Upregulated Ras/Raf/ERK1/2 signaling pathway: a new hope in the repair of spinal cord injury

Tao Liu, Fu-jiang Cao, Dong-dong Xu, Yun-qiang Xu, Shi-qing Feng

Department of Orthopedics, General Hospital of Tianjin Medical University, Tianjin, China

Abstract

An increasing number of studies report that the Ras/Raf/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway has a death-promoting apoptotic function in neural cells. We hypothesized that the Ras/Raf/ERK1/2 signaling pathway may be abnormally regulated in rat injured spinal cord models. The weight drop method was used to establish rat spinal cord injury at T12, Western blot analysis and immunohistochemical staining revealed Ras expression was dramatically elevated, and the phosphorylations of A-Raf, B-Raf and C-Raf were all upregulated in the injured spinal cord. Both mitogen-activated protein kinase kinase 1/2 and ERK1/2, which belong to the Ras/Raf signaling kinases, were upregulated. These results indicate that Ras/Raf/ERK1/2 signaling may be upregulated in injured spinal cord and are involved in recovery after spinal cord injury.

Key Words: nerve regeneration; Ras/Raf/Erk1/2 signaling pathway; spinal cord injury; apoptosis; repair; regulation; inhibition; neural regeneration

Funding: This work was funded by the National Natural Science Foundation for Young Scholars of China, No. 81101362 and 81401784; and the Key Project of National Natural Science Foundation of China, No. 81330042.

Liu T, Cao FJ, Xu DD, Xu YQ, Feng SQ (2015) Upregulated Ras/Raf/ERK1/2 signaling pathway: a new hope in the repair of spinal cord injury. Neural Regen Res 10(5):792-796.

Introduction

Ras/Raf/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling plays an essential role in cell proliferation, differentiation and growth (Liu et al., 2001; Lo et al., 2001). A death-promoting role of ERK1/2 both in vitro and in vivo has been confirmed (von Gise et al., 2001; Yang et al., 2013). Glutamate- or camptothecin-induced neuronal injury can be abolished by U0126, an inhibitor of the ERK1/2 signaling pathway (Stanciu et al., 2000; Lesuisse and Martin, 2002). After inhibiting the activation of reactive oxygen species-dependent ERK1/2 using either PD98059 or U0126, we found that glutathione depletion-induced neuronal death was abolished (De Bernardo et al., 2004). One study claimed that ERK1/2 activation was associated with dopamine-induced death of striatal neurons (Chen et al., 2009). This suggests that activation of ERK1/2 has a vital role in neuronal death.

Up to now, few reports have mentioned the mechanisms underlying ERK1/2-mediated neuronal death. Studies have found that ERK1/2 can be activated by oxidants and cytokines either directly on Src-tyrosine kinase or by acting on receptors or calcium channels. By interacting with cytoplasmic components or translocating to the nucleus, activated ERK1/2 can cause neuronal death (Stanciu and DeFranco, 2002; Subramaniam et al., 2004; Subramaniam and Unsicker, 2006; Mebratu and Tesfaigzi, 2009). Furthermore, it is reported that there is a strong connection between spinal cord injury (SCI) and oxidative stress (Sun et al., 2014), and oxidative stress may be involved in the pathogenesis of SCI by inducing autoimmunity (Hassanshahi et al., 2013). A few studies have shown that neuroprotection is achieved by inhibiting ERK signaling, including suppressing mechanical trauma, seizure-like activity and oxidative stress (Murray et al., 1998; Satoh et al., 2000; Chaudhary et al., 2013). Several studies showed that inhibition of mitogen-activated protein kinase kinase (MEK)/ERK signaling significantly reduces pro-inflammatory cytokine expression and area of infarct caused by ischemia in the central nervous system, indicating that MEK/ERK signaling could be a vital controller of inflammatory activities in SCI (Wang et al., 2004). In this study, we aim to clarify the function of MEK/ERK signaling pathway in SCI, and the relationship between Raf/Erk1/2 pathway and Ras in rats with SCI.

Materials and Methods

Establishment of SCI models

Twenty-four specific-pathogen-free female Wistar rats aged 13–14 weeks and weighing 200–250 g were raised from Jackson Laboratories (Bar Harbor, ME, USA). Rats were housed with water and food to ease stress before sacrifice. The procedures of Care and Use of Laboratory Animals were conducted in compliance with the NIH Guidelines and approved by the Tianjin Medical University (Approved code: 2000-01-3179) and National Committee of Science and Technology of China (ID: 1999-05-6941). All rats were equally and randomly divided into sham and SCI groups.
Rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g), fixed on a wooden plate, and subjected to spinal cord contusion as previously described (Zhou et al., 2012). A 10-g rod was dropped onto the spinal cord at level T9 from a height of 25 mm using the NYU impactor (New York University, New York, USA). This device has shown to inflict a well-calibrated contusive injury of the spinal cord. Surgical access to spinal cord was achieved with a laminectomy of the T9 vertebral body. The contusive injury was inflicted at this level as well. Subsequently, the animals were sutured with absorbable Dexon and nylon thread. Animals were housed under controlled humidity and temperature conditions. Injured rats were kept on sterilized sawdust and were provided with filtered water, both of which were changed every day. Their bladder was evacuated by means of an abdominal massage, 2–3 times daily until normal urination was regained. The rats of the sham group only achieved a laminectomy of the T9 vertebral body. All animals were carefully looked after to avoid and detect infections of urinary tract or any other sign of systemic infection.

**Spinal cord tissue preparation**

Animals were sacrificed at 24 hours (six rats in each group) and 7 days (six rats in each group) after injury. Rats were quickly sacrificed by cervical dislocation, and spinal cords were subsequently dissected. The frozen spinal cord samples taken from the T9 section of both rats were homogenized (10% w/v) in cold buffer containing 8.5% sucrose, a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), 10 mM β-mercaptoethanol, 2 mM ethylenediamine tetraacetic acid, and 50 mM Tris-HCl (pH 7.4). The protein concentration was calculated by a previous method (Bradford et al., 2009). Paraffin sections (6 μm) of T9 spinal cord specimens were made at the same time for immunohistochemical staining.

**Western blot analysis**

For western blot analysis, spinal cord homogenate samples in sodium dodecylsulfate sample buffer, containing 2.5% sodium dodecylsulfate (w/v), pH 6.8, 0.05% w/v bromophenol blue, 100 mM Tris, 20% glycerol, 250 mM DL-Dithiothreitol, were denatured by heating at 100°C for 3 minutes. Twenty to sixty micrograms of protein per lane per subject were loaded onto a 10% acryl-bisacrylamide gel and electrophoresed for 2 hours at 120 V at room temperature. The proteins were electroblotted onto a polyvinylidene difluoride membrane for 1 hour at 100 V and 4°C. Protein blots were then blocked with 5% milk in PBS with 0.1% Tween-20. After blocking, the blots were incubated with rabbit anti-rat Ras monoclonal antibody (1:1,000; Cell Signaling, New York, NY, USA), rabbit anti-rat phosphor-A-Raf monoclonal antibody (1:1,000; Cell Signaling), mouse anti-rat phospho-B-Raf monoclonal antibody (1:500; Cell Signaling), rabbit anti-rat phosphor-C-Raf monoclonal antibody (1:1,000; Cell Signaling), mouse anti-rat phosphor-Erk1/2 monoclonal antibody (1:1,000; Cell Signaling), and rabbit anti-rat phospho-MEK1/2 monoclonal antibody (1:1,000; Cell Signaling) overnight at 4°C followed by horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:5,000, Sigma-Aldrich) for 1 hour at room temperature. After three washes in PBS with 0.1% Tween-20 (each time for 10 minutes), the blots were visualized using the enhanced chemiluminescence detection system (Amer sham Pharmacia Biotech, QC, Canada) and exposed to hyper film enhanced chemiluminescence (Amer sham Pharmacia Biotech). Sample densities were analyzed with Image J software (National Institutes of Health, Bethesda, MD, USA). The target protein expression was expressed as the optical density ratio of target protein to β-actin.

**Immunohistochemical staining**

Paraffin sections (6 μm) of T9 spinal cord specimens were fixed with 10% formalin, washed with Tris-buffered saline, and then deparaffinized with xylene (2×), and graded concentrations of ethanol (100% (2×), 80%, 50%, and 25%), and then washed in Tris-buffered saline (5 minutes). The sections were then incubated with mouse anti-rat phospho-MEK1/2 monoclonal antibody (1:100; Cell Signaling) or rabbit anti-rat phospho-ERK1/2 monoclonal antibody (1:50; Cell Signaling) overnight at 4°C. After washing in Tris-buffered saline for 5 minutes, the sections were incubated with sheep anti-rabbit IgG or sheep anti-mouse IgG (1:1,000; Vector Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature. Subsequently, the sections were incubated with avidin-biotinylated peroxidase (Vector Laboratories) for 45 minutes at room temperature and in 30% H2O2, 0.05 M Tris-buffered saline/1 drop 0.0125 g 3,3′-diaminobenzidine/25 mL for 10 minutes at room temperature. After washing in Tris-buffered saline and increasing concentrations of ethanol (25%, 50%, 80%, 100% (2×)), followed by xylene (2×), sections were mounted for microscopy. Immunostaining images were visualized and obtained through a laser scanning confocal microscope (400× magnification; Nikon Eclipse 90i, Tianjin Medical University–Basic Research Center, China). The area and optical density calculations were carried out with Image J software.

**Statistical analysis**

Data were expressed as the mean ± SEM. To compare each parameter, we determined the statistical significance between groups with the unpaired t-test using the StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). A value of P < 0.05 was considered statistically significant.

**Results**

**Expression of Ras protein was enhanced in the injured spinal cord**

Western blot analysis showed that the mean value of Ras expression in SCI rats was increased by ~2.1 fold 24 hours after injury and ~1.8 fold 7 days after injury, compared with the sham group (P < 0.01; Figure 1).

**Expression of A-Raf, B-Raf and C-Raf was dramatically upregulated in the injured spinal cord**

Ras exerts its effect through Raf kinases, which are the downstream targets of Ras kinases (De Bernardo et al., 2004). There
are three types of Raf kinases: A-Raf, B-Raf, and C-Raf, and we examined their protein expression by western blot analysis. We found that the phosphorylation of A-Raf, B-Raf and C-Raf was dramatically enhanced by a mean value of 169% \((P < 0.01)\), 248% \((P < 0.01)\) and 40% \((P < 0.05)\) respectively in rats 7 days after surgery compared with the sham group (Figure 2), but we did not observe any changes in the total protein expression of A-Raf, B-Raf or C-Raf (data not shown).

**Phosphorylation of C-Raf was significantly upregulated in the injured spinal cord**

Western blot analysis showed that phosphorylation of C-Raf was significantly enhanced by an average value of 155% in the injured spinal cord of rats compared with the sham group at 24 hours after surgery \((P < 0.05)\); Figure 3). However, we did not observe any significant changes of expression and phosphorylation of A-Raf, B-Raf and C-Raf in the spinal cord of the sham group (data not shown). This may indicate that Raf kinases are abnormally upregulated in the injured spinal cord.

**MEK1/2 expression was significantly upregulated in the injured spinal cord**

As a downstream target, MEK1/2 can be phosphorylated by...
Raf kinases (Rubinfeld and Seger, 2005). Because expression of Raf kinases were significantly upregulated in SCI rats, we examined the expression and activity of MEK1/2 protein. Using western blot analysis, we found that at 7 days after surgery, protein levels of phosphorylated MEK1/2 were significantly upregulated in the spinal cord of SCI rats by an average value of ~4 fold compared with the sham group (P < 0.05; Figure 4A, B). However, we did not find any change in the total protein expression (data not shown). Furthermore, we examined the phosphorylation of MEK1/2 in injured spinal cord using immunohistochemistry and found that phosphorylation of MEK1/2 was significantly enhanced in neural cells in the spinal cord of SCI rats compared with sham rats (P < 0.01; Figure 4A, D), which was consistent with western blot analysis. We also examined MEK1/2 protein expression and phosphorylation 24 hours after injury. However, in both groups, protein expression and phosphorylation of MEK1/2 did not show any significant changes in western blot analysis results (data not shown).

Phosphorylation/activation of ERK1/2 was dramatically upregulated in the injured spinal cord

ERK1/2 can be activated by MEK1/2 kinase and we further examined its expression and activation by western blot analysis. We found a mean increase in phosphorylation of ERK1/2 of 151% in the spinal cord of SCI rats as compared with sham group at 7 days after surgery (P < 0.05; Figure 5A, B). However, we did not find any changes in total ERK1/2 protein expression (data not shown), which is consistent with the immunohistochemistry results. We demonstrated that the expression of phospho-ERK1/2 was dramatically enhanced in neural cells in the spinal cord of SCI rats compared with the sham group, which is consistent with western blot analysis (Figure 5C, D). Moreover, we examined the expression of ERK1/2 24 hours after injury. We did not find any change in the expression of phosphorylated ERK1/2 in SCI rats when compared with the sham group in the western blot results (data not shown).

Discussion

This study demonstrates that Ras is upregulated in SCI rats, and is associated with the upregulation of the three Raf proteins. A-Raf and B-Raf show significant upregulation of activation, and increased activation of C-Raf to a lesser extent. Increased Ras only stimulates C-Raf activation and results in an upregulation of C-Raf activity 24 hours after injury. The differences in the functions of A-Raf, B-Raf and C-Raf are not known. B-Raf is a far stronger activator of ERKs than C-Raf, which has been confirmed in studies in mice with targeted mutations of the Raf genes (Mercer and Pritchard, 2003), and this result is consistent with ours. Our studies showed that in response to elevated B-Raf activity, phospho-MEK1/2, which is the downstream target, is dramatically increased by ~4 fold in the injured spinal cord of rats; we did not observe significant stimulated activation phosphorylation of MEK1/2, but found an enhanced activity of C-Raf 24 hours after injury. These results also indicate that Ras/Raf/ERK1/2 signaling is differently stimulated at different time points in injured spinal cord rats.

Because neural cell death can be promoted by Ras/Raf/ERK1/2, the enhanced expression of ERK1/2 and the upregulation of Ras/Raf/ERK1/2 signaling in the spinal cord of injured rats could explain the agenesis of spinal cord neurons and immaturity of the spinal cord neurons in injured rats through promoting apoptotic mechanisms. By over-expressing B-Raf or ERK1/2 in the spinal cord of injured rats, future studies will be carried out to study the specific mechanism of Ras/Raf/ERK1/2 signaling on rat behavior and neural cell properties.

In summary, the entire Ras/Raf/ERK1/2 signaling pathway was dramatically upregulated in the spinal cord of injured rats. Our study implies a possible common pathogenic mechanism that could be shared by SCI patients and rats. The upregulation of Ras/Raf/ERK1/2 signaling has a vital role in the regulation of abnormal apoptosis in the spinal cord after injury, and partially explains the difficulty of axonal recovery observed in injured spinal cords of both human subjects and animals.

Author contributions: TL and FJC performed experiments and wrote the paper. DDX and YQX analyzed the data. SQF designed the experiment. All authors approved the final version of the paper.

Conflicts of interest: None declared.

References

Bradford BJ, Mamedova LK, Minton JE, Drouillard JS, Johnson BJ (2009) Daily injection of tumor necrosis factor-α [alpha] increases hepatic triglycerides and alters transcript abundance of metabolic genes in lactating dairy cattle. J Nutr 139:1451-1456.

Chaudhary SC, Siddiqui MS, Athar M, Alam MS (2013) Geraniol inhibits murine skin tumorigenesis by modulating COX-2 expression. Ras-ERK1/2 signaling pathway and apoptosis. J Appl Toxicol 33:828-837.

Chen J, Rusnak M, Lombroso PJ, Sidhu A (2009) Dopamine promotes striatal neuronal apoptotic death via ERK signaling cascades. Eur J Neurosci 29:287-306.

De Bernardo S, Canals S, Casarojos MJ, Solano RM, Menendez J, Mena MA (2004) Role of extracellular signal-regulated protein kinase in neuronal cell death induced by glutathione depletion in neuron/glia mesencephalic cultures. J Neurochem 91:667-682.

Hassanshahi G, Amin M, Shunnugavel A, Vazirinejad R, Vakilian A, Sanji M, Shamsizadeh A, RafatPanah H, Poor NM, Moosavi SR, Taheri S (2013) Temporal expression profile of CXC chemokines in serum of patients with spinal cord injury. Neurochem Int 63:363-367.

Lesuisse C, Martin LJ (2002) Immature and mature cortical neurons engage different apoptotic mechanisms involving caspase-3 and the mitogen-activated protein kinase pathway. J Cereb Blood Flow Metab 22:935-950.

Liu A, Prenger MS, Norton DD, Mei L, Kusiak JW, Bai G (2001) Nerve growth factor uses Ras/ERK and phosphatidylinositol 3-kinase cascades to up-regulate the N-methyl-D-aspartate receptor 1 promoter. J Biol Chem 276:45372-45379.

Lo LW, Cheng JJ, Chiu JJ, Wang BS, Liu YC, Wang DL (2001) Endothelial exposure to hypoxia induces Egr-1 expression involving PKCα-mediated Ras/Raf/ERK1/2 pathway. J Cell Physiol 188:304-312.

Mebratu Y, Tesfaigzi Y (2009) How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? Cell Cycle 8:1168-1175.

Mercer KE, Pritchard CA (2003) Raf proteins and cancer: B-Raf is identified as a mutational target. Biochem Biophys Acta 1653:25-40.
Figure 4 Phosphorylation of MEK1/2 in the injured spinal cord of rats 7 days after surgery.
(A) Western blot assay of phospho-MEK1/2 protein expression in the injured spinal cord of rats. Samples 1–6: Sham group; samples 7–12: SCI group. Relative expression of MEK1/2 is expressed as the optical density ratio of MEK1/2 to β-actin. Squares and triangles represent MEK1/2 expression in the rat spinal cord. Transverse lines represent the mean value in each group. (B) Immunoreactivity of phospho-MEK1/2 in the rat spinal cord (immunohistochemical staining, × 400). Arrows show positive reactions. (C) Semi-quantitative analysis of phospho-MEK1/2 immunoreactivity in the rat spinal cord. (A, C) Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, vs. sham group (unpaired t-test). SCI: Spinal cord injury; MEK1/2: mitogen-activated protein kinase kinase 1/2.

Figure 5 Phosphorylation of ERK1/2 in the injured spinal cord of rats 7 days after surgery.
(A) Western blot assay of phospho-ERK1/2 expression in the injured spinal cord of rats. Samples 1–6: Sham group; samples 7–12: SCI group. Relative expression of ERK1/2 is expressed as the optical density ratio of ERK1/2 to β-actin. Squares and triangles represent ERK1/2 expression in the rat spinal cord. Transverse lines represent the mean value in each group. (B) Immunoreactivity of phospho-ERK1/2 in the rat spinal cord (immunohistochemical staining, × 400). Arrows show positive reactions. (C) Degree of phospho-ERK1/2 immunoreactivity in the rat spinal cord. (A, C) Data are expressed as the mean ± SEM. *P < 0.05, vs. sham group (unpaired t-test). SCI: Spinal cord injury; ERK1/2: extracellular signal-regulated kinase 1/2.

Murray B, Alessandrini A, Cole AJ, Yee AG, Furshpan EJ (1998) Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell-culture model of seizure activity. Proc Natl Acad Sci USA 95:11975-11980.
Rubinfeld H, Seger R (2005) The ERK cascade: a prototype of MAPK signaling. Mol Biotechnol 31:151-174.
Satoh T, Nakatsuka D, Watanabe Y, Nagata I, Kikuchi H, Namura S (2000) Neuroprotection by MAPK/ERK kinase inhibition with U0126 against oxidative stress in a mouse neuronal cell line and rat primary cultured cortical neurons. Neurosci Lett 288:163-166.
Stanciu M, DeFranco DB (2002) Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by oxidative toxicity or proteasome inhibition in a neuronal cell line. J Biol Chem 277:4010-4017.
Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, Reynolds I, Klaun E, Angioli MR, Johnson JW, DeFranco DB (2000) Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. J Biol Chem 275:12200-12206.
Subramaniam S, Unsicker K (2006) Extracellular signal-regulated kinase as an inducer of non-apoptotic neuronal death. Neuroscience 138:1055-1065.
Subramaniam S, Zirrgeibel U, von Bohlen und Halbach O, Strelau J, Laliberté C, Kaplan DR, Unsicker K (2004) ERK activation promotes neuronal degeneration predominantly through plasma membrane damage and independently of caspase-3. J Cell Biol 165:357-369.

Liu T, et al. / Neural Regeneration Research. 2015;10(5):792-796.

Sun YQ, Chen XS, Cao D, Zhu W, Jia LS (2014) Hydrogen-rich saline can inhibit apoptosis of spinal cord motor neurons in rabbits with spinal cord ischemia-reperfusion injury. Zhongguo Zuzhi Gongcheng Yanyu 18:2861-2866.
von Gise A, Lorenz P, Wellbrock C, Hemmings B, Berberich-Siebelt F, Rapp UR, Troppmair J (2001) Apoptosis suppression by Raf-1 and MEK1 requires MEK- and phosphatidylinositol 3-kinase-dependent signals. Mol Cell Biol 21:2324-2336.
Wang QZ, Wu DC, Huang FP, Yang GY (2004) Inhibition of MEK/ERK 1/2 pathway reduces pro-inflammatory cytokine interleukin-1 expression in focal cerebral ischemia. Brain Res 996:35-46.
Yang K, Cao F, Sheikh A, Malik M, Wen G, Wei H, Ted Brown W, Li X (2013) Up-regulation of Ras/Raf/ERK1/2 signaling impairs cultured neuronal cell migration, neurogenesis, synapse formation, and dendritic spine development. Brain Struct Funct 218:669-682.
Zhou XH, Ning GZ, Peng SQ, Kong XH, Chen JT, Zheng YF, Ban DX, Liu T, Li H, Wang P (2012) Transplantation of autologous activated Schwann cells in the treatment of spinal cord injury: six cases, more than five years of follow-up. Cell Transplant 21 Suppl 1:S39-47.

Copyedited by Paul P, Stow A, Yu J, Qiu Y, Li CH, Song LP, Zhao M