Involvement of MAPK/ERK kinase-ERK pathway in exogenous bFGF-induced Egr-1 binding activity enhancement in anoxia-reoxygenation injured astrocytes

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Abstract: **Objective** Intravenous administration of basic fibroblast growth factor (bFGF) is effective to reduce the volume of cerebral infarct due to ischemia. This study was designed to investigate the molecular mechanism, especially the signal transduction pathways, involved in this protective role of bFGF. **Methods** Anoxia-reoxygenation treated astrocytes were used to study the role of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK kinase, MEK)-ERK signaling pathway after exogenous bFGF administration by Western blot. Electrophoretic mobility shift assay was used to detect the binding activity of early growth response factor-1 (Egr-1), an important transcription factor for endogenous bFGF. **Results** bFGF could protect some signal transduction proteins from the oxygen-derived free radicals induced degradation. ERK1/2 was activated and involved in Egr-1 binding activity enhancement induced by exogenous bFGF. **Conclusion** MEK-ERK MAPK cascade may be an important signal transduction pathway contributed to bFGF induced enhancement of Egr-1 binding activity in anoxia-reoxygenation injured astrocytes.

Keywords: extracellular signal-regulated kinase; mitogen-activated protein kinase; free radicals; fibroblast growth factor 2; early growth response protein 1; astrocyte

1 Introduction

Basic fibroblast growth factor (bFGF), a multifunctional cytokine in the central nervous system produced by a variety of neural cells including astrocytes, is effective to protect neurons from many kinds of insulting factors. Our previous *in vivo* study found that after ischemia the endogenous bFGF increased, but it expressed later than its receptor^[1]. This may be the reason why bFGF could not fulfill its neuroprotective function though it is abundant in the central nervous system.

Many researches have found that exogenous bFGF is helpful to reduce the cerebral ischemic volume in rats^[2], and the protective mechanisms are being studied, though not fully illustrated. Sliveman *et al.* found that early growth response factor-1 (Egr-1) was rapidly induced at the edge

of endothelial wound^[3]. Our previous study also revealed that Egr-1 was enhanced at the margin of ischemic area after brain ischemia. After exogenous bFGF being given, the Egr-1 was expressed stronger and earlier at the margin of the brain ischemic lesion, implying that the exogenous bFGF might affect the expression of Egr-1^[1].

Extracellular stimulations including growth factors, hormones and neurotransmitters could induce the expression of Egr-1 dramatically and rapidly. Egr-1 is then capable of activating the transcription of many genes including PDGF-A, PDGF-B, FGF-2, TNF- α , IL-2, tissue factor, u-PA, etc^[4]. The products of these genes are responsible for the physiological alterations. Thus Egr-1 is essential for the growth, proliferation and differentiation of various cell types in the central nervous system. It is a critical transcription mediator to produce long-term responses to the extracellular signals by altering the expression of Egr-1 target genes^[5].

The Egr-1 gene promoter contains five serum response elements (SREs)^[6], and could be activated by the continuously active mitogen-activated protein kinase (MAPK) kinase (MAPKK) responsible for phosphorylation and activa-

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tion of extracellular signal-regulated protein kinase (ERK)^[7].

Different MAPK signal pathways are involved in the biological effects of bFGF in different cell types (including astrocytes) of the central nervous system^[8,9]. This study investigated whether MAPK/ERK kinase (MEK)-ERK signaling pathway was activated by the exogenous bFGF in anoxia-reoxygenation insulted astrocytes, aiming to throw light on the protective mechanism of exogenous bFGF in the brain ischemia.

2 Materials and methods

2.1 Primary astrocyte culture Primary astrocytes from the prefrontal cortices of 2-4 day old Sprague-Dawley rats (brought from Experimental Animal Department of Fudan University, China) were cultured as previously described^[10]. Briefly, prefrontal cortex was dissected out and digested with 0.25% trypsin for 10 min at 37 °C. After trituration, cells were seeded onto poly-lysine-coated plates and cultured in DMEM with 10% heated-inactivated fetal bovine serum. In most cases, 12-13 day old cells were used for study. Astrocytes in primary cultures were determined by indirect immunostaining with antibodies against glial fibrillary acidic protein (GFAP). The purity of astrocytes was up to about 95%.

2.2 Cell treatment The oxygen consumption reagent and sealed reservoir (bioMerieux Inc., France) were used to decrease the concentration of O₂ to 1%–1.5% in one hour. After 2-h hypoxia, oxygen was resumed and cells were divided into three groups: bFGF (10 ng/mL) group, glutathione (1 mmol/L) group, and bFGF (10 ng/mL) + U0126 (MEK inhibitor, 10 μmol/L) group. Each group was then divided into subgroups of 10 min, 20 min, 30 min, 1 h, 2 h, 4 h, 6 h, 12 h, and 24 h after treatment. In each group, the astrocytes without chemical administration were allocated to the control subgroup.

2.3 Western blot Western blot analysis was performed as previously described^[11]. Briefly, after the protein contents of the cell lysates were determined, the samples were incubated with insodium dodecyl sulfate (SDS)-contained buffer at 100 °C for 5 min prior to electrophoresis in 10% SDS polyacrylamide gel. The resolved band was electro-transferred onto a PVDF membrane, blocked with 10% skim milk, and then incubated with polyclonal antibody against MEK, ERK1/2 or phospho-ERK1/2 (all diluted to 1:500; Cell Signaling Technology, Inc., USA) for 2 h at room temperature.

2.4 Electrophoretic mobile shift assay for detection of Egr-1 binding activity DNA binding proteins were extracted as previously described^[12]. The astrocytes were cultured

in DMEM/F-12 containing 0.5% fetal bovine serum for 24-48 h when 80% confluency was reached. Remove the medium and wash the cells twice with ice-cold PBS (pH 7.4). The cells were then scraped into 600 μL ice-cold PBS, pelleted for 10 s, and then resuspended in 400 μL ice-cold Buffer A [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, and 0.5 mmol/L DTT]. The cells were incubated on ice for 10 min, followed by vortexed for 10 s and centrifuged for 10 s, then suspended in 10 μL ice-cold Buffer C [10 mmol/L HEPES-KOH (pH 7.9), 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, and PMSF]. Samples were incubated on ice for 20 min and cellular debris was removed by centrifugation for 20 min at 13 600 r/min at 4 °C. The supernatants were stored at -70 °C for later analysis. The concentration of nuclear protein was analyzed by the bicinchoninic acid (BCA) assay (ranging about 1-2 μg/μL). The bFGF oligonucleotide probe containing Egr-1 binding site was synthesized by BioAsia Ltd. (Shanghai, China). The sequences of these oligonucleotides used in the binding reactions were: 5'-GGATCCAGCGGGGGCGAGCGGGGGCGA-3' and 5'-TCGCCCCTCGCTGCCCGCTGGA TCC-3'.

AP-1 oligonucleotides for competition studies were also obtained from BioAsia Co. Ltd., Shanghai. Each oligonucleotide was annealed to its complementary strand and end-labeled with digoxin (DIG) using terminal transferase (Roche Molecular Biochemicals). Competition studies were performed with molar excesses of unlabeled double-stranded oligonucleotides. The DNA binding reaction were carried out at room temperature by incubating the nuclear protein fractions (10 μg) from astrocytes with double-stranded poly[d(I-C)] (2 μg), poly-L-lysine, and binding buffer for 5 min, followed by adding 1 μL DIG-labeled oligonucleotide for additional 20 min. Protein-DNA complexes was resolved from free probes in non-denaturing 5% polyacrylamide gels. The gels were then transferred to a positively charged nylon membrane by electro-blotting, and exposed to X-ray film for 2-3 h at room temperature.

2.5 Statistical analysis Data were expressed as means ± SEM, gained from three repeated tests. The significance of difference between mean values was evaluated by two-tailed Student's test and *P* < 0.05 was considered statistically significant.

3 Results

3.1 MEK-ERK signaling pathway was not activated by exo-

genous bFGF in normal astrocyte MEK and ERK were detected after exogenous bFGF being given to the normal astrocytes, and showed no significant changes ($P > 0.05$). Although the expression of phospho-ERK1/2 was enhanced at 30 min after exogenous bFGF administered, it

had no statistical significance either ($P > 0.05$, Fig. 1).

3.2 Anoxic-reoxic insult induced degradation of MEK, ERK1/2 and phospho-ERK1/2 in astrocytes After 2-h ischemia-like insult, the expression of the signal transduction proteins MEK and ERK1/2 decreased in the time win-

Fig. 1 Dynamic expression of MEK, ERK1/2, and phospho-ERK1/2 (p-ERK1/2) in normal primary astrocytes after bFGF (10 ng/mL) administration. The grey density ratio of each subgroup to β -actin had no significant change compared with Control (the subgroup without bFGF treatment) ($P > 0.05$).

Fig. 2 Dynamic expression of MEK, ERK1/2 proteins in the anoxia-reoxygenation insulted astrocytes. The grey density ratios (grey density of each band to actin) of MEK and ERK1/2 were significantly decreased when the oxygen given again in the time window of 20 min to 2 h, while phospho-ERK1/2 showed degradation from 20 min to 4 h. * $P < 0.05$ vs Control.

dow from 20 min to 2 h after oxygen resumed, while phospho-ERK1/2 showed degradation from 20 min to 4 h ($P < 0.05$) (Fig. 2).

3.3 Glutathione protected MEK and ERK1/2 from degradation After glutathione (1 mmol/L) was added to the anoxia-reoxygenation treated astrocytes, MEK and ERK1/2

showed no degradation (Fig. 3).

3.4 bFGF induced phosphorylation of ERK1/2 in astrocytes suffered from anoxic-reoxic insult and protected it from degradation The endogenous ERK1/2 had no changes as determined by western blot (Fig. 4). The phospho-ERK1/2 was activated within 30 min after bFGF was given and then

Fig. 3 Expression of MEK and ERK1/2 in the anoxia-reoxygenation insulted astrocytes after administration of glutathione had no significant change.

Fig. 4 Dynamic expression of MEK, ERK1/2, and phospho-ERK1/2 (p-ERK1/2) in the anoxia-reoxygenation insulted astrocytes after administration of bFGF (10 ng/mL). p-ERK1/2 (the active forms of ERK1/2) were enhanced within 30 min after bFGF treatment. * $P < 0.05$ vs Control.

Fig. 5 The dynamic expressions of MEK and ERK1/2 in the anoxia-reoxygenation insulted astrocytes after administration of bFGF (10 ng/mL) + U0126 (100 μ mol/L). After U0126 was added, the expression of MEK and ERK1/2 had no change, while the phospho-ERK1/2 was totally blocked.

declined (Fig. 4).

U0126, a MEK-specific inhibitor, could inhibit the activation of ERK1/2 (Fig. 5). But the total ERK1/2 was not changed at all.

3.5 bFGF induced higher activity of Egr-1 in the astrocytes insulted by anoxia-reoxygenation: time course and inhibitive effect of MEK A series of time course experiments were carried out to determine whether Egr-1 was activated by bFGF in anoxia-reoxygenation insulted astrocytes. The results showed that Egr-1 binding activity decreased significantly during 0.5 h to 2 h after the anoxic-reoxic insult (Fig. 6A), but the decreased binding activity was enhanced during 0.5 h to 24 h after bFGF was added (Fig. 6B). U0126 (100 μ mol/L) did significantly block the binding activity of Egr-1 (Fig. 6C).

4 Discussion

The exogenous bFGF has been used to treat cerebral ischemia. Previous studies found that bFGF could protect neurons from injuries of hypoxia, hypoglycemia, calcium overload, excitatory amino acids (EAA), nitrogen monoxide and free residuals^[13]. Irina *et al.* found that the infarct volume of the *Fgf2* knock-out mice increased 75% compared with the wild type littermates^[14]. bFGF enhanced the

survival and outgrowth of neurons both *in vitro* and *in vivo*^[15], and improved the functional recovery in focal cerebral ischemia rats when administered within 24 h^[16]. However, the protective mechanism of bFGF in the brain ischemia remains to be elucidated.

In this study, some signal transduction proteins (MEK, ERK1/2 and phospho-ERK1/2) in the cultured astrocytes suffered from anoxic-reoxic insult were significantly degraded. After bFGF was administered to the insulted astrocytes, the degradation was inhibited as done by glutathione, implying that bFGF blocked the damage of oxygen-derived free radicals to these signal transduction proteins. Some researchers have found that *in vitro*, bFGF could stabilize the mitochondrial membranes, which might maintain the balance of intracellular Ca^{2+} ^[17], enhance the level of calcium binding proteins, activate the superoxide dismutase, and inhibit the expression of *N*-methyl-D-aspartic acid receptor (NMDAR)^[18]. U0126, a selective inhibitor of MEK, could not influence the protective effect of bFGF on the proteins from degradation, suggesting that the MEK-ERK signal pathway may not be involved in the protective function of bFGF.

Our experiment showed that phospho-ERK1/2 increased after bFGF was administered for 10-30 min in the

Fig. 6 The Egr-1 binding activity was influenced by bFGF in the anoxia-reoxygenation insulted astrocytes. **A:** Egr-1 binding activity decreased obviously after anoxia-reoxygenation injury. **B:** After bFGF was administrated, Egr-1 binding activity was enhanced during 0.5 h to 24 h. **C:** Egr-1 binding activity was blocked by the MEK specific inhibitor U0126.

anoxia-reoxygenation insulted astrocytes, indicating that MEK-ERK pathway was activated by the exogenous bFGF. The inductive effect was attenuated in the presence of U0126, a selective MEK-ERK pathway inhibitor. In addition, bFGF could increase Egr-1 binding activity in the anoxia-reoxygenation insulted astrocytes. These results revealed a direct relation between ERK activation and Egr-1 binding activity (both induced by bFGF). *In vivo*, the expression of Egr-1 protein was higher in bFGF group than in control group^[1]. Here we found that exogenous bFGF could also increase the Egr-1 binding activity *in vitro*.

Santiago *et al.* found that Egr-1 was rapidly induced at the edge of endothelial wound before growth factor in-

creased at the same location^[19]. We found in previous study that at the margin of the ischemic lesion of rat brain, Egr-1 expressed earlier than endogenous bFGF, and the exogenous bFGF may enhance the expression of endogenous bFGF^[1]. Biesiada *et al.* identified Egr-1 as the first transcription factor to enhance the transcriptional activity of bFGF^[20]. Thus, Egr-1 may play an important role in mediating the expression of endogenous bFGF triggered by exogenous bFGF.

The stimulus-induced syntheses of Egr-1 by exogenous bFGF was transient, indicating that there are some negative feedback pathways in cells to limit the long-term function of exogenous bFGF. Literatures reported that the

feedback pathways may involve either upregulation of NAB2 (the special co-repressor of Egr-1 in the cells)^[21,22] or inhibition of the Egr-1 promoter via Egr-1 itself binding to the Egr-1 binding sequence on its gene^[23].

In short, this study indicated that MEK-ERK MAPK cascade was an important signal transduction pathway contributed to bFGF induced Egr-1 binding activity in anoxia-reoxygenation insulted astrocytes. We considered that it would be one of the protective mechanisms of exogenous bFGF against brain ischemia.

References:

- [1] Liu Y, YE ZR, Lu JB, Shi JY. Permeability of injured blood brain barrier for exogenous bFGF and protection mechanism of bFGF in rat brain ischemia. *Neuropathology* 2006, 26: 257-266.
- [2] Kawamata T, Ren J, Cha JH, Finklestein SP. Intracisternal antisense oligonucleotide to growth associated protein-43 blocks the recovery-promoting effects of basic fibroblast growth factor after focal stroke. *Exp Neurol* 1999, 158: 89-96.
- [3] Sliverman ES, Collins T. Pathways of Egr-1-mediated gene transcription in vascular biology. *Am J pathol* 1999, 154: 665-670.
- [4] Ehrengreuber MU, Muhlebach SG, Sohrman S, Leutenegger CM, Lester HA, Davidson N. Modulation of early growth response transcription factor (Egr) -dependent gene expression by using recombinant adenovirus. *Gene* 2000, 258: 63-69.
- [5] Shi TY, Kim M. Inhibitory effect of tumor necrosis factor- α secretion from rat astrocytes by chilbokeum. *Immunopharmacol Immunotoxicol* 2001, 23: 97-106.
- [6] Sakamoto KM, Bardeleben C, Yates KE, Raines MA, Golde DW, Gasson JC. 5'upstream sequence and genomic structure of the human primary response gene, Egr-1/TIS8. *Oncogene* 1991, 6: 867-871.
- [7] Kaufmann K, Thiel G. Epidermal growth factor and platelet-derived growth factor, induce expression of Egr-1, a zinc finger transcription factor, in human malignant glioma cell. *J Neurol Sci* 2001, 189: 83-91.
- [8] Abe K, Saito H. Neurotrophic effect of basic fibroblast growth factor is mediated by the p42/p44 mitogen-activated protein kinase cascade in cultured rat cortical neurons. *Dev Brain Res* 2000, 122: 81-85.
- [9] Zathehkaja DS, Lou MF. Studies of the mitogen-activated protein kinase and phosphatidylinositol-3 kinase in the lens 1 the mitogenic and stress response. *Exp Eye Res* 2002, 74: 703-717.
- [10] Liang HZ, Liu Y, Ye ZR. Growth enhancement effect of BzATP on primary cultured astrocytes from rat brain. *Neurosci Bull* 2006, 22: 151-158.
- [11] Adams DG, Coffee RL, Zhang H, Pelech S, Strack S, Wadzinski BE. Positive Regulation of Raf1-MEK1/2-ERK1/2 Signaling by Protein Serine/Threonine Phosphatase 2A. *J Biol Chem* 2005, 280: 42644-42654.
- [12] Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 1991, 19: 2499.
- [13] Mattson MP, Scheff SW. Endogenous neuroprotection factors and traumatic brain injury: mechanisms of action and implications for therapy. *J Neurotrauma* 1994, 11: 3-33.
- [14] Kiprianova I, Schindowski K, von Bohlen und Halbach O, Krause S, Dono R, Schwaninger M, *et al*. Enlarged infarct volume and loss of BDNF mRNA induction following brain ischemia in mice lacking FGF-2. *Exp Neurol* 2004, 189: 252-260.
- [15] Ramirez J, Finklestein SP, Keller J, Abrams W, George MN, Parakh T. Basic fibroblast growth factor enhances axonal sprouting after cortical injury in rats. *Neuroreport* 1999, 10: 1201-1204.
- [16] Li Q, Stephenso D. Postischemic Administration of basic fibroblast growth factor improves sensorimotor function and reduces infarct size following permanent focal cerebral ischemia in the rat. *Exp Neurol* 2002, 177: 531-537.
- [17] Mattson MP, Zhang Y, Bose S. Growth factors prevent mitochondrial dysfunction, loss of calcium homeostasis, and cell injury, but not ATP depletion in hippocampal neurons deprived of glucose. *Exp Neurol* 1993, 121: 1-13.
- [18] Cheng B, Mattson MP. NGF and bFGF protect rat hippocampal and human cortical neurons against hypoglycemic damage by stabilizing calcium homeostasis. *Neuron* 1991, 7: 1031-1041.
- [19] Santiago FS, Lowe HC, Day FL, Chesterman CN, Khachigian LM. Early growth response factor-1 induction by injury is triggered by release and paracrine activation by fibroblast growth factor-2. *Am J Pathol* 1999, 154: 665-670.
- [20] Biesiada E, Razandi M, Levin ER. Egr-1 activates basic fibroblast growth factor transcription. *J Biol Chem* 1996, 271: 18576-18581.
- [21] Houston P, Campbell CJ, Svaren J, Milbrandt J, Braddock M. The transcriptional corepressor NAB2 block Egr-1-mediated growth factor activation and angiogenesis. *Biochem Biophys Res Commun* 2001, 283: 480-486.
- [22] Lucerna M, Mechtcheriakova D, Kadl A, Schabbauer G, Schafer R, Gruber F, *et al*. NAB2, a corepression of Egr-1, inhibits vascular endothelial growth factor-mediated gene induction and angiogenic response of endothelial cell. *J Bio Chem* 2003, 278: 11433-11440.
- [23] Thiel G, Cibelli G. Regulation of life and death by the zinc finger transcription factor Egr-1. *J Cell Physiol* 2002, 193: 287-292.

MAPK/ERK 激酶-ERK 信号通路参与外源性 bFGF 对缺氧-复氧损伤后星形胶质细胞内 Egr-1 结合活性的调节

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摘要: **目的** 静脉注射碱性成纤维细胞生长因子(basic fibroblast growth factor, bFGF)可以明显降低实验性脑缺血大鼠的脑梗死面积, 但该作用的分子机制尚不清楚。本文旨在研究外源性 bFGF 作用的信号转导通路。**方法** 缺氧-复氧损伤星形胶质细胞。Western blot 检测外源性 bFGF 作用后丝裂原活化蛋白激酶/细胞外信号调节激酶激酶(mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, MEK)-细胞外信号调节激酶(extracellular signal-regulated kinase, ERK) 信号通路活化。电泳变动迁移率分析实验检测外源性 bFGF 作用后核转录因子早期生长反应因子-1(early growth response factor 1, Egr-1)的结合活性变化。**结果** 外源性 bFGF 可以保护胞外信号调节激酶 MEK-ERK 信号通路蛋白不被氧自由基降解。MEK-ERK 信号通路在外源性 bFGF 作用后活化。这一信号通路进一步使 Egr-1 结合活性升高。**结论** 外源性 bFGF 可能通过激活 ERK 信号通路, 促进内源性转录因子 Egr-1 的结合活性升高, 进而促进内源性 bFGF 的表达。

关键词: 细胞外信号调节激酶; 丝裂原活化蛋白激酶; 自由基; 碱性成纤维细胞生长因子; 早期生长反应因子-1; 星形胶质细胞