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ENDOTHELINS-1/3 AND ENDOTHELIN-A/B RECEPTORS EXPRESSING GLIAL CELLS WITH SPECIAL REFERENCE TO ACTIVATED MICROGLIA IN EXPERIMENTALLY INDUCED CEREBRAL ISCHEMIA IN THE ADULT RATS

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Abstract—We reported previously that amoeboid microglial cells (AMC) in the developing brain exhibited endothelins (ETs) expression which diminished with advancing age and was undetected in microglia in the more mature brain. This study sought to explore if microglia in the adult would be induced to express ETs in altered conditions. By immunofluorescence microscopy, ETs and endothelin (ET)-B receptor were undetected in microglial cells in sham-operated and normal control rats. However, in adult rats subjected to middle cerebral artery occlusion (MCAO), lectin labeled activated microglia which occurred in large numbers in the marginal zones in the ischemic cortex at 3 days and 1 week intensely expressed ETs specifically endothelin (ET)-1 and ET-B receptor; ET-3 and ET-A receptor were absent in these cells. By RT-PCR and ELISA, ET-1 and -3 mRNA and protein expression level was progressively increased in the ischemic cerebral cortex after MCAO compared with the controls. ET-A and ET-B receptor mRNA and protein levels were concomitantly up-regulated. It is suggested that increased release of ET-1 following MCAO by massive activated microglia can exert an immediate constriction of local blood vessels bearing ET-A receptor. ET-1 may also interact with activated microglia endowed with ET-B receptor via an autocrine manner that may be linked to chemokines/cytokines production. ET-1, ET-3 and ET-B receptor were also localized in reactive astrocytes along with some oligodendrocytes. We conclude that activated microglia together with other glial cells in the marginal zone after MCAO are the main cellular source of ETs that may be involved in regulation of vascular constriction and glial chemokines/cytokines production. However, dissecting the role of individual component of the endothelin system in the various glial cells, notably activated microglia, would be vital in designing of an effective therapeutic strategy for clinical treatment of stroke in which microglial cells have been implicated.

Key words: endothelins, ET-1/ET3, endothelin receptors, activated microglia, ischemia, rats.

The endothelin (ET) isopeptides (ET-1, ET-2 and ET-3) are a family of small, structurally related, vasoactive peptides that may be responsible for maintaining the tone of the cerebral vasculature. In the CNS, endothelins (ETs) are expressed by endothelial cells (Yoshimoto et al., 1990; Tsang et al., 2001), neurons (Lee et al., 1990), and astrocytes (MacCumber et al., 1990; Tsang et al., 2001). These peptides exert various actions by binding to two specific G-protein-coupled receptors subtypes, ET-A and ET-B receptors. ET-A and -B have their own characteristic tissue localization, and their wide distribution in the brain suggests their important role in CNS functions (Loo et al., 2002). ET-B receptors are predominantly expressed in astrocytes, while ET-A receptors are expressed in vascular smooth muscle in the brain (Hori et al., 1992). Stimulation of ET-B receptors by ET causes activation of astrocytes (Barone et al., 1995b; Ishikawa et al., 1997; Uesugi et al., 1996), increase in cytosolic Ca^{2+} level, stimulation of extracellular-regulated kinase pathways (Lazarini et al., 1996; Marsault et al., 1990) and modulation of cytoskeletal actin organization (Cazaubon et al., 1997; Koyama and Baba, 1994). Therefore, it has been assumed that ET would induce astrocytic activation and proliferation (Sasaki et al., 1998).

There is accumulating evidence that ETs may play a role in ischemic/hypoxic injuries in the adult brain. In transient ischemic rat brain, ET-1 and -3-like immunoreactivities were detected in the astrocytes in the damaged neural tissues (Yamashita et al., 1993, 1994). In the damaged region of hypoxia/ischemic mouse brain, both astrocyte-like and endothelial cells lining cerebral vessels expressed high level of ET-1 mRNA (Tsang et al., 2001). ET-like immunoreactivity increases in the cerebral spinal fluid and glia in various animal models of global and focal cerebral ischemia (Yamashita et al., 1994; Barone et al., 1995a). These studies suggest that increased production of ET may contribute to brain injury by limiting the vasodilatory response to ischemia, and initiation of gliosis after CNS damage.

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Abbreviations: AMC, amoeboid microglial cells; CNPase, 2′,3′cyclic nucleotide 3′-phosphodiesterase; ELISA, enzyme-linked immunosorbent assay; ET, endothelin; ET-A, endothelin receptor A; ET-B, endothelin receptor B; ETs, endothelins; ET-1, endothelin-1; ET-3, endothelin-3; GFAP, glial fibrillar acidic protein; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MCP-1, monocyte chemoattractant protein-1; PB, phosphate buffer; PBS, phosphate buffered saline; RT-PCR, real time-polymerase chain reactions; TBS, tris buffer saline; TTC, tetrazolium blue chloride.

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We have previously demonstrated expression of ETs and ET-B receptor in amoeboid microglia cells (AMC), the embryonic form of microglia in the developing brain (Wu et al., 2006). It was suggested that ETs and ET-B localized in AMC may be linked to regulation and release of chemokines and cytokines by the cells (Wu et al., 2006). A remarkable change in ETs expression in microglia during the brain development was the progressive diminution of ETs and ET-B receptor expression with advancing age and was absent in these cells as the brain matures. The present study was aimed to determine if microglia in the adult brain when activated would be elicited to express ETs and their receptors as their precursor cells. It is well established that a hallmark in brain injury is the accumulation of activated microglia and brain macrophages many of them are described to be derived from microglia (Ling et al., 2001). In the present study, we have used an experimentally induced cerebral ischemia model in rats described previously to investigate the activation of microglia in response to ischemia (Wu et al., 1998), and to ascertain if ETs specifically ET-1 and -3 and their receptors are expressed in activated microglia. The expression patterns of ETs and ET-receptors were analyzed in temporal sequence both qualitative and quantitatively by immunohistochemistry, immunofluorescence double labeling, real time-polymerase chain reactions (RT-PCR), Western blot, enzyme-linked immunosorbent assay (ELISA). The information will help to better understand the roles of activated microglia in cerebral ischemia especially with reference to expression of ETs and their receptors by these cells which have been regarded as one of the key therapeutic targets in neuroinflammation (Dheen et al., 2007).

EXPERIMENTAL PROCEDURES

Animals and animal surgery

All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of laboratory Animals (NIH publications No. 80-23). All efforts were made to reduce the number of rats and their suffering. The experimental project was approved by Kunming Medical College and National Natural Science Foundation of China (Project Number: 30760093).

Eighty-one male Sprague–Dawley (SD) rats weighing between 250 and 280 g were used. The rats were anaesthetized by an i.p. injection of pentobarbital sodium (Ceva Sante Animale, Libourne, France; 50mg/kg), and were fixed in the left lateral position. Following anesthesia, the rats were subjected to middle cerebral artery occlusion (MCAO). The surgical procedure followed that described previously by us (Wu et al., 1998). Briefly, following incision of the skin, the right temporal muscle was excised and cleared until the underlying zygomatic arch was exposed. A circular hole 3 mm in diameter was burred in the right parietal bone with a dental drill and cold saline drip. With a rongeur the circular opening was enlarged by removing additional bone at the periphery to expose the main trunk of the middle cerebral artery (MCA). The pieces of bone removed were kept in cold saline. Care was taken not to damage the underlying cerebral cortex during craniotomy. The MCA was cauterized using a small vessel cauterizer (Fine Science Tools. Canada), after which the bone flaps were replaced and the muscle and skin sutured separately, layer to layer. The rectal temperature was monitored and maintained between 37.5 and 38.5 °C during the operation. On recovering from anaesthesia, the rats showed signs of paresis of both the left limbs, especially the hind limb. In sham-operated rats the same surgical procedure was carried out but the MCA was not cauterized. Along with MCAO and sham operated rats, normal rats (n=9) of equivalent body weight were also used as controls.

Real time-polymerase chain reactions (RT-PCR)

Under deep anesthesia, the rats were killed by decapitation. The ischemic cortex at the marginal zone bordering the primary area of infarct from rats sacrificed at 2, 6, 12 h, 1, 2, 3 and 7 days after MCAO (n=3 at each time point), their matching shams (n=3) and controls (n=3) was removed and immediately frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Total RNA was extracted from the control and ischemic rat cortex using RNAesy mini kit (Qiagen, CA, USA) according to the manufacturer's protocol. The amount of total RNA was quantified with a Biophotometer (Eppendorf, CA, USA). Quantitative RT-PCR was carried out on a Light Cycler 3 instrument using a FastStart DNA Master plus SYBR Green I kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The amplified PCR products were separated on a 1.5% agarose gel staining with eithidium bromide and photographed (Syngene, Chemi Genius2 Bio Imaging System, Cambridge, UK). The cDNA was used to amplify, respectively, a 159, 207, 186 and 187-bp fragment using specific primers for ET-1 (forward 5′-gtcgtcccgtatggactagg-3′; reverse 5′-ggctcggagttctttgtctg-3′), ET-3 (forward 5′-gactgtccaaccacagagga-3′; reverse 5′-gacctccagtctcctgcttc-3′), ET-A (forward 5′-atcgggatccccttgattac-3′; reverse 5′-ccagtccttcacgtcttggt-3′) and ET-B (forward 5′-gcaggattctgaagctcacc-3′; reverse 5′-cagcagcacaaacacgactt-3′). For the control and normalizing the quantities of each sample, rat β-actin was adopted. The β-actin forward primer 5′-tcatgaagtgacgttgacatccgt-3′ and reverse primer 5′-cctagaagcatttgcggtgcaggatc-3′, lead to amplification of a 285-bp DNA fragment. The amplified PCR products were separated on a 1.5% agarose gel staining with Eithidium Bromide and photographed (Syngene, Chemi Genius2 Bio Imaging System, Cambridge, UK). Gene expression was quantified using a modification of the $2^{-\Delta\Delta ct}$ method as previously described (Livak and Schmittgen, 2001).

Western blotting

The normal control (n=3), sham (n=3) and MCAO (n=3 for each time point) rats from the cerebral cortex at the marginal zones were snap-frozen in liquid nitrogen and stored at −80 °C. Tissue samples were homogenized with protein extraction reagent (Pierce Biotechnology, IL, USA) containing protease inhibitors. The protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as a standard. Samples of supernatants containing 40 μg protein were heated to 95 °C for 5 min and were separated by sodium dodecyl sulphate-poly-acrylamide gel electrophoresis in 10% gels, in a Mini-Protein II apparatus (Bio-Rad, CA, USA). Protein bands were electroblotted onto 0.45 μm polyvinylindene difluoride membrane and blocked with 5% (w/v) non-fat dried milk. The membranes were then incubated with dilutions of the polyclonal ET-A and −B antibodies (1:200, Chemicon, CA, USA) in blocking solution overnight at 4 °C. They were then incubated with the secondary antibodies, HRP conjugated anti-rabbit IgG (dilution 1:5000). Specific binding was revealed by an enhanced chemiluminescence kit (GE Healthcare, UK Limited, Bucks, UK) following the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

The protein samples collected above for Western blot analysis were used for determination of protein concentrations at different time points after MCAO using ET-1 (Catalog #900-020A. Assay Designs, Ann Arbor, Michigan, USA) and ET-3 (Immuno-biological laboratories Co. Ltd, Gunma, Japan) ELISA kits according to the manufacturers' instructions.