Research Paper

Involvement of endothelins in deoxycorticosterone acetate–salt hypertension through the modulation of noradrenergic transmission in the rat posterior hypothalamus

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Experimental Physiology

New Findings

- **What is the central question of this study?**
  Does *ex vivo* administration of endothelin-1 and endothelin-3 regulate noradrenergic transmission in the posterior hypothalamus of deoxycorticosterone acetate–salt hypertensive rats compared with normotensive rats?
- **What is the main finding and its importance?**
  Endothelin-1 and endothelin-3 enhanced diverse mechanisms leading to increased noradrenergic transmission in the posterior hypothalamus of deoxycorticosterone acetate–salt hypertensive rats. Unveiling the role of brain endothelins in hypertension would probably favour the development of new therapeutic targets for the treatment of essential hypertension, which still represents a challenging disease with high mortality.

Brain catecholamines participate in diverse biological functions regulated by the hypothalamus. We have previously reported that endothelin-1 and endothelin-3 (ET-1 and ET-3) modulate catecholaminergic activity in the anterior and posterior hypothalamus of normotensive rats. The aim of the present study was to evaluate the interaction between endothelins and noradrenergic transmission in the posterior hypothalamus of deoxycorticosterone acetate (DOCA)–salt hypertensive rats. We assessed the effects of ET-1 and ET-3 on tyrosine hydroxylase activity and expression, neuronal noradrenaline (NA) release, neuronal NA transporter (NAT) activity and expression, monoamine oxidase activity and NA endogenous content and utilization (as a marker of turnover) in the posterior hypothalamus of DOCA–salt hypertensive rats. In addition, levels of ET_A and ET_B receptors were assayed in normotensive and hypertensive rats. Results showed that tyrosine hydroxylase activity and total and phosphorylated levels, NAT activity and content, NA release, monoamine oxidase activity and NA utilization were increased in DOCA–salt rats. Both ET-1 and ET-3 further enhanced all noradrenergic parameters except for total tyrosine hydroxylase level and NA endogenous content and utilization. The expression of ET_A receptors was increased in the posterior hypothalamus of DOCA–salt rats, but ET_B receptors showed no changes. These results show that ET-1 and ET-3 upregulate noradrenergic activity in the posterior hypothalamus of DOCA–salt hypertensive rats. Our findings suggest that the

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interaction between noradrenergic transmission and the endothelinergic system in the posterior hypothalamus may be involved in the development and/or maintenance of hypertension in this animal model.

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Introduction

Although the deoxycorticosterone acetate (DOCA)–salt animal model of hypertension has been widely studied, the underlying mechanisms leading to blood pressure elevation still remain controversial. The possible causes have been divided into neural and non-neural mechanisms (Abrams & Osborn, 2008). Within the former and supported by strong evidence is the centrally mediated increase in sympathetic activity. Ablation of the area postrema or the anteroventral third ventricle (AV3V) area of Brody prevents blood pressure elevation in DOCA–salt rats and, furthermore, it decreases noradrenaline (NA) in the brainstem, suggesting that the central inhibitory effect evoked by sympathetic activity is attenuated (Oparil *et al.* 1995; Yemane *et al.* 2010). The non-neural components include several peptides, such as vasopressin, angiotensin, natriuretic peptides and endothelins (ETs), which regulate cardiovascular activity and are widely distributed in the body, including the CNS (Oparil *et al.* 1995; Yemane *et al.* 2010). Endothelins are a family of three isoforms, termed ET-1, ET-2 and ET-3, which were initially identified as potent vasoconstrictor agents (Davenport & Maguire, 2006; Kohan *et al.* 2011). Endothelins are involved in the regulation of numerous biological functions in physiological and pathophysiological situations (Davenport & Maguire, 2006; Kohan *et al.* 2011; Kaoukis *et al.* 2013).

Diverse studies were performed to determine the role of the CNS in the development and/or maintenance of hypertension. It has been clear for many years that the CNS is essential in the short-term regulation of blood pressure; however, current evidence strongly supports that it also participates in its long-term control (Dampney *et al.* 2005; Malpas, 2006). Within of CNS, the hypothalamus is an integrative centre that regulates diverse biological functions, including cardiovascular activity (Oparil *et al.* 1995; Kasparov & Teschemacher, 2008; Blaustein *et al.* 2012). The posterior hypothalamus (PH) is recognized as a sympatho-excitatory area intimately related to the central regulation of cardiovascular function (Oparil *et al.* 1995; Kasparov & Teschemacher, 2008). It receives a large number of afferents not only from other hypothalamic areas but also from the brainstem and, in particular, from the locus coeruleus (LC), which is an important noradrenergic region of the CNS (De Wardener, 2001). Impairment of NA metabolism in the PH has been associated with the development and/or maintenance of hypertension (Oparil *et al.* 1995; Kasparov & Teschemacher, 2008). Also, lesions in the PH of DOCA–salt and spontaneously hypertensive (SH) rats reduce blood pressure (Buñag & Eferakeya, 1976). Nevertheless, the precise role of the PH in the genesis and/or development of DOCA–salt hypertension remains to be elucidated fully.

Both the noradrenergic and the endothelinergic systems are widely expressed in the hypothalamus (Stojilkovic & Catt, 1996; Kueaki *et al.* 1997; Tanaka *et al.* 2000; Itoi & Sugimoto, 2010). The central effects of ETs on the cardiovascular function are mediated by changes in the sympathetic nervous system (Kueaki *et al.* 1997). In this sense, we reported that ETs modulate noradrenergic neurotransmission (biosynthesis, neuronal release and uptake of NA) in different regions of the hypothalamus in normotensive rats (di Nunzio *et al.* 2002, 2004; Morgazo *et al.* 2005; Perfume *et al.* 2007; Hope *et al.* 2008).

On this basis, the aim of the present study was to evaluate the interaction between ETs and noradrenergic transmission in the PH of DOCA–salt hypertensive rats in order to determine whether this interaction in the PH is implicated in this animal model of experimental hypertension. In addition, ET_A and ET_B receptor expression was also assayed. The present findings show that ET-1 and ET-3 increase tyrosine hydroxylase (TH) activity and its phosphorylation at Ser-31 and Ser-40 sites in the PH of DOCA–salt rats, without affecting total and Ser-19 TH. Furthermore, neuronal NA uptake and release, neuronal NA transporter (NAT) expression and monoamine oxidase (MAO) activity were also increased, whereas NA endogenous content and utilization were not modified. Also, levels of ET_A receptor were increased in the PH of DOCA–salt rats, but no changes were observed in ET_B receptor expression.

Methods

Animals and experimental design

Male Sprague–Dawley rats weighing 100–130 g (from the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires) were used in the experiments. Animals

were housed in steel cages and maintained in conditions of constant temperature and humidity, with a 12 h–12 h light–dark cycle, and fed *ad libitum*. Experiments were performed following the recommendations of the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, 1985, revised 1996) and approved by the Institutional Animal Care and Use Committee of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. All efforts were made to minimize the number and suffering of used rats. The DOCA–salt hypertension was induced by weekly S.C. injections of 30 mg kg^{-1} DOCA (MP Biomedicals, Santa Ana, CA, USA) dissolved in sesame seed oil (as vehicle) and the administration of 1% NaCl in the drinking water for 5 weeks. Control animals were injected with vehicle and given tap water. Systolic blood pressure (by tail plethysmography, PowerLab, ADInstruments, Colorado Spring, CO, USA) and body weight were determined weekly. The experimental procedures involved the use of 156 animals.

After 5 weeks, animals were killed by decapitation (between 09.00 and 12.00 h), given that general anaesthetics differentially affect catecholamine metabolism (Shimokawa *et al.* 1998; Pashkov & Hemmings, 2002; Kushikata *et al.* 2011). Brains were quickly removed and the PH immediately dissected under a magnifier according to the microdissection atlas of Palkovits & Brownstein (1988).

Experimental protocols

In all experiments, PH were pre-incubated in a Dubnoff incubator for 30 min at 37°C in Krebs bicarbonate solution, pH 7.4, and bubbled with a gas mixture (95% O_2 and 5% CO_2) under continuous shaking.

Tyrosine hydroxylase activity. Following the pre-incubation period, the PH from control or DOCA–salt rats treated with or without ET-1 or ET-3 (American Peptides, Sunnyvale, CA, USA) was incubated for 30 min and then homogenized in 500 μl buffer (5 mM KH_2PO_4 and 0.2% Triton X-100, pH 7.0). An aliquot was saved for protein determination. Samples were centrifuged for 10 min at 10,000*g* at 4°C and TH activity was determined according to Reinhard *et al.* (1986). Briefly, an aliquot of the supernatant was incubated for 20 min at 37°C with 50 mM Hepes (pH 7.0), containing 15 nmol L-tyrosine (containing 0.5 μCi ^{3}H[3,5]L-tyrosine from PerkinElmer Life and Analytical Sciences, Waltham, MA, USA), 420 mM β-mercaptoethanol, 1000 U catalase and 0.75 mM 6-methyl-tetrahydrobiopterin. The reaction was stopped by the addition of 1 ml 7.5% (w/v) activated charcoal suspension in 1 N HCl. The final mixture was centrifuged at 500*g* for 10 min and ^3H_2O assessed in the supernatant by conventional scintillation methods. Blank values were obtained by omitting 6-methyl-tetrahydrobiopterin from the mixture. Recovered ^3H_2O was determined as described by Reinhard and co-workers (1986). Results were expressed as a percentage of the control group value ± SEM.

Neuronal NA uptake. Noradrenaline uptake was assessed according to Vatta *et al.* (1996) with minor modifications. Briefly, following the pre-incubation period the NA stores in the PH from the different experimental groups (control; DOCA–salt; DOCA–salt + ET-1; and DOCA–salt + ET-3) were labelled with 2.5 μCi ml^{-1} [^{3}H]-NA (PerkinElmer Life and Analytical Sciences) for 5 min followed by three consecutive washes (10 min each) with cold Krebs solution. The MAO activity and extraneuronal NA uptake were inhibited by the addition of 50 μM pargyline and 100 μM hydrocortisone, respectively. The tissues were then homogenized and [^{3}H]-NA uptake was assessed by the usual scintillation counting methods. Data were expressed as a percentage of the control group value ± SEM.

Neuronal NA release. Neuronal NA release was measured according to Vatta *et al.* (1996) with minor modifications. Briefly, the PH of control and DOCA–salt rats in the absence of ETs was submitted to the same experimental procedures as described for NA uptake except that 10 μM desipramine was added before the last wash, in order to inhibit neuronal NA uptake. Tissues were incubated for 35 min, and seven consecutive samples of the incubation medium were collected every 5 min. The first samples corresponded to the basal period, while the remaining samples corresponded to to the experimental period during which the PH of control (alone) and DOCA–salt rats (with or without ET-1 or ET-3) were incubated. The [^{3}H]-NA release was measured in the incubation medium by conventional scintillation counting methods. Results were expressed as the area under the curve corresponding to the 30 min experimental period [in disintegrations per minute (dpm) per microgram of protein ± SEM].

Western blot assay for TH, NAT and ET receptors. The PH from the different groups (control; DOCA–salt; DOCA–salt + ET-1; and DOCA–salt + ET-3) were incubated for 30 min (TH) or 5 min (NAT) and homogenized in lysis buffer (20 mM Tris–Cl pH 7.4, 1 mM PMSF, 5 mM EDTA, 25 mM NaF, 1% Triton X-100 and 1% protease inhibitor cocktail). Following centrifugation for 20 min at 4°C 10,000 *g*, the supernatant was mixed with Laemmli buffer (62.5 mM Tris–Cl pH 6.8, 2% SDS, 5% 2-mercaptoethanol (B-ME), 10% glycerol and 4% Bromophenol Blue), boiled for 5 min and then subjected to SDS-PAGE gel at 100 V for 2 h and 30 min. The gels were then transferred onto polyvinylidene

difluoride (PVDF) membranes (GE Healthcare Life Sciences, Buckinghamshire, UK) at 100 V for 75 min. The membranes were blocked at 4°C overnight in blocked solution [5% non-fat powder milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T)], and the gels were stained overnight at 4°C with Coomasie Blue. After washing with TBS-T, the transfers were incubated with anti-TH monoclonal antibody (TH-Ab; from Sigma, St Louis, MO, USA), rabbit anti-Phospho (Ser 19) TH, phospho (Ser 31) TH, and phospho (Ser 40) TH. (19 Ser-P, 31 Ser-P and 40 Ser-P; all from Chemicon, EMD Millipore, Billerica, MA, USA) and rabbit anti-NAT (NAT-Ab, from Chemicon, EMD Millipore, Billerica, MA, USA. 1:1000 dilution overnight at 4°C); rabbit anti-actin (Sigma; 1:1500 dilution for 1 h at room temperature); anti-mouse or anti-rabbit peroxidase-conjugated antibody (Pierce, Rockford, IL, USA; 1:5000 dilution for 1 h at room temperature). After the final wash with TBS-T, the bands were detected with a Bio-lumina kit (Kalium Technologies, Buenos Aires, Argentina). For ET_A and ET_B receptor immunoblotting, anti-ET_A polyclonal and anti-ET_B polyclonal antibodies (1:200 dilution; Alomone Labs, Jerusalem, Israel) were used in samples from control and DOCA–salt rats following the same experimental procedure. Bands were analysed by densitometry and normalized to β-actin. Results were expressed as a percentage of the control group value ± SEM.

Monoamine oxidase activity. Following the pre-incubation period, the PH of control (alone) or DOCA–salt rats (with or without ET-1 or ET-3) were incubated for 30 min and MAO activity was measured as previously reported by Holt *et al.* (1997) with minor changes. Briefly, tissues were homogenized in 0.2 M phosphate buffer and centrifuged at 1000*g* for 10 min at 4°C. Supernatants were then centrifuged at 10,000*g* for 30 min at 4°C and pellets (crude mitochondrial extract) suspended in 0.3 M sucrose in 0.2 M phosphate buffer and stored at 4°C for 48 h. The MAO activity was assessed by incubating samples with 4 U ml^{-1} peroxidase, 1 mM vainillinic acid, 500 μM 4-aminoantipyrine, 10 nmol H_2O_2 and 0.2 M phosphate buffer for 3 min. The reaction was stopped by adding 50 μM pargyline and 50 μM clorgyline to inhibit MAO-A and MAO-B activity, and the reaction product was determined by spectrometry at 498 nm. The MAO activity was calculated in triplicate using the molar absorption coefficient (4654 cm^{-1} M^{-1}) and normalized to micrograms of protein in each sample. Results were expressed as a percentage of the control value ± SEM.

Endogenous content and utilization of NA. Control and DOCA–salt animals were randomly divided into two groups. One was injected I.P., 24 and 2 h before the experiments, with α-methyl-p-tyrosine (α-MPT) [200 mg (kg body weight)$^{-1}$] to inhibit catecholamine synthesis, and the other was injected with the same volume of vehicle (saline). The NA content was measured in PH samples by HPLC with a Phenomenex Luna 5 μm, C18, 100 mm × 1 mm column (Phenomenex, Torrance, CA, USA) and LC-4C electrochemical detector with glassy carbon electrode (BAS, West Lafayette, IN, USA). The working electrode was set at +0.70 V with respect to an Ag–AgCl reference electrode. The mobile phase contained 0.76 M $NaH_2PO_4.H_2O$, 0.5 mM EDTA, 1.2 mM 1-octane sulfonic acid and 5% acetonitrile (pH 2.8).

Statistical analysis

Results were expressed as means ± SEM. The statistical analysis was performed by ANOVA followed by the Student–Newman–Keuls test. Values of $P \leq 0.05$ were considered statistically significant.

Results

Systolic blood pressure and the left ventricular weight ratio (left ventricular weight relative to total body weight) were significantly elevated in DOCA–salt rats compared with control rats (control *versus* DOCA–salt, 108 ± 7 *versus* 160 ± 5 mmHg, $P < 0.001$, and 2.20 ± 0.08 *versus* 2.74 ± 0.10 mg g^{-1}, $P < 0.001$, respectively). However, no changes were observed in either body weight or relative right ventricular weight between the two groups. The DOCA-only and salt-only groups did not show modifications in blood pressure compared with control rats (data not shown).

To determine the effect of exogenous administration of ETs on TH activity and expression, tissues were incubated as described in the Methods. The results showed that DOCA–salt treatment increased TH activity, total TH protein level and TH phosphorylation at Ser-19, Ser-31 and Ser-40 sites compared with control rats (Figs 1 and 2). However, in a previous study we reported that in the PH of normotensive rats, ET-1 and ET-3 (10 nM) diminished TH activity and TH phosphorylation at Ser-19, Ser-31 and Ser-40, without changing the total enzyme level (Perfume *et al.* 2007). In the present study, the PH from DOCA–salt rats incubated with ET-1 or ET-3 showed enhanced TH activity and levels of TH phosphorylation at Ser-31 and Ser-40 with no changes in total TH and TH Ser-19 levels (Figs 1 and 2).

We next addressed the effects of ETs on NA release in the PH of DOCA–salt rats and found that hypertensive animals showed increased neuronal NA release compared with the control group (Fig. 3). We previously reported that both ETs enhanced NA release in the PH of normotensive rats (di Nunzio *et al.* 2004). Likewise, in

DOCA–salt rats, ET-1 and ET-3 increased the neuronal release of the amine (Fig. 3).

The studies on neuronal NA uptake and NAT expression in the PH of DOCA–salt rats showed that neuronal NA uptake was increased in DOCA–salt rats *versus* normotensive rats (Fig. 4). Endothelins reduced NA uptake in normal rats, as previously reported, but increased it in the PH of hypertensive animals (Fig. 4). On this basis, NAT expression was determined by Western blot, where the two characteristic bands at ∼80 and ∼50 kDa were observed (Fig. 5*A*). The expression of NAT 50 kDa was not modified in DOCA–salt rats and, furthermore, it was not affected by exposure to ETs (Fig. 5*B*). In hypertensive rats, however, NAT 80 kDa expression was increased with respect to normotensive animals (Fig. 5*C*). Exposure to ETs reduced NAT 80 kDa (data not shown) in the PH of normotensive rats but increased it in DOCA–salt rats (Fig. 5*C*).

Given that the results suggested that NA catabolism could be affected, MAO activity was measured. The results showed that DOCA–salt treatment increased MAO activity, and exposure to ET-1 and ET-3 enhanced it further (Fig. 6).

In order to summarize the status of NA transmission, we studied the effects of both ETs on NA endogenous content and utilization (as an index of NA turnover) in DOCA–salt rats (Summers & Phillips, 1988). The results showed that the NA endogenous content was diminished in the PH of hypertensive rats; however, no further changes were observed in the presence of ETs when compared with hypertensive animals (Fig. 7*A*). In addition, NA utilization was increased in DOCA–salt rats compared with control animals, but it was diminished by both ETs (ET-1, 25% and ET-3, 23%), although this decrease was not statistically significant when compared with hypertensive rats (Fig. 7*B*).

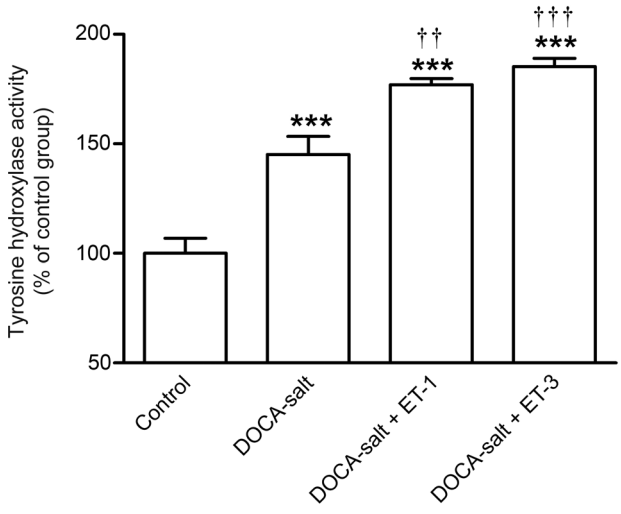

Figure 1. Tyrosine hydroxylase (TH) activity in the posterior hypothalamus of deoxycorticosterone acetate (DOCA)–salt hypertensive rats
Exogenous administration of endothelin-1 and endothelin-3 (ET-1 and ET-3) increases TH activity in the posterior hypothalamus of DOCA–salt hypertensive rats. Tyrosine hydroxylase activity was assessed as detailed in the Methods and are shown as a percentage of the control value ± SEM. $^{***}P < 0.001$ *versus* control; $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ *versus* DOCA–salt. There were six or seven animals in each experimental group.

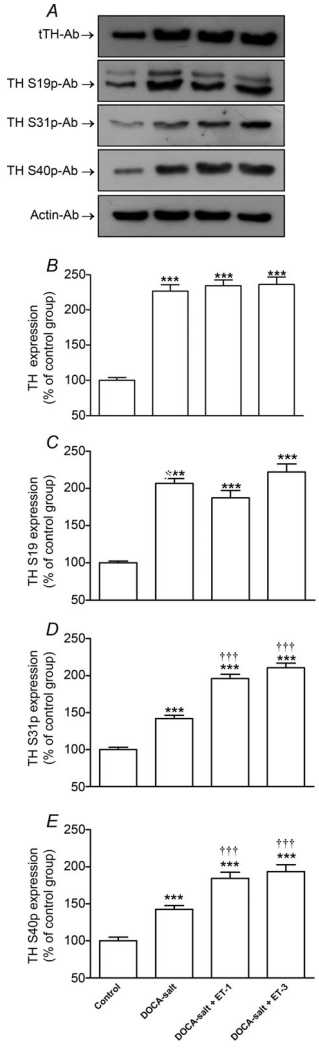

Figure 2. Tyrosine hydroxylase protein level in the posterior hypothalamus of DOCA–salt hypertensive rats
Effect of exogenous administration of ET-1 and ET-3 on the levels of total TH and its phosphorylated forms in the posterior hypothalamus of DOCA–salt hypertensive rats.
A, representative blots. *B*, total TH expression. *C*, TH Ser-19 (TH-S19p). *D*, TH Ser-31 (TH-S31p). *E*, TH Ser-40 (TH-S40p). The expressions of total TH and phosphorylated forms of TH were determined by Western blot as described in the Methods and are shown as a percentage of the control group value + SEM. $^{***}P < 0.001$ *versus* control; $^{\dagger\dagger\dagger}P < 0.001$ *versus* DOCA–salt. There were six animals in each experimental group.

The expression of ET_A and ET_B receptors was assessed by immunoblotting in the PH of normotensive and hypertensive rats. The DOCA–salt animals showed increased ET_A receptor expression (48 kDa), whereas no changes were observed in ET_B receptor level (50 kDa; Fig. 8*A*–*C*).

Discussion

The major finding of the present study was that ET-1 and ET-3 regulate noradrenergic transmission in the PH of DOCA–salt hypertensive rats. Both peptides exhibited differential effects on the activity and expression of TH and NAT compared with their effects in normotensive animals, although similar responses were observed for both ETs on NA release and MAO activity. Furthermore, DOCA–salt animals showed enhanced ET_A receptor expression in the PH.

The PH is a sympatho-excitatory centre; its stimulation increases blood pressure and sympathetic outflow and decreases baroreflex-induced bradycardia (Smith & Barrow, 1989), whereas lesions in this area decrease blood pressure in animal models of hypertension, including the DOCA–salt model (Buñag & Eferakeya, 1976). Diminished brainstem inhibitory activity as a consequence of reduced noradrenergic output is observed in DOCA–salt hypertension, which results in enhanced sympathetic activity (Oparil *et al.* 1995). However, in this animal model the role of noradrenergic transmission in the hypothalamus remains controversial and elusive. In the present study, we show that TH activity and expression as well as NA release were increased in the PH of DOCA–salt hypertensive rats compared with normotensive animals, supporting enhanced noradrenergic activity. Although dopamine β-hydroxylase is the enzyme that catalyses the conversion of dopamine into NA, we measured TH activity because it represents the rate-limiting step in catecholamine biosynthesis and it is tightly regulated by short- and long-term mechanisms (Flatmark, 2000; Dunkley *et al.* 2004; Stanford, 2013). Owing to its elevated affinity for dopamine and high maximal velocity, dopamine β-hydroxylase is not a rate-limiting enzyme in the biosynthetic pathway of catecholamines and, although it may increase in diverse circumstances, it requires stronger stimuli and longer periods of time compared with TH (Sabban & Nankova, 1998; Flatmark, 2000).

Noradrenaline uptake and NAT expression were also increased in the PH of hypertensive rats, as was MAO activity, whereas NA endogenous content was diminished. These findings confirm the existence of a balance between the synthesis and release of NA and the mechanisms that terminate the action of NA at the synaptic cleft in the PH. Although NA utilization was increased, other studies show no changes in hypothalamic NA turnover or utilization in salt-dependent models of hypertension such as DOCA–salt and Dahl rats (Fujita & Sato, 1984; Cheng *et al.* 1990). The apparent discrepancies with our findings may be related to the animal models, because the rats

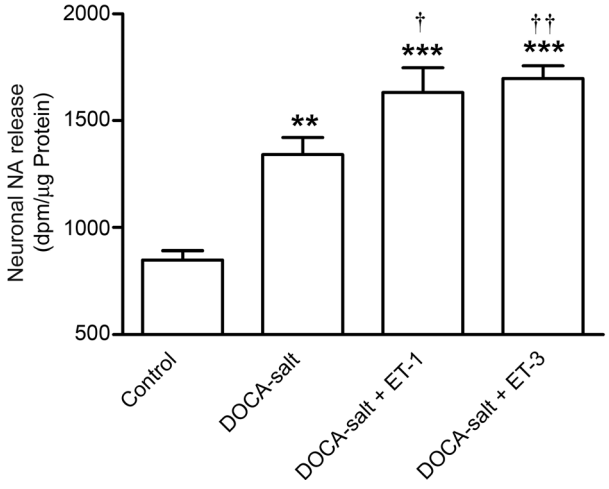

Figure 3. Neuronal noradrenaline (NA) release in the posterior hypothalamus of DOCA–salt hypertensive rats
Endothelin-1 and and ET-3 stimulate neuronal NA release in the posterior hypothalamus of DOCA–salt hypertensive rats. Noradrenaline release was assessed as detailed in the Methods and is shown as a percentage of the control group value + SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* control; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ *versus* DOCA–salt. There were six or seven animals in each experimental group.

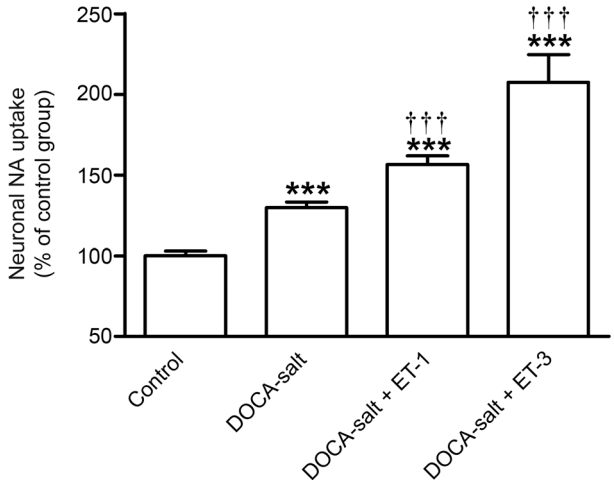

Figure 4. Neuronal NA uptake in the posterior hypothalamus of DOCA–salt hypertensive rats
Exogenous administration of ET-1 and ET-3 enhances neuronal NA uptake in the posterior hypothalamus of DOCA–salt hypertensive rats. Noradrenaline uptake was assessed as described in the Methods and is shown as a percentage of the control group value + SEM. $^{***}P < 0.001$ *versus* control; $^{\dagger\dagger\dagger}P < 0.001$ *versus* DOCA–salt. There were six or seven animals in each experimental group.

used in the present study were not nephrectomized as in other studies. However, in accordance with our findings it was reported that the electric or NA stimulation of the PH results in increased sympathetic nerve firing in DOCA–salt rats (Takeda & Buñag, 1980). In addition, PH lesions in the same animal model lead to a decrease blood pressure (Buñag & Eferakeya, 1976). It is therefore possible to assume that in DOCA–salt hypertensive rats (5 weeks treatment) an increase in noradrenergic activity maintains the blood pressure elevation. Nevertheless, enhanced noradrenergic transmission seems to be involved in triggering hypertension. In this sense, preliminary data from our laboratory show that blood pressure increases slightly and NA uptake is reduced after the first week of treatment (DOCA + 1% NaCl), but TH activity and NA release remain unchanged (Abramoff T, Guil MJ, Bianiotti LG & Vatta MS, unpublished data). However, at week 3 the blood pressure is significantly elevated and noradrenergic activity is clearly enhanced in the PH, as supported by increased TH activity and NA release. These findings suggest that enhanced noradrenergic transmission would be one of the underlying mechanisms triggering blood pressure elevation in DOCA–salt hypertension.

Although blood pressure elevation results from a significant stimulation of sympathetic activity and is correlated with enhanced plasma NA levels, excessive DOCA and NaCl administration may also contribute to increase blood pressure. Deoxycorticosterone acetate binds to peripheral as well as brain receptors, the latter being localized in areas such as the hypothalamus, hippocampus and organum vasculosum lamina terminals (Gómez-Sánchez, 1997; Abrams & Osborn, 2008). In addition, the rise in osmolality activates peripheral osmoreceptors as well as those localized in the circumventricular organs (organum vasculosum of the lamina terminals, subfornical organ and median preoptic

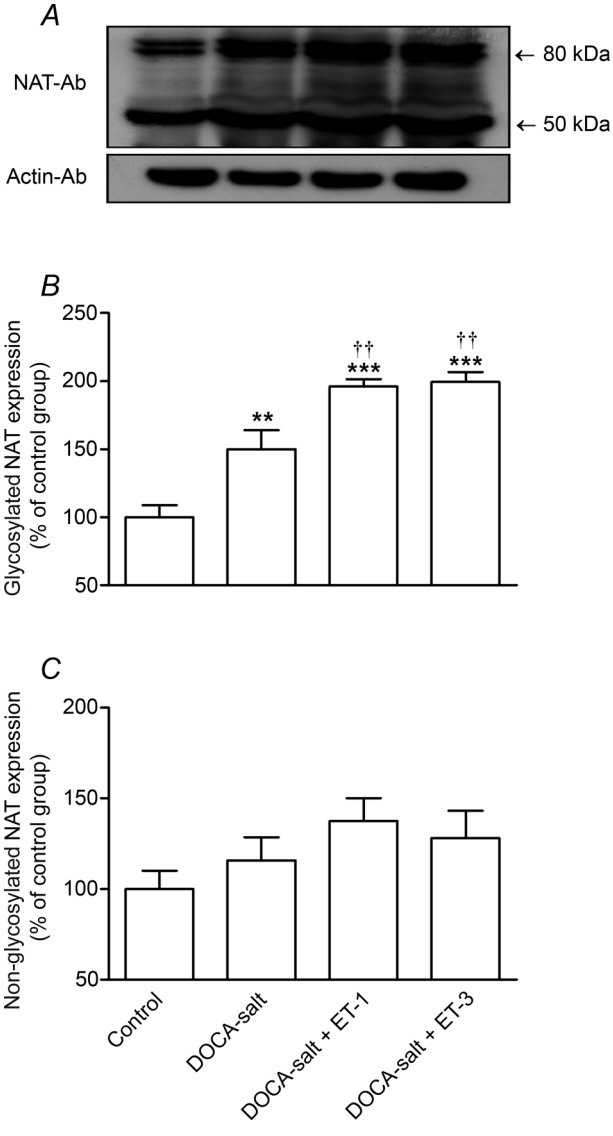

Figure 5. Neuronal noradrenaline transporter (NAT) expression in the posterior hypothalamus of DOCA–salt hypertensive rats
Effect of exogenous ET-1 and ET-3 administration on neuronal NAT in the posterior hypothalamus of DOCA–salt hypertensive rats. *A*, representative blot. The expression of glycosylated NAT (80 kDa; *B*) and non-glycosylated NAT (50 kDa; *C*) was determined by Western blot as detailed in the Methods and is shown as a percentage of the control group value + SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* control; $^{\dagger\dagger}P < 0.01$ *versus* DOCA–salt. There were six animals in each experimental group.

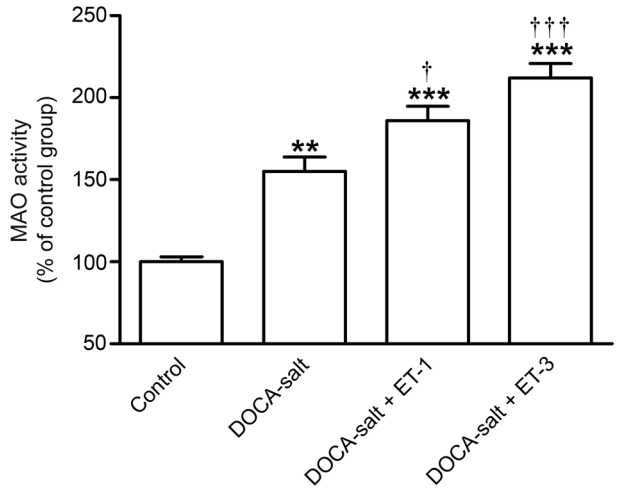

Figure 6. Monoamine oxidase (MAO) activity in the posterior hypothalamus of DOCA–salt hypertensive rats
Endothelin-1 and ET-3 increase MAO activity in the posterior hypothalamus of DOCA–salt hypertensive rats. The MAO activity was assessed as described in the Methods and is shown as a percentage of the control group value + SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* control; $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ *versus* DOCA–salt. There were five or six animals in each experimental group.

nucleus) and the AV3V region (Toney *et al.* 2003; Toney & Stocker, 2010). These complementary mechanisms lead to enhanced sympatho-excitation and blood pressure elevation in DOCA–salt hypertension, although other mechanisms may also contribute to increase it further (Abrams & Osborn, 2008; Yemane *et al.* 2010). The PH connects with brain osmosensitive areas expressing mineralocorticoid receptors, such as the median preoptic nucleus, the paraventricular nucleus and the amygdala (Gómez-Sánchez, 1997). Previous and present findings clearly support the suggestion that the PH plays a major role by increasing sympatho-excitation at different stages of DOCA–salt hypertension.

To gain further understanding of the complexity of blood pressure elevation in DOCA–salt hypertension, diverse vasoactive peptides should be considered. Endothelins are a family of related peptides widely expressed in the brain and involved not only in the physiological regulation of blood pressure but also in most

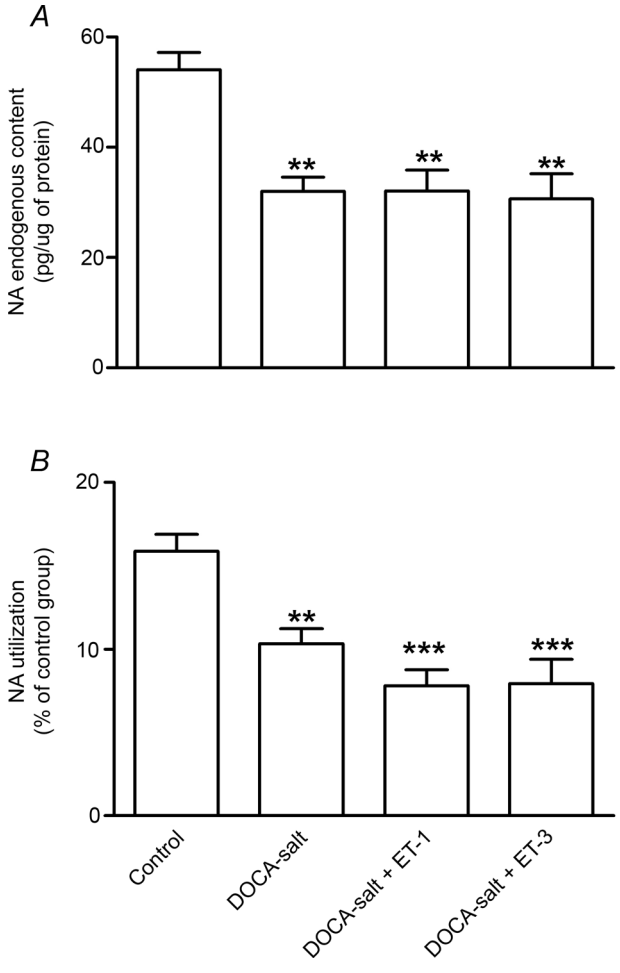

Figure 7. Endogenous content and utilization of NA in the posterior hypothalamus of DOCA–salt hypertensive rats
Effect of exogenous administration of ET-1 and ET-3 on the endogenous content (*A*) and utilization of NA (*B*) in the posterior hypothalamus of DOCA–salt hypertensive rats. These parameters were determined as described in the Methods and are shown as picograms per microgram of protein + SEM (endogenous content of NA) and as a percentage of the control group value + SEM (NA utilization). $^{**}P < 0.01$ and $^{***}P < 0.001$ *versus* control. There were four animals in each experimental group.

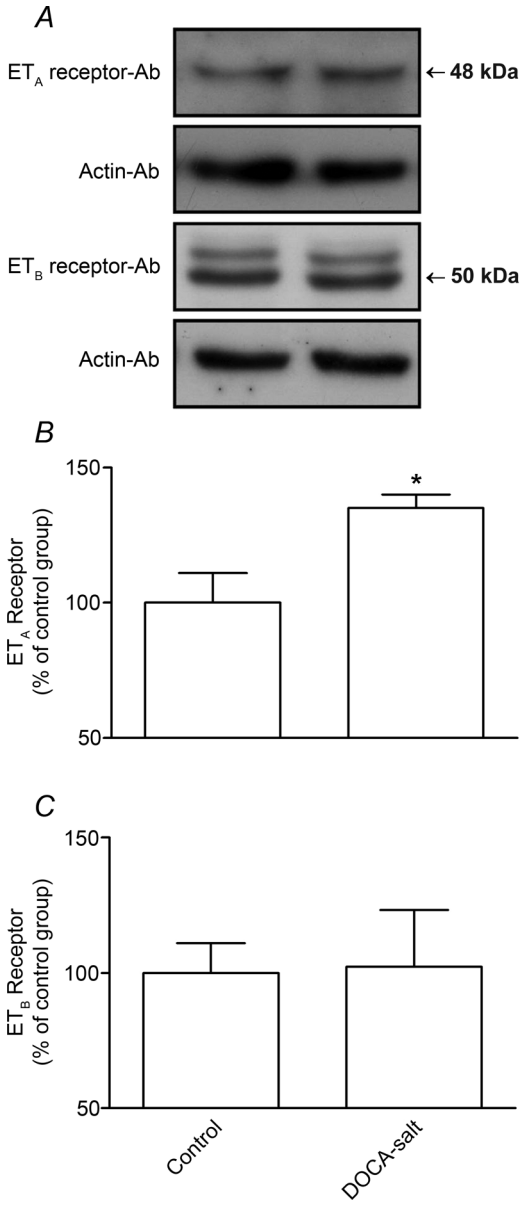

Figure 8. Expression of ET_A and ET_B receptors in the posterior hypothalamus of DOCA–salt hypertensive rats
There was enhanced ET_A receptor expression in the posterior hypothalamus of DOCA–salt hypertensive rats compared with control normotensive animals. *A*, representative blot. The expression of ET_A receptor (48 kDa; *B*) and ET_B receptor (50 kDa; *C*) was determined by Western blot as detailed in the Methods and shown as a percentage of the control group value + SEM. $^{*}P < 0.05$ *versus* control. There were three animals in each experimental group.

cardiovascular diseases (Oparil *et al.* 1995; Kueaki *et al.* 1997). We previously reported that exogenous ET-1 and ET-3 increase NA release and diminish NAT activity in the PH of normotensive rats (di Nunzio *et al.* 2004; Hope *et al.* 2008). However, short-term regulation (30 min) by ETs reduces TH activity and phosphorylation (Perfume *et al.* 2007). The present findings show that ETs have a different action within the PH of DOCA–salt rats. Both peptides enhanced TH activity and its phosphorylation at Ser-31 and Ser-40 sites. Furthermore, NA uptake and release and MAO activity were also increased by ETs and, although the peptides induced no changes in NA endogenous level, NA utilization showed an increasing trend in the presence of ET-1 (25%) and ET-3 (23%). These findings are likely to be related to stimulation of MAO, the enzyme that catabolizes NA at the presynaptic nerve ending. The upregulatory effects of both ETs on noradrenergic transmission in the PH may result from increased expression of ET_A receptors found in DOCA–salt rats.

Endothelins are involved in the regulation of cardiovascular activity through mechanisms other than effects on the vasculature (Kueaki *et al.* 1997; Kohan *et al.* 2011). These peptides regulate different biological effects at the hypothalamic level, such as water and electrolyte balance, neurosecretions and cardiovascular function (Kueaki *et al.* 1997; Kohan *et al.* 2011). Centrally applied ETs elevate blood pressure by increasing sympathetic outflow and vasopressin release (Oparil *et al.* 1995; Kueaki *et al.* 1997; Hynes & Webb, 1998; Kohan *et al.* 2011). Blockade of ET response by intracerebroventricularly or intracisternally applied ganglionic blockers and/or α_1-adrenergic antagonists supports the close relationship between ETs and noradrenergic transmission in the control of cardiovascular function (Kueaki *et al.* 1997). Levels of ETs in the cerebrospinal fluid are correlated with peripheral alterations in the cardiovascular activity and increase by ∼40% following baroreflex activation triggered by blood pressure elevation (Kueaki *et al.* 1997). Furthermore, upregulation of the ET system has been well documented in different animal models of hypertension and in patients with essential hypertension (Hynes & Webb, 1998; Naruse *et al.* 2000; Davenport & Maguire, 2006; Dhaun *et al.* 2008, Kohan *et al.* 2011). Endothelin-1 mRNA is upregulated in adrenal glands, lungs, kidneys and brain of different animal models of hypertension (Hynes & Webb, 1998; Naruse *et al.* 2000). In addition, ET_A receptor blockade lowers blood pressure in Dahl salt-sensitive and DOCA–salt hypertensive rats (Hynes & Webb, 1998; Naruse *et al.* 2000; Okada *et al.* 2000; Di Filippo *et al.* 2002).

In the present study, we evaluated diverse aspects of noradrenergic transmission, except for TH, which is involved not only in NA but also in dopamine and adrenaline biosynthesis. It is important to point out that the topographical distribution of ETs, and particularly ET_A receptors, in the CNS is similar to that of catecholamines, suggesting a functional association between the endothelinergic and catecholaminergic systems (Kurokawa *et al.* 1997). Diverse areas and nuclei of the hypothalamus, including the PH, express ETs and ET receptors (Kurokawa *et al.* 1997; Sluck *et al.* 1999; van den Buuse & Webber, 2000).

The PH not only expresses ET receptors but also receives diverse afferent projections from monoaminergic inputs, originating from the adrenergic, noradrenergic and serotonergic cell groups of various brain regions, such as the anterior hypothalamus, periaqueductal grey matter, rostral raphe nuclei, parabrachial nucleus, nucleus of the solitari tract and LC (Cavdar *et al.* 2001; de Waderner, 2001; Kasparov & Teschemacher, 2008). In particular, the LC (A6 noradrenergic cell group) is closely related with the PH. In this sense, it has been reported that catecholamine administration in the PH elevates blood pressure and administration of L-glutamic acid in the LC enhances NA content in the PH, resulting in blood pressure elevation (Nakata *et al.* 1990). The response is attenuated by 6-hydroxydopamine injection in the PH (Kawasaki *et al.* 1991; De Wardener, 2001). These findings clearly show that changes in the PH are influenced by changes in the LC activity (De Wardener, 2001). Furthermore, baroreflex activation diminishes NA release not only in the LC but also in the PH of normotensive animals (Schneider *et al.* 1995). In addition, inputs originating in the chemoreceptors of the carotid bodies enhance NA release in the LC and the PH (Kaehler *et al.* 1999).

In DOCA–salt hypertension, ETs are increased in the periphery, but little is known about the role of these neuropeptides in the brain. Experimental evidence supports the existence of an endogenous ET system in brain regions intimately related to the regulation of autonomic function (Kuwaki *et al.* 1999). Various studies support the participation of brain sympathetic activity and, in particular, that of the LC in the development of DOCA–salt hypertension (Chida *et al.* 1983; Olpe *et al.* 1985; Berecek *et al.* 1987).

In summary, the present study shows that in the PH of DOCA–salt rats, ETs enhanced NA turnover and increased the synthesis, release and catabolism of NA. These findings clearly show that ET-1 and ET-3 stimulate different mechanisms leading to enhanced noradrenergic transmission in the PH of DOCA–salt hypertensive rats and suggest that it may result from increased expression of ET_A receptors in this brain area. Although numerous studies support the contribution of brain ETs to hypertension, their role still remains to be elucidated fully. Unveiling the role of brain ETs in hypertension would probably favour the development of new therapeutic targets for the treatment of essential hypertension, which still represents a challenging disease with high morbidity and mortality.

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Additional information

Competing interests

None declared.

Author contributions

All authors approved the final version of the manuscript, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. Conception and design of the experiments: MS Vatta and LG Bianciotti Collection, analysis and interpretation of data: T Abramoff, MJ Guil, VP Morales, SI Hope, and C Höcht Drafting the article or revising it critically for important intellectual content: MS Vatta, and LG Bianciotti.

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