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ENDOTHELINS STIMULATE THE EXPRESSION OF NEUROTROPHIN-3 IN RAT BRAIN AND RAT CULTURED ASTROCYTES

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Abstract—Endothelins play a role in the regulation of astrocytic functions in brain pathologies such as hyperplasia and neurotrophic factor production. The present study examined the effects of endothelins on production of neurotrophin-3, a member of the neurotrophin family of neurotrophic factors, in cultured astrocytes and rat brain. Quantitative reverse transcription-PCR analysis of mRNA copy numbers showed that cultured astrocytes expressed comparable numbers of neurotrophin-3 and neurotrophin-4/5 mRNA copies to nerve growth factor and brain-derived neurotrophic factor. Endothelin-1 (100 nM) and Ala^{1,3,11,15}-endothelin-1 (an endothelin$_B$ receptor agonist, 100 nM) caused a transient increase in neurotrophin-3 mRNA levels, but not in neurotrophin-4/5 levels, in cultured astrocytes. The increases in mRNA levels were accompanied with that in extracellular release of neurotrophin-3. The effects of endothelin-1 on neurotrophin-3 mRNA levels were reduced by BQ788, an endothelin$_B$ receptor antagonist. I.c.v. administration of 500 pmol/day Ala^{1,3,11,15}-endothelin-1 increased mRNA and peptide levels of neurotrophin-3 in rat caudate putamen and cerebrum. On the other hand, neurotrophin-3 production in hippocampus was not affected by Ala^{1,3,11,15}-endothelin-1. Immunohistochemical examination of Ala^{1,3,11,15}-endothelin-1-infused rats showed that neurotrophin-3 was mainly expressed in glial fibrillary acidic protein-positive astrocytes in caudate putamen and cerebrum. endothelin-induced increases in neurotrophin-3 expression in cultured astrocytes were inhibited by chelation of intracellular Ca^{2+} and PD98095 (an ERK inhibitor). These results suggest that endothelin is an extracellular signal that stimulates astrocytic neurotrophin-3 production in brain pathologies. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: endothelin-1, growth factor, gene transcription, neurotrophin, glial cell.

Neurotrophin-3 (NT-3) belongs to the neurotrophin family of neurotrophic factors like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-4/5 (NT-4/5). NT-3 has a role both in physiological and pathological conditions of adult brain, similar to the other members of the neurotrophin family (Chalazonitis, 1996). In hippocampus, cerebellum, and spinal cord, subpopulations of neuronal cells constitutively produce NT-3 (Ernfors et al., 1990; Ceccatelli et al., 1991; Lindholm et al., 1993; Henderson et al., 1993; Tokumine et al., 2003). Because electrophysiological studies showed that application of NT-3 enhanced synaptic excitation (Lohof et al., 1993; Mendell et al., 2001), an acute modulation of nerve transmission by NT-3 has been suggested. Furthermore, treatment with NT-3 promoted survival and neurogenesis of several types of cultured neurons (Friedman et al., 1993; Henderson et al., 1993; Cheng and Mattson, 1994). Studies on nerve injury models showed that administration of NT-3 reduced neuronal cell death and promoted synaptic regeneration in spinal cord, septum, and striatum (Schnell et al., 1994; Fischer et al., 1994; Zhou et al., 2003; Galvin and Oorschot, 2003). These observations indicate a role of NT-3 in protection and regeneration of damaged neurons in pathological conditions. In several pathological conditions, conversion of resting astrocytes to reactive astrocytes is commonly observed (Eddleston and Mucke, 1993; Ridet et al., 1997) and characterized by hypertrophy and increased expression of glial fibrillary acidic protein (GFAP). Histological observations showed that production of astrocytic NT-3 increased with the conversion to reactive astrocytes (Tokumine et al., 2003). While regulation of NT-3 production in neuronal cells has been studied, little is known about molecular signals stimulating astrocytic NT-3 production in pathological conditions.

Endothelin (ET) peptide family, that was originally found to have potent vasoconstrictive properties, also has multiple actions in non-vascular tissues as well as in the nervous system. ET-1 was up-regulated in brain pathologies and involved in several pathophysiological responses of the nervous system (Nie and Olsson, 1996). Receptors for ETs are classified into ET_A and ET_B types, both of which are G-protein-coupled receptors. Because astrocytes possess receptors for ET-1, especially ET_B receptors (Hori et al., 1992; Rogers et al., 2003), astrocytes are thought to be the main target for ET-1 in the brain. An involvement of ET_B receptors in the conversion to reactive astrocytes was shown by administration of ET receptor agonists and antagonists in animal models of nerve injury (Uesugi et al., 1996; Ishikawa et al., 1997; Koyama et al., 1999b). In cultured astrocytes, ET-1 reproduced several properties of reactive astrocytes, including proliferation (Lazarini et al., 1996; Koyama et al., 2004), morphological alteration (Hama et al., 1992; Koyama and Baba,

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Abbreviations: BDNF, brain-derived neurotrophic factor; CREB, cAMP-responsing element binding protein; ERK, extracellular signal regulated kinase; ET, endothelin; GFAP, glial fibrillary acidic protein; NGF, nerve growth factor; NT-3, neurotrophin-3; NT-4/5, neurotrophin-4/5; PKC, protein kinase C; PLC, phospholipase C.; RT-PCR, reverse transcription-PCR.

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1994), and production of neurotrophic factors. Production of NGF and BDNF was increased by ETs in cultured astrocytes and in rat brain (Ladenheim et al., 1993; Koyama et al., 2003, 2005). These findings suggest an involvement of ET-1 in astrocytic NGF and BDNF production under pathological conditions. In this study, effects of ETs on NT-3 production in rat cultured astrocytes and rat brain were examined to show a further involvement of ETs in astrocytic neurotrophic factor production.

EXPERIMENTAL PROCEDURES

Preparation of primary cultured astrocytes from rat brain

Astrocytes were prepared from cerebrum of 1–2 day-old Wistar rats as described previously (Koyama et al., 1999a). Cells were seeded at 1×10^4 cells/cm^2 in 75 cm^2 culture flasks and grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum. To remove small process-bearing cells on the protoplasmic cell layer (mainly oligodendrocyte progenitors and microglia), culture flasks were shaken at 250 r.p.m. overnight 10–14 days after seeding. The monolayer cells were trypsinized and plated into six-well culture plates and further cultured for 7–10 days. At this stage, approximately 95% of cells showed immunoreactivity for GFAP.

I.c.v. administration of an ET_B receptor agonist

All experimental protocols conformed to the Guiding Principles for the Care and Use of Animals approved by the Japanese Pharmacological Society. All efforts were made to minimize the number of animals used. Male-Wistar rats, weighing 250–300 g, were anesthetized with sodium pentobarbital (50 mg/kg) and placed in a stereotaxic frame. I.c.v. administration of an ET_B receptor agonist was performed as described before (Koyama et al., 2003). Briefly, Ala^{1,3,11,15}-ET-1 (Peninsula Laboratory, San Carlos, CA, USA), an ET_B agonist, was dissolved in sterile artificial CSF (150 mM NaCl, 1.2 mM $MgSO_4$, 1 mM K_2HPO_4, and 10 mM D-glucose). After the skull was exposed, a burr hole was made at 0.5 mm posterior and 2.0 mm right lateral to the bregma. A mini-osmotic pump was implanted s.c., and a 28 gauge stainless cannula (Alzet 2002 and brain infusion kit, Durect Co., Cupertino, CA, USA) was inserted through the burr hole to infuse the experimental solution into the cerebral ventricle (4.0 mm under the surface of the skull). Ala^{1,3,11,15}-ET-1 was administered at a dosage of 500 pmol/day. Control animals were infused with artificial CSF in the same manner. For determination of neurotrophin mRNA and peptides, rats were decapitated under deep ether anesthesia. After the brains were removed, hippocampal tissue was dissected from the right hemisphere. Caudate putamen was dissected from coronal blocks of the right hemisphere at approximately similar levels between 2.0 and −2.0 mm from the bregma. Cerebral tissue was dissected from the left hemisphere between 1.0 and −5.0 mm from the bregma. Dissected brain tissue was stored at −80 °C. For the immunohistochemical study, rats were intracardially perfused with 3% paraformaldehyde under pentobarbital anesthesia. Brains were removed, immersed in 30% sucrose for 2–3 days, and stored at −80 °C.

Determination of immunoreactive NT-3 by ELISA

For determination of immunoreactive NT-3 in rat brain, tissue was homogenized in 10% V/W of Tris-buffered saline containing 1% NP-40, 2 mM EDTA, 2 mM phenylmethyl sulfanyl fluoride, and 10 μg/ml aprotinin. The homogenate was kept at 4 °C for 30 min and subsequently centrifuged. The supernatant was acidified to pH 3–4 by 1 N HCl and then neutralized by 1 N NaOH. The amount of immunoreactive NT-3 in the brain extract was determined by an ELISA kit (Promega, Madison, WI, USA) according to the supplier's protocol. For determination of NT-3 released from cultured astrocytes, culture medium was directly applied to the ELISA kit. Protein content in each well was determined with a BCA protein assay kit (Pierce, IL, USA).

Measurement of neurotrophin mRNA levels by quantitative RT-PCR

Total RNA was extracted from cultured astrocytes and brain tissue according to an acid–phenol method followed by repeated isopropanol precipitation as described previously (Koyama et al., 2003). First strand cDNA was synthesized from total RNA (1 μg) using MMLV reverse transcriptase (200U, Invitrogen, Carlsbad, CA, USA), random hexanucleotides (0.2 μg, Invitrogen) and RNase inhibitor (20U, Takara, Japan) in 10 μl of a buffer supplied by the enzyme manufacturer. Neurotrophin mRNA levels in each sample were determined by real-time PCR using the Syber Green fluorescent probe. The reverse transcription product was included in the DyNAmo SYBR Green master mix (Finnzymes, Espoo, Finland) with rat neurotrophin primers, and the mixture was applied to a real-time PCR thermal cycler (Opticom 2, MJ Research, Waltham, MA, USA). The following primer pairs were used: NT-3; 5′-GCTGATCCAGGCGGATATCT-3′ and 5′-ATGGCTGAGGACTTGTCGGT-3′, NT-4/5; 5′-CCCTGCGTCAGTACTTCTTCGAGAC-3′ and 5′-CTGGACGTCAGGCACGGCCTGTTC-3′, NGF; 5′-TGGACCCAAGCTCACCTCA-3′ and 5′-TGGACCCAAGCTCACCTCA-3′, BDNF; 5′-ATGACCATCCTTTTCCTTACTATGGT-3′ and 5′-TCTTCCCCTTTTAATGGTCAGTGTAC-3′, G3PDH; 5′-CTCATGACCACAGTCCATGC-3′ and 5′-TACATTGGGGGTAGGAACAC-3′. As a standard, serial concentrations of each PCR fragment were amplified in the same manner. The amount of target cDNA in each sample was presented as copy numbers in reverse transcription product equivalent to 1 μg RNA and the values were normalized to the values for G3PDH.

Immunohistochemistry

Serial frozen sections (15 μm) of Ala^{1,3,11,15}-ET-1-infused rats were cut with a cryostat and mounted onto gelatin-coated slides. NT-3 was stained with anti-NT-3 rabbit IgG (Chemicon, Temecula, CA, USA). Astrocytes and neurons were labeled with anti-GFAP mouse monoclonal (Sigma, St. Louis, MO, USA) and anti-NeuN mouse (Chemicon) antibodies, respectively. Labeled cells were visualized by secondary antibodies, conjugated to FITC or rhodamine.

Statistical analysis

Statistical analysis of experimental data were performed by Student's *t*-test.

RESULTS

ET-induced NT-3 production in rat cultured astrocytes

Previous studies showed the expression of all neurotrophins, i.e. NGF, BDNF, NT-3 and NT-4, in cultured astrocytes by northern blotting analysis (Condorelli et al., 1994; Rudge et al., 1992). However, because northern blot-based determinations are not essentially quantitative, a comparison of the mRNA expression levels among astro-