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Mindin is a critical mediator of ischemic brain injury in an experimental stroke model☆

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ABSTRACT

Background: Stroke is the second leading cause of death among adults worldwide. Mindin is an ECM protein that plays important roles in regulating inflammation, angiogenesis and neuronal outgrowth. The role of mindin in the context of brain ischemia has not been examined.

Methods and results: Transient occlusion of the middle cerebral artery was performed on mindin knockout (KO) mice, mice that carried a neuron-specific constitutively active mindin transgene (TG) and the appropriate controls. The outcome of the ischemia was evaluated by examination of the infarct and edema volumes and by neurological score assessments. The brains were collected 24 h or 3 days following the induced stroke. Compared with the control mice, the mindin KO mice exhibited lower infarct volumes and better outcomes in the neurological tests. Mindin-deficient mice exhibited low expression levels of stroke-induced inflammatory mediators, an attenuated recruitment of inflammatory cells, and inhibited activation of NF-κB. The neuronal apoptosis levels were also lower in the brains of the mindin KO mice than in those of the control mice. The mice that expressed a neuron-specific, constitutively active mindin transgene exhibited effects following the cerebral ischemic injury that were the opposite of those that were observed in the mindin KO mice. Moreover, Akt signaling activation was elevated in the ischemic brains of mindin KO mice.

Conclusions: Mindin KO mice exhibited minor infarctions, an attenuated inflammatory response and low levels of neuronal apoptosis following an ischemic insult. These data demonstrate that mindin is a critical mediator of ischemic brain injury in an experimental stroke model. Akt signaling most likely mediates the biological function of mindin in this model of cerebral ischemia.

Introduction

Stroke is the second leading cause of death and the most frequent cause of disability in adults worldwide (Bronner et al., 1995). More than 15 million individuals suffer from stroke annually, and ischemic stroke accounts for approximately 80–85% of these cases. The pathological processes of stroke are complex, involving cellular bioenergetic failure, excitotoxicity, oxidative stress, neuronal apoptosis, inflammation, and blood–brain barrier dysfunction (Brouns and De Deyn, 2009; Doyle et al., 2008). In recent decades, a number of innovative approaches have targeted the various pathological processes that occur during stroke with the aim of attenuating brain injury following the onset of ischemia. Certain approaches have demonstrated excellent therapeutic effects in experimental stroke models; however, none has reached the clinical realm, with the exception of rt-PA (Hacke et al., 1995; NINDS, 1995). More efforts are required to explore novel molecular targets for stroke therapy. In this study, we report that mindin is a

Abbreviations: ECM, extracellular matrix; NF-κB, nuclear factor kappa B; Akt, protein kinase B; tPA, tissue plasminogen activator; TSRs, thrombospondin type 1 repeats; PDGF, platelet-derived growth factor; PCR, polymerase chain reaction; MCA, middle cerebral artery; CBF, cerebral blood flow; tMCAO, transient middle cerebral artery occlusion; TTC, 2,3,5-triphenyl-2H-tetrazolium chloride; OCT, ornithine carbamyl transferase; PBS, phosphate buffer solution; TNF, tumor necrosis factor; IL, Interleukin; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; eNOS, endothelial nitric oxide synthase; GSK3, glycogen synthase kinase 3; mTOR, mammalian target of rapamycin; TLRs, toll-like receptors.

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critical mediator of ischemic brain injury and that it acts by altering both the post-ischemic inflammatory response and neuronal apoptosis.

Mindin (also referred to as Spondin 2) is a member of the mindin-/F-spondin family and identified as a secreted ECM protein in previous study (He et al., 2004). The mindin protein contains TSRs in its C-terminal portion. Two highly conserved domains among mindin-F-spondin family members, FS1 (F-spondin 1 domain) and FS2 (F-spondin 2 domain), are located in the N-terminal portion of mindin. The FS domains mediate integrin binding, which is required for inflammatory cell recruitment and T-cell priming (Jia et al., 2005; Li et al., 2006). The TSR domain recognizes pathogen-associated molecular patterns and initiates innate immune responses (He et al., 2004; Li et al., 2009). TSR-containing proteins, including mindin, are endogenous angiogenesis inhibitors (Feinstein and Klar, 2004). The functional roles of mindin have been demonstrated in the contexts of infectious diseases, cancer and diabetic nephropathy (Jia et al., 2008; Manda et al., 1999; Murakoshi et al., 2011; Parry et al., 2005). Recently, our laboratory reported that mindin is an important mediator of cardiac hypertrophy. In this context, mindin blocks Akt/GSK3β and TGF-β1–Smad signaling (Bian et al., 2012; Yan et al., 2011). These previous results illustrate that mindin is a potential target that links extracellular signaling with tissue injury and remodeling. Mindin has been demonstrated to be expressed in the brain and is involved in embryonic neuronal development (Feinstein et al., 1999). However, little is known regarding the role of this protein in neurological disease. In this study, we demonstrate that mindin deficiency protects against brain ischemic injury by activating Akt signaling, whereas the constitutive expression of mindin in the brain results in the opposite response to ischemic injury. Our studies of mindin KO mice and brain-specific mindin transgenic mice suggest that mindin is a crucial mediator of brain ischemic injury.

Materials and methods

Animals

All of the animal procedures were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. The animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication Nos. 80-23), revised in 1996. The mindin KO mice were described previously (Bian et al., 2012). Male, 10- to 12-week-old wild-type (WT) and mindin KO C57BL/6 background mice were used in this study.

To generate mindin transgenic mice, full-length mouse mindin cDNA was cloned downstream of the neuron-specific promoter PDGF. The resulting construct drives mindin expression preferentially within neuronal cell bodies in the cortex, hippocampus, and cerebellum (Sasahara et al., 1991). TG mice were then produced via microinjection of the PDGF-mindin construct into fertilized mouse embryos (C57BL/6 background). Four independent transgenic lines were established and examined. The TG mice were identified using PCR analysis of tail genomic DNA (forward primer: 5′-AAGGGTGGCAACTTCTCCTC-3′; reverse primer: 5′-ATAAGGAATGGACAGCAGGG-3′). The functional data and gene expression levels were analyzed in pairs of PDGF-mindin (TG) and non-transgenic (NTG) male littermates that ranged in age from 7 to 8 weeks.

Mouse transient focal cerebral ischemia model

The procedures for tMCAO were described previously (Wang et al., 2012a, 2012b). Briefly, the animals were anesthetized with 2.5–3% isoflurane in O_2. The rectal temperature was maintained at 37 ± 0.5 °C using a heating pad. A probe was affixed to the skull (2 mm posterior and 5 mm lateral to the bregma) and connected to a laser-Doppler flowmetry instrument (Periflux System 5010; Perimed, Sweden) for the continuous monitoring of the CBF. To achieve the tMCAO, a 6-0 Silicon-coated monofilament surgical suture (Doccol, Redland, CA) was inserted into the left external carotid artery, advanced into the internal carotid artery, and wedged into the cerebral arterial circle to obstruct the origin of the MCA. An interruption of the cerebral blood flow in the MCA territory was confirmed by documenting a >80% decline in relative cerebral blood flow. The filament was left in place for 60 or 45 min and then withdrawn. A return to >70% of basal cerebral blood flow within 10 min of suture withdrawal confirmed a reperfusion of the MCA territory.

Neurological deficit scores

24 h or 72 h after the tMCAO, neurological deficits were tested using a 9-point scale (Xia et al., 2006). No neurological deficit was scored as 0; left forelimb flexion when suspended by the tail or failure to extend the right forepaw fully was scored as 1; left shoulder adduction when suspended by the tail was scored as 2; reduced resistance to a lateral push toward the left was scored as 3; spontaneous movement in all directions, with circling to the left exhibited only if pulled by the tail, was scored as 4; circling or walking spontaneously only to the left was scored as 5; walking only when stimulated was scored as 6; no response to stimulation was scored as 7; and stroke-related death was scored as 8.

Measurement of the infarct volume

The infarct volume and swelling were measured 24 h and 72 h following the tMCAO using TTC staining. The brains were cut into 1-mm-thick coronal sections using a mouse brain matrix. The sections were stained with 2% TTC in phosphate buffer (pH 7.4) for 15 min at 37 °C. The sections were then transferred to a 10% formalin solution and fixed overnight. The fixed sections were photographed, and the volume of the infarct area was quantitated using Image-Pro Plus 6.0. To correct for the effects of edema, the area of the infarction was measured by subtracting the area of the nonlesioned ipsilateral hemisphere from that of the contralateral hemisphere. The volume of the infarction was calculated by the integration of the lesion areas at the seven measured levels of the brain.

Immunofluorescence staining

The mice were anesthetized at 24 h or 72 h after the tMCAO using sodium pentobarbital. The mice were then perfused via the left ventricle with 0.1 mol/L sodium phosphate buffer under 100 mm Hg of pressure for 5 min. This step was followed by perfusion with a fixative solution that contained 4% paraformaldehyde in a 0.1 mol/L phosphate buffer (pH 7.4) for 15 min. The brains were carefully removed and postfixed for 6–8 h in the same fixative solution at room temperature. Following this step, the brains were immersed overnight at 4 °C in a 0.1 mol/L phosphate buffer that contained 30% sucrose. The brains were embedded in OCT, and serial frontal sections were cut with a cryostat microtome. For the immunofluorescence staining, the sections were washed in PBS that contained 10% goat serum. Next, the sections were incubated with primary antibody

Table 1
The primers for PCR reaction. Sites for restriction enzyme are underlined.

Primer name	Primer sequence (5′–3′)
Mindin-F	CCGGAATTCATGGAAAACTTGAGTCTTGCC
Mindin-R	CCGCTCGAGTTAGACGCAGTTATCTGGGGC
	ACGCGTCGACGGATCCGAATTCATGAGCGACGTGGCT
Akt-F	ATTGTGAA
Akt-R	ACGCCAATTGCTCGAGTCAGGCCGTGCCGCTGGCCGA