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Protein kinase B/Akt is required for complete Freund's adjuvant-induced upregulation of Nav1.7 and Nav1.8 in primary sensory neurons

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Abstract

Voltage-gated sodium channels (Nav) are essential for the generation and conduction of action potentials. Peripheral inflammation increases the expression of Nav1.7 and Nav1.8 in dorsal root ganglion (DRG) neurons, suggesting that they participate in the induction and maintenance of chronic inflammatory pain. However, how Nav1.7 and Nav1.8 are regulated in the DRG under inflammatory pain conditions remains unclear. Using a complete Freund's adjuvant (CFA)-induced chronic inflammatory pain model and Western blot analysis, we found that phosphorylated Akt (p-Akt) was significantly increased in the ipsilateral L4/5 DRGs of rats on days 3 and 7 after intraplantar CFA injection. Immunohistochemistry showed that the percentage of p-Akt-positive neurons in the DRG was also significantly increased in the ipsilateral L4/5 DRGs at these times. Moreover, CFA injection increased the colocalization of p-Akt with Nav1.7 and Nav1.8 in L4/5 DRG neurons. Pretreatment of rats with an intrathecal injection of Akt inhibitor IV blocked CFA-induced thermal hyperalgesia and CFA-induced increases in Nav1.7 and Nav1.8 in the L4/5 DRGs on day 7 after CFA injection. Our findings suggest that the Akt pathway participates in inflammation-induced upregulation of Nav1.7 and Nav1.8 expression in DRG neurons. This participation might contribute to the maintenance of chronic inflammatory pain.

Perspective—This article presents that inhibition of Akt blocks CFA-induced thermal hyperalgesia and CFA-induced increases in dorsal root ganglion Nav1.7 and Nav1.8. These findings have potential implications for use of Akt inhibitors to prevent and/or treat persistent inflammatory pain.

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Disclosures

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Keywords

Nav1.7; Nav1.8; Akt; Dorsal root ganglion; Inflammatory pain

Introduction

Tissue injury and bacteria or virus infections are always accompanied by inflammatory pain. Inflammatory mediators, such as prostaglandin E₂, nerve growth factor, and bradykinin, act on nociceptors in the peripheral nerve terminals of dorsal root ganglion (DRG) neurons. Evidence has shown that these mediators can alter the expression and function of receptors, enzymes, and voltage-dependent ion channels in the DRG.²² For example, tetrodotoxin-insensitive and tetrodotoxin-resistant currents are increased in DRG neurons after inflammation,^{6, 9, 35, 41} and prostaglandin E₂ can act through protein kinase A and protein kinase C to increase the current density of voltage-gated sodium channel (Nav) 1.8.^{12, 15} Evidence also has shown that the mRNA and protein of Nav1.7 and Nav1.8 are increased after inflammation and that this increase occurs predominantly in small- and medium-size DRG neurons.^{8, 9, 16, 33, 40} However, the mechanism by which inflammation increases DRG sodium channel expression is not yet fully understood.

Protein kinase B/Akt is activated during the formation of neuronal plasticity in brain.^{21, 29} It can also be activated in primary sensory neurons after peripheral nerve injury and acute inflammation. In a previous study, unilateral L5 spinal nerve ligation led to a significant increase in the number of phosphorylated Akt (p-Akt)-labeled neurons in the ipsilateral L5 DRG and adjacent L4 DRG.⁴² Akt inhibition with Akt inhibitor IV attenuated nerve injury-induced thermal hyperalgesia and mechanical allodynia.⁴² It has also been shown that peripheral inflammation induced by capsaicin, nerve growth factor, or carrageenan activates Akt in the DRG.^{30, 34, 46} Furthermore, intrathecal injection of the Akt inhibitor attenuates the second phase of formalin-induced flinching behavior, as well as carrageenan-induced thermal hyperalgesia and mechanical allodynia.⁴⁴ However, whether and how the Akt signaling pathway participates in chronic inflammatory pain is unknown.

In this study, we investigated whether chronic inflammation induced by intraplantar injection of complete Freund's adjuvant (CFA) increases expression of Nav1.7 and Nav1.8 and activates the Akt signaling pathway in the DRGs. We also examined whether blockade of Akt activation can decrease CFA-evoked thermal hyperalgesia and upregulation of Nav1.7 and Nav1.8 in the DRG. Finally, we defined whether Akt is activated in DRG neurons positive for Nav1.7 and Nav1.8 after CFA injection.

Methods

Animals

Adult male Sprague Dawley rats (250–300 g) were used in protocols approved by the Animal Care and Use Committee at the Johns Hopkins University. Animal procedures were consistent with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain and the ethical guidelines to investigate experimental pain in a conscious animal. Efforts were made to minimize animal suffering and to reduce the number of animals used.

Intrathecal catheter implantation and drug administration

Intrathecal catheters were implanted before drug administration. Briefly, rats were anesthetized with 2% isoflurane, and a polyethylene 10 (PE10) catheter was inserted into the subarachnoid space as described previously.^{36–39} Seven days later, chronic inflammatory

pain was induced by injecting 100 μ l of undiluted CFA (Sigma-Aldrich, St. Louis, MO, USA) into the plantar surface of one hind paw. Saline injection into a hind paw was used as a control. In some experiments, the Akt inhibitor Akt inhibitor IV (5 μ g in 10 μ l of 20% DMSO; Calbiochem, Philadelphia, PA, USA) or vehicle (20% DMSO; Sigma-Aldrich) was administered intrathecally 30 minutes before CFA injection and once daily for 7 days. The dosages of these drugs were based on those used in previous studies.^{34, 42, 44, 46}

Thermal test

Paw withdrawal latencies (PWL) were measured with a Model 336 Analgesia Meter (IITC Life Science Instruments, Woodland Hills, CA, USA). Each animal was placed in a Plexiglas chamber on a glass plate located above a light box. Radiant heat was applied by aiming a beam of light through a hole in the light box through the glass plate to the middle of the plantar surface of each hind paw. When the animal lifts its paw in response to the heat, the light beam is turned off. The length of time between the start of the light beam and the foot lift was defined as the PWL. Each trial was repeated five times at 5-minute intervals for each paw. A cutoff time of 20 seconds was used to avoid paw tissue damage^{36–39}.

Western blot analysis

After the rats were euthanized by an overdose of isoflurane, L4 and L5 DRGs were dissected out and quickly frozen in liquid nitrogen. The L4 and L5 DRGs were homogenized together in extraction buffer [10 mM Tris-HCl, 5 mM MgCl₂, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 40 μ M leupeptin, 1:100 phosphatase inhibitor cocktail II (Sigma-Aldrich; only for antibody to phosphorylated protein), 1:100 protease inhibitor cocktail III (Sigma-Aldrich; only for antibody to phosphorylated protein)]. All procedures were carried out on ice. The crude homogenates were centrifuged at 1000 $\times$ g for 15 minutes (4°C), and the supernatants were collected. Protein concentration was measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Samples were combined with loading buffer and heated for 5 minutes at 99°C. Then equal amounts of protein were loaded onto 4–20% Tris-HCl polyacrylamide gels. Separated proteins were transferred to a nitrocellulose membrane electrophoretically. Membranes were first incubated in Tris-buffered saline containing 0.1% Tween 20 and 3% nonfat dry milk for 1 hour at room temperature (RT) and then incubated with p-Akt rabbit monoclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), Akt rabbit polyclonal antibody (1:1000; Cell Signaling), Nav1.7 mouse monoclonal antibody (1:1,000; NeuroMab, Davis, CA, USA), Nav1.8 mouse monoclonal antibody (1:1,000; NeuroMab), or β -actin mouse monoclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. β -actin was used as an internal control. Finally, the membranes were incubated with anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (1:3000; Jackson ImmunoResearch, West Grove, PA, USA) for 2 hours at RT. Signal was detected by enhanced chemoluminescence with the ECL system (Denville, Metuchen, NJ, USA). After exposure of membranes, films were scanned and the blot densities quantified. The blot density from normal control tissue was set as 100%. After normalization of the corresponding β -actin, the relative density values from other groups were calculated by dividing the optical density values from these groups by the control value.

Immunohistochemistry

Under deep anesthesia with isoflurane, the rats were perfused intracardially with normal saline followed by cooled 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 4°C). L4 and L5 DRGs were harvested, post-fixed, and dehydrated. Each DRG was frozen-sectioned at 20 μ m. For single labeling, three sets of DRG sections (4–5 sections/DRG) were collected. Sections were blocked with 10% goat serum in 0.01 M phosphate-buffered saline with 0.3% Triton X-100 for 2 h at room temperature. Then one set each was incubated

with Nav1.8 mouse monoclonal antibody (0.25 µg/ml; NeuroMab) overnight at 4°C, p-Akt rabbit monoclonal antibody (1:200; Cell Signaling) overnight at 4°C, and Nav1.7 mouse monoclonal antibody (25 µg/ml; NeuroMab) for 3 nights at 4°C. The sections were then incubated with goat anti-rabbit antibody conjugated with Cy3 (1:200; Jackson ImmunoResearch) or donkey anti-mouse antibody conjugated with Cy2 (1:200; Jackson ImmunoResearch) for 2 h at RT and covered with Fluoromount™ Aqueous Mounting Medium (Sigma-Aldrich). All labeled sections were viewed with an epifluorescence microscope under appropriate filter for Cy3 (excitation 550 nm; emission 570 nm) or Cy2 (excitation 492 nm; emission 510–520 nm). For double staining, animals were perfused, and four sets of sections were collected in the same manner as described above. One set of sections was incubated with p-Akt rabbit monoclonal antibody plus Nav1.7 mouse monoclonal antibody, the second with p-Akt rabbit monoclonal antibody plus Nav1.8 mouse monoclonal antibody, and the third with NeuN mouse monoclonal antibody (1:50; GeneTex Inc., Irvine, CA, USA) plus p-Akt rabbit monoclonal antibody or with NeuN rabbit polyclonal antibody (1:1,000; EMD Millipore Corp., Billerica, MA, USA) plus Nav1.7 or Nav1.8 mouse monoclonal antibody. Sections were then incubated with a mixture of goat anti-rabbit secondary antibody conjugated with Cy2 or Cy3 and donkey anti-mouse secondary antibody conjugated with Cy2 or Cy3. The fourth set of sections was used for control experiments, which included pre-absorption of the primary antibodies with excess of the corresponding antigens, substitution of normal serum, and omission of the primary antibodies.

For single labeling analysis of p-Akt, Nav1.7, and Nav1.8 within the DRG, 12–15 sections from three rats were randomly selected for each protein. All labeled and unlabeled cells with nuclei were counted. For the analysis of double-labeled sections from the DRG, single-labeled (Nav1.7, Nav1.8, or p-Akt) and double-labeled (Nav1.7/p-Akt or Nav1.8/p-Akt) neurons were counted. The percentages of single-labeled neurons and double-labeled neurons in the DRG were calculated.

Statistical analysis

All statistical analysis was carried out with SigmaStat 2.03 software. Data were analyzed by Student's *t*-test or one-way ANOVA followed by the Tukey post hoc test. In each group, five to six rats were included for behavioral tests and three to six rats were used for Western blots or immunohistochemistry. The results are presented as mean ± SEM. *P* < 0.05 was considered statistically significant.

Results

CFA injection increases expression of p-Akt in DRG neurons

Consistent with previous studies,^{13, 25, 26} CFA injection produced thermal hyperalgesia as evidenced by significant decreases in PWLs on the ipsilateral, but not contralateral, side (Fig. 1A). This pain hypersensitivity appeared at 30 minutes, reached a peak between 2 hours and 1 day, and was maintained for at least 7 days. Thus, the period starting at 1 day after CFA injection is considered as the maintenance phase of CFA-induced inflammatory pain. We found that CFA injection activated the Akt signaling pathway as indicated by time-dependent increases in expression of p-Akt in the ipsilateral L4/L5 DRGs (Fig. 1B; *n* = 3/time point). A marked increase in p-Akt began on day 3 after CFA injection (1.94 ± 0.22 fold that of control group; *P* < 0.05) and was maintained until at least day 7 (1.99 ± 0.22 fold that of control group; *P* < 0.05; Fig. 1B). This increase was absent in the contralateral L4/5 DRGs (Fig. 1C). CFA injection did not lead to a significant change in the level of total Akt in the ipsilateral L4/5 DRGs within the 7-day period (Fig. 1B). As expected, saline injection did not alter the level of either p-Akt or total Akt in the L4/5 DRGs during the observation

period (data not shown). These findings indicate that CFA-induced inflammation alters Akt phosphorylation status rather than total Akt protein expression in DRG.

Immunohistochemical analysis also demonstrated an increase in p-Akt expression in DRG neurons after CFA injection. Consistent with the results of previous studies,^{27, 30} p-Akt was expressed exclusively in DRG neurons (Fig. 2A), and most p-Akt-positive neurons were small or medium (Fig. 2A). CFA injection significantly increased the number of p-Akt-positive neurons in L4/5 DRGs on days 3 and 7 (Fig. 2B). On day 3 after CFA injection, $37.4 \pm 1.7\%$ of L4 DRG neurons and $37.7 \pm 1.4\%$ of L5 DRG neurons were positive for p-Akt. The corresponding values were only $28.4 \pm 1.7\%$ and $26.5 \pm 3.0\%$, respectively, in the saline-injected animals (Fig. 2C). On day 7 after CFA injection, $37.2 \pm 0.4\%$ of L4 DRG neurons and $40.4 \pm 1.2\%$ of L5 DRG neurons were positive for p-Akt. In the saline-injected animals, the corresponding values were only $24.3 \pm 1.9\%$ and $29.3 \pm 1.4\%$, respectively (Fig. 2C). These findings indicate that the Akt pathway is activated in DRG neurons during the maintenance period of CFA-induced inflammatory pain.

Intrathecal Akt inhibitor attenuates CFA-induced thermal hyperalgesia during the maintenance period

Next, we examined whether activation of the Akt pathway is required for the maintenance of CFA-induced inflammatory pain. To that end, we observed the effects of intrathecally administered Akt inhibitor IV on CFA-induced thermal hyperalgesia on day 7 post-CFA injection. As expected, ipsilateral PWL of vehicle-treated rats (4.88 ± 0.31) was significantly less than that of basal PWL (9.40 ± 0.57 s; $n = 6$; $P < 0.01$; Fig. 3) on day 7 after CFA injection. Treatment with intrathecal Akt inhibitor IV increased the ipsilateral PWL by 61% compared to vehicle treatment ($n = 5$; $P < 0.05$; Fig. 3). Akt inhibitor IV alone did not alter basal paw withdrawal responses in the saline-injected rats ($n = 5$; Fig. 3). These results suggest that Akt participates in the maintenance of CFA-induced thermal hyperalgesia.

Intrathecal Akt inhibitor blocks CFA-induced increases in DRG Nav1.7 and Nav1.8

Previous studies have demonstrated that CFA-induced peripheral inflammation upregulates Nav1.7 and Nav1.8 expression in DRG neurons, particularly in small-diameter neurons.^{8, 16, 33, 40} We also found that intraplantar CFA injection time-dependently increased the expression of Nav1.7 and Nav1.8 in the ipsilateral L4/5 DRGs (Figs. 4A and 5A; $n = 3$ /time point). Nav1.7 and Nav1.8 levels began to increase significantly at 2 hours after CFA injection (Nav1.7: 1.97 ± 0.24 fold that of control group, Nav1.8: 1.92 ± 0.16 fold that of control group; $P < 0.01$) and were maintained until at least day 7 (Nav1.7: 3.72 ± 0.54 fold that of control group, Nav1.8: 4.29 ± 0.71 fold that of control group; $P < 0.01$). Immunohistochemical analysis showed that both Nav1.7 and Nav1.8 were located exclusively in DRG neurons (Figs. 4B and 5B). The percentage of Nav1.7-positive and Nav1.8-positive neurons in the ipsilateral L4 and L5 DRGs was significantly increased on day 7 after CFA injection (Figs. 4C and 5C). Immunohistochemical analysis of tissues from rats administered intrathecal vehicle showed that $64.43 \pm 2.33\%$ of L4 DRG neurons and $61.33 \pm 2.82\%$ of L5 DRG neurons had positive labeling for Nav1.7 ($n = 3$; Fig. 4D) on day 7 post-CFA injection. These values were significantly higher than those from vehicle-treated control rats that received intraplantar injections of saline (L4, $38.70 \pm 2.64\%$; L5, $41.70 \pm 0.47\%$; $n = 3$; Fig. 4D). Similarly, $61.83 \pm 1.84\%$ of L4 DRG neurons and $63.08 \pm 1.43\%$ of L5 DRG neurons from vehicle-treated rats were positive for Nav1.8 ($n = 3$; Fig. 5D) on day 7 post-CFA injection, whereas only $41.02 \pm 1.63\%$ of L4 DRG neurons and $43.20 \pm 1.42\%$ of L5 DRG neurons were positive for Nav1.8 on day 7 post-saline injection ($n = 3$; Fig. 5D). The majority of these “new” populations of neurons that were positive for Nav1.7 or Nav1.8 were small (Figs. 4C and 5C).

We further examined whether blockade of the Akt signaling pathway affected CFA-induced upregulation of Nav1.7 and Nav1.8 in DRG. Western blot analysis showed that intrathecal administration of Akt inhibitor IV markedly blocked the CFA-induced increase in expression of Nav1.7 and Nav1.8 in the ipsilateral L4/5 DRGs on day 7 after CFA injection. The level of Nav1.7 in the group ($n = 3$) treated with Akt inhibitor IV plus CFA was reduced by 71% ($P < 0.01$) compared to the corresponding group ($n = 3$) treated with vehicle plus CFA (2.38 ± 0.19 ; Fig. 4E). Similarly, the level of Nav1.8 in the group ($n = 3$) treated with Akt inhibitor IV plus CFA was reduced by 73% ($P < 0.01$) compared to the corresponding group ($n = 3$) treated with vehicle plus CFA (4.95 ± 1.18 ; Fig. 5E). In addition, immunohistochemical analysis revealed that 7 days after CFA injection, treatment with Akt inhibitor IV reduced the percentage of Nav1.7-positive neurons by 19% ($P < 0.05$) in the ipsilateral L4 DRG and 20% ($P < 0.05$) in the ipsilateral L5 DRG compared with vehicle treatment (Fig. 4D). Similarly, Akt inhibitor IV reduced the percentage of Nav1.8-positive neurons by 22% ($P < 0.05$) in the L4 DRG and 17% ($P < 0.05$) in the L5 DRG (Fig. 5D). As shown in Figs. 4 and 5, intrathecal administration of Akt inhibitor IV ($n = 3$) alone did not alter basal expression of Nav1.7 or Nav1.8 or basal percentage of neurons positive for Nav1.7 or Nav1.8 in the L4 and L5 DRG neurons on day 7 post-saline injection. Our findings suggest that Akt participates in the regulation of Nav1.7 and Nav1.8 expression in the DRG during the maintenance of chronic inflammatory pain.

Colocalization of p-Akt with Nav1.7 and Nav1.8 in DRG neurons

Finally, we investigated whether p-Akt colocalizes with Nav1.7 and Nav1.8 in DRG neurons. As described above, p-Akt, Nav1.7, and Nav1.8 were expressed predominantly in small DRG neurons. Double labeling showed that on day 7 post-saline injection, approximately 39.5% of p-Akt-labeled neurons in L4 DRGs and 47.1% of p-Akt-labeled neurons in L5 DRGs were positive for Nav1.7. Likewise, approximately 47.6% of p-Akt-labeled neurons in L4 DRGs and 46.6% of p-Akt-labeled neurons in L5 DRGs were positive for Nav1.8 ($n = 4$; Fig. 6). Interestingly, CFA injection increased p-Akt expression in both Nav1.7- and Nav1.8-positive DRG neurons. The percentage of neurons that double labeled for p-Akt and Nav1.7 increased by 9.2% and 3.2% in the L4 DRG and L5 DRG, respectively, on day 7 after CFA injection (Fig. 6A). The percentage of neurons that double labeled for p-Akt and Nav1.8 increased by 13.6% and 8.9% in the L4 DRG and L5 DRG, respectively, on day 7 after CFA injection (Fig. 6B). These data indicate that p-Akt has a high degree of coexpression with Nav1.7 and Nav1.8 in DRG neurons under normal and CFA-induced peripheral inflammatory pain conditions.

Discussion

Cumulative evidence suggests that Akt activation in brain is critical for neuronal plasticity.^{21, 29} However, the role of Akt in peripheral sensitization associated with chronic inflammatory pain is much less understood. Here, we showed that Akt is activated in the DRGs after CFA-induced inflammation. This activation was required for CFA-induced upregulation of DRG Nav1.7 and Nav1.8 and was causally linked to the maintenance of CFA-induced inflammatory pain.

Akt activity is increased in the primary afferent neurons under chronic pain conditions. A previous study showed that the number of p-Akt-positive neurons increased significantly in ipsilateral L5 DRG and adjacent L4 DRG beginning at 12 hours after rat L5 spinal nerve ligation and remained elevated until the third day (it returned to basal level on day 7 post-injury).⁴² Our study demonstrated that the activity of Akt in DRG neurons was augmented in response to chronic peripheral inflammation. The amount of p-Akt was markedly increased in ipsilateral L4/5 DRGs beginning on day 3 post-CFA injection and remained elevated at least until the seventh day. We did not observe any significant changes in p-Akt

in DRG during the early period of CFA-induced inflammatory pain. Interestingly, Akt is also activated in DRG neurons after acute peripheral inflammation, but this activation occurs early and transiently. Sun et al.³⁴ reported that the p-Akt level increased in rat DRG 5 minutes after intradermal injection of capsaicin. The p-Akt level increased significantly between 40 minutes and 1 hour after injection and then declined toward control levels over the next 2 hours.³⁴ Carrageenan was reported to produce a significant increase in p-Akt in mouse DRG at 1 hour after intraplantar injection.³⁰ The differences between our study and previous work could be explained by distinct experimental models and/or species differences.

DRG Akt activation may be involved in the maintenance of inflammation-induced thermal hyperalgesia. We found that intrathecal Akt inhibitor IV alone did not affect basal response to thermal stimulation, indicating that basal level of Akt in the DRG and spinal cord does not participate in noxious information transmission and modulation. However, intrathecal administration of this inhibitor at the same dose attenuated thermal hyperalgesia on day 7 post-CFA injection. Given that CFA injection activated Akt in the DRG but not spinal cord (data not shown), our findings suggest the Akt inhibitor attenuates thermal hyperalgesia by blocking the CFA-induced increase in Akt activation in the DRG neurons. Hence, the DRG Akt pathway contributes to maintenance of chronic inflammatory pain.

An important finding of our study is that Akt is involved in regulating DRG Nav1.7 and Nav1.8 expression during the maintenance of chronic inflammatory pain. Nav1.7 and Nav1.8 are expressed predominantly in small-diameter DRG neurons and on C- and Aδ-nociceptive fibers. They contribute to amplification of generator potentials and set the gain on nociceptors.^{4, 5, 10, 11, 41} Peripheral inflammation upregulates mRNA and protein expression of Nav1.7 and Nav1.8 in the DRG.^{5, 8, 24, 33} Moreover, knockdown of DRG Nav1.7 or Nav1.8 significantly prevents inflammation-induced pain hypersensitivity.^{20, 45} Mice lacking Nav1.7 or Nav1.8 also fail to develop hyperalgesia in several inflammatory pain models.^{1, 23} These results suggest that inflammation-induced upregulation of DRG Nav1.7 and Nav1.8 participates in the induction and maintenance of inflammatory pain. Our work further demonstrates that this upregulation requires activation of DRG Akt. Intraplantar CFA injection increased the percentage of DRG neurons (predominantly in small DRG neurons) that co-expressed activated Akt and Nav1.7 or Nav 1.8. Inhibition of Akt attenuated CFA-induced increases in the expression of Nav1.7 and Nav1.8 and in the number of Nav1.7-labeled and Nav1.8-labeled neurons in L4/5 DRGs on day 7 post-CFA injection. Our evidence indicates that Akt may contribute to the maintenance of inflammatory pain through upregulation of DRG Nav1.7 and Nav1.8 expression.

The mechanism by which Akt increases the expression of Nav1.7 and Nav1.8 in DRG neurons during the maintenance of inflammatory pain is still unclear. However, mammalian target of rapamycin (mTOR), a serine-threonine protein kinase that is highly expressed in small-diameter DRG neurons,⁴³ may be involved. Intrathecal administration of mTOR inhibitors was shown to significantly block the development of inflammatory and neuropathic pain.^{2, 14, 19, 28} Given that mTOR is important for control of protein translation and that Akt can activate mTOR in central neurons,^{3, 17} it is possible that mTOR participates in Akt-dependent induction of Nav1.7 and Nav1.8 expression in DRG neurons after inflammation. It is worth noting that mTOR could not be activated in the DRG in models of neuropathic and bone cancer-induced pain.^{14, 31} Whether DRG mTOR is activated after inflammation remains to be determined. Akt activation may also trigger the expression of fragile X mental retardation protein and the nuclear translocation of two transcription factors, NF-κB and FOXO,^{7, 18, 32} all of which regulate translation of mRNA. Whether these proteins are involved in Akt-mediated increases in DRG Nav1.7 and Nav1.8 in inflammatory pain maintenance will be determined in our future studies. Data from previous

studies and our present work also showed that the expression of Nav1.7 and Nav1.8 in the ipsilateral L4/5 DRG was significantly increased earlier than the third day after CFA injection. The mechanisms that underlie this increase are unknown and will be further investigated.

In this study, we explored the regulatory mechanisms of sodium channels Nav1.7 and Nav1.8 in inflammatory pain. We found that upregulation of DRG Nav1.7 and Nav1.8 during the maintenance of chronic inflammatory pain required functional activation of the Akt signaling pathway. Thus, the Akt pathway may present a novel pharmacologic target for prevention or treatment of chronic inflammatory pain.

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Figure 1.

CFA-induced thermal hypersensitivity and Akt activation in L4 and L5 DRGs. (A) Thermal hypersensitivity was demonstrated by significant decreases in paw withdrawal latency (PWL) on the side ipsilateral (Ipsi) to intraplantar CFA injection. ** $P < 0.01$ vs the corresponding baseline (0 h). $n = 5-6$ /group; Contra, contralateral. (B) The level of p-Akt, but not total Akt, was significantly increased in the ipsilateral L4/5 DRGs on days 3 and 7 after CFA injection. The upper panels depict representative Western blots. The lower panel shows the statistical summary of the densitometric analysis expressed relative to basal level (0 h) after normalization to the corresponding β -actin. * $P < 0.05$ vs the corresponding basal level. $n = 3$ /group. (C) The expression of p-Akt did not change significantly in the contralateral DRGs after intraplantar CFA injection at any time point examined. The upper panels depict representative Western blots. The lower panel shows the statistical summary of the densitometric analysis expressed relative to basal level after normalization to the corresponding β -actin. $n = 3$ /group.

Figure 2.

Effect of intraplantar CFA injection on number of p-Akt-labeled neurons in L4 and L5 DRGs. (A) Co-localization of p-Akt with NeuN (a neuronal marker) in L5 DRG. Scale bar: 50 μ m. (B) Representative images depict p-Akt-labeled neurons in the ipsilateral L4 and L5 DRGs on days 3 and 7 after saline or CFA injection. Scale bar: 50 μ m. (C) Statistical analysis shows that the percentage of p-Akt-labeled neurons was significantly greater in the ipsilateral L4 and L5 DRGs of CFA-injected rats than in those of saline-injected rats on days 3 and 7 after injection. ** $P < 0.01$ vs the corresponding saline-treated group. $n = 3$ /group.

Figure 3.

Effects of intrathecal Akt inhibitor on CFA-induced thermal hypersensitivity. Intrathecal treatment with Akt inhibitor IV (Akt IV) significantly attenuated the CFA-induced decrease in paw withdrawal latency (PWL) on day 7 after CFA injection. Intrathecal injection of Akt IV in the absence of CFA injection had no effect on basal PWL. ** $P < 0.01$ vs the group injected with saline and treated with vehicle (DMSO). # $P < 0.05$ vs the group injected with CFA and treated with vehicle. $n = 5-6$ /group.

Figure 4.

Effects of intrathecal Akt inhibitor on the CFA-induced increase in Nav1.7 expression in the ipsilateral L4/5 DRGs on day 7 after CFA injection. (A) The amount of Nav1.7 was time-dependently increased in the ipsilateral L4/5 DRGs after CFA injection. The upper panels depict representative Western blots. The lower panel shows the statistical summary of the densitometric analysis expressed relative to basal level (0 h) after normalization to the corresponding β -actin. * $P < 0.05$, ** $P < 0.01$ vs the corresponding basal level. $n = 3/\text{group}$. (B) Colocalization of Nav1.7 with NeuN in L5 DRG. Scale bar: 50 μm . (C) Representative images depicting Nav1.7-labeled neurons in the ipsilateral L5 DRG on day 7 after intraplantar injection with saline (S) or CFA. Rats were treated with intrathecal injections of vehicle (V) or Akt inhibitor IV (AI). Scale bar: 50 μm . (D) Statistical summary showing the percentages of Nav1.7-labeled neurons in the ipsilateral L4 and L5 DRGs of each group. ** $P < 0.01$ vs the group injected with saline and treated with vehicle. # $P < 0.05$ vs the group injected with CFA and treated with vehicle. $n = 3/\text{group}$. (E) Intrathecal Akt inhibitor IV significantly blocked the CFA-induced increase in Nav1.7 expression in the ipsilateral L4/5 DRGs on day 7 after CFA injection. The upper panels depict representative Western blots. The lower panel shows the statistical summary of the densitometric analysis expressed relative to the group injected with saline and treated with vehicle after normalization to the corresponding β -actin. * $P < 0.05$ vs the group injected with saline and treated with vehicle. # $P < 0.05$ vs the group injected with CFA and treated with vehicle. $n = 3/\text{group}$.

Figure 5.

Effects of intrathecal Akt inhibitor on the CFA-induced increase in Nav1.8 expression in the ipsilateral L4 and L5 DRGs on day 7 after CFA injection. (A) The level of Nav1.8 was time-dependently increased in the ipsilateral L4/5 DRGs after CFA injection. The upper panels depict representative Western blots. The lower panel shows the statistical summary of the densitometric analysis expressed relative to basal level (0 h) after normalization to the corresponding β -actin. $**P < 0.01$ vs the corresponding basal level. $n = 3/\text{group}$. (B) Colocalization of Nav1.8 with NeuN in L5 DRG. Scale bar: 50 μm . (C) Representative images depicting Nav1.8-labeled neurons in the ipsilateral L5 DRG on day 7 after intraplantar injection with saline (S) or CFA. Rats were treated with intrathecal injections of vehicle (V) or Akt inhibitor IV (AI). Scale bar: 50 μm . (D) Statistical summary showing the percentages of Nav1.8-labeled neurons in the ipsilateral L4 and L5 DRGs of each group. $*P < 0.01$ vs the group injected with saline and treated with vehicle. $\#P < 0.05$ vs the group injected with CFA and treated with vehicle. $n = 3/\text{group}$. (E) Intrathecal Akt inhibitor IV significantly blocked the CFA-induced increase in Nav1.8 expression in the ipsilateral L4/5 DRGs on day 7 after CFA injection. The upper panels depict representative Western blots. The lower panel shows the statistical summary of the densitometric analysis expressed relative to the group injected with saline and treated with vehicle after normalization to the corresponding β -actin. $*P < 0.05$ vs the group injected with saline and treated with vehicle. $\#P < 0.05$ vs the group injected with CFA and treated with vehicle. $n = 3/\text{group}$.

Figure 6.

Colocalization of p-Akt with Nav1.7 and Nav1.8 in L4 and L5 DRGs. (A) The left panels depict representative labeling for p-Akt and Nav1.7 and their merged images in the ipsilateral L5 DRG on day 7 after saline or CFA injection. The right panel shows the statistical summary of the percentage of neurons that double labeled for p-Akt and Nav1.7 on day 7 after saline or CFA injection. $n = 3/\text{group}$. Scale bar: 50 μm . (B) The left panels depict representative labeling for p-Akt and Nav1.8 and their merged images in the ipsilateral L5 DRG on day 7 after saline or CFA injection. The right panel shows the statistical summary of the percentage of neurons that double labeled for p-Akt and Nav1.8 on day 7 after saline or CFA injection. * $P < 0.05$ vs the corresponding saline-treated group. $n = 3/\text{group}$. Scale bar: 50 μm .