>Low NaCl Diet, n=8</th>
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<tbody>
<tr>
<td>ΔARNA, %·sec</td>
<td>6750±430*</td>
<td>11840±3080‡**</td>
</tr>
<tr>
<td>ΔERSNA, %·sec</td>
<td>6450±890**</td>
<td>11060±1770‡**</td>
</tr>
<tr>
<td>ΔMAP, mmHg ·sec</td>
<td>4040±1050**</td>
<td>3950±840*</td>
</tr>
</tbody>
</table>

ΔARNA, ΔERSNA and ΔMAP responses expressed as area under the curve of ARNA, ERSNA or MAP vs. time; *, ** P<0.05 and P<0.01, respectively vs. baseline; † P<0.05, high sodium diet: vehicle-1 vs vehicle-2 responses; ‡ P<0.05 and P<0.01 vehicle-1 or vehicle-2 responses in low vs. high sodium diet rats.
Table 3. Effects of norepinephrine 1250 pM on substance P and PGE$_2$ release from isolated renal pelvic wall preparations derived from low sodium diet rats.

<table>
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<th>PGE$_2$, pg/min</th>
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<tr>
<td></td>
<td>Control</td>
<td>NE, 1250pM</td>
</tr>
<tr>
<td>Vehicle</td>
<td>5.4±0.6</td>
<td>5.0±0.7</td>
</tr>
<tr>
<td>BQ123</td>
<td>5.7±0.9</td>
<td>6.2±0.6</td>
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</tbody>
</table>

** P<0.01 vs. average of control and recovery.
Table 4. Effects of BQ123, 1 μM, on the release of substance P produced by norepinephrine 50 pM in isolated renal pelvic wall preparations derived from low sodium diet rats. PGE$_2$, 0.03 μM, was present throughout the experiment.

<table>
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<tr>
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<th>Control</th>
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<tr>
<td>Vehicle+PGE$_2$</td>
<td>8.1±1.1</td>
<td>8.9±1.3</td>
<td>7.1±0.9</td>
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<tr>
<td>BQ123+PGE$_2$</td>
<td>7.9±1.0</td>
<td>15.5±2.2‡**</td>
<td>7.1±1.2</td>
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</table>

N=13, ‡ P<0.01 increase in substance P release produced by NE in the presence of BQ123 vs vehicle; ** P<0.01 vs average of control and recovery.
Figure 1

Gene Expression of ETB- and ETA-Receptors in Dorsal Root Ganglia and Renal Pelvic Wall

**ETB-Receptors**

- High NaCl diet
- Low NaCl diet

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<thead>
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</tr>
<tr>
<td>Low NaCl diet</td>
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**ETA-Receptors**

- High NaCl diet
- Low NaCl diet

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<tr>
<td>Low NaCl diet</td>
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Protein Expression of ETA and ETB Receptors in Dorsal Root Ganglia and Renal Pelvic Wall

**Figure 2**

<table>
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<th>Hi NaCl</th>
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**ETA-Receptors**

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*P< 0.05

n=4
Figure 3
ERSNA and ARNA Responses to Thermal Cutaneous Stimulation

Figure 7

Diet: High NaCl Normal NaCl Low NaCl
n=20 n=20 n=22

\(47^\circ \text{C} \quad 47^\circ \text{C} \quad 49^\circ \text{C} \quad 49^\circ \text{C} \quad 49^\circ \text{C} \quad 49^\circ \text{C} \quad 49^\circ \text{C} \)
Figure 8

**ARNA Responses to Renal Pelvic Administration of Norepinephrine**

- **High NaCl, n=11**
- **Normal NaCl, n=8**
- **Low NaCl, n=11**
Effects of BQ788, ETB-R Antagonist, and BQ123, ETA-R Antagonist, on the ERSNA and ARNA Responses to Thermal Cutaneous Stimulation

**High NaCl Diet**

- **Vehicle**
- **BQ788**

**Low NaCl Diet**

- **Vehicle**
- **BQ123**

Figure 9
Effects of BQ788 and BQ123 on the ARNA Responses to Thermal Cutaneous Stimulation

Figure 10

Effects of BQ788 and BQ123 on the ARNA Responses to Thermal Cutaneous Stimulation

Normal NaCl Diet

Vehicle
BQ788
BQ123

\( \Delta \text{ ARNA, } \% \text{, sec (AUC)} \)

n=10
n=10

**
Isolated renal pelvises from High, Normal and Low Sodium Diet rats

**Figure 11**

**Substance P**

- **Vehicle**
- **NE, 250 pM**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Substance P release, pg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>High NaCl n=8</td>
<td>25 <strong>,</strong></td>
</tr>
<tr>
<td>Normal NaCl n=8</td>
<td>15 <strong>,</strong></td>
</tr>
<tr>
<td>Low NaCl n=12</td>
<td>10</td>
</tr>
</tbody>
</table>

**PGE₂**

- **Vehicle**
- **NE, 250 pM**

<table>
<thead>
<tr>
<th>Diet</th>
<th>PGE₂ release, pg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>High NaCl n=8</td>
<td>200 <strong>,</strong></td>
</tr>
<tr>
<td>Normal NaCl n=6</td>
<td>150 <strong>,</strong></td>
</tr>
<tr>
<td>Low NaCl n=12</td>
<td>100 <strong>,</strong></td>
</tr>
</tbody>
</table>

Note: *Significant difference from Vehicle group. **Significant difference from Normal NaCl group. ***Significant difference from Low NaCl group.
Effects of the ETB-R Antagonist BQ788 - Isolated Renal Pelvis

High NaCl diet

**Figure 12A**

Effects of the ETB-R Antagonist BQ788 on Substance P and PGE$_2$ release.

**Substance P**
- Vehicle
- BQ788

**PGE$_2$**
- Vehicle
- BQ788

*Note:* Values are mean ± SEM, n=12
Effects of the ETA-R Antagonist BQ123 - Isolated Renal Pelvis

**Low NaCl diet**

**Substance P**

- Vehicle
- BQ123

**PGE\(_2\)**

- Vehicle
- BQ123

Figure 12B
Effects of the ETB-R Antagonist BQ788 and the ETA-R Antagonist BQ123 - Isolated Renal Pelvis

**Normal NaCl diet**

Figure 13
Figure 14

**Hypothesis**

- ERSNA
  - NE
    - ETB-R
      - LNa diet
        - cAMP
          - ETA-R
            - ARNA
            - Substance P
          - PGE₂