Endothelin-1 and -3 diminish neuronal NE release through an NO mechanism in rat anterior hypothalamus

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Di Nunzio, Andrea S., María S. Jaureguiberry, Valeria Rodano, Liliana G. Bianciotti, and Marcelo S. Vatta. Endothelin-1 and -3 diminish neuronal NE release through an NO mechanism in rat anterior hypothalamus. Am J Physiol Regul Integr Comp Physiol 283: R615–R622, 2002.—The existence of endothelin binding sites on the catecholaminergic neurons of the hypothalamus suggests that endothelins (ETs) participate in the regulation of noradrenergic transmission modulating various hypothalamic-controlled processes such as blood pressure, cardiovascular activity, etc. The effects of ET-1 and ET-3 on the neuronal release of norepinephrine (NE) as well as the receptors and intracellular pathway involved were studied in the rat anterior hypothalamus. ET-1 (10 nM) and ET-3 (10 nM) diminished neuronal NE release and the effect blocked by the selective ET type B receptor antagonist BQ-788 (100 nM). Nω-nitro-L-arginine methyl ester (10 μM), methylene blue (10 μM), and KT5823 (2 μM), inhibitors of nitric oxide synthase activity, guanylate cyclase, and protein kinase G, respectively, prevented the inhibitory effects of both ETs on neuronal NE release. In addition, both ETs increased nitric oxide synthase activity. Furthermore, 100 μM picrotoxin, a GABA_A-receptor antagonist, inhibited ET-1 and ET-3 response. Our results show that ET-1 as well as ET-3 has an inhibitory neuromodulatory effect on NE release in the anterior hypothalamus mediated by the ET type B receptor and the involvement of a nitric oxide-dependent pathway and GABA_A receptors. ET-1 and ET-3 may thus diminish available NE in the synaptic gap leading to decreased noradrenergic activity.

endothelin receptor; soluble guanylyl cyclase; BQ-610; BQ-788; GABA_A receptor, nitric oxide, norepinephrine

ENDOTHELIN (ET) is a potent vasoconstrictor peptide that was originally isolated from the supernatant of cultured porcine aortic endothelial cells (21). The ETs comprise a family of 21-amino acid peptides, ET-1, ET-2, and ET-3, that are encoded by three different genes (21). Two receptors for ETs have been cloned, ET_A and ET_B receptors. The ET_A receptor has higher affinity for ET-1 and ET-2 than for ET-3, whereas the ET_B receptor displays similar affinity for all three ETs (21, 43). Specific distribution of the ETs (ET-1 and ET-3) in the central nervous system (CNS) has been demonstrated. The hypothalamus contains an elevated expression of ET-1 and ET-3 mRNA and high densities of ET-binding sites (9, 21, 36, 43, 45).

The hypothalamus is involved in the regulation of cardiovascular activity because certain hypothalamic regions and nuclei receive cardiovascular sensory input (26). The anterior hypothalamus plays an important role in the regulation of blood pressure as electrolytic lesions in this area produce hypertension in normotensive rats. Current evidence supports that the anterior hypothalamus primarily subserves a sympathoinhibitory role (26).

Several studies suggest that ET-1 and ET-3 act as neurotransmitters or neuromodulators within the CNS, supported by findings showing direct interactions between ETs and brain neurons. ET-1 induces dopamine release mediated by the ET_B receptor in rat striatum (43). Furthermore, ET-3 evokes the release of catecholamines from cortical and striatal brain slices in the rat (7).

Diverse factors modulate noradrenergic neurotransmission by altering the release, the synthesis, and the uptake of the neurotransmitter. On the basis of the existence of high density ET receptors on catecholaminergic neurons of the hypothalamus and the importance of hypothalamic catecholamines in blood pressure regulation (26, 20), we sought to establish the effect of ET-1 and ET-3 on the spontaneous neuronal release of NE in rat anterior hypothalamus. Furthermore, the mechanisms underlying ET-1 and ET-3 response as well as the receptors involved were also investigated.

Our findings show that ET-1 and ET-3 reduced neuronal NE release in the rat anterior hypothalamus through the ET_B receptor and a mechanism involving the nitric oxide (NO) pathway and GABA_A receptors. The sympathoinhibitory response elicited by this region would be diminished by ETs resulting in an increase of blood pressure.
METHODS

Animals and chemicals. Male Sprague-Dawley rats weighing 250–300 g were used (from Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina).

The following drugs were used: 1-[7,8-3H]NE (1.18 TBq/mmol of specific activity, Amersham Pharmacia Biotech); 1-[2,3,4-3H]arginine (53.4 Ci/mmol of specific activity, New England Nuclear); ET-1, ET-3, BQ-610, and BQ-788 (Peninsula Lab); pargyline, hydrocortisone, desipramine HCl, tetrahydrobiopterin, suramin (SMN), minimum essential media (MEM) amino acid solution, and basal medium Eagle vitamin solution (ICN Biomedicals); N nitro-1-arginine methyl ester (l-NAME), methylene blue (MeB), picrotoxin (PTX), l-arginine (l-Arg), d-arginine (D-Arg), and β-NADPH (Sigma Chemical, St. Louis, MO); Dowex-AG50W-X28 resin (sodium form, 200–400 mesh, BioRad Lab); DTT (Promega); and KT5823 (Alomone Labs, Jerusalem, Israel). Other reagents were of analytic purity and were obtained from standard sources. All drugs were dissolved in Krebs solution, except for KT5823 and PTX that were dissolved in DMSO and ethanol, respectively. Spontaneous neuronal NE release as well as ET-1 and ET-3 response were not affected by DMSO or ethanol (data not shown).

Experimental protocol. Animals were killed by decapitation and brains were quickly removed and anterior hypothalami were immediately dissected under a magnifier. [3H]NE release was measured according to the method described by Vatta et al. (44) with minor modifications. Briefly, anterior hypothalami slices were preincubated at 37°C for 30 min in gassed standard Krebs solution supplemented with MEM amino acid solution and basal medium Eagle vitamin solution (KSS). Monoamine-oxidase activity and extraneuronal NE uptake were inhibited by the addition of 50 μM pargyline and 100 μM hydrocortisone, respectively. NE stores were labeled with 2.5 μCi/ml [3H]NE (100 nM) for 30 min followed by three consecutive washes (10 min each) with KSS. In the last wash, 10 μM desipramine was added to inhibit neuronal NE uptake. The tissues were then incubated for 15 min, and three consecutive samples of the incubation medium were collected every 5 min. The first sample corresponded to the basal release period (B), the second to the inhibitory period (I), and the third to the experimental period (E). ET-1 and ET-3 (0.1, 1, and 10 nM) were added at the beginning of the E period, whereas the following drugs were added at the beginning of the I period: 100 nM BQ-610 (ETA receptor antagonist), 100 nM BQ-788 (ETB receptor antagonist), 500 nM SMN (G proteins inhibitor), 10 μM l-NAME [NO synthase (NOS) inhibitor], 100 μM l-Arg (NO precursor), 10 μM d-Arg (inactive enantiomer), 10 μM MeB (guanylate cyclase inhibitor), 2 μM KT5823 (selective protein kinase G inhibitor, PKG), and 100 μM PTX (GABAA receptor antagonist). [3H]NE release was measured in the incubation medium by conventional scintillation counting methods. Results are expressed as the ratio of the radioactivity released in the inhibitory (I) or experimental (E) and basal periods (I/B and E/B, respectively).

NOS activity assay. The anterior hypothalami were preincubated at 37°C for 30 min with gassed KSS. Tissues were then incubated for 5 min in the absence (control) or in the presence of 10 nM ET-1 or 10 nM ET-3. Reaction was stopped by three consecutive washes (5 min each) with cold KSS. The activity of NOS was measured according to the method described by Tsuchiya et al. (41). Briefly, tissues were quickly homogenized with 20 mM HEPES buffer (pH 7.4) and then centrifuged at 10,000 g for 10 min at 4°C. One aliquot of the supernatant was used for protein assay by the method of Lowry et al. (12), whereas another aliquot was incubated at 37°C for 10 min in the buffer reaction [1 μM l-Arg, 20 nM [3H]arginine, 20 mM EDTA, 1 mM DTT, 1 mM β-NADPH, 10 μM tetrahydrobiopterin (H4B), 20 mM HEPES, and 1.25 mM CaCl2]. Reaction was stopped by lowering the temperature to 4°C, and samples were then loaded onto 1-ml columns containing Dowex-AG50W-X28 resin preequilibrated with 200 mM citrulline. Columns were eluted with distilled water, and 2-ml fractions containing the [3H]citrulline were collected and the radioactivity was determined by usual scintillation counting methods. NOS activity is expressed as the percentage of the control group ± SE.

Statistical analysis. All values are expressed as the means ± SE. Differences among groups were statistically assessed using the ANOVA test followed by the t-test modified by Bonferroni (GraphPad, San Diego, CA). In all cases, P values of 0.05 or less were considered statistically significant.

RESULTS

To determine the effects of ET-1 and ET-3 on neuronal NE release, a concentration-response study was previously performed. Data given in Fig. 1, A and B, show that both 10 nM ET-1 and 10 nM ET-3 diminished neuronal NE release, whereas 1 and 0.1 nM ET-1 or ET-3 did not modify neuronal secretion of NE (Fig. 1, A and B).

None of the inhibitors used (BQ-610, BQ-788, SMN, l-NAME, MeB, KT5823, or PTX) modified [3H]NE release per se in the I period (data not shown).

To study the type of ET receptor coupled to the inhibitory effect of ET-1 and ET-3 on neuronal NE release, the effect of selective antagonists for ETA and ETB receptor types was assessed. Results showed that neither 100 nM BQ-610 nor 100 nM BQ-788 (selective ETA and ETB receptor antagonists respectively) modified spontaneous neuronal output of NE in the I and E periods (Fig. 2). The reduction of NE release evoked by
10 nM of ET-1 and ET-3 was not blocked by BQ-610 (Fig. 2, A and B) but it was inhibited in the presence of 100 nM BQ-788 (Fig. 2, C and D). ET receptors belong to the superfamily of G protein-coupled receptors (21, 43). Therefore, we examined the effects of 500 nM SMN, a G protein inhibitor at this concentration, on ET-1 and ET-3 inhibitory response. SMN modified spontaneous NE release neither in the I period nor in the E period, but it completely inhibited the decrease in neuronal release produced by ET-1 and ET-3 (Fig. 3, A and B).

It has been reported that ET B activation is followed by an increase of NOS activity (38). To determine the role of NO as possible mediator of ET-1 and ET-3 response in the rat anterior hypothalamus, experiments were carried out in the presence of 10 µM L-NAME, an inhibitor of NOS activity. L-NAME (10 µM) did not modify neuronal NE release in the I and E periods, but it abolished the decrease in NE secretion induced by 10 nM ET-1 as well as by 10 nM ET-3 (Fig. 4). Furthermore, 100 µM L-Arg, the precursor of NO synthesis, decreased neuronal NE release in the anterior hypothalamus, supporting the participation of NO in ET-1 and ET-3-induced reduction of NE release (Fig. 5).

As NO induces activation of soluble guanylyl cyclase leading to generation of cGMP, which activates PKG (42), we used an inhibitor of guanylyl cyclase and an inhibitor of PKG to confirm the activation of this pathway by both ETs. The results showed that 10 µM MeB and 2 µM KT5823 per se did not modify neuronal NE release but both prevented the inhibitory response induced by ET-1 and ET-3 (Fig. 7).

As NO has been involved in the modulation of GABA<sub>A</sub> receptors and GABA has been related to the release of NE (17), we studied the effect of PTX, a
GABA<sub>A</sub>-receptor antagonist, on the inhibitory effect of ET-1 and ET-3 on NE release. PTX (100 μM) did not alter spontaneous NE release in the I and E periods, but it abolished ET-1 and ET-3 inhibitory response on neuronal NE release (Fig. 8). To confirm the interaction between the GABA<sub>A</sub> receptor and the NO pathway, we investigated the effect of L-Arg in the presence of PTX. The reduction of neuronal NE release induced by L-Arg was antagonized by PTX (Fig. 9).

**DISCUSSION**

The present study reports that ET-1 as well as ET-3 diminished neuronal release of NE from the anterior hypothalamus. Previous studies showed that a number of hypothalamic regions and nuclei containing different neurotransmitters, including NE, participate in the regulation of several biological processes such as cardiovascular activity (4, 26, 37). The anterior hypothalamus plays an important role in blood pressure control. The electrical stimulation of this area causes a decrease in blood pressure and heart rate in normotensive animals (26). Studies in conscious, freely moving rabbits revealed that a reduction of blood pressure leads to a decrease in the release of NE in the anterior hypothalamus, whereas a rise in blood pressure increases the release of NE from this area (32). Furthermore, studies to characterize regional brain NE turnover in NaCl-sensitive spontaneously hypertensive rats (SHR-S), Wistar-Kyoto rats, and NaCl-resistant spontaneously hypertensive rats (SHR-R) fed with high and basal NaCl diets, showed that dietary NaCl loading elevates blood pressure in SHR-S by reducing NE released from the adrenergic nerve terminals in the anterior hypothalamus, thus decreasing the activation of sympathoinhibitory neurons in the anterior hypothalamus and increasing peripheral sympathetic nervous system activity (26).

The existence of ETs, their receptors, and ET-converting enzyme activity has been shown in the brain by immunohistochemistry, autoradiography, and in situ hybridization studies. ET binding sites were described in the hypothalamus and the brain stem [nucleus of the solitary tract (NTS) and the ventrolateral medulla] (36, 45), suggesting that ETs may contribute to the control of sympathetic nerve activity and blood pressure. Intracisternal administration of ET-1 evokes an initial decrease followed by an increase in blood pressure (22). Microinjection of ET-1 into the rostral ventrolateral medulla evokes pressor and bradycardic effects followed by sustained decreases in blood pressure, bradycardia, and respiratory depression (23). In the caudal ventrolateral medulla, ET-1 decreases blood pressure, renal sympathetic nerve activity, respiratory frequency, and phrenic nerve activity, whereas it increases heart rate (23). In addition, the neural activity of NTS neurons is increased by ET-1 (35).

In addition to the physiological importance of ETs in the regulation of blood pressure, its role in the pathogenesis of hypertension has also been suggested. Exogenous ET-1 enhances the release of catecholamines...
in the adrenal medulla from both normotensive and DOCA-salt hypertensive rats (4). When the adrenal gland is stimulated in the presence of ET-1, NE release is inhibited in DOCA-salt hypertensive rats but not in normotensive rats, suggesting two different roles for ET in the adrenal gland of DOCA-salt hypertensive rats. On one hand, endogenous ET may protect the adrenal gland from rapid depletion of catecholamines in times of stress during DOCA-salt hypertension. On the other hand, the increased level of exogenous ET in the DOCA-salt hypertension may serve to enhance the basal release of catecholamines from the adrenal medulla (10). Therefore, our findings as well as these data support the role of ET in increasing blood pressure.

The inhibitory effect of ET-1 and ET-3 on NE release was blocked by the ET_B receptor antagonist BQ-788 but not by the ET_A receptor antagonist BQ-610, showing that ET_B receptors mediate ET-1 and ET-3 response in the anterior hypothalamus. Both ET_A and ET_B receptors are members of the G protein-coupled family of rhodopsin-like receptors (21, 43). Our results show that in the ET-1 and ET-3 response, a G protein is involved because ET-1 and ET-3 effects were abolished in the presence of SMN that at a concentration of 500 nM acts as an inhibitor of G protein. The ET_B receptor has both pressor and depressor effects in vivo (2). Thus the activation of the ET_B receptor on vascular smooth muscle produces a pressor response through vasoconstriction, whereas its activation, on the vascular endothelium, induces a depressor response mediated by the release of endothelium-derived vasodilators NO and prostacyclin (2, 24). Current evidence indicates that the ET_B receptor is normally present in a wide variety of tissues in the adult rat, including those related to the regulation of blood pressure such as the glia, neurons in regions of the brain stem involved in the control of cardiovascular function and the hypothalamus, as well as endothelial cells and vascular smooth muscle (8). An endothelial ET_B receptor-mediated pathway of vascular relaxation involving the
release of NO seems to be active under basal conditions and may protect against excessive vasoconstriction and increased blood pressure particularly during a high-salt diet (7). The elevation of blood pressure was reported in ETβ-deficient mice, suggesting that ETβ may play an essential depressor role in maintaining blood pressure within the normal range (25). In addition, ETβ receptor subtype also appears to have a protective role in the development of hypertension and cardiovascular hypertrophy in different experimental models of hypertension (6, 14, 25).

It is well known that the activation of ETβ receptor is coupled to the generation of NO. In the endothelium, the activity of NOS is increased on ETβ stimulation (24). Our findings show that the decrease in neuronal NE release evoked by ET-1 and ET-3 was inhibited in the presence of 10 μM L-NAME, supporting the participation of NO in ETs response. Moreover, the participation of NO in ETs response was further confirmed by the evidence that both ET-1 and ET-3 increased NOS activity in the anterior hypothalamus. In the L-arginine-NO-guanylyl cyclase pathway, NOS from L-arginine forms NO (19, 28, 29, 31) that in turn activates guanylyl cyclase (1, 18, 27, 39). Several reports show that NO is involved in the modulation of catecholamine secretion in different tissues such as the adrenal medulla and the CNS (34, 40). The inhibition of catecholamine secretion produced by the endothelial cells and the autoinhibition of catecholamine output induced by chromaffin cells are both reversed by N(G)-monomethyl-L-arginine, L-NAME, and MeB, supporting that NO from endothelial and chromaffin sources regulates catecholamine secretion through the activation of guanylyl cyclase (40). As expected, the inhibitory action of ET-1 and ET-3 on neuronal NE release was prevented by MeB, supporting that guanylyl cyclase activation mediates ETβ activation through NO release. Guanylyl cyclase catalyzes the formation of cGMP, which in turn activates PKG, resulting in the phosphorylation of target proteins (42). In the present work, the reduction in NE release evoked by ET-1 and ET-3 was blocked in the presence of a PKG inhibitor (KT5823), thus supporting PKG activation in ETs response in the anterior hypothalamus. Our findings are in agreement with previous reports showing that NO inhibits basal and potassium-stimulated release of NE from the medial basal hypothalamus (33). Furthermore, both ET-1 and NE release evoked by ET-1 and ET-3 was inhibited in the presence of 10 μM L-NAME, supporting the participation of NO in ETs response. Moreover, the participation of NO in ETs response was further confirmed by the evidence that both ET-1 and ET-3 increased NOS activity in the anterior hypothalamus. In the L-arginine-NO-guanylyl cyclase pathway, NOS from L-arginine forms NO (19, 28, 29, 31) that in turn activates guanylyl cyclase (1, 18, 27, 39). Several reports show that NO is involved in the modulation of catecholamine secretion in different tissues such as the adrenal medulla and the CNS (34, 40). The inhibition of catecholamine secretion produced by the endothelial cells and the autoinhibition of catecholamine output induced by chromaffin cells are both reversed by N(G)-monomethyl-L-arginine, L-NAME, and MeB, supporting that NO from endothelial and chromaffin sources regulates catecholamine secretion through the activation of guanylyl cyclase (40). As expected, the inhibitory action of ET-1 and ET-3 on neuronal NE release was prevented by MeB, supporting that guanylyl cyclase activation mediates ETβ activation through NO release. Guanylyl cyclase catalyzes the formation of cGMP, which in turn activates PKG, resulting in the phosphorylation of target proteins (42). In the present work, the reduction in NE release evoked by ET-1 and ET-3 was blocked in the presence of a PKG inhibitor (KT5823), thus supporting PKG activation in ETs response in the anterior hypothalamus. Our findings are in agreement with previous reports showing that NO inhibits basal and potassium-stimulated release of NE from the medial basal hypothalamus (33). Furthermore, both ET-1 and and

![Diagram showing the mechanism of ET-1 and ET-3-induced reduction of neuronal NE release](http://ajpregu.physiology.org/)

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**Fig. 9.** Antagonism by a selective GABA<sub>α</sub> receptor antagonist, PTX, of the L-Arg-induced reduction in neuronal [3H]NE release in the rat anterior hypothalamus. E/B represents the factor above basal release of [3H]. Values are means ± SE. Number of experiments: 6–10. *P < 0.001 vs. control.

**Fig. 10.** Schematic illustration of the proposed mechanism for ET-1- and ET-3-induced reduction of neuronal NE release in the rat anterior hypothalamus. Both ET-1 and ET-3 bind to the ETβ receptor-G protein complex increasing NOS activity. NO through soluble guanylyl cyclase activation leads to generation of cGMP, which activates PKG. Subsequently, PKG would activate GABA<sub>α</sub> receptors, that would induce membrane hyperpolarization leading to decreased NE release. G-Ps, G proteins; sGC, soluble guanylyl cyclase.

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ET-3 increase cGMP formation through ETB activation and NO generation in rat adrenal medulla (13). Several studies suggest the existence of an interaction between NO and GABA receptors. Both ETs increase NO formation through ETB receptor activation. Brain NO-containing neurons stimulate the release of GABA (33, 34). Moreover, GABAAergic neurons of the brain have been implicated in the central regulation of cardiovascular activity. Microinjection of adrenergic agonists, such as clonidine, into the anterior hypothalamus produces hypotensive responses. These effects result from an increase of GABA content and [3H]GABA binding (3). On the basis of these findings, we studied if GABA receptors were involved in ETs-evoked decrease in NE release in the anterior hypothalamus. The inhibitory response of both ET-1 and ET-3 on NE output was blocked by the GABA antagonist PTX. In agreement with our results, NE release was reported to be increased in the locus ceruleus after onist PTX. In agreement with our results, NE release was increased in the locus ceruleus after treatment with PTX (30). However, in the hippocampus, GABA was found to enhance NE release by activating GABA receptors located on noradrenergic terminals (5). The discrepancy of results regarding the interaction between GABA receptors and NE release may be related to functional as well as morphological differences between the brain areas studied. Our findings are in agreement with those reported by Czyzewska-Szafran et al. (3) that showed that PTX abolished the depressor response of clonidine.

Binding of an agonist to the GABA receptor-channel complex leads to the opening of an anion channel permeable to chloride. In presynaptic nerve terminals, one of the mechanisms proposed for the inhibition of transmitter release by the activation of chloride channels is a decrease in the amount of transmitter released by each action potential, which precedes in reaching the terminal (16). The opening of the GABA receptor channels results in the inhibition of the neurotransmitter release in preparations such as supraoptic nucleus, olfactory cortex, peptidergic nerve in posterior pituitary, central nucleus of the inferior colliculus, etc. The activity of the GABA channels can be modulated by NO, phosphorylation by protein kinase A, and calcium/phospholipid-dependent protein kinase C (16). Other authors reported that there are specific sites of phosphorylation for PKG within GABA receptor subunits (11, 15), suggesting that GABA receptors can be regulated by the cGMP-dependent protein kinase. These findings are in agreement with our results because the inhibition of PKG activity with KT5823 blocked the ET-1 and ET-3 effect on neuronal NE release. Present results suggest that ET-1 and ET-3 decrease NE release, likely through the mechanism depicted in Fig. 10. In the anterior hypothalamus both ET-1 and ET-3 bind to the ETB receptor-G protein complex and activate NOS, increasing NO generation and leading to increased cytosolic cGMP levels by guanylyl cyclase stimulation. Subsequently, cGMP activates PKG, and the activation of this intracellular pathway triggers the activation of the GABA receptors that would induce membrane hyperpolarization leading to decreased NE release. The proposed mechanism may occur in a single noradrenergic neuron or may also involve the participation of other nonnoradrenergic neurons.

In conclusion, our results show that ET-1 and ET-3 have a neuromodulatory inhibitory effect on neuronal NE release in the anterior hypothalamus, mediated by ETB activation signaling through NO and the participation of GABAA receptors. ET-1 and ET-3 may thus diminish NE availability in the synaptic gap leading to decreased noradrenergic activity. The decrease of neuronal NE release in the anterior hypothalamus evoked by ETs would reduce the sympathoinhibitory response elicited by this area on stimulation. Thus it is likely that both ET-1 and ET-3 may participate in the development and/or maintenance of hypertension.

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