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Endothelin 1 and 3 enhance neuronal nitric oxide synthase activity through ET_B receptors involving multiple signaling pathways in the rat anterior hypothalamus

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Abstract

We have previously reported that endothelin 1 and 3 (ET-1, ET-3) through the ET_B receptor decrease norepinephrine release in the anterior hypothalamus and activate the nitric oxide (NO) pathway. In the present work we sought to establish the receptors and intracellular mechanisms underlying the increase in nitric oxide synthase (NOS) activity stimulated by ET-1 and ET-3 in the rat anterior hypothalamus. Results showed that ETs-stimulated NOS activity was inhibited by a selective ET_B antagonist (BQ-788), but not by a selective ET_A antagonist (BQ-610). In addition, NOS activity was not altered in the presence of an ET_A agonist (sarafotoxin 6b), but it was enhanced in the presence of a ET_B agonist (IRL-1620). Both N^{ω}-nitro-L-arginine methyl ester (NOS inhibitor), and 7-nitroindazole (neuronal NOS inhibitor) diminished ETs-stimulated NOS activity. The stimulatory effect of ETs on NOS activity was inhibited in the presence of PLC, PKC, PKA and CaMK-II inhibitors (U-73122, GF-109203X, H-89 and KN-62, respectively), and the IP_3 receptor selective antagonist, 2-APB. Our results showed that both ET-1 and ET-3 modulate neuronal NOS activity through the ET_B receptor in the rat anterior hypothalamus involving the participation of the PLC-PKC/IP_3 pathway as well as PKA and CaMK-II.

Keywords: Endothelin 1; Endothelin 3; ET_B receptor; Neuronal NOS; Anterior hypothalamus; PKA; PKC; PLC; CaMK-II; IP_3 receptor

1. Introduction

Endothelins (ETs) are a family of potent vasoactive peptides of 21 amino acids. Three isoforms of ETs have been described, ET-1, ET-2 and ET-3 that exert different biological effects mainly in an autocrine/paracrine manner [5,28]. Molecular cloning techniques revealed the existence of two receptor subtypes, ET_A and ET_B, widely distributed in mammalian tissues [5,28,29]. ET_A displays higher affinity for ET-1 than for ET-2 and ET-3 whereas ET_B binds the three isoforms with similar affinity [5,28,29]. A third receptor subtype, termed ET_C that displays higher affinity for ET-3 was cloned from *Xenopus laevis* oocytes [12]. Although functional studies support its existence, molecular biology and binding studies failed to described it in mammalian tissues. ETs are involved in the regulation of cardiovascular function as well as water and salt homeostasis [9,19,26].

Nitric oxide (NO) is synthesized from L-arginine by the homodimer nitric oxide synthase (NOS) [22]. One inducible and two constitutive isoforms of the enzyme have been described [1,33]. The constitutive isoforms are calcium dependent and expressed in endothelial cells (eNOS or NOS 3) as well as in neurons (nNOS or NOS 1) whereas the inducible isoform (iNOS or NOS 2) is expressed in various cell types and can be activated by IL-1β and TNFα [1,33]. The regulation of NOS is complex and involves several mechanisms mediated by kinases, Ca^{2+} and NO levels [10,11,14]. Second messenger dependent kinases (PKA, PKC and PKG) as well as CaMK-II have been shown to regulate NOS activity by inducing phosphorylation of different serine/threonine residues [10,11,14]. In addition, variations in intracellular

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calcium levels regulate NOS by inducing conformational changes of the enzyme [10,11].

In the central nervous system (CNS), the anterior hypothalamus is an important sympathoinhibitory area closely related to blood pressure regulation that expresses high levels of ETs and ETs receptors [19,20,31]. We have previously reported that both ET-1 and ET-3 diminish norepinephrine (NE) release in the rat anterior hypothalamus through a nitric oxide (NO) pathway [6]. On the other hand, high levels of NO are found in neurons where this free radical plays a role in the regulation of neuronal activity by stimulating soluble guanylyl cyclase and increasing cGMP formation [33].

Based upon our previous findings showing that the NO pathway mediated the effect of ETs on neuronal NE release in the anterior hypothalamus, the aim of the present study was to determine the receptors and the underlying mechanisms promoting the activation of the NO pathway in such process. For this purpose we investigated the ET receptors and the NOS isoform involved as well as different kinases known to participate in the regulation of the enzyme.

2. Materials and methods

2.1. Animals and chemicals

Male Sprague–Dawley rats (from Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina) weighing 250–300 g were used in the experiments.

The following drugs were used: L-[2,3-^{3}H]arginine (53.4 Ci/mmol of specific activity, New England Nuclear); ET-1, ET-3, BQ-610, and BQ-788 (Peninsula Lab); N^{ω}-nitro-L-arginine methyl ester (L-NAME), L-arginine, β-NADPH, Citrulline, U-73122 (Sigma Chemical, St. Louis, MO, USA); Dowex-AG50W-X28 resin (sodium form, 200-400 mesh, Bio Rad Lab); Dithiothreitol (DTT), KN-62, H-89, GF-109203X, tetrahydrobiopterin (H_4B), minimum essential media (MEM) amino acid solution and basal medium Eagle vitamin solution (ICN Biomedicals Inc., OH, USA). 7-Nitroindazole (7-NI), 2-APB, sarafotoxin 6b (SRTX 6b) and IRL-1620 (Calbiochem, La Jolla, CA, USA). Other reagents were of analytical purity and obtained from standard sources. All drugs were dissolved in Krebs solution, except for 2-APB, H-89, GF-109203X and U-73122 that were dissolved in DMSO, IRL-1620 dissolved in 2.5% NH_4OH and SRTX 6b dissolved in 5% acetic acid. These vehicles did not affect NOS activity and ET-1 and ET-3 responses were sustained in the presence of DMSO, NH_4OH and acetic acid (data not shown).

2.2. Experimental protocol

Animals were decapitated, brains quickly removed and anterior hypothalami (11.4 ± 0.9 mg) dissected under a magnifying glass [21]. Anterior hypothalami were preincubated at 37 °C for 30 min in gassed (carbogen, 95% O_2 and 5% CO_2) standard Krebs solution supplemented with MEM amino acid solution and basal medium Eagle vitamin solution (KSS). Tissues were then incubated for 5 min in the absence (control) or in the presence of ETs and/or the different inhibitors: ET-1 and ET-3 (10 nM), 100 nM BQ-610 (ET_A receptor antagonist), 100 nM BQ-788 (ET_B receptor antagonist), 300 nM SRTX 6b (ET_A receptor agonist), 1 μM IRL-1620 (ET_B receptor agonist), 20 μM KN-62 (CaMK-II

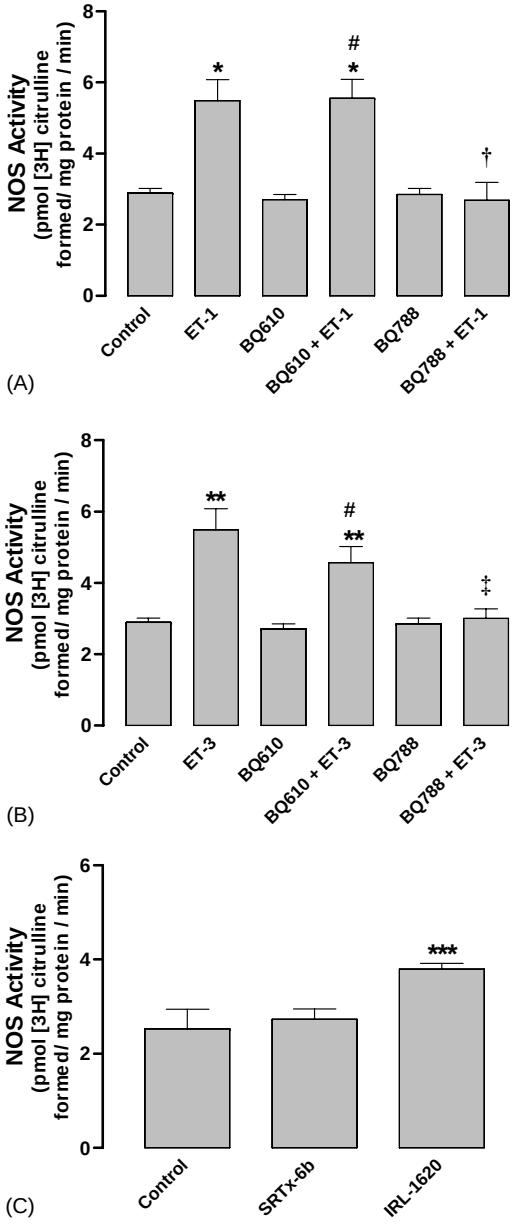

Fig. 1. Effects of ET_A selective antagonist BQ-610 (100 nM) and of ET_B selective antagonist BQ-788 (100 nM) on NOS activity increase induced by 10 nM ET-1 (A) and 10 nM ET-3 (B), in rat anterior hypothalamus. Effects of ET_A selective agonist 300 nM SRTX 6b and of ET_B selective agonist 1 μM IRL-1620 (C) on NOS basal activity, in rat anterior hypothalamus. Values are expressed as means ± S.E.M. Number of experiments: $n = 5$–7. $^{*}P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.05$ vs. control; $^{\#}P < 0.001$ vs. BQ-610; $^{\dagger}P < 0.001$ vs. ET-1; $^{\ddagger}P < 0.05$ vs. ET-3.

inhibitor), 10 μM L-NAME (NOS inhibitor), 10 μM 7-NI (nNOS inhibitor) [2,8,35], 500 nM H-89 (PKA inhibitor), 100 nM GF-109203X (PKC inhibitor), 10 μM U-73122 (PLC inhibitor), 42 μM 2-APB (IP_3 receptor antagonist). Reaction was stopped by three consecutive washes of 5 min each with KSS at 4 °C. Inhibitors were added in the last 5 min of the preincubation and during the incubation.

2.3. NOS activity assay

NOS activity was measured according to Tsuchiya et al. [30], with modifications. Briefly, tissues were quickly homogenized in 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 saved for protein assay, whereas another aliquot was incubated at 37 °C for 10 min in the reaction buffer [1 μM L-Arg, 20 nM L-[2,3-^{3}H]arginine, 20 mM EDTA, 1 mM DTT, 1 mM β-NADPH, 10 μM H_4B, 20 mM HEPES and 1.25 mM $CaCl_2$]. Reaction was stopped by lowering the temperature to 4 °C, and samples were then loaded onto 1-ml columns containing Dowex-AG50W-X28 resin, pre-equilibrated with 200 mM citrulline. Columns were eluted with distilled water, 2-ml fractions containing the [^{3}H]citrulline were collected, and radioactivity was determined by usual scintillation counting methods. NOS activity was expressed as pmol [^{3}H]citrulline formed/mg of protein/min ± S.E.M.

2.4. Statistical analysis

All values are expressed as the mean ± S.E.M. Differences among groups were statistically assessed by ANOVA followed by the *t*-test modified by Bonferroni (Graph Pad, San Diego, CA). In all cases, *P* values of 0.05 or less were considered statistically significant.

3. Results

Our previous findings showed that ET-1 and ET-3 increase NOS activity in the rat anterior hypothalamus [6]. In order to determine the ET receptor subtype involved, experiments were carried out in the presence of selective antagonists and agonists of ET_A and ET_B receptors. Blockade of the ET_A receptor subtype with BQ-610 affected neither basal nor ET-1 or ET-3-evoked NOS activity (Fig. 1A and B). However, ET_B receptor blockade with BQ-788 prevented the increase in NOS activity induced by ET-1 and ET-3 without

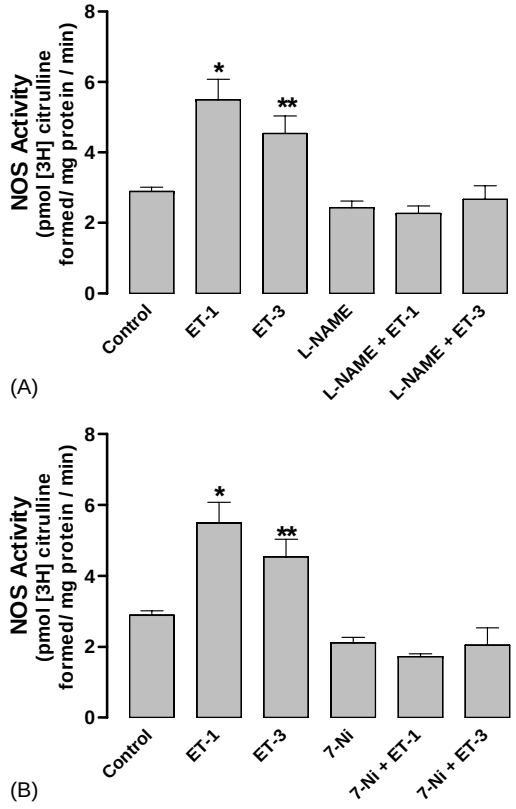

Fig. 2. Effects of NOS inhibitor 10 μM L-NAME (A) and of nNOS-specific inhibitor 10 μM 7-NI (B) on NOS activity induced by 10 nM ET-1 and 10 nM ET-3, in rat anterior hypothalamus. Values are means ± S.E.M. Number of experiments: $n = 5$–7. $^*P < 0.001$, $^{**}P < 0.01$ vs. control.

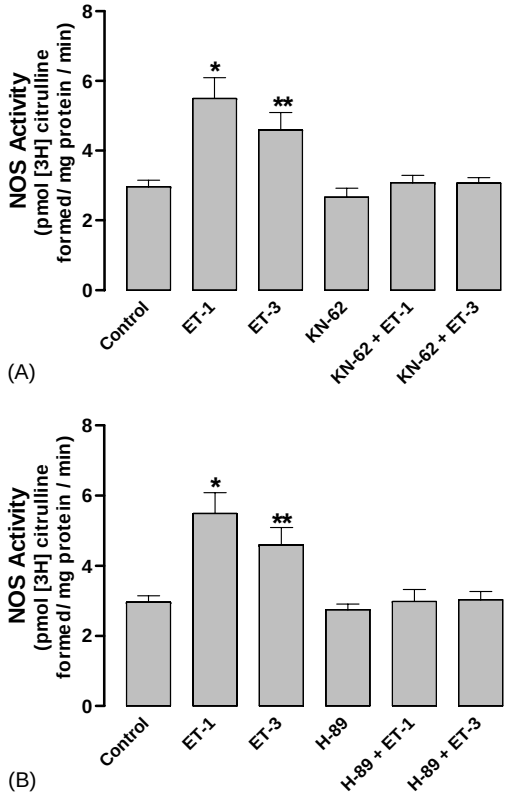

Fig. 3. Effects of CaMK-II (A) and PKA (B) inhibitors (20 μM KN-62 and 500 nM H-89, respectively) on NOS activity induced by 10 nM ET-1 and 10 nM ET-3, in rat anterior hypothalamus. Values are means ± S.E.M. Number of experiments: $n = 5$–7. $^*P < 0.001$, $^{**}P < 0.01$ vs. control.

affecting the basal activity of the enzyme (Fig. 1A and B). In order to confirm the participation of the ET_B receptor in NOS activation, we investigated the effect of selective ETs receptor agonists. The specific ET_A agonist, SRTX 6b, did not modify NOS activity whereas the selective ET_B agonist, IRL-1620, increased it (Fig 1C).

With the aim to identify the NOS isoform involved, ET-1 and ET-3 effect on NOS activity was studied in the presence of 10 μM L-NAME (non-specific inhibitor of NOS) or 10 μM 7-NI (specific inhibitor of nNOS isoform). Both inhibitors prevented the increase in NOS activity induced either by ET-1 or ET-3 (Fig. 2A and B).

The regulation of NOS activity is mediated by several kinases such as CaMK-II, PKA and PKC. In order to investigate the participation of these kinases, we studied the effects of both ETs on NOS activity in the presence of specific inhibitors of CaMK-II (20 μM KN-62), PKA (500 nM H-89) or PKC (100 nM GF-109203X). Results showed that KN-62, H-89 and GF-109203X did not affect basal NOS activity but they all prevented the increase induced by ET-1 and ET-3 (Fig. 3A–C). In addition, The inhibition of PLC by 100 μM U-73122 or the blockade of IP_3 sensitive stores by 42 μM 2-APB did not affect basal nNOS activity but prevented the activation of the enzyme evoked by ET-1 and ET-3 (Fig. 4A and B).

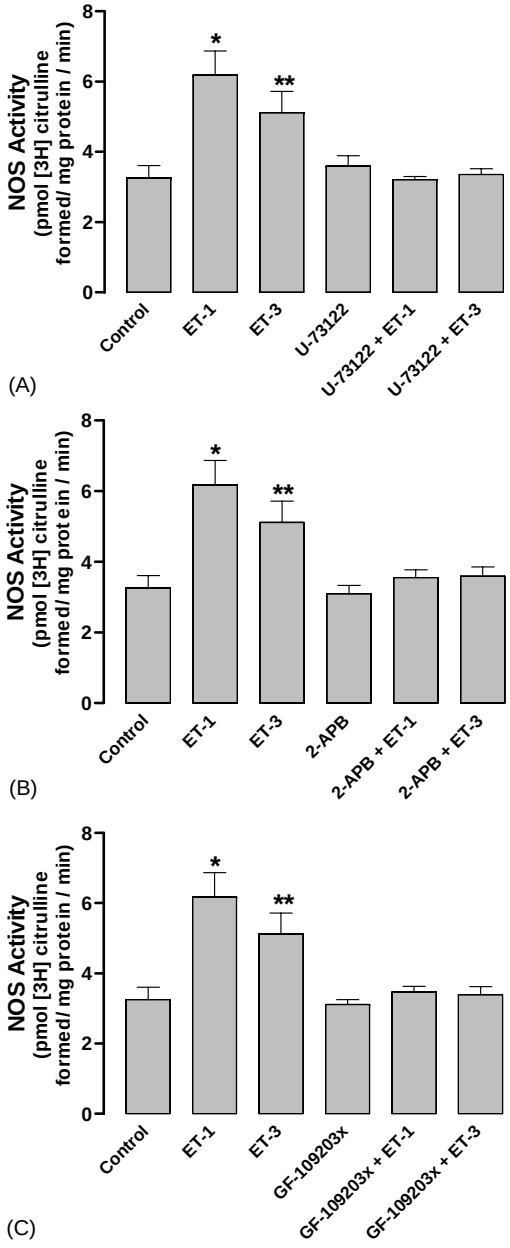

Fig. 4. Effects of PLC (A), IP_3-induced Ca^{2+} release (B) and PKC (C) inhibitors (10 μM U-73122, 42 μM 2-APB and 100 nM GF-109203X, respectively) on NOS activity induced by 10 nM ET-1 and 10 nM ET-3, in rat anterior hypothalamus. Values are means ± S.E.M. Number of experiments: $n = 6$–8. $^{*}P < 0.001$, $^{**}P < 0.01$ vs. control.

4. Discussions

We have previously reported that in the anterior hypothalamus ET-1 and ET-3 diminish neuronal NE release through a NO pathway [6]. In the present work we studied the receptors and intracellular mechanisms involved in the stimulation of NOS activity by ET-1 and ET-3 in the anterior hypothalamus.

The major findings of the present work was that both, ET-1 and ET-3, increased NOS activity by stimulating the neuronal isoform through activation of the ET_B receptor subtype coupled to multiple signaling pathways.

The presence of ETs and their receptors in different regions and areas of the CNS including the hypothalamus have been determined by autoradiography, immunocytochemistry and *in situ hybridization* studies [19,20,29,31]. The ET_B receptor is distributed in both neuronal and glial cells and participates in central as well as peripheral mechanisms related to blood pressure lowering [4,15,19,20,29,31]. Thus, the ET_B receptor is coupled to NO and prostacyclin production in several cell types [4].

As the regulation of NOS activity involves the participation of Ca^{2+} and several kinases, we also investigated the intracellular pathways that might be involved in NOS activation by ET-1 and ET-3. Different kinases, such as CaMKs, participate in the regulation of nNOS activity by phosphorylating its serine/threonine residues [10,11,14]. CaMK-II was shown to be the most effective in changing the K_m and V_{max} of the enzyme [10]. The results obtained in the present work show that CaMK-II is involved in the regulation of nNOS activity produced by ET-1 and ET-3. In accordance with our results Rodriguez-Alvarez et al. [25] demonstrated that in striatal neurons, the stimulation of NO synthesis induced by NMDA activation is also inhibited by KN-62, supporting the view that CaMK-II activation is relevant for NOS stimulation. However, discrepancies arise in NG108-15 neuronal cells where the phosphorylation of nNOS by CaMK-II results in diminished enzymatic activity [13]. Nevertheless it

should be considered that observations made in tumoral cell lines may not always match those made in normal tissues as striatal neurons or hypothalamic slices, probably due to the characteristics of immortal cells cultures. nNOS is also stimulated following the activation of neurotransmitter receptors linked to the regulation of intracellular Ca^{2+} [11]. Receptors coupled to ion channels, as the NMDA receptor, activate guanylyl cyclase by increasing intracellular Ca^{2+} [7]. Later, this process was shown to be mediated by NO synthesis [3]. Other kinases, such as PKC and PKA, also regulate nNOS by phosphorylation [11,14]. Our results showed that both kinases, PKC and PKA, participate in the increase of nNOS activity induced by ET-1 and ET-3.

Another mechanism involved in the regulation of NOS activity is the physiological changes in intracellular calcium in response to the phosphoinositide pathway activation [11]. PLC activation induces phosphoinositide hydrolysis leading to the formation of IP_3 and DAG. In turn, IP_3 increases intracellular Ca^{2+} by releasing it from IP_3-sensitive stores of the endoplasmic reticulum, whereas DAG activates PKC that phosphorylates different substrates including nNOS [11]. These findings show that the activation of nNOS by ET-1 and ET-3 is dependent upon the activation of the PLC pathway that leads to Ca^{2+} release from IP_3 sensitive stores. The activation of this intracellular signaling pathway also induces PKC activation that is another kinase involved in nNOS regulation.

Several studies reported that various hypothalamic regions and nuclei containing different neurotransmitters participate in the regulation of various biological processes such as the cardiovascular activity [20]. The anterior hypothalamus plays an important role in arterial pressure regulation as a sympathoinhibitory area [20]. In accordance, we have previously reported that ET-1 and ET-3 diminish neuronal NE release in the anterior hypothalamus through the ET_B receptor subtype coupled to the NO-cGMP-PKG pathways resulting in decreased sympathoinhibitory response of this area [6]. In agreement with our results, several studies showed a relationship between ET_B receptor activation and NO formation. Thus, Yamada et al. [36], demonstrated that the increase in NO formation induced by the activation of the ET_B receptor could be involved in the ET-induced preganglionic inhibition in canine stellate ganglion. In addition, Mathison and Israel [18], reported that ET-1 and ET-3 activated ET_B receptor stimulating NO/cGMP pathways in the rat median eminence. All of these findings support the role of NO as a neuronal messenger in both the CNS and the peripheral nervous system.

NOS is widely distributed in the hypothalamus as well as other regions and areas of the CNS [16,24]. NOS coexists with different neurotransmitters and neuromodulators in neurons and glial cells [16,24]. Various studies show that NOS is found with tyrosine hydroxylase, acetylcholinesterase, angiotensin, NE as well as hormones like oxytocin and vasopressin [16,24,30,32]. A great body of evidence supports a relevant role for NO in the regulation of diverse physiological processes controlled by the CNS including those regulated by the hypothalamus [16,32,33].

ETs receptors are coupled to different G-proteins and may activate multiple signaling pathway simultaneously in one cell [17,27]. ETs receptor subtypes belong to the family of Ca^{2+}-mobilizing receptors coupled to PLC activation that leads to DAG-PKC and IP_3 formation [4,28,29]. Furthermore, in studies using rat cerebral slices and immortalized Schwann cells, ET-1 enhances cAMP levels through ET_B subtype receptor [23,34]. All these intracellular mechanisms are also involved in the regulation of nNOS activity.

In conclusion, present results suggest that in the rat anterior hypothalamus ET-1 and ET-3 activate the ET_B receptor subtype coupled to different G-proteins and increase nNOS activity through several kinases such as CaMK-II, PKC and PKA. Both, previous [6] and present findings support the participation of ET-1 and ET-3 in the regulation of diverse biological processes controlled by the anterior hypothalamus through the activation of ET_B receptors coupled to the NO pathway.

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