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VASOACTIVE INTESTINAL PEPTIDE IN RATS WITH FOCAL CEREBRAL ISCHEMIA ENHANCES ANGIOGENESIS

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Abstract—We studied the effect of vasoactive intestinal peptide (VIP) on angiogenesis in the ischemic boundary area after focal cerebral ischemia. Adult male Sprague–Dawley rats underwent middle cerebral artery occlusion for 2 h. A single dose of VIP was given via i.c.v. injection at the beginning of reperfusion. Immunohistochemistry and Western blotting were performed to assay angiogenesis and brain levels of vascular endothelial growth factor (VEGF) protein, respectively. In addition, the expression of VEGF and its receptors (flt-1 and flk-1), as well as endothelial proliferation, was measured using rat brain microvascular endothelial cells. Immunohistochemical analyses revealed significant ($P<0.05$) increases in the numbers of bromodeoxyuridine (BrdU) positive endothelial cells and microvessels at the boundary of the ischemic lesion in rats treated with VIP compared with rats treated with saline. Western blotting analysis showed that treatment with VIP significantly ($P<0.05$) raised VEGF levels in the ischemic hemisphere. In addition, treatment with VIP increased flt-1 and flk-1 immunoreactivity in endothelial cells. *In vitro*, incubation with VIP significantly ($P<0.01$) increased the proliferation of endothelial cells and induced the expression of VEGF, flt-1 and flk-1 in endothelial cells. The stimulatory effect of VIP on the proliferation of endothelial cells was significantly ($P<0.01$) inhibited by SU5416, a selective inhibitor of VEGF receptor tyrosine kinase. Our data suggest that treatment with VIP enhances angiogenesis in the ischemic brain, and this effect may be mediated by increases in levels of VEGF and its receptors.

Key words: vasoactive intestinal peptide, angiogenesis, vascular endothelial growth factor, cerebral ischemia, rats.

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Abbreviations: BrdU, bromodeoxyuridine; DAB, diaminobenzidine; DAPI, 4′,6′-diamidino-2-phenylindole; ECA, external carotid artery; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; HIF, hypoxia-inducible factor; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; MTT, 3-(4,5-dimethyl-triazolyl-2)2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; RBMECs, rat brain microvascular endothelial cells; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide.

Blood is the only source of glucose and oxygen for neurons, hence, new microvasculature is necessary for brain development and repair. Cerebral ischemia induces angiogenesis (Zhang et al., 2000; Hayashi et al., 2003; Zadeh and Guha, 2003). Remarkable amounts of neovascularization develop in stroke patients (Krupinski et al., 1994). The neovascularization in these patients can be beneficial for the functional recovery from ischemic stroke. Therefore, an enhancement of angiogenesis can be a valuable therapeutic option for cerebral ischemia.

Angiogenesis, the process through which new blood vessels arise from pre-existing ones, is mediated by numerous factors that include vascular endothelial growth factor (VEGF), platelet-derived growth factor, angiopoietin-2, thrombospondin-1 and hypoxia-inducible factor (HIF) (Zadeh and Guha, 2003). Among these, VEGF, which binds to its receptors and results in endothelial cell activation, proliferation, migration, invasion, and survival (Marti et al., 2000; Ferrara et al., 2003; Donovan and Kummar, 2006), is though to be the primary regulator of angiogenesis. In recent years, a growing body of evidence indicates that many other endogenous peptides play an important regulatory role in angiogenesis, especially under pathological conditions (Ribatti et al., 2007). However, the mechanisms underlying proangiogenic action of these peptides have not been fully elucidated.

Vasoactive intestinal peptide (VIP), a neuropeptide belonging to the secretin/glucagon family of peptides, is expressed in distinct subpopulations of neurons in most major structures of both the CNS and peripheral nervous system (Fahrenkrug and Said, 2000; Moody et al., 2003). This neuropeptide, through G protein–coupled membrane receptors (VPAC1 and VPAC2 receptors) expressed in neurons, astrocytes, and endothelial cells (Lange et al., 1999; Joo et al., 2004), exerts multiple biologically active roles in processes that include exocrine and endocrine secretions, immunomodulation, muscle relaxation, and cell proliferation and differentiation (Fahrenkrug and Said, 2000; Voice et al., 2002). Some reports from *in vitro* and *in vivo* models revealed that VIP regulates VEGF expression and secretion in lung cancer cells (Casibang et al., 2001), increases VEGF mRNA expression in human prostate cancer LNCaP cells (Collado et al., 2004), and induces angiogenesis in the xenograft (Collado et al., 2007) and rat sponge (Hu et al., 1996) models. These observations suggest an important role for VIP as a proangiogenic factor, contributing to the induction of the angiogenic phenotype by stimulating VEGF. However, to our knowledge there have been no reports of the effects of VIP on angiogenesis following cerebral ischemia. Accordingly, the purpose of

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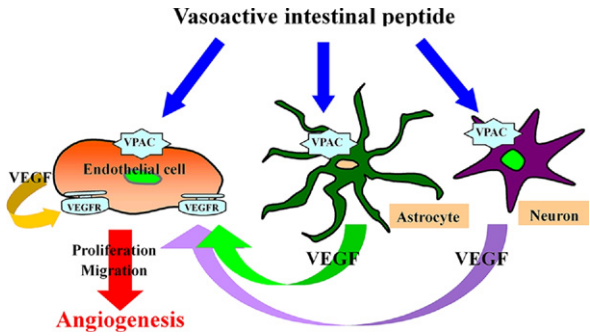

Fig. 1. Schematic representation of VEGF mediating VIP-enhanced angiogenesis after cerebral ischemia. VIP binds with VIP receptors (VPAC) on neurons, astrocytes and endothelial cells, and increases brain levels of VEGF (Western blotting analysis), and upregulates expression of VEGF in neurons, astrocytes and endothelial cells and expression of VEGF receptors in endothelial cells (immunohistochemical analyses). The released VEGF can affect proliferation (MTT assay) and migration of endothelial cells through its receptors in a paracrine manner and in an autocrine manner, consequently promoting angiogenesis in the ischemic brain (immunohistochemical analyses).

the present study was to test the hypothesis that VIP treatment enhances VEGF and thereby promotes angiogenesis in the ischemic brain (Fig. 1).

EXPERIMENTAL PROCEDURES

Animal model

Adult Sprague–Dawley male rats weighing 240–280 g (Animal Breeding Center, Xi'an Jiaotong University, Xi'an, China) were used in this study. The animals were quarantined for at least 7 days before the experiment. All animal care and treatment were conducted in accordance with the regulations of Xi'an Jiaotong University for the use of experimental animals in research, and in conformity with the NIH *Guide for the Care and Use of Laboratory Animals*. Transient middle cerebral artery occlusion (MCAO) was induced using the suture method of Longa (1989). Briefly, the animals were anesthetized with chloral hydrate (300 mg/kg). The right carotid artery was exposed through a midline cervical incision, the external carotid artery (ECA) was ligated distally. A 3-0 monofilament nylon suture, whose tip had been rounded by heating near a flame and coated with poly-*l*-lysine (Sigma, St. Louis, MO, USA), was introduced from the stump of ECA into the internal carotid artery until a mild resistance was felt (17–18 mm past the common carotid artery bifurcation), thereby occluding the origin of the middle cerebral artery (MCA). Two hours after MCAO, a neurological score was performed in each rat as reported by Bederson (1986). The rats with successful occlusion of the MCA showed gait disturbances with circling or walking to the left (corresponding to grade 3 by Bederson et al.), and were subjected to reperfusion (Memezawa et al., 1992). Reperfusion was established by gentle withdrawal of the suture until the rounded tip cleared the lumen of the ECA (under the same anesthetic conditions as surgery). Rectal temperature was maintained at 37±0.5 °C using a heating lamp and a heating pad during surgery.

Drug administration

After onset of reperfusion, the rats were randomly assigned to either the VIP-treated group (n=12) or the control group (n=12) and were immobilized in a stereotaxic instrument. A microsyringe was placed in the right lateral ventricle 3.5 mm deep as measured from the dural surface, 0.8 mm posterior and 1.4 mm lateral to the bregma. Rats in the VIP-treated group were injected with 2 μg VIP (Sigma) dissolved in 10 μl physiological saline. In our preliminary experiment, the ischemic rats treated with 2 μg VIP showed a higher expression for VEGF in the brain and a lower mortality rate than rats treated with 0.5 or 1 or 4 μg VIP, therefore, animals were treated only with 2 μg VIP in the present study. In the control group, the vehicle alone (10 μl physiological saline) was given via the same routes.

Bromodeoxyuridine (BrdU) labeling

BrdU (Sigma) was used for mitotic labeling. BrdU (50 mg/kg) was intraperitoneally injected daily for 14 consecutive days into VIP-treated rats (n=4) and control rats (n=4) starting at 24 h after MCAO.

Specimen preparation

For histologic analyses, at 3 (n=4 per group) or 21 days (n=4 per group, all BrdU-injected groups) after MCAO, animals were deeply anesthetized with chloral hydrate (600 mg/kg), and perfused through the heart with 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS). After post-fixation overnight in 4% paraformaldehyde, the brain blocks were cut at the coronal level (bregma +1 to 3 mm), embedded in paraffin and cut into 6 μm coronal sections. The sections obtained from BrdU-injected groups were processed for BrdU and CD34 immunostaining, whereas the other sections were prepared for VEGF, flt-1 and flk-1 immunostaining. For Western blotting analysis, rats (four per group) were euthanized by decapitation at 3 days after MCAO. Their brains were quickly removed and the left and right hemispheres were dissected and frozen immediately on dry ice to be stored at −80 °C until use.

Immunohistochemistry and quantification

The sections were immunostained for the following antibodies: mouse anti-VEGF (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit anti-flt-1 (1:100; Santa Cruz), mouse anti-flk-1 (1:100; Santa Cruz), mouse anti-BrdU (1:200; LabVision Corporation NeoMarkers, Fremont, CA, USA) and rabbit anti-CD34 (1:100; Bioster Biotech, WuHan, China). For CD34, VEGF, flt-1 and flk-1 antigen retrieval, a microwave oven was used to heat dewaxed paraffin sections for 10 min in 0.01 M citrate buffer solution (PH6.0). For BrdU immunostaining, sections were first pretreated to denature DNA by incubating them in 4 N HCl for 30 min at room temperature followed by digestion in 0.1% trypsase at 37 °C for 10 min. After being blocked in endogenous peroxidase 3% of H_2O_2 for 10 min, sections were incubated with 5% normal goat serum for 30 min, and reacted with the primary antibody overnight at 4 °C. After washing in PBS, the sections were incubated with biotinylated anti-rabbit IgG or antimouse IgG (Bioster Biotech) diluted 1:200 in PBS for 20 min at 37 °C. Consequently, the sections were incubated in a solution of avidin-biotin complex (ABC, Bioster Biotech) for 20 min at 37 °C. Labeling was visualized with a diaminobenzidine (DAB) kit (Bioster Biotech). The sections were then counterstained with hematoxylin. In each staining, other sections of the same brain were used for the negative controls, which included omission of primary antibodies and replacement of polyclonal antibodies with normal serum from the appropriate species or monoclonal antibodies with isotypic immunoglobulins, as well as pre-absorption (for 24 h at 4 °C) of the primary antibodies with the corresponding antigens obtained from Sigma for BrdU and VEGF, Santa Cruz for flt-1 and flk-1 and Lifespan (USA) for CD34, and no immunostaining was observed in such sections.

To evaluate VEGF immunohistochemistry, three VEGF-immunostained sections at 100 μm interval were analyzed from each rat. The numbers of VEGF immunoreactive cells in eight fields of view from the ischemic boundary area (Fig. 2) in each section were counted under a ×20 objective. The average cell