Lumbar 5 spinal nerve ligation induced PI3K-PKB/Akt signal pathway activation in dorsal root ganglia in rats☆

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Abstract

Several lines of evidence indicate that phosphatidylinositol 3-kinase (PI3K)-protein kinase B/Akt (PKB/Akt) signal pathway mediate the pain hypersensitivity induced by intradermal injection of capsaicin, and nerve growth factor induced upregulation of vanilloid receptor 1 in dorsal root ganglia (DRG) neuron via the activation of PI3K. While, the roles of PI3K-PKB/Akt signal pathway activation in neuropathic pain still remained unknown. Used the L5 spinal nerve ligation (L5 SNL) and immunohistochemistry, we found that the percentage of phospho-PKB/Akt-immunoreactive (p-PKB/Akt-IR) positive staining neuron significantly increased in ipsilateral L5 DRG 12 hours after L5 SNL, reached peak on day 1 and maintained to the 3rd day after operation. Results of double immunofluorescence staining showed that p-PKB/AKT expressed entirely in DRG neurons, especially in IB4 positive staining small size neurons. Intrathecal injection of wortmannin, a potent inhibitor of PI3K, for 2 days clearly reduced magnitude of p-PKB/Akt-IR level in DRG. The above data indicate that L5 SNL induced a PI3K-PKB/Akt signal pathway activation in DRG neurons and might be involved in the development of neuropathic pain. [Life Science Journal. 2007; 4(2): 19 – 24] (ISSN: 1097 – 8135).

Keywords: phosphatidylinositol 3-kinase; protein kinase B/Akt; neuropathic pain; dorsal root ganglia; immonohistochemistry

1 Introduction

Peripheral nerve injury often results in neuropathic pain, which manifested as allodynia and hyperalgesia. Previous studies showed that the activation of several signal pathways, including PKA, PKC, MAPK, etc, induced by nerve injury, play important roles in the development of neuropathic pain[1,2]. Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase that phophorylates the D3 position of phosphatidylinositol lipids to produce PI(3,4,5)P3, acting as a membrane-embedded second messenger[3]. Serine/threonine protein kinase B/Akt (PKB/Akt) is a pivotal downstream target of PI3K[4] and mediates the key functions of the PI3K dependent survival pathway through its phosphorylation and regulation of apoptotic proteins and transcription factors[5,6]. Several lines of evidence indicates that PI3K and PKB/Akt were crucial mediators which lead to transcription factor nuclear factor kB (NF-kB) activation induced by interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α)[7–12]. Our recent study[13] as well as many other groups reported that cytokines, especially IL-1 and TNF-α, involved in the development of neuropathic pain[14–17]. Ample evidence shows that PI3K is also upstream of growth factor-induced PKB/Akt activation[18–20]. Recently, several groups reported that PKB/Akt contributes to the pain hypersensitivity induced by intradermal injection of capsaicin in rats[21–23]. The PI3K is also playing a pivotal role in NGF-induced transient receptor potential vanilloid type 1 (TRPV1) expression and sensitization[24–27].

Nerve injury often induces the synthesis and release of NGF as well as cytokines and contributes to the induction and maintenance of the pain facilitation[17,28,29,30]. But little is known whether the PI3K-PKB/Akt signal pathway was activated following peripheral nerve injury. Therefore,

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in the present study we explored the expression of phosphorylated-PKB/Akt (p-PKB/Akt) in dorsal root ganglia (DRG) with the method of immunofluorescence staining after L5 spinal nerve ligation (L5 SNL) in rats.

2 Materials and Methods

2.1 Animal preparation

Male Sprague-Dawley rats weighing 180 – 250g were used. The rats were housed in separated cages with free access to food and water. The room temperature was kept at 23±2ºC under a 12:12 light-dark cycles. All animals experimental procedures were approved by the local animal care committee and were carried out in accordance with the guidelines of the National Institutes of Health of America on animal care and the ethical guidelines for investigation of experimental pain in conscious animal.

2.2 Surgical procedures and drug deliver

The animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, *i.p.*). One group of rats received a unilateral L5 SNL followed the method described by Kim and Chung[31]. Briefly, to produce a L5 SNL, a skin incision was made in the midline lumbar region (L4 –S1). The S1 transverse process was identified, freed of muscular attachments and partially removed. The L5 spinal nerve was tightly ligated with silk suture and transected distal to the ligature after it has been exposed and isolated from the adjacent nerves. And then the wound was washed with saline and closed in layers (fascia and skin) with 3 – 0 silk thread. In sham-operated rats, the left L5 spinal nerve was isolated, but without ligation.

Drug delivers were performed through a PE-10 catheter, which has been implanted intrathecally in rats according to the method described by Obata *et al*[32]. Briefly, a laminectomy of the L5 vertebra was performed under adequate anesthesia with sodium pentobarbital. The dura was cut, and a soft tube (PE-10) was inserted into the subarachnoid space of the spinal cord at the L4/5 DRG level. The position of the catheter was checked postmortem. In one group of the rats, the PI3K inhibitor wortmannin (Sigma, 0.5 μg/10μl) were injected intrathecally and flushed with 10 μl of saline started 30 minutes before L5 SNL and once daily thereafter for 2 days. The control group received same volume of vehicle (saline contained 3% DMSO) injection at same time as above.

2.3 Immunohistochemistry

Immunofluorescence staining was performed following the procedures described by Ji *et al*[29]. Briefly, after defined survival times, control and nerve injured rats were terminally anesthetized and perfused through the ascending aorta with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the L5 DRG were removed and post fixed in the same fixative for 3 hours and then replaced with 30% sucrose overnight. The DRG sections (16 μm) were cut in a cryostat (LEICA CM1900) and processed for immunostaining with immunofluorescence. All of the sections were blocked with 3% donkey serum in 0.3% Triton X-100 for 1 hour at room temperature and incubated over two nights at 4ºC with primary antibody (phospho-Akt antibody, 1:200; Cell Signaling Technology). The sections were then incubated for 1 hour at room temperature with Cy3-conjugated secondary antibody (1:300; Jackson ImmunoResearch). For double immunofluorescence staining, the DRG sections were incubated with a mixture of anti-phospho-Akt antibody and neuroflament-200 (NF-200) (a marker for myelinated A-fibers, 1:200; Chemicon), Isolectin B4 (FITC conjugated, IB4) (a marker for unmyelinated C-fibers, 20 μg/ml; Sigma), and GFAP (a marker for satellite glial cell, 1:200; Chemicon) over two nights at 4ºC. Except IB4 treated DRG sections, which only treated by Cy3-conjugated secondary antibody, all of above sections treated by a mixture of FITC- and Cy3-conjugated secondary antibody for 1 hour at room temperature. The stained sections were examined with an Olympus IX71 (Olympus Optical, Tokyo, Japan) fluorescence microscope and images were captured with a CCD spot camera.

The quantification of the immunofluorescence staining in the DRG was performed by counting the number of phospho-PKB/Akt-immunoreactive (p-PKB/Akt-IR) positive neurons in each section. The proportion of p-PKB/Akt-expressing neurons was determined by counting the neuronal profiles that showed distinctive labeling in the DRG sections. In each rat, every fourth section was picked up from a series of consecutive DRG sections, and four sections were counted for each DRG. An average percentage of p-PKB/Akt-IR neurons relative to the total number of neurons were obtained for each animal across the different tissue sections, and then the mean ± SE across animals was determined[29].

2.4 Statistical analysis

Differences in changes of values over time were tested using one-way ANOVA followed by individual post hoc comparisons (Tukey post hoc tests). The data between two groups were analyzed with *t* test. Statistical test were performed with SPSS 10.0 (SPSS Inc, USA). All data are ex-

pressed as mean ± SE. $P < 0.05$ was considered significant.

3 Results

3.1 PKB/Akt was activated in DRG following L5 SNL

Phosphorylation at threonine 308 or at serine 473 is a marker of PKB/Akt activation[26]. So in the present study a specific antibody to serine 473 was used to detect the activation of PKB/Akt with the immunofluorescence staining. The results showed that the phospho-PKB/Akt immunoreactive (p-PKB/Akt IR) staining neurons could be observed in DRG of naïve rat and sham group, but the percentage less than 10%. (Figure 1A, G). Compared with sham group, the significant increase of p-PKB/Akt IR staining neurons in ipsilateral L5 DRG were evident 12 hours after the animal received L5 SNL ($P < 0.05$), reached a peak on day 1 ($P < 0.001$), and remained at significant levels until the third day after operation ($P < 0.01$) (Figure 1 A – G). To confirm the above results, a specific antibody to threonine 308 was also used and the similar results were obtained (data not shown).

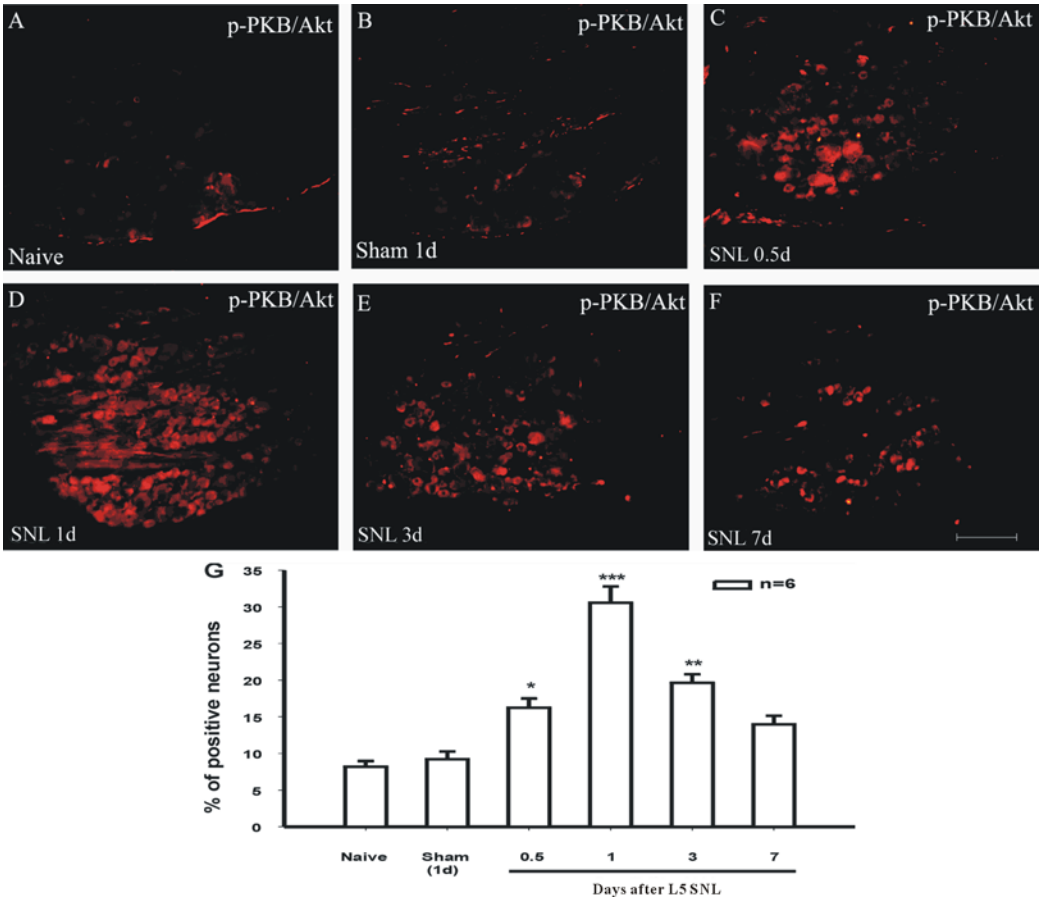

Figure 1. L5 spinal nerve ligation induced PKB/Akt activation in ipsilateral L5 DRG. A – F: Representative experiments showed the changes of p-PKB/Akt IR in L5 DRG from 0.5 day to 7 days following L5 SNL or sham operation. G: Showed the results of quantification of p-PKB/Akt positive staining neurons. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with sham group. Scale bars: A – F = 100μm.

3.2 The cell types that expressed p-PKB/Akt in DRG after L5 SNL

To identify the cell types that express p-PKB/Akt IR after L5 SNL, we performed double immunofluorescence staining of p-PKB/Akt with several cell-specific markers: NF-200 (A-type neuron), IB4 (C-type neuron) and GFAP (satellite glial cells). The results showed that p-PKB/Akt colocalized with NF-200 and IB4 but not with GFAP (Figure 2 A – I). The percentage of p-PKB /NF-200 and p-PKB/IB4 double-labeled neurons relative to the total number of p-PKB/Akt staining neurons was 21.3 ± 2.9% and 63.9 ± 5.2%, respectively.

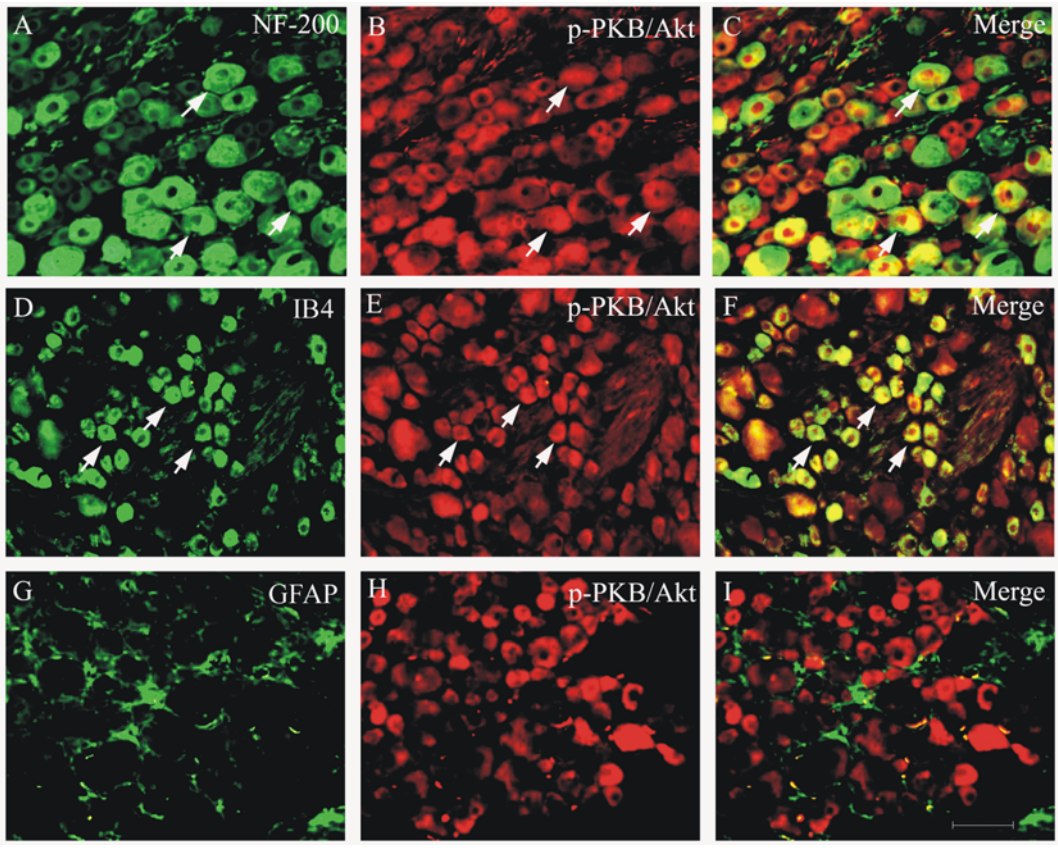

Figure 2. p-PKB/Akt IR was detected in DRG neurons, especially in IB4 positive staining small diameter neurons. A – I: show the results of double immunofluorescence staining in ipsilateral L5 DRG between p-PKB/Akt (red; B, E, H) and NF-200, A-type neuronal marker (green; A); IB4, C-type small size neuronal marker (green; D); GFAP, satellite glial cell marker (green; G) 1 day after L5 SNL. Images between A and B, D and E, G and H were merged in C, F and I. The results indicated that p-PKB/Akt co-localized with neuronal cells (C and F), especially C-type small size nociceptive neurons. Scale bars: (A – I) = 50 μm.

3.3 Wortmannin, an inhibitor of PI3k, intrathecal injection decreased the activation of PKB/Akt in DRG

To verify the effect of PI3K on the activation of PKB/Akt in the present study, we performed immunofluorescence staining of p-PKB/Akt in ipsilateral L5 DRG after the rats had received wortmannin (0.5 μg/10μl) intrathecal injection for 2 days. The results showed that wortmannin treatment significantly decreased the magnitude of PKB/Akt activation in DRG induced by L5 SNL (Figure 3). The percentage of p-PKB/Akt-IR staining neuron in wortmannin and vehicle treated group was (13.29±1.86)% and (31.62 ± 3.92)%, respectively (Figure 3 A – C).

4 Discussion

In the present study, we found that L5 SNL induced activation of PKB/Akt obviously in DRG neurons occurred at the 12th hour, reached to the peak at the 1st day and persisted until the 3rd day after operation. Intrathecal injection of Wortmannin, an inhibitor of PI3K, started at 30 minutes before surgery and once daily thereafter for 2 days, significantly reduced the magnitude of p-PKB/Akt level in DRG following L5 SNL. It suggests that L5 SNL induced the activation of PI3K-PKB/Akt signal pathway in DRG.

PI3K and PI3K-PKB/Akt signal pathway is usually activated by some neurotrophin[18] as well as other physiological stimuli[33]. It has been implicated in a variety of cellular processes, including glucose metabolism, transcription, apoptosis, proliferation, migration and angiogenesis involved in the activation of PI3K or PI3K-PKB/Akt signal pathway[34]. In the nervous system, the PI3K-PKB/Akt signal pathway is activated by growth factors, hormones, or neurotransmitters, and participates in cellular activity that underlies development[5,35]. Ample and growing evidence indicates that the PI3K-PKB/Akt pathway is involved in synaptic plasticity such as long-term potentiation (LTP), long-term depression (LTD)[36–39] and

brain-derived neurotrophic factor (BDNF)-dependent spatial memory formation[40]. Recently, it has been reported that PI3K and the PI3K-PKB/Akt pathway mediates the thermal hyperalgesia induced by capsaicin or by NGF intradermal injection[21,26,27,41], and there is an activity-dependent phosphorylation of PKB/Akt in adult DRG neurons[42]. However, the activation of PI3K or PI3K-PKB/Akt signal pathway in the neuropathic pain induced by peripheral nerve injury remained unexplored. Used a L5 SNL pain model, we found that PKB/Akt was activated in primary afferent neurons, especially in IB4 positive staining small nociceptive neurons, from 0.5 day to 3 days after surgery in the present study.

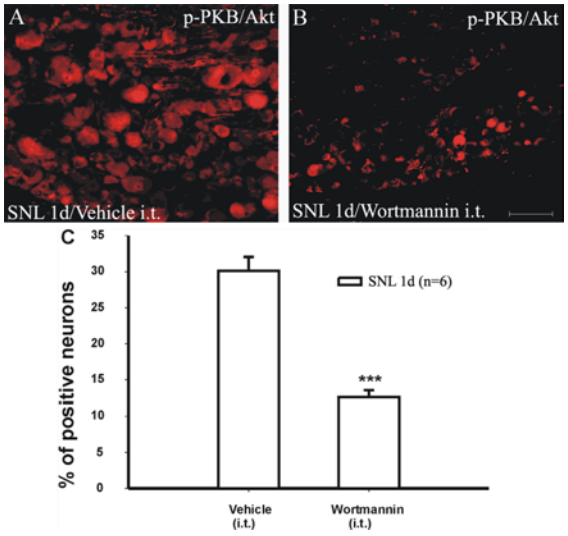

Figure 3. Intrathecal injection of wortmannin significantly blocked the activation of PKB/Akt in ipsilateral L5 DRG after L5 SNL. A and C show the effect of intrathecal injection of vehicle on the activation of PKB/Akt in DRG after L5 SNL. B and C represent the action of intrathecal injection of wortmannin on the activation of PKB/Akt in DRG following L5 SNL. ***$P < 0.001$. Scale bars (A – B) = 100μm.

The p-PKB/Akt is usually referred as the marker of PI3K activation. So we further observed the effect of wortmannin, a potent inhibitor of PI3K, on the activation of PKB/Akt in DRG after L5 SNL. The results showed wortmannin treatment for 2 days significantly reduced the magnitude of the p-PKB/Akt level. It confirmed the previous study that PI3K activation lead to the phosphorylation of PKB/Akt. Very recently, several groups reported that intradermal injection of capsaicin induced PKB/Akt activation in primary afferent started as early as 5 minutes and maintained for more than 1 hour after the treatment, and wortmannin effectively block the capsaicin-induced increase of p-PKB/Akt level[22,26]. The different time course of PKB/Akt activation between our study with Zhuang or Sun had reported might be due to the different pain model were used.

Therefore, in the present study we clearly showed for the first time that L5 SNL in rats induced a PI3K-PKB/Akt signal pathway activation in DRG neurons. While, the role of PI3K-PKB/Akt signal pathway in the neuropathic pain still remained to further study.

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