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REGIONAL CHANGES IN CARDIAC AND STELLATE GANGLION NOREPINEPHRINE TRANSPORTER IN DOCA-SALT HYPERTENSION

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Abstract

Uptake of norepinephrine via the neuronal norepinephrine transporter is reduced in the heart during deoxycorticosterone (DOCA)-salt hypertension. We hypothesized that this was due to reduced norepinephrine transporter mRNA and/or protein expression in the stellate ganglia and heart. After 4-weeks of DOCA-salt treatment there was no change in norepinephrine transporter mRNA in either the right or the left stellate ganglia from hypertensive rats (n=5–7, p>0.05). Norepinephrine transporter immunoreactivity in the left stellate ganglion was significantly increased (n=4, p<0.05) while the right stellate ganglion was unchanged (n=4, p>0.05). Whole heart norepinephrine content was significantly reduced in DOCA rats consistent with reduced uptake function; however, when norepinephrine was assessed by chamber, a significant decrease was noted only in the right atrium and right ventricle (n=6, p<0.05). Cardiac norepinephrine transport binding by chamber revealed that it was only reduced in the left atrium (n=5–7, p>0.05). Therefore, 1) contrary to our hypothesis reduced reuptake in the hypertensive heart is not exclusively due to an overall reduction in norepinephrine transporter mRNA or protein in the stellate ganglion or heart, and 2) norepinephrine transporter regulation occurs regionally in the heart and stellate ganglion in the hypertensive rat heart.

Keywords

hypertension; noradrenaline; NAT; NET; stellate ganglion; cardiac

Introduction

The sympathetic innervation from the heart arises primarily from the stellate ganglia. Bilateral sympathetic block at T3 (stellate ganglion) in humans reduces heart rate, systolic

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blood pressure and diastolic blood pressure in hypertensive patients, but has no effect on blood pressure in normotensive patients (Chou et al., 2005). This supports the notion that there are changes in the stellate ganglion and/or its cardiac-projecting axons during hypertension that may play a role in the disease. While hypertension is a multi-factorial condition, a common finding is that there is a reduction in uptake of norepinephrine (NE) in the sympathetic innervation of the hypertensive heart. Since the actions of released NE in the heart are terminated primarily by neuronal reuptake of NE into sympathetic nerve terminals (Hertting, 1961), one possibility is that expression of neuronal norepinephrine transporter (NET) mRNA and protein in the stellate ganglion and associated cardiac sympathetic terminals are decreased in hypertension underlying the observed reduction in NET functionality.

Many of the changes observed in NE metabolism, which are associated with hypertension, can be accounted for by reduced function of NET. An inverse correlation exists in multiple models of hypertension between blood pressure and [^{3}H] NE accumulation in the heart *in vivo* (de Champlain et al., 1967, Kazda et al., 1969,). This loss of NE occurs in parallel with elevating blood pressure and increasing inotropic response to NE (LeLorier et al., 1976). Lean hypertensive patients have increased spillover of NE from the heart, reduced fractional extraction of infused [^{3}H] NE, increased NE extraneuronal metabolites (due to elevated junctional NE) and reduced release of the intraneuronal metabolite [^{3}H] DHPG (as a result of decreased reuptake into nerve terminals) (Rumantir et al., 2000). Additionally, in some hypertensive patients, an increase in muscle sympathetic nerve activity, elevated total and cardiac-specific NE spillover, reduction of DHPG in plasma and a diminished effect of NET blockade with despiramine was observed (Schlaich et al., 2004). Moreover, reductions in cardiac NE reuptake and reductions in NE content of the heart have been observed in numerous animals models of hypertension (Iversen, 1963, de Champlain et al., 1966, de Champlain et al., 1967, Kazda et al., 1969, Gudeska et al., 1976, LeLorier et al., 1976, Rascher et al., 1981, Krakoff et al., 1985) and in human subjects (Grassi et al., 1999, Shannon et al., 2000, Esler et al., 2001, Goldstein et al., 2002).

Furthermore, there is a relationship between the amount of NE in the heart and the reuptake capacity in the organ. Lee et al, showed a positive relationship between NE content and NET such that NET binding sites are reduced when NE is depleted with reserpine, while NET binding is increased when NE levels are raised by treatment with monoamine oxidase inhibitors (Lee et al., 1983). Consequently, the reported reduction in NE content in the heart during hypertension (Krakoff et al., 1967, Kazda et al., 1969, de Champlain et al., 1967) could also be related to a reduction in NET.

Therefore, there are compelling reasons to further investigate the role of cardiac NET in hypertension. The purpose of this study was to perform molecular and biochemical studies on NET mRNA and protein in the cardiac sympathetic nerves in hypertension. We hypothesized that NET mRNA and protein would be reduced in the bilateral stellate ganglia and heart chambers in DOCA-salt hypertensive rats (HT) offering a mechanistic explanation for the well-established reduction in NE uptake function and NE content as has been described for many years in the hypertensive heart.

Materials and Methods

Animals

Adult male Sprague Dawley rats (250–300 g; Charles River Laboratories, Inc., Portage, MI, n=42) were used. The hypertensive group (HT) underwent uninephrectomy and subcutaneous implantation of deoxycorticosterone acetate salt (DOCA, 200 mg kg^{-1}) under isoflurane anesthesia. Post-operatively, the rats were given drinking water containing 1%

NaCl and 0.2%KCl. Herein, the DOCA-salt treated group is referred to as hypertensive (HT). Normotensive (NT) controls to the HT rats were uninephrectomized but were not given DOCA implantation or salt drink. Four weeks after surgery, the arterial blood pressure was measured using the tail cuff method. Rats with a mean systolic arterial pressure above 150 mmHg were considered hypertensive. All animal experiments were performed in accordance with the "Guide for the Care and Usage of Laboratory Animals" (National Research Council) and were approved by the Animal Use and Care Committee of Michigan State University. Animals were housed two per cage in temperature and humidity-controlled rooms with a 12 hour/12 hour light-dark cycle. Standard pellet rat chow and water were given *ad libitum*.

Tissue Collection

Rats were anesthetized with sodium pentobarbital (Sigma-Aldrich Corp, St. Louis, MO, 65 mg/kg, intra-peritoneal) followed by thoracotomy. Hearts were removed and placed into chilled (4 °C) phosphate buffered saline (PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM sodium phosphate dibasic and 1.4 mM potassium phosphate monobasic) for rinsing and separation of chambers. Frozen tissues were stored at −80° C until use.

Stellate Immunohistochemistry

Stellate ganglia were fixed in 10% neutral buffered formalin for 2 hours then transferred to 70% ethanol, routinely processed, embedded in paraffin and sectioned on a rotary microtome at 5μM. Standard avidin-biotin complex staining steps were performed at room temperature. The polyclonal primary antibody NET 48411 was used (1:250 in Scytek normal antibody diluent (Scytek, Logan, UT)). Biotinylated goat anti-rabbit IgG H+L (Vector, Burlingame CA) in normal antibody diluent 1:200 was applied for 30 minutes followed by application of Vectastain® Elite ABC Reagent (Vector, Burlingame CA) for 30 minutes. Slides were developed using Nova Red Peroxidase substrate kit (Vector, Burlingame CA) for 15 minutes. Sections (15μm) of right and left stellate ganglia from an animal were mounted on the same slide for a direct comparison under the same experimental conditions. Brightfield images were digitized using a SPOT RT Slider camera (Diagnostic Instruments, Inc, Sterling Heights, MI) at a resolution of 1520 × 1080 pixels and 24 bit RGB. NET staining in ganglia was quantified by assessing all individual neuron cell bodies in a single section of stellate ganglia using NIH Image J Version 1.37 software. The nucleus did not stain positive for NET and was not included in the determination of staining intensity. The arbitrary intensity of NET staining was determined on color inverted images by using the straight line measurement tool and drawing a line in a region defined by the user to contain cytoplasmic NET staining in every cell in a section from each ganglion. The mean intensity values in arbitrary units for individual neurons were then averaged to determine total NET staining intensity per ganglia. Staining intensity of right stellate ganglia was compared to left stellate ganglia using a paired t-test.

RNA isolation and real time PCR

Frozen ganglia in TRIzol® were thawed on ice and homogenized using an RNase-free Kontes Pellet Pestle® with hand-held motor in a compatible RNase-free Kontes microcentrifuge tube (Fisher Scientific, Hanover Park, IL). Total RNA was isolated from all tissues using the standard TRIzol® procedure (GIBCO Life Technologies, Carlsbad, CA) with the use of glycogen as an RNA carrier for ganglia. The concentration and purity/integrity of RNA was ascertained spectrophotometrically (A260/A280 and A260/A230) using a Nanodrop ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE). To eliminate residual genomic DNA in the preparation, total RNA samples were treated with diluted RNase-free DNase I (10U/μl, Roche Diagnostics, Nutley, NJ) for 30 minutes at 37°C.

DNase I was inactivated by heating for 10 minutes at 75°C. Primers were tested to determine efficiency and this value was used in calculations of fold-change. NET forward primer: 5′-GCC TGA TGG TCG TTA TCG TT-3′, NET reverse primer: 5′-CAT GAA CCA GGA GCA CAA AG-3′, GAPDH forward primer: 5′-ATC ACT GCC ACT CAG AAG-3′, GAPDH reverse primer: 5′-AAG TCA CAG GAG ACA ACC-3′. A two-step RT–PCR was performed. The first strand complementary DNA (cDNA) was synthesized from a starting amount of 70 ng total DNase treated RNA for real time PCR by adding the following components into a nuclease-free microcentrifuge tube (20 μl reaction volume): Oligo (dT) (500 μg/ml) (Invitrogen, Carlsbad, CA), 10mM dNTP mix (Invitrogen, Carlsbad, CA), 5X first strand buffer, 0.1 M DTT (dithiothreitol), RNase inhibitor (Roche Diagnostics, Indianapolis, IN) and Superscript II RNase H- reverse transcriptase (Invitrogen, Carlsbad, CA). Samples were mixed, incubated at 42°C for 60 minutes and Superscript II was inactivated by heating at 70°C for 15 minutes. Additional samples were run without reverse transcriptase enzyme (no RT) to rule out contamination. A negative control was run with no cDNA template added to the PCR reaction (No Template Control).

For real time PCR, a 25 μl PCR reaction volume was prepared with cDNA (70ng/μl) from the first-strand reaction, forward primer (20mM), reverse primer (20 mM), SYBR Green Supermix (Applied Biosystems, Bedford, MA) and DEPC-treated distilled water (Ambion, Austin, TX). Real time PCR thermal profile was set up according to manufacturer's instructions for SYBR green and run for 40 cycles. A dissociation protocol (60–95°C melt) was done at each end of the experiment to verify that only one amplicon was formed during the process of amplification. Relative quantification of mRNA was measured against the internal control, GAPDH. End point, used in qPCR quantification and Cycle threshold (Ct) value is defined as the PCR cycle number that crosses a calculated signal threshold. Relative quantification of mRNA was measured against the internal control, GAPDH. Relative expression value calculation was done using raw Ct value and statistical analysis was performed by Pair Wise Fixed Reallocation Randomization Test© (http://www.gene-quantification.info) using Relative Expression Software Tool (REST 384) (Grassi et al., 1999). All samples compared were run on a single 96 well plate whenever possible to allow for accurate comparisons. NET expression in NT animals from both right and left stellate ganglia was normalized to 1 in order to calculate a relative expression ratio of NT to HT. The variability of the data is most accurately assessed using Ct values and not converted data. Raw values are provided with standard error.

[^{3}H]-nisoxetine binding

Preparation of Total Cardiac Membranes: Frozen heart chambers were pulverized on dry ice and then transferred to a mortar and pestle that had been pre-chilled with liquid nitrogen. Tissue was further processed by grinding, then suspended in the appropriate amount of homogenization buffer without detergent to keep membranes intact (50 mM Tris pH 7.4, 120 mM NaCl, 5 mM KCl). The suspended tissue in solution was transferred to an ice-cold 10 ml glass hand-held homogenizer for 10 strokes of further gentle processing in ice. For ventricular tissue, the homogenate was centrifuged (Sorvall RC 5B Plus) at 700 × g for 10 min at 4°C to remove nuclei and cellular debris. Atrial membranes were used without centrifugation. Samples were spun at 40,000 × g for 30 minutes after which the supernatant was discarded and the pellet resuspended in an additional 4 ml of buffer. A second identical spin was performed, supernatant discarded, and the pellet stored at −80°C until use.

Binding Assay

Differences in NET protein expression in cardiac membranes from individual heart chambers from NT and HT animals were determined by assessing [^{3}H]-Nisoxetine (Perkin-Elmer, Waltham, MA) binding in a manner similar to that described previously (Lee et al.,

1983, Zhen et al., 2012). Frozen membrane pellets were resuspended in ice-cold incubation buffer (50 mM Tris pH 7.4, 300 mM NaCl, 5 mM KCl) on ice just prior to use. The resuspended membranes were loaded in quadruplicate into 96-well reaction plates and aliquots were used in parallel for a Bradford protein assay to determine protein concentration. Samples were assessed for total and background binding. Cardiac membranes from both groups were incubated with 15 nM [^{3}H]-Nisoxetine in duplicate with additional duplicate wells used to determine non-specific binding with 1.5 mM desipramine. Samples were incubated on a shaker for a minimum of 4 hours at 0° C then filtered through glass fiber filters presoaked in 0.5% polyethylenimine using a 96-well Filtermate cell harvester (Packard Biosciences, Shelton, CT, USA). Standard scintillation counting was performed using Ecolite scintillation fluid (ICN Biomedicals, Irvine CA, USA).

Norepinephrine measurements

Heart chambers were homogenized in ice-cold 0.1 M perchloric acid. The homogenate was then centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was further processed by solid phase extraction using Oasis MCX cartridges, 30 mg/1cc (Waters, Milford, MA, USA) as previously described (Backs et al., 2001). NE levels in the heart chambers were determined using the eluate analyzed by capillary electrophoresis with electrochemical detection (CE-EC), as described elsewhere (Kreusser et al., 2008), using a boron-doped diamond electrode. The separation and detection was performed using the following conditions: 76 cm long, 362 μm o.d., 29 μm i.d. capillary, 250 mM boric acid − 1 M potassium hydroxide run buffer of pH 8.8, separation voltage 24 kV, detection potential +0.86 V vs Ag/AgCl and electrokinetic injection at 18 kV for 8 s.

NGF ELISA

Frozen hearts were pulverized and suspended in sample buffer consisting of 0.1M phosphate buffered saline (PBS: 0.225 M NaCl, 0.02 M NaH_2PO_4, 0.08 M $NaHPO_4$), containing 0.1% Tween-20, 0.05% bovine serum albumin (BSA), aprotinin (6.6 trypsin inhibitor unit/mL), 0.2 mM Benzamidine, 0.01 mM Benzethonium Chloride and 0.2 mM ethylenediaminetetra-acetic acid (EDTA) (all from Sigma, St. Louis, MO). The chilled suspension was homogenized for 30 s using a variable speed Tissue Tearor (Biospec Products, Inc., Bartlesville, OK) then centrifuged at 13,000 × g.

Ninety six well plates (NUNC-Immuno™, Rochester, NY) were coated with 1 μg/ml anti-NGF monoclonal antibody (R & D Systems, Minneapolis, MN) in PBS, pH 7.4, overnight at room temperature (RT). All incubations were performed in a humidified chamber at RT and plates were washed between each step. Plates were blocked with 200 μl/well of 1.0% BSA and 5% sucrose in PBS for 1 hour. 100 μl/well of NGF standard or sample was added in quadruplicate. Each well received 100 μl of biotinylated anti-NGF secondary antibody (100 ng/ml, R & D Systems, Minneapolis, MN) diluted in Tris buffered saline (TBS, pH 7.3) which contained 1% BSA, 0.05% Tween-20 and incubated for 2 hours. 100 μl of streptavidin linked β-galactosidase (100 μg/mL, Molecular Probes, Eugene, OR) diluted in TBS containing 1% BSA and 0.05% Tween-20 was added and incubated for 20 minutes. Galactosidase substrate chlorophenolred-beta-D-galactopyranoside (CPRG, Roche, Palo Alto CA) was dissolved in substrate buffer (0.1 M sodium phosphate, 1 mM $MgCl_2$, 1% BSA, pH 7.4). Room temperature plates were incubated in a humidified chamber with substrate buffer with CPRG overnight. Absorbance was measured at 575 nm using a microplate spectrophotometer.

Results

Blood pressure

Systolic blood pressure was significantly elevated following 4-weeks of DOCA-salt hypertension (HT) compared to uninephrectomized control rats (NT) (Fig 1, $p<0.001$). HT rats had a mean systolic blood pressure of 185.2 ± 6.97 mmHg (n=19) while NT had a mean systolic blood pressure of 142.6 ± 4.03 mmHg (n=23).

NET mRNA expression in stellate ganglion

NET mRNA was expressed in right and left stellate ganglia in both HT and NT groups. There was no difference in expression of NET mRNA in NT versus HT in either the left (Fig 2, left panel; 1.00 vs 1.08 ± 0.45 relative expression ratio, $p>0.05$, n=5) or right (Fig 2, right panel; 1.00 vs 1.47 ± 0.84 relative expression ratio, $p>0.05$, n=7) stellate ganglia. Cycle threshold (Ct) values were used to calculate relative expression ratio. In both NT and HT animals there was no relationship between blood pressure and the levels of NET mRNA in either the right ($r^2=0.03$, $p>0.05$) or left stellate ($r^2=0.01$, $p>0.05$) ganglia (Fig 3).

NET immunoreactivity in stellate ganglion

There was no difference in the intensity of NET staining between left and right stellate ganglia in normotensive animals (Fig 4; $p>0.05$, n=4). When comparing NT to HT for each ganglia, NET staining intensity was significantly greater in HT animals compared to NT animals in the left stellate ($p<0.05$, n=4), but there was no difference in NET staining intensity in the right stellate ganglion from HT animals compared to NT animals (Fig 4; $p>0.05$, n=4). In NT versus HT animals, there was only a significant positive correlation between blood pressure and NET immunoreactivity observed in the left stellate ganglion ($r^2=0.58$, $p<0.05$) versus the right stellate ganglion ($r^2=0.16$, $p>0.05$) (Fig 5).

Chamber NE content

Assessment of chamber NE content was performed in NT and HT rats (Fig 6A). In the right atrium, NE was significantly lower in HT (0.023 ± 0.005 μg NE) versus NT (0.052 ± 0.006 μg NE, $p<0.05$, n=5–6). Additionally, right ventricle NE levels were significantly less in HT (0.088 ± 0.012 μg NE, n=5) compared to NT (0.140 ± 0.012 μg NE, $p<0.05$, n=4–5). No significant differences in tissue NE levels were found in left atrium, left ventricle or ventricular septum ($p>0.05$). Although significant differences in NE levels were only observed in the right atrium and right ventricle, when considered together these changes do reflect that total heart NE was significantly lower in HT (0.424 ± 0.042 μg, n=5) as compared to NT (0.555 ± 0.014 μg, $p<0.05$, n=4–5) (Fig 6B). In addition to data comparisons by μg, tissue NE concentrations normalized to chamber weight (μg/g tissue) were calculated in NT and HT rats (data not shown). Using chamber-normalized concentrations (μg/g chamber tissue), only the right atrium showed a significant NE concentration decrease in HT (0.522 ± 0.083 μg NE/g tissue) compared to NT (1.040 ± 0.111 μg NE/g tissue, $p<0.05$, n=5–6).

Nerve Growth Factor

NGF protein was measured in all individual heart chambers from NT and HT rats. There was no significant difference in the amount of NGF protein expressed in NT versus HT rats (Fig 7, $p>0.05$, n=4).

Regulation of NET mRNA in hypertension

NET mRNA expression from each chamber in NT animals was normalized to 1 so a relative expression ratio of NT to HT could be calculated. The left atrium was the only chamber in

which NET mRNA was significantly lower in HT compared to NT animals (1.00 vs 0.34 ± 0.12 relative expression ratio, $p<0.05$, n=6) (Fig 8). To the contrary, NET mRNA was increased in the left (1.00 vs 3.69 ± 0.93; $p<0.05$, n=7) and right ventricles (1.00 vs 1.69 ± 0.41; $p<0.05$, n=4) from HT rats.

NET protein in heart chambers

Chamber NET protein levels between NT and HT animals were compared using single concentration [^{3}H]-nisoxetine binding in cardiac membranes. In the right atrium from HT animals, there was a significantly greater specific [^{3}H]-nisoxetine binding to NET protein compared to NT (Fig 9A: 81.49 ± 0.49 vs 306.8 ± 42.63 fmol/mg, $p<0.05$, n=7–8). In contrast, there was significantly less specific [^{3}H]-nisoxetine binding in left atrium from HT animals compared to NT (Fig 9B: 160 ± 30.89 vs 79.21 ± 17.97 fmol/mg, $p<0.05$, n=5–7). There was no difference between NT and HT [^{3}H]-nisoxetine binding in the left ventricle (Fig 9C: 121.4 ± 15.84 vs 115.6 ± 17.68 fmol/mg, $p<0.05$, n=8).

Discussion

This study aimed to determine if the well-established reduction in cardiac neuronal NE reuptake in hearts from DOCA-salt hypertensive (HT) rats was due to a decrease in NET mRNA or protein in the sympathetic innervation of the heart either in the stellate ganglia or in the nerve terminals in each heart chamber. We determined that there was no evidence for NET mRNA or protein downregulation in stellate ganglia from DOCA HT rats. In the heart chambers, there was a regional reduction of NET mRNA as well as protein only in the left atrium. Therefore, the functional reduction in whole heart reuptake in hypertension is not due to a global reduction in NET mRNA or protein isolated from the stellate ganglia or heart in DOCA HT rats. Although reduced NE uptake and NE content in the hypertensive heart occurs in the hypertension, the underlying mechanisms investigated in this study reveal that regulation of this reduction is not as simple as downregulation of NET mRNA or protein. With the elimination of this possibility, further studies are appropriate to determine the mechanism.

NET in stellate ganglion in hypertension

It is generally accepted that synthesis of mRNA and protein for the cardiac sympathetic innervation occurs in the stellate ganglion with newly synthesized protein shipped to the distant cardiac nerve terminals via axonal transport. We hypothesized that a downregulation of NET mRNA and/or protein in the stellate ganglia may be responsible for the reduction in NE uptake in the hypertensive heart. Contrary to this, however, we observed no evidence of NET mRNA reduction in stellate ganglia. This rules out the role of transcriptional repression by DNA methylation (Guo et al., 2005), or otherwise, as a regulatory mechanism for NET mRNA in the stellate ganglia in hypertension. This suggests that either NET is regulated post-transcriptionally or there are alternative sites of NET mRNA outside sympathetic cell bodies that control NET protein expression in the heart.

In the present study, NET mRNA expression in the stellate ganglion did not reflect NET protein expression in either the stellates themselves or in the heart. Likewise, studies in heart failure and myocardial infarction demonstrate that NET mRNA in the stellate ganglion is unchanged when heart NET function is reduced suggesting that the reduction in NET protein is not due to a decrease in NET mRNA but rather is a post-transcriptional change (Backs et al., 2001, Kreusser et al., 2008). While there was no difference in NET mRNA in the right or left stellate ganglia in hypertension, the left stellate ganglion exhibited an increase in NET immunoreactivity. Since NET mediated reuptake of NE increases with elevated nerve firing (Eisenhofer et al., 1990) and there is elevated sympathetic drive to the heart in hypertension

(Julius et al., 2000, Smith et al., 2002, Grassi et al., 2004), it is feasible that there would be a corresponding elevation in NET protein in the stellate ganglia related to this increase in nerve activity.

Our study shows an increase in NET protein only in the left stellate. There is substantial cross innervation from both stellate ganglia to all heart chambers (Pardini et al., 1989, Lujan et al. 2012), so changes in the left stellate alone may impact whole heart function. The left stellate is functionally important in augmenting contractility and increasing coronary artery blood flow more so than regulating rate of contraction (Janes, et al., 1984). Left stellate blockade does not alter heart rate variability or have a role in SA node innervation (Fujiki et al., 1999). If the increased expression of NET protein in the left stellate ganglion cell bodies resulted in an increase in NET protein in the nerve terminals of these same cells, there would be an increased reuptake capacity in varicosities in the heart associated with a reduction in the post-junctional effects of NE. This is contrary to the findings that enhanced inotropic response in the hypertensive heart is associated with a reduction in NE uptake (LeLorier et al., 1976). This is further evidence that the stellate ganglion expression of NET does not reflect the function of NET in the heart.

We assessed the entire stellate ganglion rather than putative cardiac neurons alone. Not all cell bodies in the stellate ganglion project axons to the heart and therefore we measured NET mRNA and protein in both cardiac and non-cardiac neurons. Cardiac neurons exist in a zone near the exit of the cardiac nerve at the medial aspect of the ganglia (Wallis et al., 1996). While these neurons do not exhibit any significant differences in morphology compared to non-cardiac stellate neurons (Wallis et al., 1996), their function and response to hypertension may be different. This is based on the idea that there are functional specific pathways in autonomic ganglia (Janig et al., 1992). There is evidence that individual neurons can project selectively to a single organ or part of the organ such as the vasculature (Gibbins et al., 1998(a), Gibbons et al., 1998(b), Browning et al., 1999). Ideally, changes in NET expression in hypertension would be performed only in cardiac projecting neurons that exit the stellate ganglion via the cardiac nerve. This type of study is feasible using retrograde tracer in neurons that exit via the cardiac nerve (Wallis et al., 1996) or by injecting tracer in the heart wall (Wallis et al., 1996, Richardson et al., 2006, Lujan et al., 2012).

Assessment of heart chambers in hypertension: NE, NGF, and NET

NE content in the heart—Using the uninephrectomized normotensive control and the uninephrectomized DOCA-salt rats, a reduction in whole heart NE content has been repeatedly observed, (de Champlain et al., 1967, Krakoff et al., 1967, Kazda et al., 1969). Any regional differences in NE content would have been masked in an examination of whole heart NE. Using the same animal model, we also observed a significant reduction in total heart NE content with hypertension then further analyzed the heart by chamber to determine regional differences in NE content with hypertension. Decreased NE content was observed only in the right atrium and right ventricle demonstrating regional regulation similar to reported chamber differences in uptake function. In fact, there is evidence of both inter- and intra-chamber differences in NE uptake capacity (Chilian et al., 1982, Pierpont et al., 1984, Beau et al., 1994, Ieda et al., 2007) substantiating regional regulation of NET as a possible mechanism.

NGF content in heart chambers—NGF-overexpressing mice have a greater heart NE content, an increased presence of neuronal NE metabolites in the plasma, and greater neuronal uptake of NE in left ventricle tissue strips (Kiriazis et al., 2005). In hypertension, there is reduction in NGF protein in the heart but not NGF mRNA that occurs prior to the reduction of NE uptake. This finding suggests that NGF reduction may be the initial event

leading to NE uptake reduction (Kreusser et al., 2006). In another model of heart failure, the reduction in NE uptake and tissue NE levels with disease is recovered by injection of NGF into the stellate ganglion (Kreusser et al., 2006). Therefore, we examined if changes in NGF are related to the reduced NE uptake in the hypertensive heart. There were no differences in NGF in any chamber in the DOCA-salt heart, indicating that it is not NGF reduction that mediates the reduction in NE reuptake capacity.

NET mRNA in heart chambers—While there are ample functional data on NE uptake in the normal and diseased heart, the genetic regulation of NET is not well understood. Since the stellate ganglion pool of NET mRNA does not correlate with the reduction in NET function in disease, we aimed to determine if a downregulation of local NET mRNA in the heart was responsible for the reduction in protein function. We examined NET mRNA by chamber in hypertension to determine if a global downregulation of NET mRNA was associated with the established reduced uptake function of the heart. In DOCA-salt hypertension, NET mRNA in the heart chambers was regionally reduced in the left atrium only, thus a global reduction of NET mRNA is not the underlying cause of reduced NET function in hypertension. Notably, cardiac tissue NET mRNA is differentially regulated by chamber and could play an important role in NET protein expression and function in pathophysiological states.

NET binding in heart chambers—Since cardiac NE uptake is reduced in hypertension (Iversen, 1963, de Champlain et al., 1966, de Champlain et al., 1967, Kazda et al., 1969, Gudeska et al., 1976, LeLorier et al., 1976, Rascher et al., 1981, Krakoff et al., 1985), we predicted that there would be a reduction in NET protein in the heart chambers. We found that in the hypertensive animals there was a reduction in NET binding in the left atrium only. Thus, the reduction of heart NE reuptake is not due to a global reduction in cardiac NET protein expression and there is regional regulation of NET protein. There is a low amount of NET in the atria compared to the ventricles (Wehrwein et al., 2008) and the ventricles have greater uptake capacity compared to the atria so it is unlikely that a reduction in left atrium NET protein in hypertension would be enough to result in a reduction in whole heart uptake. Having ruled out a loss of NET protein in the heart as a cause for reduced reuptake, other factors that could cause a decrease in NE uptake must be considered.

Limitations

The uninephrectomized DOCA-salt model is not considered “garden variety” hypertension and therefore our findings may be more relevant to mineralocorticoid or exacerbated arterial hypertension. We have presented citations that include studies on DOCA-salt hypertension and other forms in animals and humans, but our conclusions may only be directly relevant to the DOCA-salt model. There was no attempt in this study to determine if the data we report are due to DOCA, salt, hypertension, or a combination of factors.

This study has ruled out suppression of NET expression in cardiac sympathetic nerves and the loss of cardiac NGF as possible mechanisms for the reduction in NET function in hypertension. Further studies must be conducted to evaluate the sympathetic dysfunction that occurs in the heart, determine if perhaps an anatomical reason (i.e. the immediacy of the synaptic nerve endings to the cardiac muscle) may be the underlying cause of changes seen in uptake.

Conclusion

We examined the mechanisms underlying the decrease in neuronal NE uptake and NE content in the DOCA-salt hypertensive heart. We found no evidence for NET mRNA or protein downregulation in either the right or the left stellate ganglia from hypertensive rats.

In the heart chambers, there was a regional reduction of NET mRNA and binding in the LA only. These changes were unrelated to NGF levels in the heart. Therefore, the functional reduction in whole heart reuptake in hypertension is not due to a global reduction in NET protein in the stellate ganglion and heart. Furthermore, stellate ganglia expression of NET does not predict the uptake capacity of the heart or explain the regional distribution of NET protein in heart chambers. The important site of study for changes in heart NE uptake therefore is the sympathetic nerve terminals and/or the alternative cellular sites of NET in the heart and not the stellate ganglion. Perturbations in NE release, NE storage in vesicles, and/or altered regulation of NET function or trafficking must be considered since a simple reduction in mRNA or protein expression in not the mechanism for the reduction in reuptake (Zahniser et al., 2001, Bonisch et al., 2006, Shanks et al. 2013). NET function can be altered by changing the transport efficiency (Zahniser et al., 2001) without altering total NET in the membrane. Modulators of NET function that could be considered include reduction in NE reuptake in the heart by endothelin-1 (Backs et al., 2005), aldosterone (Buss et al., 2006), and oxidative stress in PC12 cells (Mao et al., 2004). NE uptake is also altered by B- and C-type natriuretic peptides and angiotensin II (Bonisch et al, 2006).

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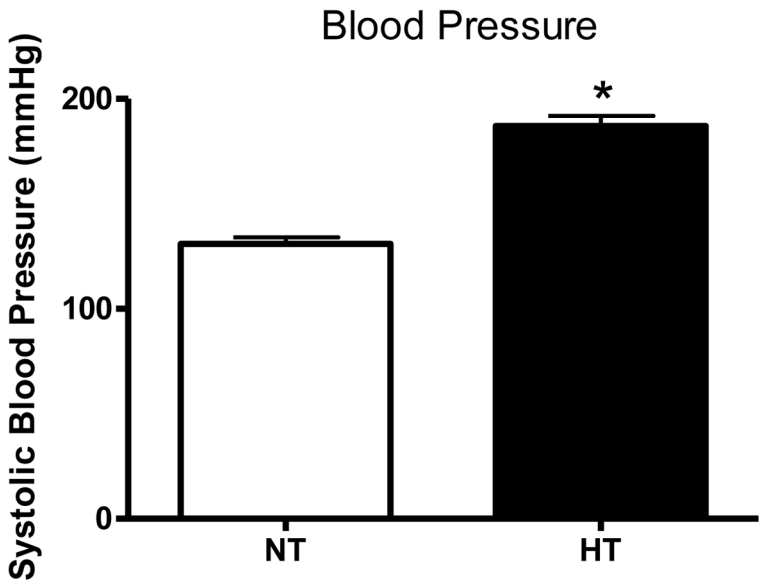

Figure 1. Blood pressure is elevated following 4-weeks of DOCA-salt treatment

Blood pressure was measured using tail cuff plethysmography and systolic blood pressure is reported as mmHg. Normotensive animals (NT) were uninephrectomized. Hypertensive animals(HT) were uninephrectomized, received an implant of DOCA, and were given saline in the drinking water to induce hypertension. (n=19–23, * indicates significance of $p<0.05$).

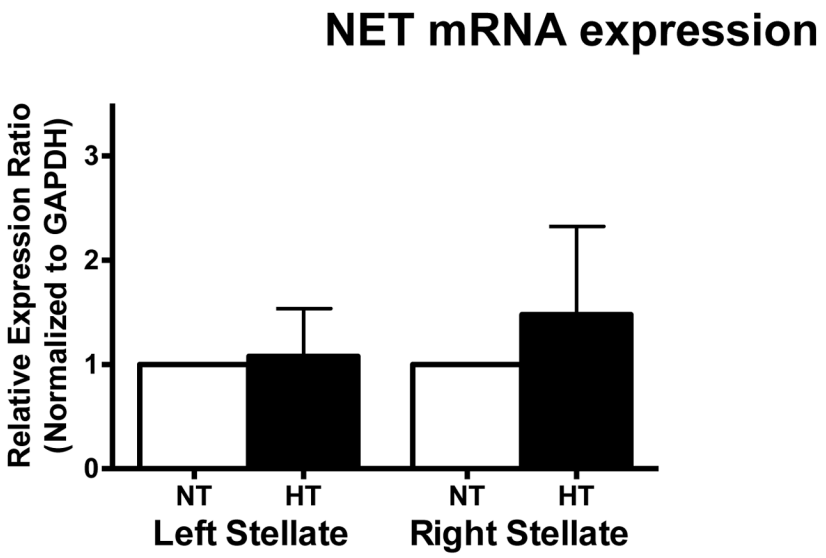

Figure 2. NET mRNA expression is unchanged in the bilateral stellate ganglia with DOCA-salt hypertension

RNA was isolated from stellate ganglia from normotensive (NT, white bar) and DOCA-salt hypertensive (HT, black bar) rats and analyzed for expression of NET mRNA. Data are shown as relative expression ratio using GAPDH as a control gene. For each ganglion, expression in NT ganglia is set to one and the HT is analyzed relative to control. Calculations of fold change and significance were run separately for each ganglion using pair-wise randomization on REST® 384 software. To eliminate intra-run error, NT and HT samples from each ganglion were run together on the same qPCR plate.

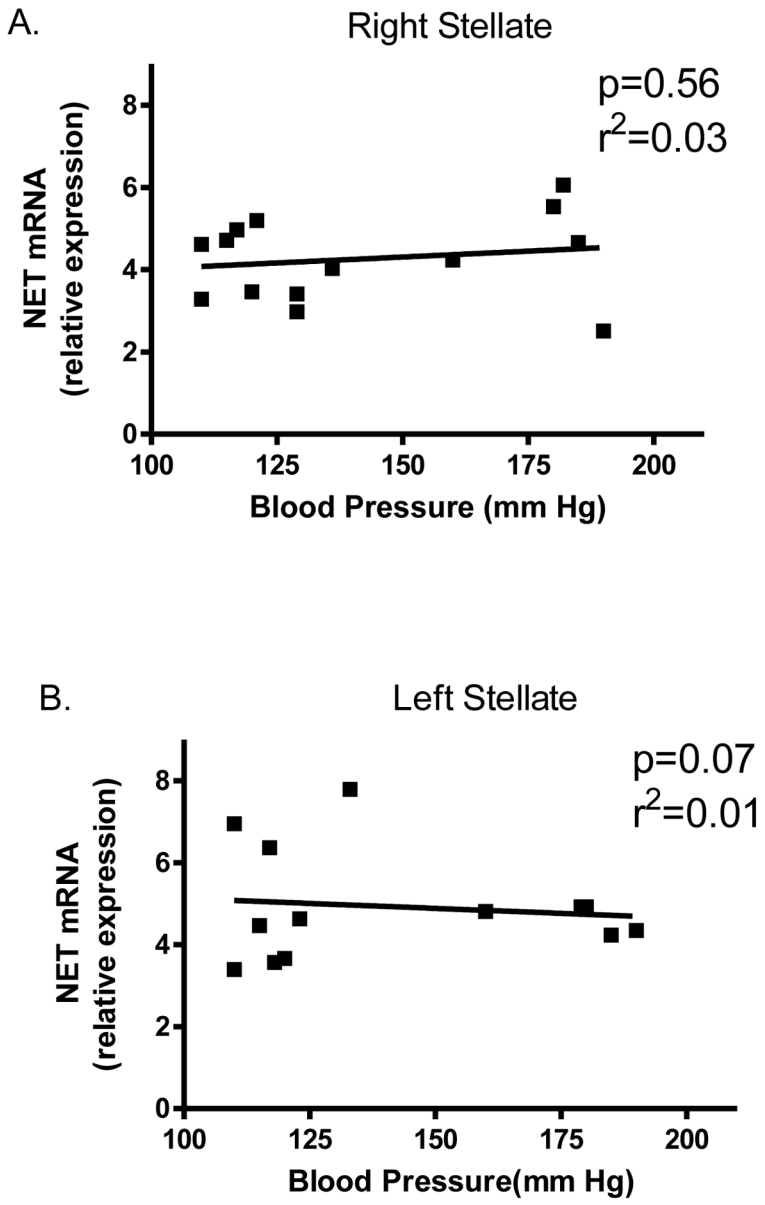

Figure 3. NET mRNA does not correlate to blood pressure in the stellate ganglia

A) Right stellate ganglia and B) Left stellate ganglia. A scatter plot of NET mRNA and systolic blood pressure is shown. A correlation analysis was performed on NET mRNA expression level (normalized NET:GAPDH expression ratio) to systolic blood pressure. There is no correlation of NET mRNA or blood pressure in the right or left stellate ganglion.

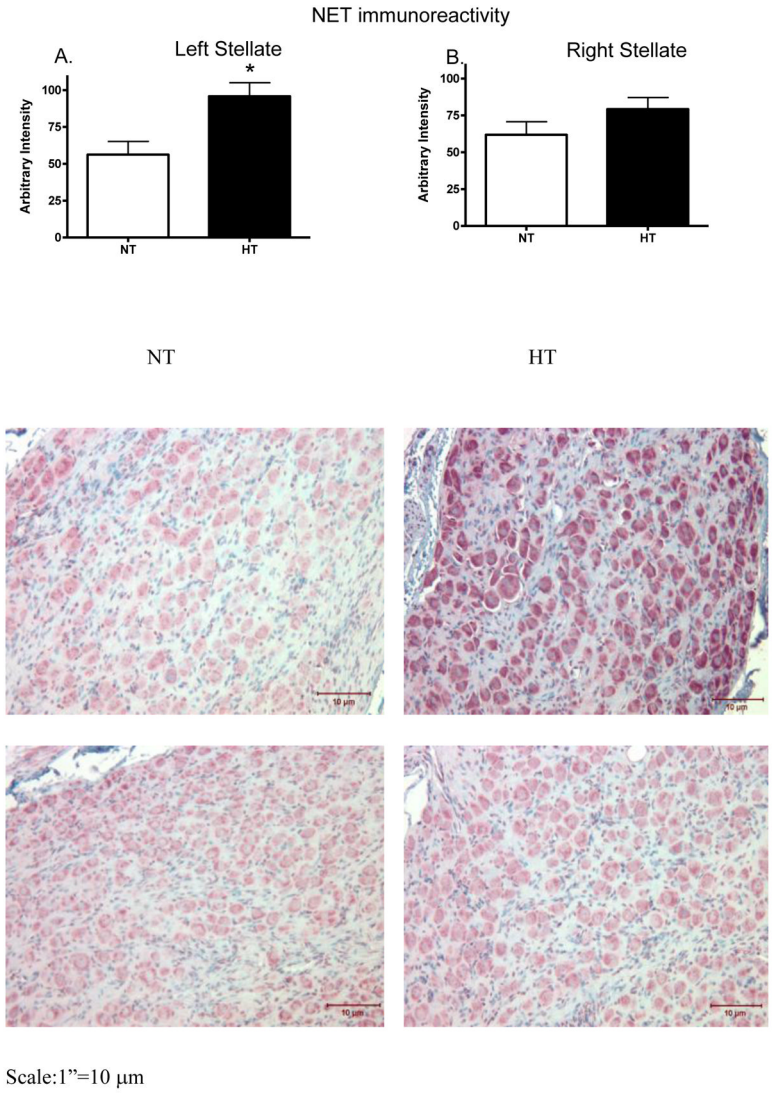

Figure 4. NET protein expression is elevated in left but not right stellate ganglia from DOCA-salt hypertensive rats

A) Left stellate ganglion and B) Right stellate ganglion from normotensive (NT, white bars) and hypertensive (HT, black bars) were fixed, sectioned, and analyzed for NET immunoreactivity (IR) using NIH Image J software as described in the methods section. A representative section (scale bar=10μM) is shown below each bar with NET IR shown in red. NET IR was determined in all neurons in a single section and then averaged per ganglia. (n=4 animals, * indicates significance $p<0.05$).

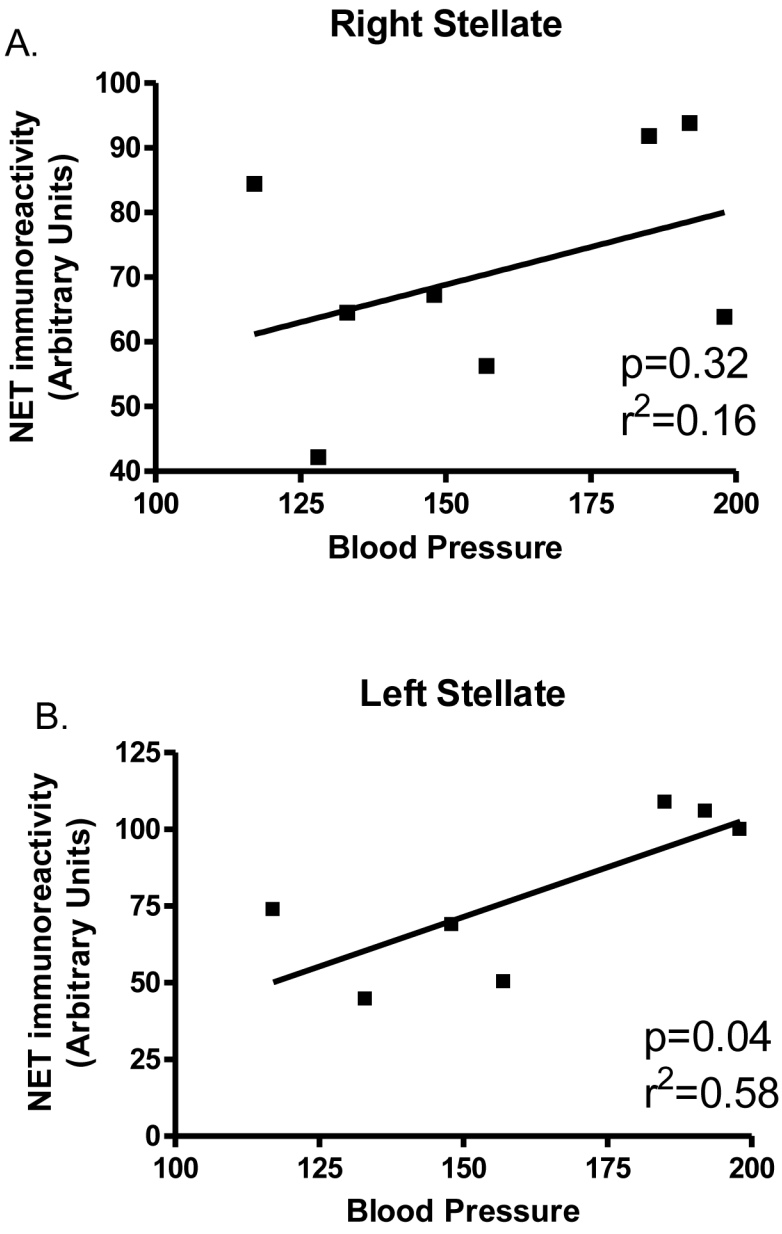

Figure 5. Left stellate ganglion NET protein positively correlates to blood pressure

A) Right stellate ganglia and B) Left stellate ganglia. A scatter plot of systolic blood pressure to quantified mean NET immunoreactivity (IR, arbitrary units) from right or left stellate ganglion neurons from normotensive (NT) and hypertensive (HT) rats is shown. A correlation analysis was performed comparing NET protein and systolic blood pressure. Left stellate NET IR (NET protein) is positively related to blood pressure (r^2=0.58, $p<0.05$).

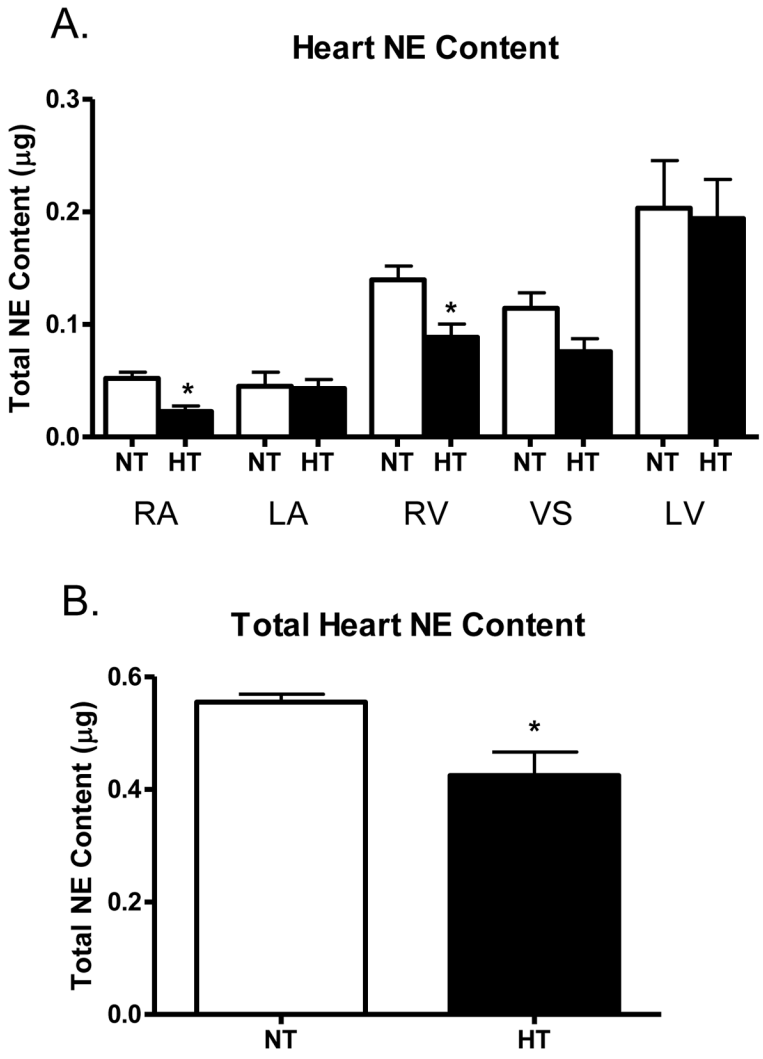

Figure 6. Chamber norepinephrine (NE) content was regionally decreased in DOCA-salt hypertension

A) Total NE content per chamber (3g) was determined by capillary electrophoresis with electrochemical detection in normotensive (NT, white bars) and DOCA-salt hypertensive (HT, black bars) rats. B) NE content in all chambers in a single heart was summed for total NE content per heart and averaged for comparison between NT and HT. * indicates significance $p<0.05$ NT versus HT.

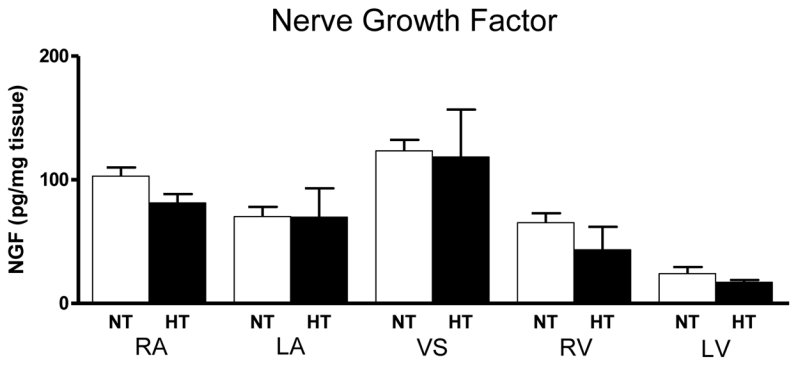

Figure 7. Nerve Growth Factor protein is unchanged in the heart chambers of DOCA-salt hypertensive rats

NGF (pg/mg) was measured using enzyme linked immunosorbant assay (ELISA) in heart homogenates of all chambers from normotensive (NT, white bars) and DOCA-salt hypertensive rats (HT, black bars). $p>0.05$, $n=4$.

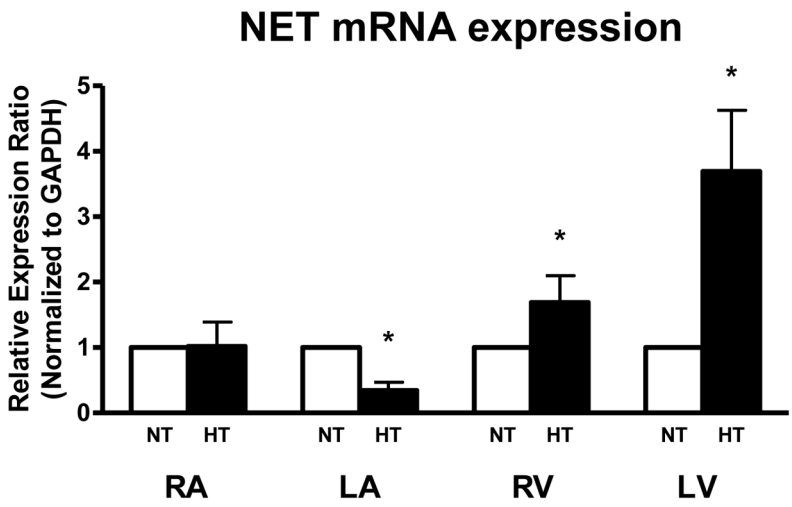

Figure 8. NET mRNA from the heart is regionally regulated in DOCA-salt hypertension

RNA was isolated from heart chambers of normotensive (NT, white bar) and DOCA-salt hypertensive (HT, black bar) rats and analyzed for expression of NET mRNA. Data are shown as relative expression ratio using GAPDH as a control gene. For each chamber, expression in NT ganglia is set to one and the HT is analyzed relative to control. Calculations of fold change and significance were run separately for each ganglion using pair-wise randomization on REST® 384 software. NT and HT samples from each chamber were run together on the same qPCR plate.

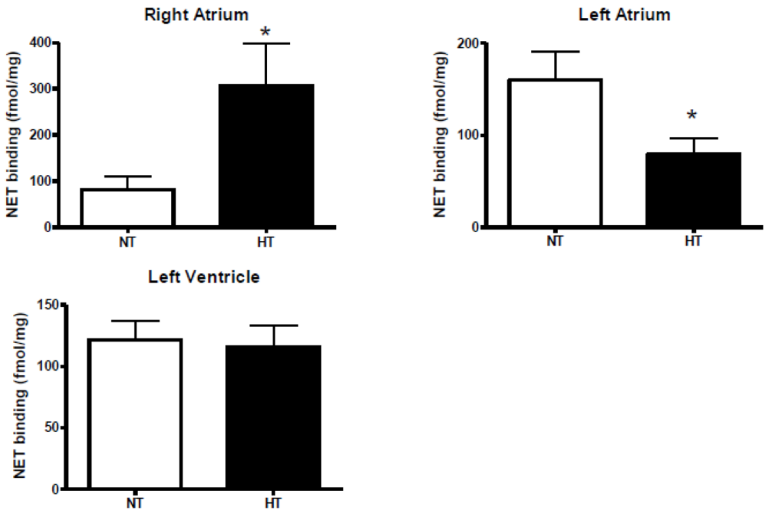

Figure 9. NET protein in cardiac membranes is regionally regulated in different heart chambers in DOCA-salt hypertensive rats

NET protein (fmol/mg) was assessed by [^{3}H]-nisoxetine binding in cardiac membranes from heart chambers of normotensive (NT, white bars) and DOCA-salt hypertensive (HT, black bars). A) right atrium, B) left atrium, and C) left ventricle. (n=7–8, * indicates significance $p<0.05$).