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FOREBRAIN SPECIFIC HEPARIN-BINDING EPIDERMAL GROWTH FACTOR-LIKE GROWTH FACTOR KNOCKOUT MICE SHOW EXACERBATED ISCHEMIA AND REPERFUSION INJURY

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Abstract—Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a hypoxia-inducible neuroprotective protein that also stimulates proliferation of neuronal precursor cells. In this study, we investigated the possible role of HB-EGF in ischemia and reperfusion injury by measuring the changes in its mRNA expression following focal cerebral ischemia. We also examined neural damage after a middle cerebral artery occlusion (MCAO) and reperfusion in ventral forebrain specific HB-EGF knockout (KO) mice. The levels of HB-EGF mRNA in the cerebral cortex of wild-type (WT) mice were significantly increased 3–24 h after MCAO and reperfusion. Cerebral infraction in HB-EGF KO mice was aggravated at 1 day and 6 days after MCAO and reperfusion compared with WT mice. The number of terminal deoxynucleotidyl transferase (TdT)-mediated dNTP nick end labeling (TUNEL) and an oxidative stress marker, 8-hydroxy-2′-deoxyguanosine (8-OHdG) positive cells, were higher in HB-EGF KO mice than in WT mice. On the other hand, fewer bromodeoxyuridine (BrdU) positive cells were found in the subventricular zone in HB-EGF KO mice compared with WT mice. These results indicate that HB-EGF may play a pivotal role in ischemia and reperfusion injury and that endogenously synthesized HB-EGF is necessary for both the neuroprotective effect and for regulation of cell proliferation in the subventricular zone.

Key words: HB-EGF, MCAO, oxidative stress, proliferation, subventricular zone.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the EGF family of growth factors that includes epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, betacelulin, and neuregulin (Higashiyama et al., 1991; Watanabe et al., 1994; Barnard et al., 1995). In the brain, EGF family members act as neurotrophic molecules, serving to enhance stem cell proliferation and neural differentiation, and they also influence synaptic plasticity (Morrison et al., 1987; Ferrari et al., 1991; Ishiyama et al., 1991). In the central nervous system, HB-EGF is widely distributed in neurons and neuroglia throughout the brain (Mishima et al., 1996). HB-EGF binds to and activates the EGF receptor (EGF receptor/ErbB1) (Higashiyama et al., 1991) and also binds to ErbB4 (Elenius et al., 1997), which has been implicated in neuronal survival and glial/stem cell proliferation (Nakagawa et al., 1998; Kornblum et al., 1999; Farkas and Krieglstein, 2002). HB-EGF also promotes the survival of dopaminergic neurons, an action mediated by mitogen-activated protein kinase (MAPK) as well as by the Akt signaling pathway (Farkas and Krieglstein, 2002). For these reasons, HB-EGF may be an important contributor to both neural development and neuroprotection.

Stroke is the third commonest cause of death after heart attack and cancer in industrialized countries, and it has profound negative social and economic effects. Neurotrophic and growth factors such as EGF (Craig et al., 1996), fibroblast growth factor-2 (FGF-2) (Kuhn et al., 1997), and brain-derived neurotrophic factor (BDNF) (Pencea et al., 2001) have been implicated in neurogenesis as well as in *in vivo* neuroprotection. For this reason, recent studies have focused on the ability of these factors to promote endogenous neurogenesis as a novel therapeutic strategy against ischemic stroke (Nakatomi et al., 2002; Teramoto et al., 2003). Similarly, EGF family growth factors such as EGF and TGF-α have been also reported to exert protective effects in rodent models of ischemic brain injury (Peng et al., 1998; Justicia and Planas, 1999; Justicia et al., 2001). Intraventricular injection of HB-EGF into rats reduced infarct size and modified subsequent neurogenesis after focal cerebral ischemia (Jin et al., 2004). However, no studies have yet examined the effects of HB-EGF deletion on the cell damage and neurogenesis induced by cerebral ischemia.

In the present study, we investigated the possible role of endogenous HB-EGF in ischemia and reperfusion injury by examining the neural damage and cell proliferation in the subventricular zone of HB-EGF knockout (KO) mice following focal ischemia.

EXPERIMENTAL PROCEDURES

Animals

Ventral forebrain specific HB-EGF KO mice were generated using the Cre-loxP system, as described previously (Oyagi et al., 2009). All procedures relating to animal care and treatment conformed to the animal care guidelines of the Animal Experiment Committee of Gifu Pharma-

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Abbreviations: BDNF, brain-derived neurotrophic factor; BrdU, bromodeoxyuridine; CaMKII, calcium/calmodulin-dependent protein kinase II; EGF, epidermal growth factor; FGF-2, fibroblast growth factor-2; HB-EGF, heparin-binding epidermal growth factor-like growth factor; KO, knockout; MAPK, mitogen-activated protein kinase; MCAO, middle cerebral artery occlusion; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction; PSD-95, post-synaptic protein-95; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TGF-α, transforming growth factor-α; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dNTP nick end labeling; WT, wild-type; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

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ceutical University. All efforts were made to minimize both suffering and the number of animals used. The animals were housed at 24±2 °C under a 12 h light-dark cycle (lights on from 8:00 to 20:00) and had *ad libitum* access to food and water. In all experiments, we used wild-type (WT) littermates as a control group for the HB-EGF KO mice.

Focal cerebral ischemia model

Mice were anesthetized with isoflurane 2%–3% (for induction) and maintained with 1.0%–1.5% isoflurane in 70% N_2O and 30% O_2 via a face mask (Soft Lander; Sin-ei Industry, Saitama, Japan). Focal cerebral ischemia was induced [using an 8-0 nylon monofilament (Ethicon, Somerville, NJ, USA) coated with silicone hardener mixture (Xantpren; Bayer Dental, Osaka, Japan)] via the internal carotid artery, as described previously (Hara et al., 1996, 1997). Briefly, the coated filament was introduced into the left internal carotid artery through the common carotid artery, and then advanced up to the origin of the anterior cerebral artery via the internal carotid artery, so as to occlude the middle cerebral artery and posterior communicating artery. At the same time, the left common carotid artery was occluded. 90 min after occlusion, the filament was withdrawn to allow reperfusion. Anesthesia did not exceed 10 min.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Gene expressions for HB-EGF, EGF, and TGF-α were quantified using real-time polymerase chain reaction (PCR) analysis. Briefly, total RNA was isolated from the hemisphere of the ischemic cortex (2–7 mm from frontal pole) at various time points (at 1, 3, 6, and 24 h after middle cerebral artery occlusion (MCAO)) using a total RNA isolation kit (BIO-RAD; CA, USA), according to the manufacturer's instructions. Template cDNA was synthesized using Superscript RT Enzyme (Takara, Shiga, Japan), an oligo (dT), and a random primer in a reaction at 37 °C for 15 min. Real-time PCR was performed with SYBER Premix Ex Taq (Takara) and Thermal Cycler Dice Real time system TP800 (Takara). The PCR primer sequences used were follows: HB-EGF, 5′-AAGTGAAGTTGGGCGTGGCTA-3′ (forward) and 5′-CGTGTAACGAACCACTGTCTCAGAA-3′ (reverse), EGF, 5′-CATCATGGTGGTGGCTGTCTG-3′ (forward) and 5′-CACTTCCGCTTGGCTCATCA-3′ (reverse), TGF-α, 5′-CTGCTTGCTGCCACTCTGAGAC-3′ (forward) and 5′-AGGCCACCTGGCCAAATTC-3′ (reverse), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGTGTCCGTCGTGGATCTG-3′ (forward) and 5′-TTGCTGTTGAAGTCGCAGGAG-3′ (reverse).

Evaluation of infarct size in the focal cerebral ischemia model

At 24 h after MCAO and reperfusion, the mice were given an overdose of pentobarbital sodium, and then decapitated. The forebrain was divided into five coronal 2 mm sections using a mouse brain matrix (RBM-2000C; Activational Systems, Warren, MI, USA). These slices were immersed for 20 min in a 2% solution of 2,3,5-Triphenyltetrazolium Chloride (TTC) (Sigma, St. Louis, MO, USA) in normal saline at 37 °C, and then fixed in 10% phosphate-buffered formalin at 4 °C. When TTC reacts with intact mitochondrial respiratory enzymes, it generates a bright red color that contrasts with the pale color of the infarction. The caudal face of each slice was photographed. The area of the infarction (unstained) in the left cerebral hemisphere was traced and measured using Image J (http://rsb.info.nih.gov/ij/download/), and the infarction volume per brain (mm^3) was calculated from the measured infarction area. Brain swelling was calculated according to the following formula: (infarct volume+ipsilateral undamaged volume−contralateral volume)×100/contralateral volume (%) (Hara et al., 1996, 1997). Mice were tested for neurological deficits at 24 h after MCAO and reperfusion. These were scored as described in our previous study (Hara et al., 1996, 1997): 0, no observable neurological deficits (normal); 1, failure to extend the right forepaw (mild); 2, circling to the contralateral side (moderate); 3, loss of walking or righting reflex (severe); and 4, deceased. Physiological parameters and cerebral blood flow were measured as previously described (Hara et al., 1996; Koumura et al., 2009). Briefly, a polyethylene catheter was inserted into the femoral artery, and arterial blood pressure, pO_2, pCO_2, pH, and plasma glucose were measured 15 min after the start of ischemia.

Histological analysis

In preparation for TUNEL staining and immunostaining, the mice were deeply anesthetized with pentobarbital sodium at 24 h after MCAO and reperfusion, and then perfused with 4% paraformaldehyde. Coronal sections (thickness: 20 μm) were obtained from the frozen brains by serial sectioning. Sections at 6 or 8 mm from olfactory bulb were chosen for staining. The TUNEL assay was performed according to the manufacturer's instructions (Roche Molecular Biochemicals Inc.; Mannheim, Germany). For immunostaining, the sections were rinsed three times in PBS and blocked with 1% serum for 30 min. Antibodies against 8-OHdG (anti-8-hydroxy-deoxyguanosine mouse monoclonal, JAICA, Shizuoka, Japan) or BrdU (anti-bromodeoxyuridine rat monoclonal, Abcam; MA, USA) were applied to sections overnight at 4 °C. Secondary antibody (M.O.M. biotinylated anti-mouse) was applied for 10 min. The avidin/biotinylated horseradish peroxidase complex (ABC Elite kit; Vector Laboratories, UK) was applied for 30 min, and the chromogen was allowed to develop for 2 min by adding 3, 3-diaminobenzidine plus nickel solution (Sigma). For immunofluorescence, sections were incubated with a secondary antibody [Alexa Fluor488 F(ab')2 fragment of goat anti-rat IgG (H+L), Invitrogen; Carlsbad, CA, USA]. The histologists (A.O. and J.H.) were blinded as to which group each mouse belonged. Frozen sections (thickness: 20 μm) were also stained with Cresyl Violet (Sigma).

BrdU injections

BrdU (Sigma; 50 mg/kg) was dissolved in saline and given i.p. twice daily at 8 h intervals for three consecutive days, starting 24 h after MCAO and reperfusion. The animals were killed at 6 days after MCAO and reperfusion and then perfused with 4% paraformaldehyde. Coronal sections (thickness: 20 μm) were obtained from frozen brains by serial sectioning.

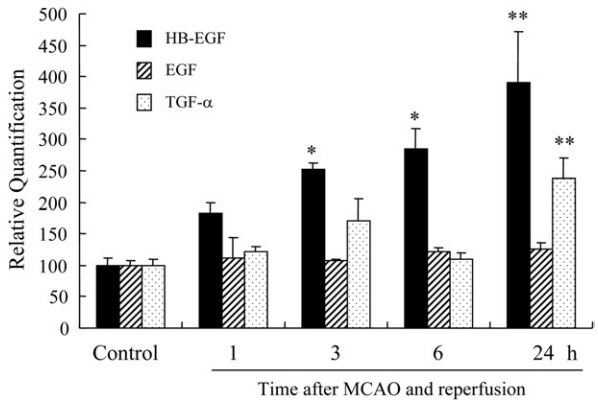

Fig. 1. Changes in mRNA levels for HB-EGF, EGF, and TGF-α in the cerebral cortex after ischemia and reperfusion. Expression of EGF family mRNAs in the cortex was measured in wild-type (WT) mice using quantitative RT-PCR performed 1, 3, 6, and 24 h after 90 min middle cerebral artery occlusion (MCAO) and reperfusion. n=5 for each group. Values are means±SEM. * $P<0.05$, ** $P<0.01$ vs. control.