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Injury-Induced Janus Kinase/Protein Kinase C-Dependent Phosphorylation of Growth-Associated Protein 43 and Signal Transducer and Activator of Transcription 3 for Neurite Growth in Dorsal Root Ganglion

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Elevation of corticosteroids and excessive glutamate release are the two major stress responses that occur sequentially during traumatic CNS injury. We have previously reported that sequential application of corticosterone and kainic acid (CORT + KA) mimicking the nerve injury condition results in synergistic enhancement of neurite outgrowth and expression of growth-associated protein 43 (GAP-43) in cultured dorsal root ganglion (DRG). GAP-43 is known to promote neurite extension when phosphorylated by protein kinase C (PKC). In addition, PKC can phosphorylate the signal transducer and activator of transcription 3 (STAT3) at Ser727, which is phosphorylated primarily by Janus kinase (JAK) at Tyr705. In this study, we further examine the role of PKC in this stress-induced growth-promoting effect. In the cultured DRG neurons, the JAK inhibitor AG-490 and the PKC inhibitor Ro-318220 reduced the CORT + KA-enhanced neurite growth effect when applied prior to CORT and KA treatment, respectively. Both AG-490 and Ro-318220 diminished the CORT + KA-enhanced GAP-43 expression, phosphorylation, and axonal localization. Furthermore, CORT + KA treatment synergistically phosphorylated STAT3 at Ser727 but not at Tyr705. Similar phenomena were observed in an animal model of acute spinal cord injury (SCI), in which phosphorylation of GAP-43 and phospho-Ser727-STAT3 was elevated in the injured DRG 4 hr after the impact injury. Further treatment with the therapeutic glucocorticoid methylprednisolone enhanced the phosphorylation of GAP-43 in both the DRG and the spinal cord of SCI rats. These results suggest that elevated glucocorticoids and overexcitation following CNS injury contribute to nerve regeneration via induction of JAK/PKC-mediated GAP-43 and STAT3 activities.

Key words: corticosterone; kainic acid; spinal cord injury

Glucocorticoids (GCs), such as corticosterone and cortisol, are the major stress hormones known to be elevated immediately after traumatic nerve injury. Ele-

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vated GCs activate the glucocorticoid receptor (GR), which in turn binds to glucocorticoid responsive elements in various gene promoter regions to regulate the transcription of stress- and inflammation-related genes (Schoneveld et al., 2004). GC receptor expression was found to be elevated in the area of injury, implying that the injured neurons are more susceptible to GC-mediated responses (Yan et al., 1999). The local effect of GCs on injured neurons is controversial, with most evidence suggesting a detrimental effect (Diem et al., 2003; Qian et al., 2004). Nevertheless, the synthetic GCs methylprednisone (MP) and dexamethasone are frequently used for treating the acute phase of CNS injury. With the exception of their anti-inflammatory effect, limited information is available with respect to the potentially beneficial effects of GCs on nerve injury.

Trauma to the CNS often leads to excessive glutamate-mediated neural transmission, which can result in secondary neuronal damage (Zipfel et al., 2000). The kainic acid (KA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are the most abundant subtype of glutamate receptor in the dorsal root ganglia (DRG) and spinal cord, respectively (Sato et al., 1993; Tsai et al., 2002). Activation of AMPA/KA receptors results in intracellular calcium elevation, with subsequent activation of protein kinase C (PKC). PKC is important for neuronal survival and synaptic plasticity in hippocampal and cortical neurons (McNamara and Lenox, 2000; Kaasinen et al., 2002; Melyan et al., 2002), suggesting that activation of AMPA/KA receptors in DRG and spinal cord during nerve injury may be beneficial for survival and growth.

Signal transducer and activator of transcription 3 (STAT3) is a cytokine-related transcription factor transactivated by the GR (Zhang et al., 1997). The STAT3 protein belongs to the Janus kinase (JAK)/STAT signaling pathway involved in cytokine receptor-mediated signaling, such as interleukin 6, ciliary neurotrophic factor, and cardiotrophin-1 (Dolcet et al., 2001; Yamauchi et al., 2006). The JAK-phosphorylated STAT3 can be further phosphorylated by PKC-δ at Ser727 to enhance its transcriptional activity (Jain et al., 1999). GCs are believed to attenuate the proinflammatory cytokine activities, and subsequently reduce JAK/STAT signaling (Bianchi et al., 2000; Ishida-Takahashi et al., 2004). On the other hand, recent studies show that STAT3 is important for neuronal survival and spinal axonal regeneration after a conditioning injury (Ihle, 1995; Okada et al., 2004; Qiu et al., 2005).

The membrane protein growth-associated protein 43 (GAP-43) is a frequently used marker for nerve regeneration in spinal cord injury (Oestreicher et al., 1997; Teng et al., 2002). GAP-43 is located in the growth cones of growing neurites, and its growth-promoting effect is regulated by phosphorylation. Phosphorylation of GAP-43 by PKC results in its translocation from the cytosolic membrane compartment to the nerve terminus, where it interacts with F-actin associated adhesion molecule and/or extracellular matrix complexes to promote neurite extension (He et al., 1997; Shen et al., 2002). Recent studies show that STAT3 can transactivate GAP-43 expression in the in vivo and in vitro systems (Schwaiger et al., 2000; Wu and Bradshaw, 1996). The above-mentioned findings imply that PKC activity and JAK/STAT3 signaling may be involved in expression and phosphorylation of GAP-43 to regulate neurite outgrowth.

In our previous study, we found that corticosterone (CORT) and KA synergistically promote neurite outgrowth and GAP-43 expression when applied sequentially with CORT at day 1 in vitro culture (1 DIV) and KA at day 2 in primary cultured DRG neurons (Tsai et al., 2002). Although the sequential engagement of these two factors is a simulation of the condition in traumatic CNS injury, there is no clue regarding the mechanism of their synergy in promoting neurite growth. Taking the fact that activation of GAP-43 to promote neurite extension requires PKC phosphorylation, we herein hypothesize that PKC signaling might be involved in the synergistic effect of CORT and KA on neurite outgrowth. In addition, STAT3 is phosphorylated subsequently by JAK and PKC to exert its transcriptional activity, which leads to the possibility that JAK activity and STAT3 phosphorylation might also be involved in the neurite growth enhanced by CORT and KA treatment. To test this hypothesis, CORT and KA were used to mimic stress and overexcitation in the injured tissue, respectively. Neurite outgrowth and GAP-43 expression were examined in primary cultured DRG neurons. The acute phase of signaling events, such as phosphorylation of GAP-43 and STAT3, was examined in the isolated DRG ex vivo. For in vivo study of traumatic CNS injury, an acute spinal cord injury (SCI) animal model in rats was used to investigate the activities of GAP-43 and STAT3 in injured DRG and spinal cord. Furthermore, the effect of GC therapy on the phosphorylation of GAP-43 and STAT3 in acute SCI rats was examined.

MATERIALS AND METHODS

Materials

The compounds and antibodies used in this study were obtained from the following sources. Sevoflurane was from Abbott Laboratories Ltd. (Kent, United Kingdom). Zoletil was from Virbac Laboratories (Carros, France). Rompun was from Bayer AG (Leverkusen, Germany). Collagenase, trypsin, and F-12 nutrient mixture were from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). GeneStrips hybridization tubes were from RNAture Inc. (Irvine, CA). SuperScript II reverse transcriptase was from Invitrogen Life Technologies (Carlson, CA). Horseradish peroxidase (HRP)-reactive chemiluminescence reagent kit was from Amersham Pharmacia Biotech (Piscataway, NJ). Percoll, corticosterone, and methylprednisolone were from Sigma-Aldrich Co. (St. Louis, MO). KA was from Tocris Cookson Ltd. (Bristol, United Kingdom). AG490 and Ro-318220 were from Calbiochem (San Diego, CA). Mouse anti-GAP-43

monoclonal antibody, rabbit anti-pSer41-GAP-43 polyclonal antibody, and rabbit anti-neurofilament H polyclonal antibody were from Chemicon International (Temecula, CA). Mouse antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was from Biogenesis (Poole, United Kingdom). Rabbit anti-STAT3 antibody, rabbit anti-pSer727-STAT3 polyclonal antibody, and rabbit anti-pTyr705-STAT3 polyclonal antibody were from Cell Signaling Technology (Danvers, MA). Fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat anti-rabbit IgG (H + L) and Cy5-conjugated affinity-purified goat anti-mouse IgG (H + L) were from Jackson Immunoresearch Laboratories (West Grove, PA).

Animals

Male Sprague Dawley (SD) rats (2 months old) weighing approximately 200 g and female Long-Evans rats (9 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Upon arrival in the laboratory, the animals were housed in groups of three in a room with a 12:12 hr light:dark cycle beginning with the lights on at 5:00 AM and controlled temperature and humidity at 19–21°C and 50–60%, respectively. All animals had free access to food and water at all times. For animal care and surgical procedures, we followed the *Guide for the care and use of laboratory animals* published by National Institutes of Health (Kunihara et al., 2004). The University Laboratory Animal Care and Use Committee of Taipei Medical University approved all surgical protocols.

Isolation of Rat Axotomized DRG (AX-DRG) for Primary Culture and Ex Vivo Studies

Male SD rats 2–3 month old were sacrificed after anesthesia overdose with Sevoflurane inhalation. Approximately 35–40 DRGs in one rat were isolated from the cervical to the sacral levels of the spinal column for either primary culture or ex vivo studies as follows. For primary culture of the rat DRG neurons, isolated ganglia were digested with 0.15% collagenase and 0.25% trypsin. The cell mixture was fractionated in 30% percoll and centrifuged at 1,500 rpm for 5 min. The pellet, which was enriched with DRG neurons, was plated onto poly-L-lysine-coated 35 mm culture dishes and cultured in F-12/10% FBS medium in a humidified tissue culture incubator with 5% CO_2 maintained at 37°C. The day of plating was counted as day 0 in vitro (0 DIV). For ex vivo experiment, isolated ganglia were chopped and incubated initially with 10 μM CORT for 2 hr, followed by treatment with 1 mM KA for another 2 hr in a humidified culture incubator with 5% CO_2 maintained at 37°C. Tissue was then subjected to Western blot analysis for GAP-43 and STAT3 phosphorylations.

SCI Animal Model for In Vivo Study

Female Long-Evans rats were used in the SCI study. We induced SCI in female Long-Evans rats based on procedures published previously (Liu et al., 1997; Xu et al., 1998). Female rats were anesthetized by intraperitoneal injections of Zoletil (50 mg/kg) and Rompun (10 mg/kg). Animals were randomly assigned to three groups: the sham control, SCI, and SCI + methylprednisolone (MP; 30 mg/kg body weight) groups. The T9–T10 laminectomy was performed in all animals under a dissecting microscope and the dura mater was left intact. For the sham control group, animals received a T9–T10 laminectomy, followed by an immediate intravenous injection of vehicle. After T9–T10 laminectomy, SCI was induced in animals in the SCI and SCI + MP groups by a New York University (NYU) impactor with a 10-g weight dropped from a height of 25 mm. Vehicle or MP was administered via an intravenous route immediately after the SCI. Four hours after vehicle or MP treatment, animals were anesthetized with the anesthetics described above. A 5-mm section of the spinal cord with the lesion, or a similar area in the sham control group, was collected for protein extraction. DRG and spinal cord located near the lesion (T8–T11) were collected as injured/adjacent tissue for protein extraction and Western blot analysis. For perioperative care, we followed the MASCIS guidelines that have been described in previous publications (Liu et al., 1997; Xu et al., 1998).

Determination of Neurite Outgrowth

Neurite length of cultured DRG neurons was measured every day from 1 DIV to 5 DIV by randomly selecting neurites and visualizing their length under an inverted phase contrast microscope with a microruler. Phase contrast photomicrographs were taken in parallel. In one culture batch, approximately 10–20 neurites for one experimental condition were measured. At least three separate batches of cultures were performed for each condition. Data of neurite length from separate batches of culture of the same experimental condition were pooled for statistical analysis.

Immunofluorescent Double Labeling

Cultured DRG neurons were plated onto poly-L-lysine-coated glass coverslips in 35-mm dishes and cultured in F12/10% FBS medium in a humidified tissue culture incubator with 5% CO_2 maintained at 37°C. After 3 DIV, DRG neurons were washed twice with an Earle's balanced salt solution (EBSS; pH 7.4; containing in mM: 117 NaCl, 1 NaH_2PO_4, 5.3 KCl, 26 $NaHCO_3$, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 5.6 D-glucose), followed by incubation with fixative solution (4% formaldehyde in 20 mM PBS) for 30 min at room temperature. Fixed cells were then permeabilized with prechilled EtOH/CH_3COOH (95%:5%) for 15 min at −20°C, followed by incubation sequentially with blocking solution, properly diluted mouse anti-GAP-43 monoclonal antibody (1:1,000) and rabbit antineurofilament H polyclonal antibody (1:200). Immunofluorescence double labeling was performed by using Cy5-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG. Fluorescent micrographs were captured by confocal laser scanning microscope (Zeiss; LSM510), and digital images were merged by Zeiss LSM510 operating software.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from DRG neurons at 3 DIV by using GeneStrips hybridization tubes according to the

manufacturer's protocol. The mRNAs were reversed transcribed into cDNA using random primers and reverse transcriptase. Primer sets of rat GAP-43 or GAPDH genes and total RNA extracted from DRG neurons were used. The expression levels of GAP-43 and an internal control for GAPDH were detected from the same samples using designed primers. The sequences of the primer set for amplification of each gene were 5′-GCT TCC GTG GAC ACA TAA CAA GGA-3′ and 5′-CTT AAA GTT CAG GCA TGT TCT TGG T-3′ (for GAP-43 cDNA) and 5′-GAC CCC TTC ATT GAC CTC AAC-3′ and 5′-GAT GAC CTT GCC CAC AGC CTT-3′ (for GAPDH cDNA). PCR was performed with a Gibco-BRL Technology thermocycler and a cycle sequence of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. Samples were collected after 30 cycles and loaded onto 2% agarose gels for electrophoresis. The gel was visualized under UV light after staining with ethidium bromide. Band intensity was quantified by image analysis software (Kodak Digital Science 1D). The band intensity of each group was normalized by GAPDH, and values are given as the ratio to control for statistical analysis.

Western Blot Analysis

The ex vivo AX-DRGs or injured DRG and spinal cord isolated from SCI rats were harvested with ice-cold lysis buffer (50 mM Tris-HCl containing 1% glycerol, and 1% IGEPAL CA-630 in pH 7.4) and sonicated to obtain total cell lysate. To detect the expression of GAP-43 and pSer41-GAP-43, STAT3, pSer727-STAT3, and pTyr705-STAT3, 80 μg of the protein was separated by 6–10% SDS-PAGE, transferred to a Hybond ECL nitrocellulose membrane (Bio-Rad Electrophoresis System), and probed with mouse anti-GAP-43 antibody (1:1,000), rabbit anti-pSer41-GAP-43 antibody (1:1,000), mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, 1:3,000) antibody, rabbit anti-STAT3 antibody (1:1,000), rabbit anti-pSer727-STAT3 antibody (1:1,000), or rabbit anti-pTyr705-STAT3 antibody (1:1,000) at 4°C overnight. The immune complex was further probed with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibody and then visualized by HRP-reactive chemiluminescence reagents. The chemiluminescent band was detected and analyzed with the Night Owl LB 981 imaging system (Berthold Technologies GmbH & Co.). The band intensity of each group was normalized by GAPDH, and values are given as the ratio to control for statistical analysis.

Statistic Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by Dunnet's, Newman-Keuls, or Bonferroni multiple-comparisons post hoc test to compare all groups with the control group, to evaluate the difference among groups, or to compare designated pairs of groups. Statistical significance was assumed at $\star P < 0.05$ or $\star\star P < 0.01$.

RESULTS

Sequential Effect of CORT and KA on Promoting Neurite Extension in AX-DRG Neurons

In our previous study, we revealed the window of time as well as the effective concentrations for the growth-promoting effect of CORT and KA in cultured DRG neurons (Tsai et al., 2002). Sequential application of CORT and KA was to mimic the injury-induced stress response, but why this application order could promote neurite outgrowth remains to be resolved. Therefore, this study was initiated by testing whether changes of the application order of CORT (10 μM) and KA (1 mM) would affect their neurite growth effect in cultured DRG neurons. Figure 1A shows that the growth-promoting effect of CORT and KA was observed only when CORT and KA were applied sequentially at 1 DIV and 2 DIV, respectively (CORT + KA). Neither reversed application order (KA at 1 DIV and CORT at 2 DIV) nor combined treatment of CORT and KA at 1 DIV (CORT/KA) could enhance neurite outgrowth. Figure 1B further shows that single application of either CORT at 1 DIV or KA at 2 DIV did not increase neurite growth rate, suggesting that the sequential application of CORT and KA is needed to promote neurite outgrowth and acts in a synergistic manner.

Blockade of PKC and JAK Activities Attenuated CORT + KA-Enhanced Neurite Outgrowth in AX-DRG Neurons

In our hypothesis, PKC-mediated GAP-43 phosphorylation and JAK-mediated STAT3 phosphorylation might be involved in the injury-induced nerve regeneration. Therefore, we tested whether blockade of these two kinase activities could diminish the CORT-KA synergism on neurite outgrowth in DRG neurons. We attempted to apply the specific JAK inhibitor AG-490 (30 μM) and the specific PKC inhibitor Ro-318220 (1 μM) at either 30 min prior to the CORT treatment or 30 min prior to the KA treatment. Interestingly, the JAK inhibitor blocked CORT + KA-enhanced neurite growth only when applied prior to CORT, whereas the PKC inhibitor blocked the effect only when applied prior to KA (Fig. 1C,D). The concentrations of AG-490 (30 μM) and Ro-318220 (1 μM) used in this study did not influence the basal level of neurite growth rate. Representative photomicrographs further show that neurite lengths of cultured neurons at 3 DIV were apparently shorter in AG-490- and Ro-318220-treated groups (Fig. 1E), but no significant cell damage was observed. The results suggest that JAK and PKC activities contribute to the growth-promoting effect of CORT and KA, respectively, for their synergistic effect on neurite outgrowth.

Blockade of JAK and PKC Activities Attenuated CORT + KA-Enhanced GAP-43 Gene Expression and Its Axonal Localization in AX-DRG Neurons

In our previous study, we found that sequential application of CORT and KA could increase GAP-43 expression at the protein level. GAP-43 is known to promote neurite extension when translocated to regenerating axons upon PKC phosphorylation at Ser41. Therefore, we examined whether sequential application of

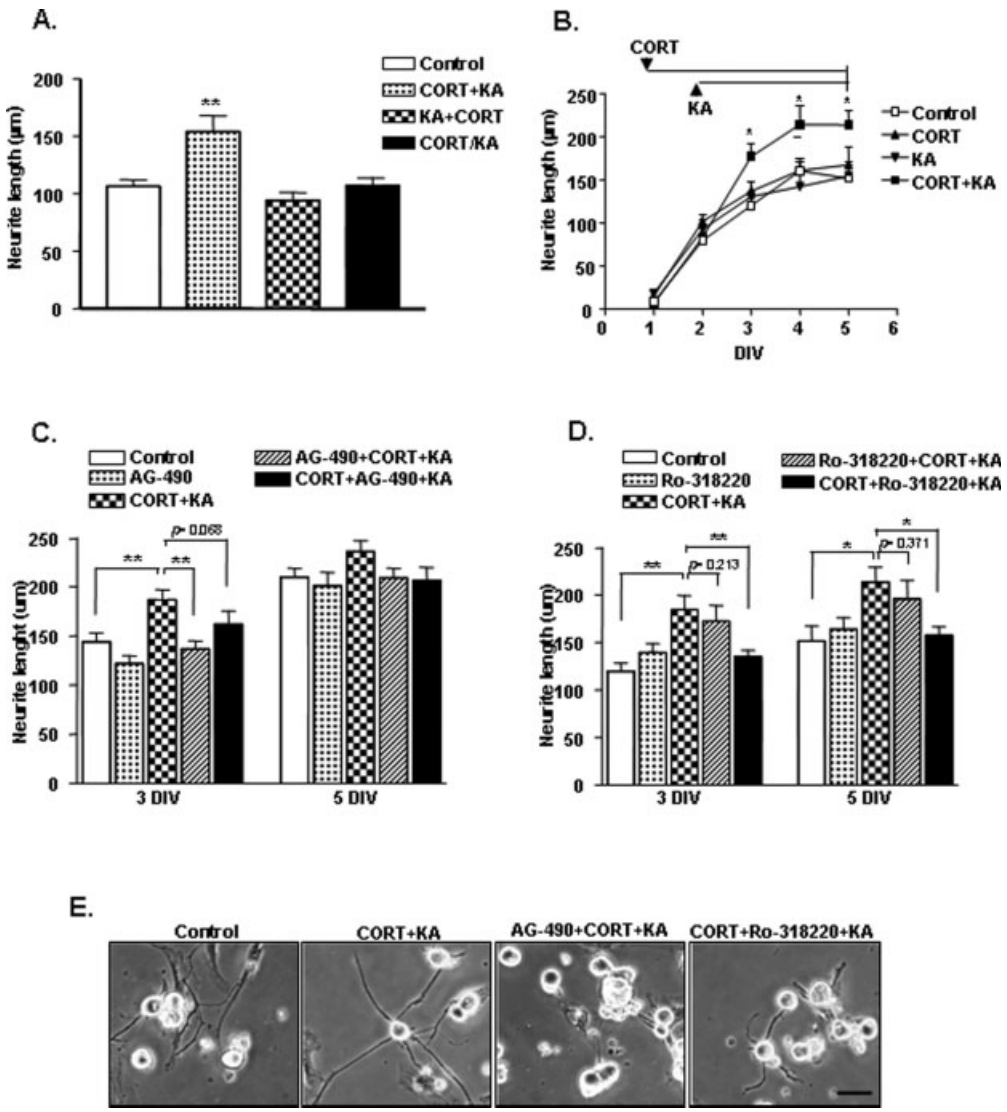

Fig. 1. Sequential application of CORT and KA enhanced neurite outgrowth in cultured DRG neurons. **A:** Cultured DRG neurons were treated with CORT (10 μM) and KA (1 mM) in different orders: CORT at 1 DIV, followed by KA at 2 DIV (CORT + KA); KA at 1 DIV, followed by CORT at 2 DIV (KA + CORT); or application of CORT and KA simultaneously at 1 DIV (CORT/KA). Bar graphs show the neurite length of DRG neurons measured under different conditions at 3 DIV. **B:** Cultured DRG neurons were treated with CORT (10 μM) at 1 DIV, followed by application of KA (1 mM) at 2 DIV. Neurite length of DRG neurons was measured once per day from 1 DIV to 5 DIV. The JAK inhibitor AG-490 (**C**; 30 μM) or PKC inhibitor Ro-318220 (**D**; 1 μM) was used prior to CORT (10 μM at 1 DIV) or KA (1 mM at 2 DIV) treatment. Bar graphs in C and D show the neurite length of DRG neurons measured at 3 DIV and 5 DIV. Data were pooled from three batches of cultures and expressed as mean ± SEM (n = 30–50). $\star P < 0.05$, $\star\star P < 0.01$ compared with the control group (A,B) or the designated group (C,D) by one-way ANOVA with Dunnet's or Bonfferoni multiple-comparisons post hoc test. **E** shows phase contrast photomicrographs of cultured DRG at 3 DIV for each condition. Scale bar = 50 μm.

CORT and KA could enhance GAP-43 mRNA expression and axonal localization. RT-PCR analysis shows that GAP-43 mRNA levels were increased by CORT + KA treatment when cells were harvested 12 hr after the KA treatment (Fig. 2A). This effect was reversed when AG-490 or Ro-318220 was added prior to CORT and KA treatments, respectively (Fig. 2A). For axonal localization of GAP-43 in DRG, we used immunofluorescent double labeling to examine the colocalization of GAP-43 and an axonal marker, namely, high-molecular-weight neurofilament protein (NF-H). Figure 2B shows that GAP-43 immunoreactivity in the vehicle-treated DRG neurons was distributed along neurites but with less colocalization with NF-H. Sequential application of

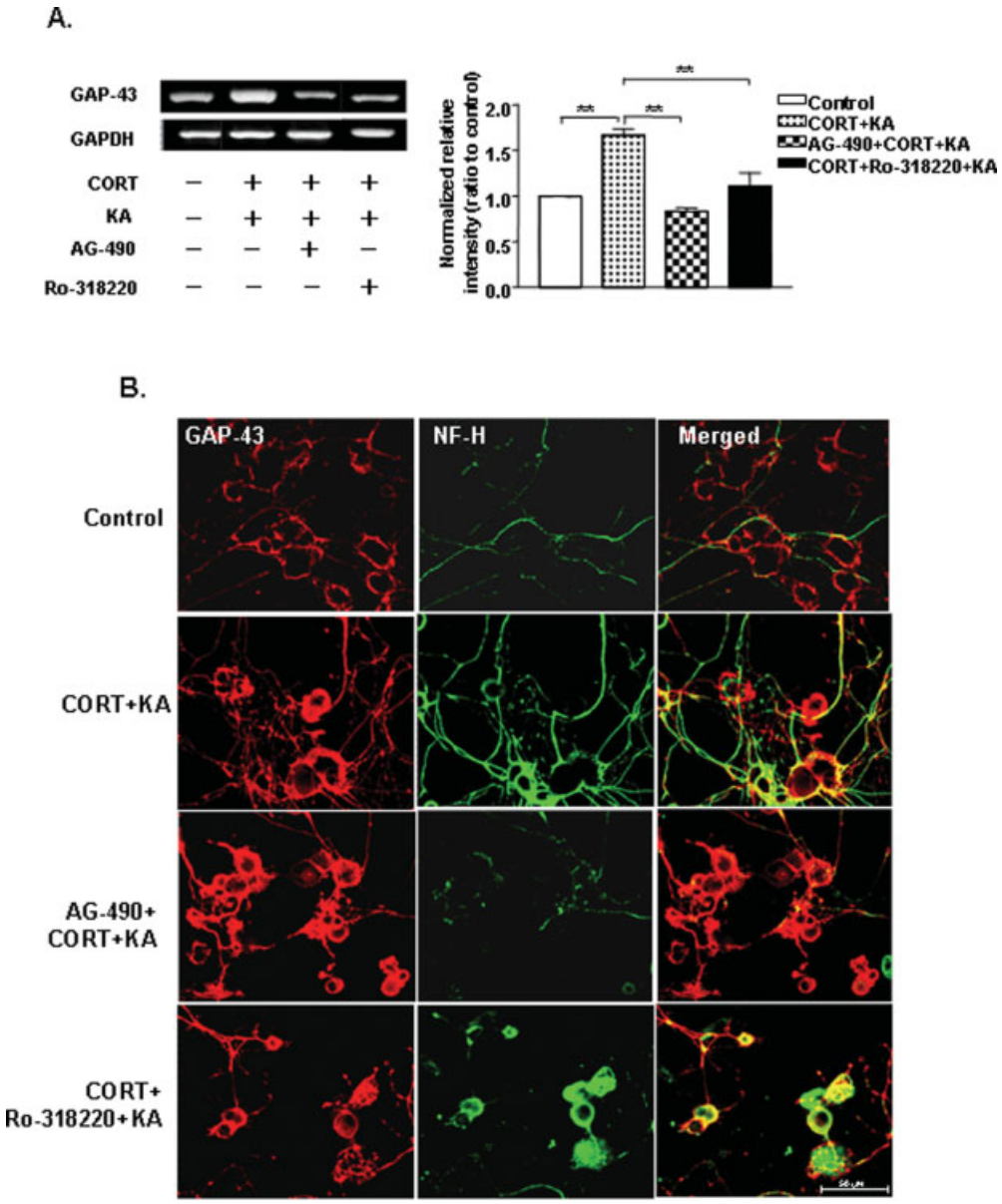

Fig. 2. Blockade of JAK and PKC activities attenuated CORT + KA-enhanced GAP-43 expression and axonal localization. **A:** Cultured DRG neurons were treated with CORT (10 μM at 1 DIV) and KA (1 mM at 2 DIV) in the presence or absence of AG-490 (30 μM) and Ro-318220 (1 μM). Total RNA extracted from DRG neurons at 3 DIV was used as a template for cDNA synthesis. The left panel shows the digitized gel image of the RT-PCR product of GAP-43 and GAPDH. Quantifications of the bands were normalized to GAPDH and the relative levels of GAP-43 were given as the ratio to control, as shown in the right panel. Data are expressed as the mean ± SEM (n = 3–5). $^{\star}P < 0.05$, $^{\star\star}P < 0.01$ compared with other groups by one-way ANOVA with Newman-Keuls multiple-comparisons post hoc test. **B:** Immunofluorescence double labeling of 3 DIV DRG neurons with GAP-43 and axonal marker neurofilament H (NF-H) was performed with mouse monoclonal antibody (anti-GAP-43) and rabbit polyclonal antibody (anti-NF-H). GAP-43 was further labeled with Cy5-conjugated anti-mouse IgG (red) and NF-H with FITC-conjugated anti-rabbit IgG (green). Pseudocolor in yellow indicates merged signal for colocalization of GAP-43 and NF-H. Scale bar = 50 μm.

CORT and KA, in which the neurite length was significantly longer than the vehicle-treated control, enhanced immunoreactivities of both GAP-43 and NF-H and their colocalization along neurites of DRG neurons. Blockade of JAK and PKC activities with AG-490 (30 μM) and Ro-318220 (1 μM) prior to CORT and KA treatment, respectively, reversed CORT + KA-enhanced immunoreactivities and colocalization of GAP-43 and NF-H. The results suggest that the sequential application of CORT and KA enhances GAP-43 gene expression and axonal localization and that the effects are mediated by JAK and PKC activities, respectively.

Blockade of PKC Activity Attenuated CORT + KA-Enhanced GAP-43 Phosphorylation in AX-DRG

Axonal localization of GAP-43 is known to be triggered by PKC phosphorylation, so we further hypothesized that KA treatment, in which PKC was found involved for its growth-promoting effect, could promote GAP-43 phosphorylation at Ser41. Acutely isolated DRG tissue was used to examine the PKC phosphorylation of GAP-43 2 hr after the KA treatment in Western blot analysis with anti-pSer41-GAP-43 antibody. Figure 3 shows that, with total GAP-43 protein levels among groups being constant, the PKC-phosphorylated form of GAP-43 was significantly increased in both the KA- and the CORT + KA treated groups but not in the CORT-only group. Application of the PKC inhibitor Ro-318220 prior to KA stimulation significantly reduced the CORT + KA-increased pSer41-GAP-43. The result suggests that KA-represented overexcitation may contribute to the growth-promoting effect in the regenerating DRG by mediating a PKC-dependent GAP-43 phosphorylation.

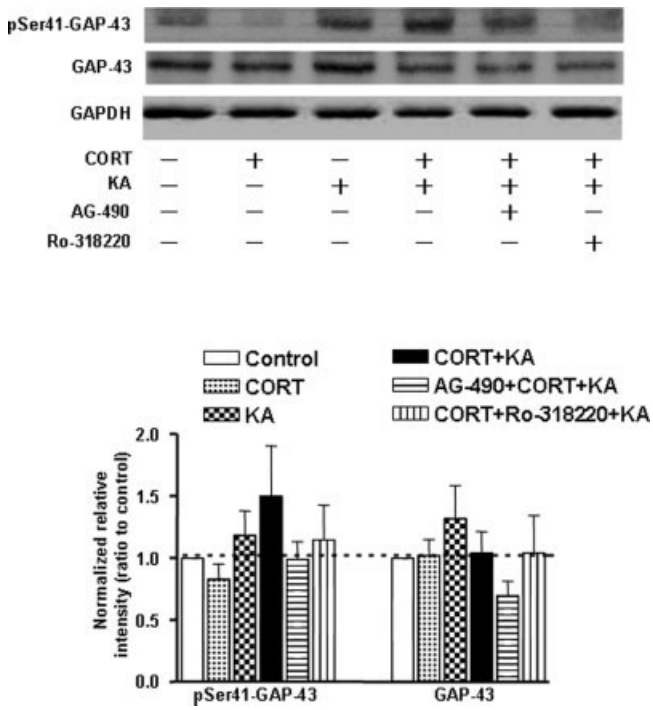

Fig. 3. Sequential application of CORT and KA enhanced GAP-43 phosphorylation in AX-DRG. Acutely isolated DRGs collected from naive rats were treated with 10 μM CORT for 2 hr, followed by 1 mM KA treatment for another 2 hr. JAK inhibitor AG-490 (30 μM) was applied 30 min before CORT, and the PKC inhibitor Ro-318220 (1 μM) was applied 30 min prior to KA application. At the end of treatment in each condition, the ganglia were homogenized and subjected to Western blot analysis with anti-GAP-43, anti-pSer41-GAP-43, and anti-GAPDH antibodies. The upper panel shows a representative blot result. The band intensity of each group was normalized by GAPDH, and values are given as the ratio to control as shown in the lower panel. Data are expressed as the mean ± SEM (n = 3–5). $\star P < 0.05$, $\star\star P < 0.01$ compared with other groups by one-way ANOVA with Newman-Keuls multiple-comparisons post hoc test.

Blockade of JAK Activity Attenuated CORT + KA-Enhanced Phosphorylation of STAT3 at Ser727 in AX-DRG

The data described above revealed that JAK activity is involved in the effect of CORT on its synergy with KA for promoting neurite growth. As reviewed in the introductory paragraphs, STAT3 is one of the major JAK-downstream transcription factors related to neuronal survival and growth and can also be phosphorylated by PKC. Therefore, we further examined how CORT and KA could affect phosphorylation of STAT3 at its JAK and PKC phosphorylation sites. Acutely isolated DRGs from rats were treated with CORT for 2 hr, followed by KA treatment for another 2 hr, and the tissue was subjected to total protein extraction and Western blot to analyze phosphorylations of STAT3 at Tyr705 and Ser727. Surprisingly, the results showed that the level of JAK-phosphorylated form of STAT3, indicated as pTyr705-STAT3, was enhanced by KA but not by CORT (Fig. 4). KA-mediated enhancement of pTyr705-STAT3 was reversed by pretreatment of cultures with CORT. Interestingly, the level of the PKC-phosphorylated form of STAT3, indicated as pSer727-STAT3, was profoundly increased in the CORT + KA group, but not by separate CORT or KA treatment. Both the JAK inhibitor AG-490 and the PKC inhibitor Ro-318220 reversed CORT + KA-increased pSer727-STAT3, but neither of these two inhibitors further suppressed the pTyr705-STAT3 in the CORT + KA group. These results indicate that the JAK-dependent STAT3 phosphorylation was elevated by the overexcitation mimicked by the KA treatment but was attenuated by GC. However, sequential application of CORT and KA profoundly enhanced PKC-dependent STAT3 phosphorylation in a synergistic manner, which correlates well with the synergy noted with these two agents in promotion of neurite growth.

PKC-Dependent Phosphorylation of GAP-43 and STAT3 Phosphorylation in DRG of Acute SCI Rats Treated With MP

We further examined whether the above-mentioned phenomena occur in the DRG in an animal model of SCI. An animal model of acute SCI with an NYU impactor was used to examine the effect of impact lesion on the phosphorylation of GAP-43 and STAT3 in injured DRG. Weight drop was applied at T10 of the spinal cord, and DRGs from T8–T11 were harvested for Western blot analysis. Figure 5A shows a profound increase in GAP-43 phosphorylation but only a slight increase in GAP-43 protein level in injured DRG 4 hr after the injury compared with the sham control group. For the phosphorylation of STAT3, the PKC-dependent

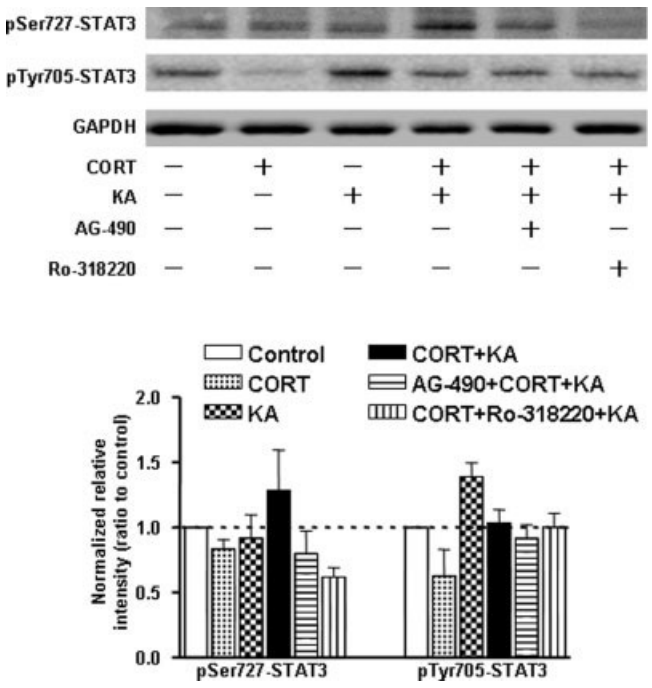

Fig. 4. Sequential application of CORT and KA enhanced the PKC-dependent phosphorylation of STAT3. Acutely isolated DRGs collected from naive rats were treated with 10 μM CORT for 2 hr, followed by 1 mM KA treatment for another 2 hr. The JAK inhibitor AG-490 (30 μM) was applied 30 min before CORT, and the PKC inhibitor Ro-318220 (1 μM) was applied 30 min prior to KA application. At the end of treatment in each condition, the ganglia were homogenized and subjected to Western blot analysis with anti-pTyr705-STAT3, anti-pSer727-STAT3, and anti-GAPDH antibodies. The upper panel shows a representative blot result. The band intensity of each group was normalized by GAPDH, and values are given as the ratio to control as shown in the lower panel. Data are expressed as the mean ± SEM (n = 3–5). $\star P < 0.05$, $\star\star P < 0.01$ compared with other groups by one-way ANOVA with Newman-Keuls multiple-comparisons post hoc test.

pSer727-STAT3 but not the JAK-dependent pTyr705-STAT3 was profoundly increased in injured DRG, with the total STAT3 remaining unchanged (Fig. 5B). The results correlate well with the ex vivo results seen in Figures 3 and 4, suggesting that injury of rat DRG by acute SCI, as was simulated by CORT and KA in vitro and ex vivo, can enhance PKC-dependent phosphorylation of GAP-43 and STAT3.

MP is the only FDA-approved agent used in treating acute SCI, so we further examined its effect on these PKC-dependent events. Figure 5A shows that the application of MP (30 mg/kg, i.v.) after SCI (SCI + MP) profoundly enhanced GAP-43 phosphorylation but had negligible effects on the protein level of GAP-43. In contrast, the pSer727-STAT3 level was lower in the SCI + MP group compared with the untreated SCI group. No significant effect of MP on pTyr705-STAT3 was observed (Fig. 5B). The results suggest that MP therapy may facilitate injury-induced GAP-43 phosphorylation but suppress injury-enhanced PKC phosphorylation of STAT3 in injured DRG.

PKC-Dependent Phosphorylation of GAP-43 and STAT3 Phosphorylation in the Spinal Cord of Acute SCI Rats Treated With MP

Finally, we assessed whether the spinal cords of the SCI rats exhibit changes in PKC-dependent phosphorylation of GAP-43 and STAT3 similar to those of injured DRG. Tissue from the injury site (5 mm around the impact point) in the spinal cord of SCI rats was harvested for Western blot analysis. Figure 6A shows that both the expression and the phosphorylation of GAP-43 were significantly elevated in injured spinal cords 4 hr after injury compared with the sham control group. As with injured DRG, MP treatment of the injured spinal cord enhanced GAP-43 phosphorylation, but without down-regulation of the GAP-43 expression. In contrast, the pSer727-STAT3 level was significantly elevated in injured spinal cords 4 hr after injury compared with the sham control group, but treatment of MP did not change the pSer727-STAT3 level induced by injury (Fig. 5B). However, no significant differences in STAT3 and JAK-dependent pTyr705-STAT3 levels were observed among the three groups. This result indicates that spinal cord is similar to injured DRG in injury- and MP-induced up-regulation of GAP-43 phosphorylation, whereas changes in STAT3 phosphorylation are different between spinal cord and DRG.

DISCUSSION

The present study used CORT and KA to mimic stress-related neurotrauma and showed that the neurotrophic effects of these agents on neurite growth are mediated, at least in part, by sequential activity from the JAK and PKC signaling pathways. This growth-promoting effect correlates well with the enhancement of PKC-dependent phosphorylation of GAP-43 and STAT3. The PKC-dependent phosphorylation of GAP-43 is associated with the application of KA, but PKC-dependent phosphorylation of STAT3 requires the synergistic effect of the CORT-KA combination. Possible mechanisms are discussed below.

The PKC-dependent expression and phosphorylation of GAP-43 were increased in CORT + KA-simulated stress-injury conditions of both cultured DRG and in an animal model of acute SCI. The immunoreactive pattern of GAP-43 along neurites shown in Figure 2 was correlated well with its phosphorylation, as shown in Figure 3. This observation coincides with the PKC-dependent phosphorylation of GAP-43, resulting in activation and translocation of GAP-43 to extend neurites. Furthermore, GAP-43 mRNA was profoundly elevated by CORT + KA treatment in a PKC-dependent manner, which is in agreement with another study on the transcriptional mechanisms of GAP-43 (Perrone-Bizzozero et al., 1993). It is noted that not only a PKC

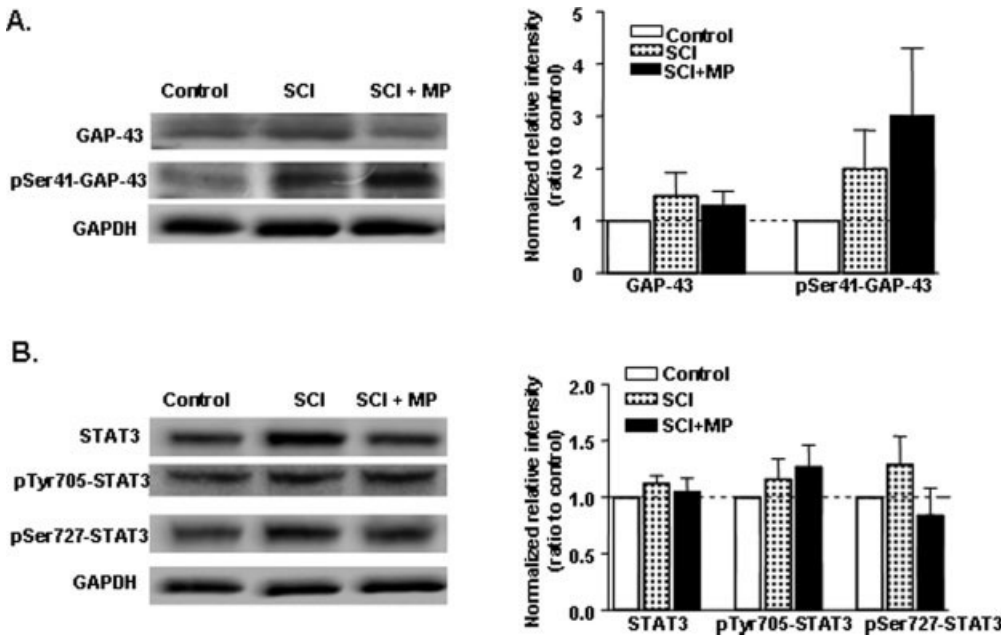

Fig. 5. Expression and phosphorylation of GAP-43 and STAT3 in DRG of SCI rats. Acute SCI was induced in rats by the NYU impactor. Exposed dorsal cord at T9–T10 level was subjected to weight-drop impact (weight of 10 g, height of 25 mm). Immediately after the injury, rats received either vehicle or MP (i.v. 30 mg/kg). Four hours after treatment, DRGs from T8–T11 were harvested for protein extraction. The levels of GAP-43 and pSer41-GAP-43 (**A**) and STAT3, pTyr705-STAT3, and pSer727-STAT3 (**B**) were analyzed by Western blot analysis. The band intensity of each group was normalized by GAPDH, and the relative intensity was obtained by the ratio to control. The DRG tissue of each rat were analyzed separately, and three to five rats were used in each experimental condition. Data are expressed as the mean ± SEM (n = 3–5). $\star P < 0.05$, $\star\star P < 0.01$ compared with other groups by one-way ANOVA with Newman-Keuls multiple-comparisons post hoc test. Control, sham control with vehicle; SCI, SCI with vehicle; SCI + MP, SCI with MP.

inhibitor but also a JAK inhibitor reduced the PKC-phosphorylated form of GAP-43 after CORT + KA treatment, suggesting that cross-talk exists between the JAK and the PKC signaling pathways.

We observed that treatment with a JAK inhibitor, but not a PKC inhibitor, attenuated CORT + KA-promoted neurite growth when applied prior to CORT. However, JAK-dependent STAT3 phosphorylation was suppressed by CORT, suggesting that the JAK activity needed for neurite growth may not be through STAT3. Alternatively, STAT3 may be transactivated by CORT and other GCs via the GR without further phosphorylation (Zhang et al., 1997). In this case, the essential role of JAK activity may function to maintain sufficient amounts of phosphorylated STAT3 capable of being transactivated. On the other hand, CORT suppression of STAT phosphorylation is known to occur through a rather indirect pathway via GR-dependent trans-repression of proinflammatory cytokine gene expression, and this subsequently reduces the JAK/STAT signaling mediated through the receptors of these cytokines (Bianchi et al., 2000). A recent study shows that dominant negative STAT3 significantly blocks 5-HT_{1A} receptor-mediated neurite outgrowth in SK-N-SH cells (Fricker et al., 2005). Whether the glucocorticoid enhancement of neurite growth is mediated via GR-STAT3 trans-activation remains to be elucidated.

Excitatory glutamate in CSF is highly elevated after acute SCI, and this may contribute to the secondary neuronal death in both gray matter and white matter resulting from excitotoxicity (Park et al., 2004). Our previous work also demonstrated that KA was neurotoxic to cultured DRG neurons if applied later than 3 DIV, but application as early as 2 DIV was not toxic (Tsai et al., 2002). Interestingly, in this study, we found that JAK-dependent phosphorylation of STAT3 was enhanced by KA in isolated AX-DRG in vitro, suggesting that overexcitation may directly induce an inflammatory signal in neurons as reported in other study (Choi et al., 2003). Because JAK-phosphorylated STAT3 can be further phosphorylated by PKC at Ser727, it is likely that the enhanced pSer727-STAT3 in CORT + KA-treated DRG, as well as in SCI-injured DRG, is mediated by both JAK and PKC activities.

In this study, we demonstrate that both the expression and the phosphorylation of GAP-43 are not only induced by SCI but also further enhanced by MP treatment. Although the effect of MP on GAP-43 expression is somewhat opposite in DRG to the effect in the spinal cord tissue, the up-regulation of GAP-43 phosphorylation found in both tissues suggests that glucocorticoid therapy might indeed improve nerve regeneration-related protein functions. GC therapy has been widely used for the treatment of acute SCI, but the high dosage

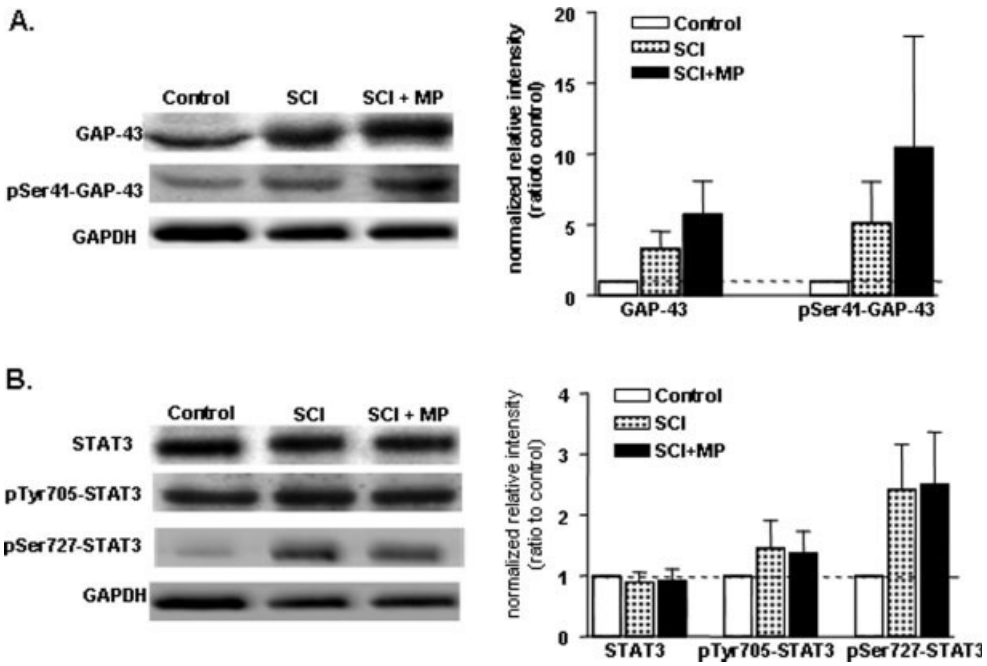

Fig. 6. Expression and phosphorylation of GAP-43 and STAT3 in the spinal cord of SCI rats. The spinal cord tissue with a 5-mm section around the impact site was harvested from acute SCI rats prepared as described in Materials and Methods. Total protein was extracted, and the levels of GAP-43 and pSer41-GAP-43 (**A**) and of STAT3, pTyr705-STAT3, and pSer727-STAT3 (**B**) were analyzed by Western blot analysis. The band intensity of each group was normalized by GAPDH, and the relative intensity was obtained by the ratio to control. The spinal cord tissue of each rat was analyzed separately, and three to five rats were used in each experimental condition. Data are expressed as the mean ± SEM (n = 3–5). $\star P < 0.05$, $\star\star P < 0.01$ compared with other groups by one-way ANOVA with Newman-Keuls multiple-comparisons post hoc test. Control, sham control with vehicle; SCI, SCI with vehicle; SCI + MP, SCI with MP.

of MP used has been questioned because of its associated side effects (Gomes et al., 2005). Indeed, a recent study shows that decreased GAP-43 expression was accompanied by decreased GR expression in the amygdala of fetal alcohol exposure-associated offspring who demonstrate behavioral and cognitive dysfunctions (Wilcoxon et al., 2005). On the other hand, GCs were also found to regulate neurite growth in connected neuronal populations by reducing amyotrophic lateral sclerosis-associated GAP-43 hyperexpression (González Deniselle et al., 2001). Therefore, the window of time following nerve injury and the maturation status of neurons are indeed critically important for the GC effect on GAP-43 gene expression.

In summary, we demonstrate in both isolated DRG and an animal model of acute SCI that the two neurotrauma-induced components, stress response-related GC and overexcitation, could enhance neurite outgrowth via JAK and PKC activities. The PKC-dependent phosphorylation of GAP-43 and STAT3 is elevated by injury and correlated well with neurite growth. Further GC therapy in SCI rats indeed enhances the PKC-dependent phosphorylation of GAP-43, but not of STAT3. The underlying molecular mechanism of the JAK/PKC signaling in GC-mediated nerve regeneration remains to be elucidated.

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