Long-term modulation of tyrosine hydroxylase activity and expression by endothelin-1 and -3 in the rat anterior and posterior hypothalamus

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Perfume G, Nabhen SL, Riquelme Barrera K, Otero MG, Bianciotti LG, Vatta MS. Long-term modulation of tyrosine hydroxylase activity and expression by endothelin 1 and 3 in the rat anterior and posterior hypothalamus. Am J Physiol Regul Integr Comp Physiol 294: R905–R914, 2008. First published December 19, 2007; doi:10.1152/ajpregu.00555.2007.—Brain catecholamines are involved in the regulation of biological functions, including cardiovascular activity. The hypothalamus presents areas with high density of catecholaminergic neurons and the endothelin system. Two hypothalamic regions intimately related with the cardiovascular control are distinguished: the anterior (AHR) and posterior (PHR) hypothalamus, considered to be sympathoinhibitory and sympathoexcitatory regions, respectively. We previously reported that endothelins (ETs) are involved in the short-term tyrosine hydroxylase (TH) regulation in both the AHR and PHR. TH is crucial for catecholaminergic transmission and is tightly regulated by well-characterized mechanisms. In the present study, we sought to establish the effects and underlying mechanisms of ET-1 and ET-3 on TH long-term modulation. Results showed that in the AHR, ETs decreased TH activity through ETα receptor activation coupled to the nitric oxide, phosphoinositide, and CaMK-II pathways. They also reduced total TH level and TH phosphorylated forms (Ser 19 and 40). Conversely, in the PHR, ETs increased TH activity through a G protein-coupled receptor, likely an atypical ET receptor or the ETc receptor, which stimulated the phosphoinositide and adenyl cyclase pathways, as well as CaMK-II. ETs also increased total TH level and the Ser 19, 31, and 40 phosphorylated sites of the enzyme. These findings support that ETs are involved in the long-term regulation of TH activity, leading to reduced sympathoinhibition in the AHR and increased sympathoexcitation in the PHR. Present and previous studies may partially explain the cardiovascular effects produced by ETs when applied to the brain.

catecholamines; endothelin receptors; central nervous system

ENDOTHELINS (ETs) ARE VASOCATIVE peptides originally isolated from cultured porcine endothelial cells (56). They constitute a family of three 21-amino acid isopeptides termed ET-1, ET-2, and ET-3 (10, 23). ETs exert their biological effects through the activation of ETα and ETβ receptors that are G protein-coupled receptors (GPCRs) (10, 34, 38). Despite these two well pharmacologically characterized GPCRs, several pharmacological and functional studies support the existence of additional ET receptors. The finding of atypical responses in the presence of selective ETα and ETβ antagonists and/or agonists observed in different studies built up the term atypical receptors (ETαX or ETβX) to refer to those receptors that mediate ET responses but fail to be identified by using selective antagonists or agonists of the conventional ETα and ETβ receptors (12, 20, 36, 38, 41). In addition, a third receptor subtype named ETc that displays high affinity for ET-3 was cloned in Xenopus laevis, and although many functional studies support its existence in mammals, it has not been cloned in this species yet (12, 24, 32, 38, 41). The conventional ETα and ETβ receptors by coupling to different G proteins stimulate multiple intracellular signaling pathways, including adenyl cyclase/PKA, nitric oxide (NO)/guanylyl cyclase/PKG, as well as the phosphoinositide pathway (30, 34, 38, 51).

The ET system (precursor peptides, endothelin-converting enzyme, and receptors) is widely distributed in different mammalian tissues, including the central nervous system (4, 30). In the hypothalamus, the ET system is highly expressed, suggesting that these peptides participate in the regulation of diverse biological functions controlled at this level (4, 30). The hypothalamus coordinates endocrine, neuroendocrine, and autonomic signals involved in the control of the cardiovascular activity, water, and sodium homeostasis, as well as hormone release (18, 21, 37, 55). Among the different areas and nuclei that form the hypothalamus, the anterior and posterior hypothalamic regions play a major role in the regulation of the cardiovascular activity (37, 55). The anterior hypothalamic region (AHR) is a sympathoinhibitory area, whereas the posterior region (PHR) behaves as a sympathoexcitatory area (37). Functional impairment of these areas has been associated with hypertension (37, 55). In addition, ETs through ETα receptors in the spinal cord also regulate cardiovascular and sympathetic activity (42).

Catecholamines [dopamine, norepinephrine (NE), and epinephrine] are widely distributed in the brain, NE being a major neurotransmitter in diverse neuronal circuits, including the hypothalamus (37, 55). Tyrosine hydroxylase (TH), the key enzyme in the biosynthesis of catecholamines, is a specific marker of catecholaminergic neurons (13, 14, 29). This enzyme catalyzes the hydrolysis of L-tyrosine to L-Dopa, which is the rate-limiting step in the biosynthetic pathway (13, 14, 29). The regulation of TH is a complex process, which involves both short and long-term mechanisms. Short-term modulatory mechanisms (seconds to minutes) include end-product feedback inhibition, polyprotein-mediated allosteric inhibition, as well as posttranslational, phosphorylation, and dephosphorylation.

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tion processes (13, 29). Long-term regulatory mechanisms (hours to days) mainly involve enzyme modifications and/or changes in its gene expression (13, 29). However, phosphorylation and dephosphorylation processes also contribute to the long-term regulation of TH activity (3, 15).

Catecholamines, ETs, and the hypothalamus, particularly the AHR and PHR, were separately reported to be intimately involved in the regulation of cardiovascular activity. However, little is known about their interaction. It was shown that ET-1 increases dopamine release in the rat striatum, whereas ET-3 augments catecholamine output in cortical, as well as striatal brain slices (27, 52). We previously reported that both ETs modulate NE release, as well as the short-term modulation of TH activity and expression of the phosphorylated forms of the enzyme in the AHR and PHR (11, 12, 33, 41).

As the role of the ETs-catecholamines-hypothalamus interaction and its regulatory mechanisms are not presently well defined, the aim of the present work was to study the effect of ETs in the long-term regulation of TH activity and expression in the AHR and PHR, as well as to determine the underlying mechanisms and receptors involved.

Results showed that in the AHR, both ETs decreased TH activity through the activation of ET type receptors coupled to the NO, phosphoinositide, and CaMK-II pathways. ETs also decreased total TH expression, as well as the phosphorylated sites of the enzyme (Ser 19, Ser 31, and Ser 40). On the other hand, in the PHR, ET-1 and ET-3 increased TH activity through a GPCR, presumably an atypical receptor or the ETC receptor, coupled to the adenyl cyclase/PKA, phosphoinositide, and CaMK-II pathways. ETs also increased total TH expression and the phosphorylated sites of the enzyme in this hypothalamic region.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing between 250 and 300 g (Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires) were used in the experiments. Animals were housed in steel cages and maintained at a temperature between 20 and 23°C in a controlled room with a 12:12-h light-dark cycle. All animals had free access to water and commercial chow.

Experimental design. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no. 85-23 1985, Revised 1996). Rats were decapitated between 9:00 a.m and 12:00 p.m., hypothalami were removed, and the AHR and PHR were dissected according to Palkovits and Brownstein (39). The AHR includes the periventricular, supraoptic, suprachiasmatic, and anterior hypothalamic nuclei, whereas the PHR includes premammillary and supramammillary, posterior hypothalamic, and prelateral mamillary nuclei, as well as the hypothalamic part of the medial forebrain bundle (39). Tissues were preincubated in a Dubnoff incubator for 30 min at 37°C in Krebs bicarbonate solution supplemented with minimal essential media amino acid solution and basal medium Eagle vitamin solution (KBSS), pH 7.4, and bubbled with a gas mixture (95% O2-5% CO2) under continuous shaking. To evaluate the long-term effect of ET-1 or ET-3 on TH activity, tissues were incubated for 360 min in the presence or in the absence of ETs (experimental and control groups, respectively). The following drugs were added 15 min before and during the incubation period: 100 nM BQ-610 (ETα receptor antagonist), 100 nM BQ-788 (ETβ receptor antagonist), 500 nM suramin (SMN) (G protein inhibitor), 10 μM Nω-nitro-arginine methyl ester (l-NNAME) [nitric oxide synthase (NOS) inhibitor], 10 μM 7-nitroindazole (7-NI) (neuronal NOS inhibitor), 10 μM 1H-[1,2,4]oxadiazololo[4,3-a]quinoxalin-1-one (ODQ) (NO-sensitive guanylyl cyclase inhibitor), 2 μM KT-5823, 500 nM H-89, 1 μM KN-62, 10 μM U-73122, and 2 μM chelerytine (CRT) (PKG, PKA, CaMK-II, PLC, and PKC inhibitors, respectively) and 42 μM 2-APB (IP3 receptor selective antagonist). In other experiments, the AHR or PHR was incubated for 360 min in the presence of 300 nM sarafotoxin S6b (SRTx-6b) or 1 μM IRL-1620 (ETα and ETβ receptor agonist, respectively). Other experiments were performed with higher concentrations of the agonists (600 nM SRTx-6b or 2 μM IRL-1620) or adding both agonists together. Drugs were dissolved in KBSS except for 7-NI, ODQ, KT-5823, H-89, KN-62, U-73122, CRT, and 2-APB that were dissolved in DMSO while IRL-1620 and SRTx-6b in 2.5% NH4OH and 500 μM HEPES (pH 7.0), in the presence of 15 nM l-tyrosine containing 0.5 μCi [3H]l-tyrosine, 420 mM β-mercaptoethanol, 1,000 U catalase, and 0.75 mM 6-methyl-tetrahydrobiopterin. The reaction was stopped by the addition of 1 ml 7.5% activated charcoal suspension in 1 N HCl. The final mixture was vortexed and centrifuged at 500 g for 10 min. Radioactivity of 3H2O was determined in the supernatant by conventional scintillation methods. Blank values were obtained by omitting 6-methyl-tetrahydrobiopterin from the reaction mixture. The determination of recovered 3H2O was performed as described by Reinhard et al. (43). Results were expressed as the percentage of control group ± SE.

TH Western blot assay. Tissues were homogenized in lysis buffer (20 mM Tris-Cl pH 7.4, 1 mM PMSF, 5 mM EDTA, 25 mM NaF, 1% Triton X-100, 1% proteases inhibitor cocktail), and then centrifuged for 20 min at 4°C. An aliquot from the supernatant was saved for protein determination, and the remaining sample mixed with Laemmli buffer (62.5 mM Tris-Cl pH 6.8, 2% SDS, 5% B-ME, 10% glycerol, 4% bromophenol blue, treated for 5 min, and then subjected to SDS-PAGE at 100 Vts for 2.30 h. Gels were transferred to polyvinylidene difluoride (PVDF) membranes at 100 Vts for 75 min. The membranes were blocked overnight at 4°C in blocked solution (5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20 [TBS-T]), and the gels were stained overnight at 4°C with Coomassie blue. After washing with TBS-T, transfers were incubated with α-TH Ab, TH S19p-Ab, TH S31p-Ab and TH S40p-Ab (1/1,000, overnight at 4°C), actin-Ab (1/1,500, 1 h at room temperature), streptavidin-alkaline phosphatase conjugate and U73122 (Sigma, St. Louis, MO), and Biocytin-NTB. Bands were analyzed by densitometry (UN-SCAN IT gel; Silk Scientific, Orem, UT) and normalized to β-actin. Results of total TH and each TH phosphorylated Ser were expressed as a percentage of control group ± SE.

Chemicals. The following drugs, reagents, and kits were used: l-[3,5-3H]tyrosine (1.70 TBq/mmol of specific activity) and PVDF membrane (GE Healthcare, New York, NY, and Amersham Biosciences, Little Chalfont, Buckinghamshire, UK); ET-1, ET-3, BQ-610, and BQ-788 (American Peptides, Sunnyvale, CA); catalase, l-Dopa, KN-62, H-89, l-tyrosine, SMN, 6 methyl-tetrahydrobiopterin, MEM amino acid solution, and basal medium Eagle vitamin solution (MP Biomedicals, Irvine, CA); anti-actin polyclonal antibody (Ab), 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT), α-NH2, ODQ, protease inhibitor cocktail, streptavidin-alkaline phosphatase conjugate and U73122 (Sigma, St. Louis, MO); 2-APB, IRL-1620, 7-NI, and SRTx-6b (Calbiochem, San Diego, CA); CRT, KT-5823 (Alomone, Jerusalem, Israel). Mouse anti-TH Ab (α-TH-Ab), rabbit anti-TH phosphor Ser –19, –31, and
−40 (TH S19p-Ab, TH S31p-Ab, and TH S40p-Ab, respectively), biotinylated anti-mouse Ab and biotinylated anti-rabbit Ab (Chemicon, Temecula, CA). Other reagents were of analytical or molecular biology quality and obtained from standard sources.

Statistical analysis. Results were expressed as means ± SE. The statistical analysis was performed by the ANOVA and the Student-Newman-Keuls test. In all cases, P values of 0.05 or less were considered statistically significant.

RESULTS

ET-1 as well as ET-3 reduced TH activity in the AHR, whereas they both increased it in the PHR at 360 min (Fig. 1). We previously reported that at 30 min ETs diminish TH activity in both hypothalamic regions (33, 41). However, the time course study on TH activity in the AHR showed that ET-1 and ET-3 (10 nM) diminished the activity of the enzyme at 60, 120, 240, and 360 min. Conversely, in the PHR, ETs showed a biphasic response, a reduction at 60 and 120 min followed by an increase in the enzymatic activity at 240 and 360 min (data not shown). To determine the receptors and intracellular signaling involved in the long-term regulatory mechanism of TH activity and expression by ETs, experiments were carried out at 360 min.

To investigate the ET receptor subtype involved in the effect of ET-1 and ET-3 on TH activity, the AHR and PHR were incubated in the presence of selective antagonists of ETA and ETB receptors (100 nM BQ-610 and 100 nM BQ-788, respectively). Results showed that neither BQ-610 nor BQ-788 modified basal TH activity (Fig. 1). In the AHR, the selective ETA antagonist failed to inhibit ETs-induced reduction of TH activity but the selective ETB antagonist inhibited ET response (Fig. 1, A and B). However, in the PHR, both antagonists abolished ET-1 and ET-3 response (Fig. 1, C and D). To further confirm the participation of ET receptors, tissues were incubated with selective ETα (300 nM SRTx-6b) and ETβ (1 μM IRL-1620) agonists. In the AHR, IRL-1620 diminished TH activity, whereas SRTx-6b did not modify it, thus confirming ETβ activation (Fig. 2A). On the other hand, neither 300 nM SRTx-6b nor 1 μM IRL-1620 modified TH activity in the PHR (Fig. 2B). Higher concentrations of the agonists (600 nM SRTx-6b and 2 μM IRL-1620) induced similar results (data not shown). Furthermore, the addition of both agonists together failed to affect the activity of the enzyme in the PHR (data not shown). These findings support that in the AHR, ET response was mediated by the ETβ receptor, whereas in the PHR, the conventional receptors were not involved. In an attempt to identify whether the receptor activated by ETs in the PHR was a GPCR, tissues were pretreated with 500 nM SMN (a G protein inhibitor at this concentration) (22). SMN modified TH activity neither in the AHR nor in the PHR, but it abolished ET-induced reduction of TH activity in the PHR and ET-induced increase of the enzyme activity in the PHR (Fig. 3, A and B).

To elucidate the intracellular signaling pathways that mediated ET response, tissues were incubated with different inhibitors or antagonists. We previously reported that the NO pathway mediates ET-1 and ET-3 short-term regulation of TH in both hypothalamic regions (33, 41). The participation of the NO/cGMP/PKG pathway in ET response was evaluated by pretreatment with 10 μM L-NAME, 10 μM 7-NI, 10 μM ODQ, and 2 μM KT 5823, (inhibitors of NOS, NOS neuronal isoform, NO sensitive guanylyl cyclase, and PKG, respectively). Results showed that in the AHR, L-NAME, 7-NI, ODQ, and KT-5823 did not affect basal TH activity (Fig. 4, A–D). However, they all abolished ET-1 and ET-3 response.
It is well known that different kinases increase NOS activity (1, 28). Furthermore, we reported that ETs increase neuronal NOS activity through multiple intracellular signaling involving various kinases (23). To evaluate the participation of cAMP/PKA pathway and CaMK-II in ET’s effects in the AHR, tissues were pretreated with 500 nM H-89 and 1 μM KN-62 (PKA and CaMK-II inhibitors, respectively). As shown in Fig. 5A, H-89 affected neither basal TH activity nor ET response. On the other hand, KN-62 did not modify basal TH activity, but it inhibited ET’s effect on the enzyme activity (Fig. 5B). To determine the involvement of the phosphoinositide pathway, tissues were pretreated with 10 nM U73122 and 2 μM CRT (PLC and PKC inhibitors, respectively) and 42 μM 2-APB (IP₃ receptor selective antagonist). The inhibitors did not modify basal TH activity, but they abolished ET response in the AHR (Fig. 6).

The intracellular pathways activated by ET-1 and ET-3 in the PHR were investigated by incubating tissues in the presence of the specific inhibitors detailed above on the basis of previous studies showing that various kinases enhance TH activity (13, 14, 29). Fig. 7 shows that U-73122, CRT, and 2-APB did not modify basal TH activity, but they did inhibit ETs response on the enzyme activity. Furthermore, PKA and CaMK-II inhibitors (H-89 and KN-62, respectively) did not affect basal TH activity but inhibited the increase induced by ET-1 and ET-3 (Fig. 8, A and B). The incubation of the PHR with NOS inhibitors modified neither basal nor ET-stimulated TH activity (Fig. 8C).

The effect of ETs at 360 min on TH protein level was analyzed by Western blot analysis. Fig. 9A shows that in the AHR, both ETs decreased total TH expression and diminished TH S40p-Ab and TH S19p-Ab without affecting TH S31p-Ab (Fig. 9, B–D). On the other hand, ET-1 and ET-3 significantly increased total expression, as well as the phosphorylated forms of TH in the PHR (Fig. 10, A–D).

DISCUSSION

ET gene expression was reported in the brain with the highest density found in the hypothalamus (4, 30). The localization of ETs immunoreactivity and mRNA shows a similar distribution, suggesting that these peptides participate in the control of sympathetic nerve activity and blood pressure (52). Thus, several reports show that ETs applied at different brain sites (intracisternal, intracerebroventricular, ventrolateral medulla, nucleus of the solitary tract) regulate different biological functions, including blood pressure, renal sympathetic nerve activity, heart rate, and respiratory frequency, among others (17, 25, 30, 46). Furthermore, ET-1 increases the neuronal activity of the nucleus of the solitary tract (49). Alterations in the interaction between catecholamines and ETs may be eventually involved in the pathogenesis of hypertension.
The major finding of the present study was that ET-1 and ET-3 were involved in the long-term regulation of TH in the AHR and PHR. In the AHR, which is a sympathoinhibitory region, ETs significantly diminished TH activity and expression through the activation of the ET_{B} receptor coupled to the NO/cGMP/PKG, phosphoinositide, and CaMK-II pathways. In addition, both ETs diminished not only TH total protein level but also the phosphorylated forms of the enzyme (Ser 19 and Ser 40). On the other hand, in the PHR, which is a sympathoexcitatory region, ETs increased TH activity through a GPCR, presumably an atypical receptor or the ET_{C} receptor, coupled to the activation of cAMP/PKA, phosphoinositide, and CaMK-II pathways. Furthermore, in this region, ETs also increased TH protein level, as well as the phosphorylated sites of the enzyme.

TH catalyzes the initial and rate-limiting step in the biosynthetic pathway of catecholamines (13, 14, 29). The enzyme uses L-tyrosine, molecular oxygen, and tetrahydrobiopterin to form L-Dopa, dihydrobiopterin, and H_{2}O in the presence of ferrous iron. Catecholamines play an important role controlling diverse physiological functions in the central, as well as the peripheral nervous system. Therefore, the regulation of TH activity and expression is highly relevant for the neuronal functions that depend on catecholamines. TH activity is controlled by well-characterized short-term and medium- to long-term mechanisms (13, 14, 29). Short-term mechanisms include phosphorylation of specific Ser sites of the enzyme, feedback inhibition, and allosteric effectors (13, 14, 29). Phosphorylation is a key posttranslational mechanism that results from the equilibrium between the activity of kinases and phosphatases and has not only short-term, but also long-term regulatory effects on the enzyme (3, 15). The four known phosphorylation sites of TH are clustered within the first 40 amino acids of the N-terminal region (Ser 8, Ser 19, Ser 31, and Ser 40). The phosphorylation of Ser 40 by PKA, PKC, CaMK-II, and MAPKs induces a significant increase in TH activity (13, 29). On the other hand, ERK1 and ERK2 phosphorylate TH at Ser 31, resulting in a modest rise of TH activity, whereas CaMK-II and MAPKs phosphorylate Ser 19, increasing TH activity only when 14-3-3 proteins are present (13, 29). However, it has been reported that TH phosphorylation increases the activity but decreases the stability of the enzyme (29). Medium- to long-term mechanisms mainly involve enzyme stability, transcription, alternative RNA splicing, RNA stability, and translation (14, 29). It is important to point out that changes in TH protein level are under the regulation of phosphorylation and dephosphorylation processes. Extensive evidence suggests that a brief rise in sympathetic activity is associated with episodic increases in TH activity, presumably via phosphorylation/dephosphorylation of existing TH molecules, whereas a more sustained sympathetic activation also recruits a more slowly developing and longer-lasting increase in TH activity through an increase in the number of TH molecules (13, 14, 29).

ET_{A} and ET_{B} receptors are coupled to multiple signaling pathways depending on the specific G protein activated (30, 34, 38, 50, 51). Various reports support that the NO pathway is coupled to ET_{B} receptor activation (9, 40). In this sense, we reported that in the AHR, both ETs decrease NE release and participate in the short-term regulation of TH activity through the activation of ET_{B} receptors coupled to the NO pathway (11, 33). Present findings show that in the AHR, ET_{B} receptors also mediated the long-term effect of ETs on TH activity since the reduction was abolished by a selective antagonist (BQ-788) and mimicked by an agonist (IRL-1620). The ET_{B} receptor is expressed in a wide variety of tissues in the adult rat, including neurons in regions of the brain involved in the control of the cardiovascular function such as the hypothalamus, as well as endothelial and vascular smooth muscle cells (9, 30). However, in the present study, the neuronal NO/cGMP/PKG and phos-
phosphoinositide pathways and CaMK-II activation mediated ET-induced decrease in TH activity in the AHR. In a previous work, we showed that ET-1 and ET-3 modulate neuronal NOS activity in the AHR through the ETB receptor coupled to the PLC-PKC/IP3 pathway, as well as to PKA and CaMK-II activation (23). These results support that in the AHR the phosphoinositide pathway and CaMK-II mediate NOS activation induced by ETs. Neuronal NOS is widely distributed in the brain, including the hypothalamus (28). ET-1 and ET-3 also diminished total TH, as well as 40 Ser-P TH and 19 Ser-P TH, without affecting 31 Ser-P TH site. Our studies support that in the AHR, diverse mechanisms would underlie ET-induced reduction of TH activity. According to current literature, the role of PKG in TH activity regulation is rather controversial. It was reported that NO induces cGMP/PKG-dependent inhibition of N-type Ca\(^{2+}\) channels in neuroblastoma cells (8). Furthermore, the NO/cGMP/PKG pathway is associated with reduced calcium entry to the cell (7, 26). Calcium acts directly on the phosphorylation mechanism and/or TH activation in adrenal chromaffin cells, PC12 cells, striatal slices, olfactory bulb cells, and superior cervical ganglion (13, 29). We also reported that atrial natriuretic factor decreases TH activity through the cGMP/PKG pathway (53). Conversely, it was reported that cGMP increases TH activity in PC12 cells, and PKG phosphorlyates TH in intact bovine chromaffin cells (44, 45). These discrepancies may arise from the intrinsic nature of the tissues involved, as well as from the agents used in the different studies. A clear interaction between NO/cGMP/PKG and phosphatase 2A, an important enzyme involved in TH inactivation, was reported by Nakasawa and coworkers (35). Atrial natriuretic factor induces the phosphorylation of phosphatase 2A through the cGMP/PKG pathway (54). Numerous studies support that TH activity is regulated by a tight equilibrium between phosphorylation and dephosphorylation, which results in increased or decreased enzyme activity (13). In the present study, the equilibrium would favor the...

![Fig. 5](image-url)

Fig. 5. Participation of PKA and CaMK-II in the long-term modulation of TH induced by ET-1 and ET-3 in the anterior hypothalamic region. Tissues were preincubated with H-89 (PKA inhibitor) or KN-62 (CaMK-II inhibitor) before the addition of ET-1 or ET-3. TH was measured as detailed in MATERIALS AND METHODS and expressed as a percentage of control. ***P < 0.001 vs. control; ††P < 0.01 and †P < 0.05 vs. H-89; †††P < 0.001 vs. ET-1; ###P < 0.001 vs. ET-3. Number of experiments: 6 or 7.

![Fig. 6](image-url)

Fig. 6. Participation of the phosphoinositide pathway in the long-term modulation of TH induced by ET-1 and ET-3 in the anterior hypothalamic region. Tissues were pretreated with U-73122 (PLC inhibitor) (A), chelerythrine (CRT) (PKC inhibitor) (B), and 2-APB (IP3 receptor antagonist) (C) before the addition of ET-1 or ET-3. TH activity was measured as detailed in MATERIALS AND METHODS and expressed as percentage of control. ***P < 0.001 vs. control; †P < 0.05 and †††P < 0.001 vs. ET-1; ###P < 0.001 vs. ET-3. Number of experiments: 6 or 7.
dephosphorylation of TH, probably inducing a more stable but less active form of the enzyme, given that phosphorylation makes the enzyme more active and less stable as previously reported (29). This may result from decreased phosphorylation at Ser 19, 31, and/or 40 sites or from increased dephosphorylation mediated by phosphatase activation. In this sense, diverse studies show that an increase in phosphatase activity decreases TH activity (5, 31, 47). In fact, phosphatase 2A and phosphatase 2C play a relevant role in TH regulation (5, 31). Present findings permit us to hypothesize that the reduction of TH activity induced by ET-1 and ET-3 in the AHR may result from an increase in phosphatase activity mediated by the NO/cGMP/PKG pathway that would lead to a reduction in TH phosphorylation. In accordance with this hypothesis, we observed that phosphatase 2A inhibition partially blocked ET-reduced TH activity (unpublished observations). Nevertheless, the inhibitory effect of nitration and peroxynitrites cannot be excluded, since peroxynitrites are generated by the interaction of NO and the superoxide anion (2, 6).

Fig. 7. Participation of the phosphoinositide pathway in the long-term modulation of TH induced by ET-1 and ET-3 in the posterior hypothalamic region. Tissues were pretreated with U-73122 (PLC inhibitor) (A), CRT (PKC inhibitor) (B), and 2-APB (IP3 receptor antagonist) (C) before the addition of ET-1 or ET-3. TH activity was measured as detailed in MATERIALS AND METHODS and expressed as percentage of control. ***P < 0.001 vs. control; ‡‡‡P < 0.001 vs. ET-1; ###P < 0.001 vs. ET-3. Number of experiments: 6 or 7.

Fig. 8. Participation of PKA, CaMK-II, and NO in the long-term modulation of TH induced by ET-1 and ET-3 in the posterior hypothalamic region. Tissues were pretreated with H-89 (PKA inhibitor) (A), KN-62 (neuronal NO synthase) (B) or L-NAME (NO synthase inhibitor) (C) followed by incubation with ET-1 and ET-3. TH activity was measured as detailed in MATERIALS AND METHODS and expressed as a percentage of control. ***P < 0.001 vs. control; ††P < 0.01 vs. L-NAME; ‡‡‡P < 0.001 vs. ET-1; ###P < 0.001 vs. ET-3. Number of experiments: 6 or 7.
On the other hand, in the PHR, ET-1, and ET-3 increased TH activity. Present experimental observations using ET agonists and antagonists support that the response was not mediated by the conventional ETA and ETB receptors. Numerous pharmacological, physiological, as well as binding studies, strongly support the existence of nonconventional receptors.

Fig. 9. Effect of ET-1 and ET-3 on the expression of total TH and the phosphorylated forms of the enzyme in the anterior hypothalamic region. The expression of total TH protein level (tTH-Ab) (A) and the enzyme phosphorylated forms at Ser 19 (B), 31 (C), and 40 (D) (TH S19p-Ab, TH S31p-Ab and TH S40p-Ab, respectively) were determined by Western blot analysis and normalized to β-actin as detailed in MATERIALS AND METHODS.*P < 0.05, ***P < 0.001 vs. control. The Western blot assays shown are representative of at least three or four independent experiments.

Fig. 10. Effect of ET-1 and ET-3 on the expression of total TH and the phosphorylated forms of the enzyme in the posterior hypothalamic region. The expression of total TH protein level (tTH-Ab) (A) and the enzyme phosphorylated forms at Ser 19 (B), 31 (C), and 40 (D) (TH S19p-Ab, TH S31p-Ab, and TH S40p-Ab, respectively) were determined by Western blot analysis and normalized to β-actin as detailed in MATERIALS AND METHODS.*P < 0.05, **P < 0.01, and ***P < 0.001 vs. control. The Western assays shown are representative of at least 3 or 4 independent experiments.

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ETS receptors may probably result from the formation of ETA...

Recent studies suggest that atypical ET receptors may problem result from the formation of ETA-ETB heterodimers (19, 48). It was reported that ETA and ETB receptors constitutively form heterodimers and homodimers in transfected cells (16). However, further studies are needed to clarify this issue. In previous studies (12, 41), we reported that an atypical ET receptor (ETAX and/or ETBX) was presumably involved in the response. Recent studies show that atypical ET receptors may problem result from the formation of ETA-ETB heterodimers (19, 48). It was reported that ETA and ETB receptors constitutively form heterodimers and homodimers in transfected cells (16). However, further studies are needed to clarify this issue. In previous studies (12, 41), we reported that an atypical ET receptor (ETAX and/or ETBX) was presumably involved in the response.

Inhibitors of PLC and of the downstream effectors of the signaling cascade abolished the stimulatory effect of ETs on TH activity in the PHR, suggesting that the ET receptor was coupled to the phosphoinositide pathway. Furthermore, CaMK-II activation and the cAMP/PKA pathway were also involved in ET response. However, the NO pathway was not stimulated in the PHR by ETs as it was observed in the AHR. ET-1 and ET-3 also increased TH protein levels and the phosphorylation of Ser 19, 31, and 40 sites of the enzyme. These findings suggest that increased TH activity by ET-1 and ET-3 in the PHR resulted from an increase in the enzyme content and its phosphorylated forms, thus generating a less stable but more active enzyme. Various authors reported that phosphorylation makes the enzyme more active but less stable (29). It is likely that a less stable TH, with a shorter half-life, may stimulate de novo synthesis of the enzyme. In accordance with these findings, we recently observed that both ETs increased mRNA-TH (unpublished observations).

As brain catecholamines play a relevant role in controlling different biological functions, it is important to fully understand the underlying mechanisms involved in TH regulation. The central nervous system’s medullary control of blood pressure stems from a tonic excitatory center, situated in a rostroventral position with spinal excitatory fibers to the spinal intermediolateral nucleus that controls sympathetic ganglia and the adrenal medulla. The medulla’s excitatory center is under the influence of the hypothalamus, the midbrain, a medullary inhibitory center slightly more caudal than the excitatory center, and the nucleus of the solitary tract (18, 37, 55). The hypothalamus is a well-known autonomic regulatory and integrative region of the brain involved in the coordination of several biological functions. The hypothalamic regions and nuclei containing different neurotransmitters, including catecholamines, participate in the regulation of the cardiovascular activity (18, 37, 55). Thus, functional alterations of hypothalamic areas increase or diminish arterial blood pressure through changes in the sympathetic tone.

Our results support that ET-1 and ET-3 regulate catecholaminergic transmission in the hypothalamus by controlling the synthesis (11, 12), uptake (unpublished observations), and release of NE (33, 41). These findings suggest that ETs modulate NE turnover. In fact, ETs decrease NE utilization in the AHR and increased it in the PHR (unpublished observations), which correlates with the observed changes in TH activity.

The present study shows that ET-1 and ET-3 are involved in the long-term regulation of TH in the AHR and PHR, suggesting that the interaction between ETs and catecholaminergic transmission may play a role in the brain control of the cardiovascular activity.

Perspectives and Significance

Relatively little is known about the role of ETs in the regulation of catecholamine biosynthesis in the different hypothalamic areas closely related to cardiovascular function. The present study gives evidence that ET-1 and ET-3, through ET receptors in the AHR and nonconventional ET receptors (presumably atypical receptors or ETc receptors) in the PHR, initiate a cascade of cellular and molecular events that differentially modulate TH activity and expression in both hypothalamic regions. Present and previous findings further support that the modulation of hypothalamic catecholaminergic transmission may be one of the mechanisms that mediates the cardiovascular effects evoked by ETs when applied to the brain. Whether ETs and catecholamine interaction, at the hypothalamic level, play a role in the genesis and/or development of cardiovascular pathologies, such as hypertension, needs to be assessed in further studies.

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