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Endothelin-1 (ET-1) causes death of retinal neurons through activation of nitric oxide synthase (NOS) and production of superoxide anion

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Abstract

Endothelin-1 (ET-1) is the most potent and long-acting vasoconstricting peptide presently known. In addition to its vascular effects, endothelin signaling pathway exists in the central nervous system (CNS), which is deeply related to neuronal degeneration. In the present study, we evaluated the effect of ET-1 on death of retinal neurons consisting mainly of amacrine cells, and its interaction with nitric oxide synthase (NOS) and superoxide production. Cultured retinal neurons from fetal rats were exposed to various doses of ET-1 (0.1, 1.0, 10 and 100 nM). Neuronal toxicity of ET-1 was assessed by trypan blue exclusion, Hoechst 33,258 staining and TUNEL assay at different times. Intracellular levels of nitric oxide (NO), superoxide and peroxynitrite were determined semiquantitatively by DAF2-DA, hydroethidine and dihydrorhodamine-123, respectively. The effects of adding SOD (100 U/ml) and L-NAME with ET-1 on these changes were evaluated. In addition, the receptor mechanisms involved in these reactions were determined by BQ-123 and BQ-788, receptor antagonists for ET_A and ET_B receptors, respectively. Exposure of cultured retinal neurons to ET-1 reduced the percentage of living cells in a dose- and time-dependent way, and the percentage of living cells was significantly increased by addition of SOD and L-NAME. Fluorometric analyses revealed that ET-1 increased the intracellular NO level in a dose- and time-dependent manner. The intracellular superoxide and peroxynitrite levels were also significantly increased 24 h after incubation with 100 nM of ET-1, and this elevation was suppressed by SOD and L-NAME. These ET-1-induced alterations were significantly suppressed when both BQ-123 and BQ-788 were added simultaneously with ET-1 to the medium. These results indicate that the neuronal death caused by ET-1 is most likely mediated by the activation of NOS in association with the formation of superoxides and peroxynitrite.

Keywords: endothelin; cultured retinal neuron; amacrine cell; nitric oxide synthase; superoxide anion

1. Introduction

The endothelins (ETs) are a family of 21-amino acid peptides with three isomers, ET-1, ET-2, and ET-3. ET-1 is the most potent and long-acting vasoconstricting peptide known (Yanagisawa et al., 1988). In the brain, ETs modulate the activity of astrocytes (Koyama et al., 1993, 2003), the proliferation of glial cells (Rogers et al., 1997, 2003; Stanimirovic et al., 1995), and the communication between glial and neuronal cells at gap junctions (Blomstrand et al., 1999, 2004). ET-1 and its G-protein-coupled receptors, ET_A and ET_B, are abundantly expressed and widely distributed in ocular tissues including the sensory retina (de Juan et al., 1995; MacCumber et al., 1991; MacCumber and D'Anna, 1994; Ripodas et al., 2001). Endothelin signaling pathways exist in the astrocytes of the optic nerve head and in retinal neurons and Mueller cells, where they play pivotal roles in the development of glaucoma (Prasanna et al., 2003), and in retinal remodeling after several kinds of photoreceptor damage (Rattner and Nathans, 2005; Torbidoni et al., 2005). Thus, the ETs act not only as vasoconstricting peptides but also as neuro-peptides in the central nervous system (CNS).

The ETs are also associated with neuronal apoptosis in the CNS (Siren et al., 2002; Yagami et al., 2002). Exposure of RGC-5 cells, a transformed cell line of retinal ganglion cells

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(RGCs), to ET-1 causes apoptotic cell death (Krishnamoorthy et al., 2005). In addition, our laboratory has demonstrated that ET-1 enhances the glutamate-induced death of cultured retinal neurons (Kobayashi et al., 2005). Furthermore, Syed et al. (2006) showed that a nonselective ET antagonist provides a neuroprotective effect in the rat retina during ischemia/reperfusion. These findings indicate that ET-1 is involved in the cell death signaling pathway in retinal neurons.

Interactions between ET-1 and nitric oxide synthase (NOS) have been well documented. For example, stimulation of ET_B receptors leads to vasodilation through the formation of nitric oxide (NO) (Kiel, 2000), and an intravitreal injection of ET-1 enhances NO production in the optic nerve head of rabbits (Okuno et al., 2003). NOS is strongly coupled to the generation of superoxide through the uncoupling of NOS (Pou et al., 1992). NO can affect mitochondrial function and increase the formation of superoxide by mitochondria (Moncada and Erusalimsky, 2002). Under these conditions, NO is inactivated by superoxide anion and changed into highly toxic peroxynitrite (Koppenol et al., 1992). Superoxide also causes neuronal apoptosis (Troy and Shelanski, 1994), and an inhibition of its formation has neuroprotective effects (Greenlund et al., 1995; Keller et al., 1998). Reactive oxygen species (ROS), e.g., nitric oxide, superoxide, and peroxynitrite, contribute to neurodegenerative diseases including Parkinson disease and amyotrophic lateral sclerosis (Shaw, 2005; Torreilles et al., 1999).

Amacrine cells are the main source of NO in the retina (Chun et al., 1999; Goto et al., 2005; Lee et al., 2003). We have shown previously that these neurons can secrete ETs and express ET_A/ET_B receptors (Kobayashi et al., 2005). Because ET-1 increases superoxide formation in the sympathetic neurons of DOCA-salt hypertensive rats (Dai et al., 2004), we hypothesized that ET-1 will act directly on the retinal neurons and enhance the formation of superoxide by interacting with NOS. To test this hypothesis, we cultured retinal neurons from fetal rats, and evaluated the alterations induced by ET-1 on NO and superoxide formation, and on the death of retinal neurons.

2. Materials and methods

2.1. Animals

Pregnant Wistar rats were purchased from Japan SLC (Shizuoka, Japan). The rats were housed in an air-conditioned room with a temperature of approximately 23 °C and humidity of 60% on a 12:12 light:dark cycle. All animals were handled in accordance with the ARVO resolution for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol was approved by the Committee of Animal Use and Care of the Osaka Medical College.

2.2. Chemicals

Chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. ET-1 (Peptide Institute, Osaka, Japan) was dissolved with 0.1% acetic acid to make 100 μM stock solution and further dilution was made by phosphate-buffered saline (PBS).

2.3. Cell culture

Cells for the primary cultures were obtained from the retinas of fetal Wistar rats (gestational age 19 days), and prepared as described in detail (Kobayashi et al., 2005). In brief, retinas were isolated and mechanically dissociated. Single-cell suspensions (1.0×10^6 cells/ml) were plated onto plastic 10 mm square cover slips in Earle's Minimal Essential Medium (MEM, Cat. No. 12360) supplemented with 2 mM glutamine, penicillin–streptomycin (100 U/ml–50 μg/ml), 25 mM *N*-[2-hydroxyethyl] piperadine-*N*′-[2-ethanesulfonic acid] (HEPES), and 10% heat-inactivated fetal bovine serum (medium A) under an atmosphere of 5% CO_2 in air. The medium and supplements were purchased from GIBCO BRL (Rockville, MD). To eliminate non-neuronal cells, 10 μM cytosine arabinoside (ara-C) was added to the cultures on day 5. No passage was performed. Primary cultures of retinal neurons were maintained for 10 days before use. Immunohistochemical studies in our laboratory have shown that the cultured retinal neurons consisted mainly of amacrine cells (>80 %), and these neurons expressed ETs and their specific ET_A and ET_B receptors (Kobayashi et al., 2005). The glial population was less than 1.0% under these culture conditions (Kobayashi et al., 2005). All of the experiments were carried out in a medium with 10% bovine serum (medium A), but in the fluorometric analyses, phenol red free MEM (GIBCO, Cat. No. 51200) containing the same supplements as medium A was used (medium B).

2.4. Assessment of neurotoxicity

The cultured retinal neurons were exposed to 0.1, 1.0, 10, and 100 nM ET-1 in medium A at 37 °C for 24 h to determine whether the cell death was dose-dependent. To determine if there was a time-dependent effect of ET-1 on the neurotoxicity, the retinal cells were assessed at 2, 6, and 24 h after exposure to 100 nM ET-1. As noted in Section 2.2 , acetic acid was used to help solubilize ET-1; in the medium containing 100 nM ET-1, which was the maximum concentration used in this study, there was 0.0001% acetic acid. In the control experiments for this study, an appropriate concentration of acetic acid was present. To determine which type of receptors mediated the effects of ET-1, BQ-123 (100 nM), a selective ET_A receptor antagonist, or BQ-788 (100 nM), a selective ET_B receptor antagonist, was added to the medium with 100 nM ET-1. L-NAME (100 μM) or Cu-Zn SOD (100 U/ml) was added to the medium with ET-1 to determine whether NOS and superoxide production were involved in the ET-1-induced cell death.

The degree of neurotoxicity caused by ET-1 was assessed quantitatively by the trypan blue exclusion method on five randomly selected cover slips. After exposure to ET-1 alone or in combination with the other agents, the cell cultures were