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Upregulation of the GEF-H1 Pathway after Transient Cerebral Ischemia

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Abstract

The microtubule-dependent GEF-H1 pathway controls synaptic re-networking and overall gene expression via regulating cytoskeleton dynamics. Understanding this pathway after ischemia is essential to developing new therapies for neuronal function recovery. However, how the GEF-H1 pathway is regulated following transient cerebral ischemia remains unknown. This study employed a rat model of transient forebrain ischemia to investigate alterations of the GEF-H1 pathway using Western blotting, confocal and electron microscopy, dephosphorylation analysis, and pull-down assay. The GEF-H1 activity was significantly upregulated by: (i) dephosphorylation and (ii) translocation to synaptic membrane and nuclear structures during the early phase of reperfusion. GEF-H1 protein was then downregulated in the brain regions where neurons were destined to undergo delayed neuronal death, but markedly upregulated in neurons that were resistant to the same episode of cerebral ischemia. Consistently, GTP-RhoA, a GEF-H1 substrate, was significantly upregulated after brain ischemia. Electron microscopy further showed that neuronal microtubules were persistently depolymerized in the brain region where GEF-H1 protein was downregulated after brain ischemia. The results demonstrate that the GEF-H1 activity is significantly upregulated in both vulnerable and resistant brain regions in the early phase of reperfusion. However, GEF-H1 protein is downregulated in the vulnerable neurons but upregulated in the ischemic resistant neurons during the recovery phase after ischemia. The initial upregulation of GEF-H1 activity may contribute to excitotoxicity, whereas the late upregulation of GEF-H1 protein may promote neuroplasticity after brain ischemia.

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Disclosures:

None

Keywords

Brain ischemia; GEF-H1; cytoskeletal dynamics; synaptic plasticity; RhoA; gene expression

INTRODUCTION

Transient cerebral ischemia leads to neuronal death that does not occur until 3 days of reperfusion, the so-called delayed neuronal death. During this 3 day delay period, all neurons destined to die appear normal under the light microscope (Smith et al., 1984). The delay period provides a window of opportunity for understanding the underlying molecular and pathological processes and for developing therapies.

The neuroplasticity associated with rewiring and repairing of the synaptic network is one of the most important adaptive mechanisms after brain ischemia (Hu et al., 1998; Martone et al., 1999). Understanding the underlying mechanisms is essential to develop therapies for functional recovery after brain ischemia. The previous studies from the authors' laboratory have shown that synapses undergo remarkable morphological and molecular reorganization after an episode of cerebral ischemia (Hu et al., 1998; Martone et al., 1999). The microtubule-associated Rho activating factor GEF-H1 is a chief regulator for axonal re-networking, dendritic spine retraction, and general gene expression (Tolias et al., 2011). The activation process includes dephosphorylation and translocation of GEF-H1 to the synaptic membrane and nuclear structure, catalyzing the GTP-to-GDP exchange of RhoA GTPase, and regulating cytoskeletal dynamics (Gao et al., 2004; Tolias et al., 2007). RhoA is a best-known Rho-GTPase regulator for axonal growth and spine retraction in the central nervous system (CNS) (Ba et al., 2013). GEF-H1 and RhoA also regulate general gene expression via regulating nuclear cytoskeletal networks (Rajakylä et al., 2014)

The objective of this study is to investigate the GEF-H1 pathway after brain ischemia. This study shows that the GEF-H1 pathway may play a neuroprotective role after brain ischemia.

MATERIALS AND METHODS

Materials

Leupeptin, pepstatin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Triton X-100 (TX100), sodium dodecyl sulfate (SDS), propidium iodide and other chemicals were purchased from Sigma (Sigma, St. Louis, MO, USA). The following antibodies were used: anti-RhoA (Cell Signaling Tech, Danvers, MA, Cat# 2117), anti-GEF-H1 (Cell Signaling Tech, Danvers, MA, Cat# 4076), anti-beta-actin (Cell Signaling Tech, Danvers, MA, #4970), anti-microtubule-associated protein 2 (MAP2, Sigma, St Louis, Cat# M9942), and anti-phospho-GEF-H1 (Ser885) (Abcam, Cambridge, MA, Cat# ab94348). The anti-mouse or anti-rabbit secondary antibodies were purchased from Jackson ImmunoResearch (PA, USA).

Ischemia Model

The 2-vessel occlusion (2VO) rat transient forebrain ischemia model was produced by occlusion of two common carotid arteries while induction of hypotension as described in our previous publications (Zhang et al., 2013). Male Wistar rats of about 3-month-old and approximately 300 g body weight were used in this study. All the experimental procedures were approved by the Animal Use and Care Committee in the University of Maryland School of Medicine. Three series of sham-operated control rats and rats subjected to 20 min of ischemia followed by at 0.5, 4, 24 or 72 h of reperfusion were prepared for biochemical, histopathological, confocal microscopic, and electron microscopic (EM) studies. At least three rats in each experimental group were used in each of these series. For biochemical studies, brains were obtained by freezing them *in situ* with liquid nitrogen while the animals were artificially ventilated (Ponten et al., 1973). The brain subregions were dissected in a -12°C glove box in the coronal plane. For histopathology and confocal microscopy, rats were perfused via ascending aorta with ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS), sectioned with a vibratome, and stored in an anti-freeze solution at -20°C until use. For EM, rats were perfused with ice-cold 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer.

Light, confocal and electron microscopy

For light microscopic examination of histopathology, 50 μm vibratome sections were stained with acid fuchsin and celestine blue. Brain sections at bregma -3.60 mm of the hippocampal level were employed to perform a quantitative analysis with the StereoInvestigator program and a custom-designed morphology and stereology software (Tomimoto et al., 1994). The computer controls the stage to randomly place the counting frame on the first counting area, and then to systematically move it until the entire delineated field is sampled. Cell numbers were quantified according to the optical fractionator method (Vereczki et al., 2006). Only cells on the top layer between 4 – 14 μm were counted. The extent of injury will be expressed as percentage of dead neurons among the total neurons in the region examined. This experiment was conducted blindly.

For confocal microscopy, double-labeled fluorescence immunocytochemistry was performed on coronal brain sections (50 μm) according to the method described in our previous studies (Hu et al., 1998).

For EM, brains after perfusion-fixation were sectioned with a vibratome at 100 μm , postfixed for 2 h in 1% osmium tetroxide in 0.1 M cacodylate buffer immediately, rinsed in distilled water, and stained with 1% aqueous uranyl acetate overnight. The tissue sections were then dehydrated in an ascending series of ethanol to 100% followed by dry acetone and embedded in Durcupan ACM. Thin sections were counterstained with lead citrate prior to examination with a transmission electron microscope.

Preparation of Subcellular Fractions

The neocortical tissues were dissected and chopped into small pieces in a -12°C glove box freezer (Liu et al., 2005). Subcellular fractions were prepared for analyzing subcellular redistribution of components of the GEF-RhoA pathway based on the method described in

our previous studies (Sabirzhanova et al., 2013). Briefly, the rat cortex tissue homogenates were centrifuged at 800 g at 4°C for 10 min to obtain P1 pellets and supernatants (S1). The S1 was centrifuged at 10,000 g at 4°C for 20 min to obtain P2 and S2. The S2 was further centrifuged at 165,000 g to obtain the cytosol S3 and a P3 fraction. In some experiments, the tissue homogenates were centrifuged at 10,000 g for 10 min to obtain a pellet fraction. This fraction was then washed with 0.5% TX100, and then centrifuged 10,000 g at 4°C for 20 min to obtain the detergent insoluble (D-I) and detergent soluble (D-S) fractions. Each subcellular fraction was assayed for total protein concentration using a detergent compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Western Blot Analysis

Equal protein amounts among samples were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA) according to method described previously (Sabirzhanova et al., 2013). In addition to the loading of the same protein amounts to every lane on SDS-PAGE, β -actin levels on immunoblots were used as an internal sample loading control. All Western blot data were normalized to β -actin and expressed as the ratio between protein of interest and the β -actin protein level. Densitometry was performed with the ImageJ software in a no blinding way (version 1.48, National Institutes of Health).

Dephosphorylation of GEF-H1 and pull-down assay

Dephosphorylation studies were performed with protein homogenates prepared without phosphatase inhibitors according to our previous report (Sabirzhanova et al., 2013). Equal protein amounts of sham cortical tissues samples (25 μ g/25 μ l) were incubated at 37°C for 1 h in the presence or absence of calf intestinal alkaline phosphatase (CIP) (New England Biolabs, MA), and then were subjected to Western blot analysis with GEF-H1 antibody or phospho-GEF-H1 (Ser885).

A pull-down assay was carried out to determine RhoA GTPase activity in the brain tissue samples after brain ischemia according to the method described previously (Sabirzhanova et al., 2013). Briefly, the cortical tissues of sham-operated rats and rats subjected to ischemia followed by 0.5 h of reperfusion were homogenized in a lysis buffer, and then cleared by 10 min centrifugation at 13,000 g at 4°C (Sabirzhanova et al., 2013). The supernatants were incubated with Rho assay reagent (Cat# 14-382) (Millipore, Billerica, MA, USA). The RhoA binding beads were collected by centrifugation and then washed three times with lysis buffer. The bead-binding complexes as well as homogenates aliquots were then subjected to Western blot analysis to determine the amount of the GTP-form of RhoA, as well as the total RhoA with antibody.

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation (SD). Four animals in each experimental group were employed for quantitative analysis of histopathology, and 3 animals in each experimental group for quantitative analysis of protein bands on Western blots. One-way ANOVAs followed by Dunnett's post-hoc tests were used for statistical analysis. *denotes

$p < 0.05$ between sham and post-ischemia groups; # $p < 0.05$, 24h vs. 72h of reperfusion; ¶ $p < 0.05$, DG area vs. CA1 or Cx region.

RESULTS

Histopathology and electron microscopy

It is well established that 10–15 min of ischemia in the rat 2VO ischemia model leads to delayed neuronal death selectively in the dorsal CA1 neurons at 3 days of reperfusion, but leaves CA3, DG and neocortical neurons largely intact (Hu et al., 1998; Martone et al., 1999; Zhang et al., 2013; Smith et al., 1984). However, the tissue quantity of the dorsal CA1 area is extremely small for multiple biochemical analyses. To study whether delayed neuronal death also occurred in neocortical neurons with a longer ischemic duration, we induced 20 min ischemia in this model followed by different periods of reperfusion and carried out the quantitative analysis of delayed neuronal death in different brain regions. Neuronal nuclei of sham-operated control neurons were round in shape and with visible nucleoli (arrowheads) and dendritic trucks (Fig. 1A, arrows). At 72 h of reperfusion following 20 min ischemia, nuclei of dead neurons were significantly shrunken, became polygonal in shape, and surrounded with acidophilic cytoplasm (Fig. 1B, arrows). The stereological histopathological analysis of brain sections at the bregma -3.60 mm level showed that neuronal death occurred in less than 3% of the cortical and CA1 neurons, and no dead neurons were found in the DG area at 24 h of reperfusion after 20 min ischemia (Fig. 1C). Delayed neuronal death occurred in significant amounts of the dorsal CA1 (about 83%), the neocortical neurons (about 25%) at 72 h of reperfusion after 20 min ischemia (Fig. 1C). In comparison, about 8% of DG neurons undergo delayed neuronal death after 20 min ischemia (Fig. 1C). In the CA1, neocortical, and DG regions, the percentage of dead among the total neurons was significantly increased at 72 h of reperfusion compared to either sham or 24 h of reperfusion group, but the increases did not quite reach the statistical significance at 24h of reperfusion relative to the sham control. Furthermore, the percentage of dead among the total neurons was significantly higher in the CA1 and neocortical regions, compared to the DG area at 72 h of reperfusion. The results shows that: (i) 20 min ischemia in this model leads to delayed neuronal death mainly at 72 h of reperfusion; (ii) CA1 and neocortical neurons are more vulnerable, relative to DG neurons; and (iii) neocortical neurons also undergo delayed neuronal death, even though to a lesser degree relative to the CA1 region.

Changes in GEF-H1 after brain ischemia

To study changes in GEF-H1 after 20 min of brain ischemia, tissue homogenate, as well as P1, P2, P3, and S3 subcellular fractions were subjected to Western blot analysis (Fig. 2). The total GEF-H1 level was not significant changed in homogenate, but appeared to be translocated from P3 to P1 and P2 fraction after ischemia (Fig. 2, A and B). The cytosolic GEF-H1 level was barely detectable as it was predominantly cytoskeleton-associated (data not shown). Different exposures of Western blot films further showed that GEF-H1 had two bands, the upper was transiently increased in P2 fraction at 30 min of reperfusion, and then decreased in homogenates and all subcellular fractions at 24 or 72 h of reperfusion (Fig. 2A). Concomitantly, the lower band density was significantly increased in P1 fraction after

brain ischemia (Fig. 2B). The results suggest that the total level of GEF-H1 may not be significantly altered, but shifted from upper to the lower bands after brain ischemia.

The shifting of GEF-H1 to lower bands may be due to its activation by dephosphorylation (Birkenfeld et al., 2007). This was further studied with Western blot analysis of phosphorylation of GEF-H1 (Ser885). While the total GEF-H1 level was not significantly changed (see Fig. 2, A and B, top panels), the phosphorylation of GEF-H1 was progressively decreased in the homogenate and all subcellular fractions at 0.5, 24, or 72 h of reperfusion (Fig. 3, A and B).

To further identify that the lower band was dephosphorylated GEF-H1, we performed an *in vitro* dephosphorylation study which showed that calf intestinal phosphatase (CIP) treatment led to shift of GEF-H1 to the lower molecular size variants similar to those seen after brain ischemia (Fig. 3C). CIP treatment also abolished the phospho-GEF-H1 antibody labeling, confirming that GEF-H1 was completely dephosphorylated (Fig. 3C).

GEF-H1 binds with microtubules to be inactive (Birkenfeld et al., 2008). Dephosphorylation leads to its dissociation from microtubules and translocation to the postsynaptic densities (PSDs) to become active (Birkenfeld et al., 2008; Sarrouilhe et al., 2006; Kang et al., 2009; Dubash et al., 2011). To study this, we performed Western blot analysis of the detergent insoluble fraction (D-I) and soluble fractions (D-S) (Fig. 4). Our previous studies show that the D-I fraction contains PSD tightly associated proteins and D-S fraction contains microtubule-associated proteins (Hu et al., 1998; Zhang et al., 2013). GEF-H1 was significantly accumulated in the D-I fraction and concomitantly decreased in the D-S fraction (Fig. 4), suggesting that GEF-H1 may be dissociated from microtubules and tightly incorporated into PSDs after brain ischemia (Hu et al., 1998).

Regional and cellular distribution of GEF-H1 after brain ischemia

We next performed confocal microscopy of brain sections with GEF-H1 or phospho-GEF-H1 antibody (green color) and propidium iodide (PI, red color) or microtubule-associated protein-2 (MAP2, red color) (Fig. 5). Three brain sections from three different rats, respectively, in each experimental group were used in the following confocal microscopic analyses. The presented are those that were highly reproducible among three different brain sections. Both GEF-H1 and phospho-GEF-H1 are located mainly in neurons (Fig. 5, A–D, green, arrows). The GEF-H1 level was either unchanged or slightly decreased in all brain areas and also translocated to the nuclei of some cortical neurons at 30 min and 4 h of reperfusion after brain ischemia (Fig. 5A, Cx, arrows). At 24 and 72 h of reperfusion, relative to the control level (Fig. 5A and 5B upper image), the GEF-H1 level was further decreased in the vulnerable CA1 and some cortical neurons, but dramatically upregulated in resistant DG neurons after ischemia (Fig. 5A and 5B lower image). At 72 h of reperfusion, most of dorsal hippocampal CA1 neurons were dead based on the morphology of shrunken and polygonal nuclei (Fig. 5, A and C, arrowheads). The GEF-H1 immunostaining was also seen in a few microglial cells in the CA1 area at 72 h of reperfusion (Fig. 5A, CA1, 72h, double-arrows).

In comparison with GEF-H1, phospho-GEF-H1 (Ser885) immunoreactivity was located in the cell body and dendrites of sham-operated control neurons (Fig. 5C, arrows), and it was gradually decreased in the CA1, DG and neocortical regions during 0.5 – 24 h of reperfusion (Fig. 5C, arrows). At 72 h of reperfusion, most of CA1 neurons and some cortical neurons were dead and lost the immunoreactivity (Fig. 5C, arrowheads). Interestingly, phospho-GEF-H1 immunostaining was decreased mainly in the neuronal soma and dendrites (Fig. 5, C and D, double-arrows), but unchanged in the peri-neuronal region of the cortical area (Fig. 5D, upper, arrows). Fig. 5D lower panel further showed no overlapping between phospho-GEF-H1 (green, arrows) and MAP2 (red, double-arrows) immunostainings, suggesting that the peri-neuronal phospho-GEF-H1 may represent presynaptic components.

Depolymerization of microtubules after brain ischemia

As shown in previous studies, microtubule depolymerization activates GEF-H1 (Birkenfeld et al., 2008; Varma et al., 2010; Chang et al., 2008; Krendel et al., 2002). To study whether activation of GEF-H1 was due to depolymerization of microtubules after brain ischemia, we performed an EM study. Electron micrographs showed that CA1 dendritic microtubules were compact structures (Fig. 6A, arrows) and were associated with normal mitochondrion (M) and ribosome rosettes (Fig. 6A, double arrows). At 4 h of reperfusion, microtubules began to flat their compact structures (arrows) and were depolymerized into fragments in some spots (Fig. 6B, asterisks); monomeric ribosomes (arrowheads) became large abnormal aggregates (Fig. 6B, double arrows). At 24 h of reperfusion, microtubules were mostly depolymerized into fragments (Fig. 6C, arrows) and protein aggregates were progressively accumulated (Fig. 6C, double arrows). In comparison, changes in microtubule morphologies were only moderate and transient, and then recovered to the normal structures at 24 h of reperfusion in DG and most cortical neurons after the same episode of brain ischemia (data not shown).

RhoA activation

RhoA is a substrate of GEF-H1 (Pathak et al., 2012). Several previous studies show that RhoA activity is persistently upregulated, and inhibition of RhoA activity protect neurons from ischemic injury (Chang et al., 2008; Krendel et al., 2002; Gisselsson et al., 2010). To reproduce this, we performed the same pull-down assay (Fig. 7). Relative to that of the sham samples, the GTP-RhoA level was significantly upregulated, whereas the total RhoA was not altered after brain ischemia (Fig. 7).

DISCUSSION

This study shows that the GEF-H1 activity or dephosphorylation is upregulated all brain regions after brain ischemia. However, in the later period of reperfusion, GEF-H1 protein was downregulated in the brain regions where neurons undergo delayed neuronal death, but markedly upregulated in neurons that were resistant to the same episode of cerebral ischemia. Consistently, RhoA activity is also upregulated and neuronal microtubules were persistently depolymerized in the brain region where GEF-H1 protein was downregulated after brain ischemia. The initial upregulation of the GEF-H1 activity may play a detrimental

role in vulnerable neurons, whereas upregulation of GEF-H1 protein during the recovery phase may promote neuroplasticity after brain ischemia.

Synaptic GEF-H1

The GEF-H1 activity is inhibited when associated with microtubules, and it is the only GEF currently known that couples microtubule dynamics to RhoA activation for regulation of synaptic spine morphology (Birkenfeld et al., 2008). In general, any pathological condition that changes in the integrity of the microtubules should upregulate GEF-H1 activity. This is consistent with the present study showing disintegrated microtubules transiently in resistant neurons, but persistent in vulnerable neurons after brain ischemia (see Fig. 2) (Tomimoto et al., 1994; Deshpande et al., 1992).

GEF-H1 is a key component of the glutamate receptor complex (Sarrouilhe et al., 2006; Kang et al., 2009). This study shows that GEF-H1 is activated and translocated into the synaptic fraction after brain ischemia. The results may explain the authors' previous observation that synaptic ultrastructure and composition are profoundly altered after transient cerebral ischemia (Hu et al., 1998; Martone et al., 1999). At present, changes in GEF-H1 after brain ischemia have not been reported in the literature. Several previous studies show that overexpression of GEF-H1 in cultured neurons changes spine length and area (Tolias et al., 2011; Ryan et al., 2005; Conde et al., 2010; Bonnekoh et al., 1990; Dubash et al., 2011). Therefore, upregulation of GEF-H1 protein during the recovery phase may be related to synaptic repairing and formation after brain ischemia.

Role of the GEF-H1 - RhoA pathway after brain ischemia

Glutamate excitotoxicity is one of the most comprehensively studied mechanisms responsible for neuronal death in several neurological disorders including stroke, trauma and neurodegenerative disease. Glutamate receptor antagonists are effective in preventing excitotoxicity, but they also induce severe adverse effects when employed as therapeutic agents for stroke patients (Aarts et al., 2003). Therefore, it is essential to explore alternative mechanisms for developing novel therapies that can alleviate excitotoxicity while circumvent the negative consequences of blocking glutamate receptors.

Several recent studies show that inhibition of the RhoA pathway provides strong neuroprotection, which was of similar magnitude as that by NMDA receptor blockade in organotypic hippocampal slices during in vitro ischemia (Gisselsson et al., 2010). Inhibition of RhoA activity also reduces cerebral infarction in focal ischemia models (Kilic et al., 2010; Koumura et al., 2011; Yamashita et al., 2007), and prevents tau hyperphosphorylation and p25/CDK5 increase after global cerebral ischemia (Castro-Alvarez et al., 2011). The present study provides new information about upregulation of the GEF-H1 activity, a key upstream regulator of the RhoA activity after brain ischemia. Furthermore, GEF-H1 also plays a critical role in a positive feedback loop for the RAS/MAPK pathway (Cullis et al., 2014). Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia (Namura et al., 2001). Therefore, GEF-H1 may be used as a new or better drug target to block both the RhoA and the RAS/MAPK pathway after brain ischemia (Gisselsson et al., 2010). The limitation is now, however, that

specific GEF-H1 inhibitory agents are not available. It remains to be studied whether inhibition of GEF-H1 also offers similar or better neuroprotection after transient cerebral ischemia with reperfusion.

It should also be pointed out that GEF-H1 is not neuron-specific, involving regulation of cell morphological and mobility changes in all cell types, including inflammatory cells. This study shows that upregulation of the GEF-H1 pathway occurs mainly in neurons after transient cerebral ischemia. It remains to be studied whether up-regulation of the GEF-H1 pathway also contributes to cell morphological and mobility changes in inflammatory cells after focal ischemia, as inflammatory response may be stronger after focal ischemia, compared to that after a brief episode of cerebral ischemia with reperfusion.

On the other hand, GEF-H1 is highly enriched in the postsynaptic density and is an important mediator of the activity-dependent structural plasticity (Kang et al., 2009). Upregulation of GEF-H1 protein in ischemic resistant neurons in the later period of reperfusion may facilitate neuroplasticity after brain ischemia. Therefore, a practical strategy may be to inhibit the GEF-H1 pathway during the early periods of reperfusion to protect neurons from ischemic injury and promote this pathway in the later periods of recovery for facilitating neuroplasticity after brain ischemia.

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Abbreviations

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
GEF	guanine nucleotide exchange factor
CNS	central nervous system
TX100	Triton X100
TBS	Tris-buffered saline
MAP2	microtubule-associated protein 2
PI	propidium iodide
CIP	calf intestinal phosphatase
DG	dentate gyrus

REFERENCES

- Aarts MM, Tymianski M. Novel treatment of excitotoxicity: targeted disruption of intracellular signalling from glutamate receptors. *Biochem Pharmacol.* 2003; 66:877–886. [PubMed: 12963474]
- Ba W, van der Raadt J, Nadif Kasri N. Rho GTPase signaling at the synapse: implications for intellectual disability. *Exp Cell Res.* 2013; 319:2368–2374. [PubMed: 23769912]

- Birkenfeld J, Nalbant P, Bohl B, Pertz O, Hahn KM, Bokoch GM. GEF-H1 modulates localized RhoA activation during cytokinesis under the control of mitotic kinases. *Developmental Cell*. 2007; 12:699–712. [PubMed: 17488622]
- Birkenfeld J, Nalbant P, Yoon SH, Bokoch GM. Cellular functions of GEF-H1, a microtubule-regulated Rho-GEF: is altered GEF-H1 activity a crucial determinant of disease pathogenesis? *Trends Cell Biol*. 2008; 18:210–219. [PubMed: 18394899]
- Bonnekoh P, Barbier A, Oschlies U, Hossmann KA. Selective vulnerability in the gerbil hippocampus: morphological changes after 5-min ischemia and long survival times. *Acta Neuropathol*. 1990; 80:18–25. [PubMed: 2360415]
- Castro-Alvarez JF, Gutierrez-Vargas J, Darnaudéry M, Cardona-Gómez GP. ROCK inhibition prevents tau hyperphosphorylation and p25/CDK5 increase after global cerebral ischemia. *Behav Neurosci*. 2011; 125:465–472. [PubMed: 21517148]
- Chang YC, Nalbant P, Birkenfeld J, Chang ZF, Bokoch GM. GEF-H1 couples nocodazole-induced microtubule disassembly to cell contractility via RhoA. *Mol Biol Cell*. 2008; 19:2147–153. [PubMed: 18287519]
- Conde C, Arias C, Robin M, Li A, Saito M, Chuang JZ, Nairn AC, Sung CH, Cáceres A. Evidence for the involvement of Lfc and Tctex-1 in axon formation. *J Neurosci*. 2010 May 12; 30:6793–800. [PubMed: 20463241]
- Cullis J, Meiri D, Sandi MJ, Radulovich N, Kent OA, Medrano M, Mokady D, Normand J, Larose J, Marcotte R, Marshall CB, Ikura M, Ketela T, Moffat J, Neel BG, Gingras AC, Tsao MS, Rottapel R. The RhoGEF GEF-H1 is required for oncogenic RAS signaling via KSR-1. *Cancer Cell*. 2014; 25:181–195. [PubMed: 24525234]
- Deshpande J, Bergstedt K, Lindén T, Kalimo H, Wieloch T. Ultrastructural changes in the hippocampal CA1 region following transient cerebral ischemia: evidence against programmed cell death. *Exp Brain Res*. 1992; 88:91–105. [PubMed: 1371756]
- Dubash AD, Guilluy C, Srougi MC, Boulter E, Burridge K, García-Mata R. The small GTPase RhoA localizes to the nucleus and is activated by Net1 and DNA damage signals. *PLoS One*. 2011; 6:e17380. [PubMed: 21390328]
- Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y. Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proc Natl Acad Sci U S A*. 2004; 101:7618–7623. [PubMed: 15128949]
- Gisselsson L, Toresson H, Ruscher K, Wieloch T. Rho kinase inhibition protects CA1 cells in organotypic hippocampal slices during in vitro ischemia. *Brain Res*. 2010; 1316:92–100. [PubMed: 20026316]
- Hu BR, Park M, Martone ME, Fischer WH, Ellisman MH, Zivin JA. Assembly of proteins to postsynaptic densities after transient cerebral ischemia. *J Neurosci*. 1998; 18:625–633. [PubMed: 9425004]
- Kang MG, Guo Y, Huganir RL. AMPA receptor and GEF-H1/Lfc complex regulates dendritic spine development through RhoA signaling cascade. *Proc Natl Acad Sci USA*. 2009; 106:3549–3554. [PubMed: 19208802]
- Kilic E, ElAli A, Kilic U, Guo Z, Ugur M, Uslu U, Bassetti CL, Schwab ME, Hermann DM. Role of Nogo-A in neuronal survival in the reperfused ischemic brain. *J Cereb Blood Flow Metab*. 2010; 30:969–984. [PubMed: 20087369]
- Koumura A, Hamanaka J, Kawasaki K, Tsuruma K, Shimazawa M, Hozumi I, Inuzuka T, Hara H. Fasudil and ozagrel in combination show neuroprotective effects on cerebral infarction after murine middle cerebral artery occlusion. *J Pharmacol Exp Ther*. 2011; 338:337–344. [PubMed: 21493751]
- Krendel M, Zenke FT, Bokoch GM. Nucleotide exchange factor GEF-H1 mediates crosstalk between microtubules and the actin cytoskeleton. *Nat Cell Biol*. 2002; 4:294–301. [PubMed: 11912491]
- Liu C, Chen S, Kamme F, Hu BR. Ischemic preconditioning prevents protein aggregation after transient cerebral ischemia. *Neuroscience*. 2005; 134:69–80. [PubMed: 15939539]
- Martone ME, Jones YZ, Young SJ, Ellisman MH, Zivin JA, Hu BR. Modification of postsynaptic densities after transient cerebral ischemia: a quantitative and three-dimensional ultrastructural study. *J Neurosci*. 1999; 19:1988–1997. [PubMed: 10066252]

- Namura S, Iihara K, Takami S, Nagata I, Kikuchi H, Matsushita K, Moskowitz MA, Bonventre JV, Alessandrini A. Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia. *Proc Natl Acad Sci USA*. 2001; 98:11569–11574. [PubMed: 11504919]
- Pathak R, Delorme-Walker VD, Howell MC, Anselmo AN, White MA, Bokoch GM, Dermardirossian C. The microtubule-associated Rho activating factor GEF-H1 interacts with exocyst complex to regulate vesicle traffic. *Dev Cell*. 2012; 23:397–411. [PubMed: 22898781]
- Ponten U, Ratcheson RA, Siesjö BK. Metabolic changes in the brains of mice frozen in liquid nitrogen. *J Neurochem*. 1973; 21:1211–1216. [PubMed: 4761706]
- Rajakylä EK, Vartiainen MK. Rho, nuclear actin, and actin-binding proteins in the regulation of transcription and gene expression. *Small GTPases*. 2014; 6:5.
- Ryan XP, Alldritt J, Svenningsson P, Allen PB, Wu GY, Nairn AC, Greengard P. The Rho-specific GEF Lfc interacts with neurabin and spinophilin to regulate dendritic spine morphology. *Neuron*. 2005; 47:85–100. [PubMed: 15996550]
- Sabirzhanova I, Liu C, Zhao J, Bramlett H, Dietrich WD, Hu B. Changes in the GEF-H1 pathways after traumatic brain injury. *J Neurotrauma*. 2013; 30:1449–1456. [PubMed: 23611588]
- Sarrouilhe D, di Tommaso A, Métayé T, Ladeveze V. Spinophilin: from partners to functions. *Biochimie*. 2006; 88:1099–1113. [PubMed: 16737766]
- Smith ML, Auer RN, Siesjö BK. The density and distribution of ischemic brain injury in the rat following 2–10 min of forebrain ischemia. *Acta Neuropathol*. 1984; 64:319–332. [PubMed: 6507048]
- Tolias KF, Bikoff JB, Kane CG, Tolias CS, Hu L, Greenberg M. The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptor-dependent dendritic spine development. *Proc Natl Acad Sci USA*. 2007; 104:7265–7270. [PubMed: 17440041]
- Tolias KF, Duman JG, Um K. Control of synapse development and plasticity by Rho GTPase regulatory proteins. *Prog Neurobiol*. 2011; 94:133–148. [PubMed: 21530608]
- Tomimoto H, Yanagihara T. Golgi electron microscopic study of the cerebral cortex after transient cerebral ischemia and reperfusion in the gerbil. *Neuroscience*. 1994; 63:957–967. [PubMed: 7700519]
- Varma H, Yamamoto A, Sarantos MR, Hughes RE, Stockwell BR. Mutant huntingtin alters cell fate in response to microtubule depolymerization via the GEF-H1-RhoA-ERK pathway. *J Biol Chem*. 2010; 285:37445–37457. [PubMed: 20858895]
- Vereczki V, Martin E, Rosenthal RE, Hof PR, Hoffman GE, Fiskum G. Normoxic resuscitation after cardiac arrest protects against hippocampal oxidative stress, metabolic dysfunction, and neuronal death. *J Cereb Blood Flow Metab*. 2006; 26:821–835. [PubMed: 16251887]
- Yamashita K, Kotani Y, Nakajima Y, Shimazawa M, Yoshimura S, Nakashima S, Iwama T, Hara H. Fasudil, a Rho kinase (ROCK) inhibitor, protects against ischemic neuronal damage in vitro and in vivo by acting directly on neurons. *Brain Res*. 2007; 1154:215–224. [PubMed: 17482584]
- Zhang F, Guo A, Liu C, Comb M, Hu B. Phosphorylation and assembly of glutamate receptors after brain ischemia. *Stroke*. 2013; 44:170–176. [PubMed: 23212166]

Highlights

The GEF-H1 is significantly activated by dephosphorylation after brain ischemia.

GEF-H1 protein level is downregulated in ischemia-vulnerable neurons.

GEF-H1 protein level is upregulated in ischemia-resistant neurons.

The early upregulation of GEF-H1 activity may contribute to the excitotoxicity.

The late increase in GEF-H1 protein may promote neuroplasticity after brain ischemia.

Fig. 1. Histopathology

Brain sections were obtained from a sham-operated control rat and a rat subjected to 20 min of ischemia following by 24 and 72 h of reperfusion. (A) Sham control neuronal nuclei were round in shape and with visible nucleoli (arrowheads) and dendritic trunks (arrows); (B) The nuclei of ischemic dead neurons were shrunken and surrounded with acidophilic cytoplasm (arrows). Significant proliferation of non-neuronal cells was seen in the dead CA1 neuronal layer (double-arrows); (C) Quantification of dead and normal neurons in the CA1, DG and cortex (Cx) with an unbiased stereology method (see Methods). The total neurons = dead

neurons + normal neurons. Data are expressed as percentage of dead neurons among the total neurons (mean $\pm$ SD, n=4). * $p<0.01$, sham vs. post-ischemia; # $p<0.05$, 24h vs. 72h of reperfusion; ¶ $p<0.05$, DG area vs. CA1 or Cx region. .

Fig. 2. Western blot analysis of GEF-H1

Samples were prepared from sham-operated control rats and rats subjected to 20 min of cerebral ischemia followed by 30 min, 24 and 72 h of reperfusion. (**A**) Western blots of both upper and lower GEF-H1 bands (*top panel*); and Western blots of a short exposure film to show only the upper band of GEF-H1 (*lower panel*) in homogenate (H), P1, P2, and P3 fraction after brain ischemia. Three separate samples in each experimental group were analyzed on the same Western blot membrane. (**B**) Quantitative analysis of the total GEF-H1 level (*top*), the level of the upper band (*middle*), and the level of the lower band (*bottom*). Quantitative data are expressed as fold of control (mean $\pm$ SD, $n=3$). One-way ANOVAs followed by Dunnett's post-hoc tests were used for statistical analysis. * $p<0.05$ between sham and ischemia groups.

Fig. 3. Phosphorylation analysis of GEF-H1

The same samples of Fig. 3 were used. (A) Western blots of phospho-GEF-H1; (B) quantitative analysis of the phospho-GEF-H1 level. Molecular size in kDa is indicated on the right; (C) Dephosphorylation of GEF-H1 *in vitro*. The sham control samples were incubated in the presence (+) or absence (-) of CIP, and then were subjected to Western blot analysis with GEF-H1 or phospho-GEF-H1 antibody. Molecular size in kDa is indicated on the left. Data are expressed as fold of control (mean $\pm$ SD, n=3). One-way ANOVA followed by Dunnett's post-hoc test were used for statistical analysis. * $p < 0.01$, sham vs. post-ischemia group; # $p < 0.01$, 24h vs. 72h of reperfusion; and ¶ $p < 0.01$, CA1 vs. Cx or DG.

Fig. 4. Translocation of GEF-H1

Left: Western blots of GEF-H1 in detergent insoluble (D-I) and detergent-soluble (D-S) fractions; Right: Quantitative analysis of the GEF-H1 band. One-way ANOVAs followed by Dunnett's post-hoc tests were used for statistical analysis. * $p < 0.05$ between sham and ischemia groups.

Fig. 5. Confocal microscopy of GEF-H1 and phospho-GEF-H1

Brain sections were from a sham-operated control rat and rats subjected to 20 min ischemia followed by 0.5, 4, 24 and 72 h of reperfusion. **(A)** The CA1, DG, and cortical regions double-labeled with anti-GEF-H1 antibody (green color) and PI (red color). Arrows indicate the normal neurons. Arrowheads point dead CA1 neurons. Double arrows denote non-neuronal cells. **(B)** Montage of 27 individual images to show the hippocampi of a sham-operated control (upper) and 24 h of reperfusion (lower). **(C)** The CA1, DG, and cortical regions labeled with anti-phospho-GEF-H1 antibody (green color). Arrows indicate the nuclear immunolabeling. Arrowheads point dead CA1 neurons. Double asterisks mark the DG dendritic area. Double arrows denote neurons lacking of the immunolabeling in the cytoplasm. **(D)** Upper: Higher magnification of the empty cytoplasmic immunostaining

(double-arrows) surrounded by normal peri-neuronal immunostaining (arrows); Lower: Double staining of phospho-GEF-H1 (green, arrows) and MAP2 (red, double arrows).

Fig. 6. Dendritic ultrastructures

Brain sections were obtained from a sham-operated control rat (**A**) and rats subjected to 20 min of forebrain ischemia followed by 4 (**B**) and 24 h (**C**) of reperfusion. (**A**) Sham control CA1 dendritic microtubules (arrows), a normal mitochondrion (M) and ribosome rosettes (double arrows); (**B**) At 4 h of reperfusion, microtubules began to lose their compact structure (arrows) and became depolymerized in some spots (asterisks). Ribosomes became either monomers (arrowheads) or large abnormal aggregates (double arrows); (**C**) By 24 h of reperfusion, microtubules were mostly depolymerized into fragments (arrows) and protein aggregates (double arrows) were accumulated.

Fig. 7. RhoA pull-down assay

The cortical tissues were obtained from sham-operated control rats and rats subjected to 20 min of ischemia followed by 30 min of reperfusion. **Left:** Western blots of the GTP-RhoA pull-down assay and the total RhoA protein level in the pull-down samples. **Right:** The optical densities of the RhoA-GTP level presented as the ratio between the RhoA-GTP and the total RhoA. Data are expressed as fold of control (mean $\pm$ SD, n=3). One-way ANOVAs

followed by Dunnett's post-hoc tests were used for statistical analysis. * $p < 0.05$ between sham and ischemia groups.