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Methylprednisolone Inhibits the Expression of Glial Fibrillary Acidic Protein and Chondroitin Sulfate Proteoglycans in Reactivated Astrocytes

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KEY WORDS
central nervous system; chondroitin sulfate proteoglycans; gliosis; glucocorticoid; spinal cord injury

ABSTRACT
Reactive gliosis caused by post-traumatic injury often results in marked expression of chondroitin sulfate proteoglycan (CSPG), which inhibits neurite outgrowth and regeneration. Methylprednisolone (MP), a synthetic glucocorticoid, has been shown to have neuroprotective and anti-inflammatory effects for the treatment of acute spinal cord injury (SCI). However, the effect of MP on CSPG expression in reactive glial cells remains unclear. In our study, we induced astrocyte reactivation using α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and cyclothiazide to mimic the excitotoxic stimuli of SCI. The expression of glial fibrillary acidic protein (GFAP), a marker of astrocyte reactivation, and CSPG neurocan and phosphacan were significantly elevated by AMPA treatment. The conditioned media from AMPA-treated astrocytes strongly inhibited neurite outgrowth of rat dorsal root ganglion neurons, and this effect was reversed by pretreatment with MP. Furthermore, MP downregulated GFAP and CSPG expression in adult rats with SCI. Additionally, both the glucocorticoid receptor (GR) antagonist RU486 and GR siRNA reversed the inhibitory effects of MP on GFAP and neurocan expression. Taken together, these results suggest that MP may improve neuronal repair and promote neurite outgrowth after excitotoxic insult via GR-mediated downregulation of astrocyte reactivation and inhibition of CSPG expression. © 2008 Wiley-Liss, Inc.

INTRODUCTION

Astrocytes are the most abundant non-neuronal cells in the central nervous system (CNS). Astrogliosis is the characteristic reactive response of astrocytes to almost all neurological insults and diseases (Eddleston and Mucke, 1993; Norenberg, 1994; Ransom et al., 2003). Glial fibrillary acidic protein (GFAP) expression by astrocytes is a prototypic marker of astroglial activation (Bignami and Dahl, 1974; Bignami et al., 1972; Dahl and Bignami, 1974, 1976) and a characteristic response to inflammation after traumatic injury (Balasingam et al., 1994; Fitch and Silver, 1997a). In addition, reactive astrogliosis generates increased expression of extracellular matrix (ECM) molecules, including chondroitin sulfate proteoglycans (CSPGs); in turn, CSPG overexpression is linked to neuronal cell death, necrosis, and glial scar formation, which inhibits neurite outgrowth and regeneration (Fitch and Silver, 1997b; Hausmann, 2003; McKeon et al., 1991; Snow et al., 1990).

CSPGs, a class of glyco-conjugates, are composed of a core protein and at least one repetitive glucuronic acid and N-acetylgalactosamine disaccharide unit glycosaminoglycan (GAG) chain, which is covalently attached to the core protein. The CSPGs, neurocan, phosphacan, versican, brevican, and NG2 present in glial scars that inhibit axon growth are secreted by reactivated astrocytes after CNS injury (Asher et al., 2000; Haas et al., 1999; Jones et al., 2003; McKeon et al., 1999; Morgenstern et al., 2002). Removal of chondroitin sulfate (CS) may block the inhibitory effect of CSPGs on neurite outgrowth. Specifically, recent studies have shown that axonal regeneration in the spinal cord can be promoted by degradation of CS with chondroitinase ABC (Barritt et al., 2006; Bradbury et al., 2002; Yick et al., 2003). However, nonselective removal of CS from CSPG may impair functional recovery. CSPGs are expressed in axon pathways in vivo during development, suggesting that intact CSPGs may be required in conjunction with growth-promoting ECM to guide axonal pathfinding (Mace et al., 2002; Powell and Geller, 1999; Snow et al., 2003).

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Methylprednisolone (MP), a synthetic glucocorticoid (GC), is the only FDA-approved therapeutic agent for the treatment of acute spinal cord injury (SCI) (Bracken, 1990, 1991; Bracken et al., 1992; Young and Bracken, 1992). MP reduces the development of severe edema and preserves spinal cord architecture adjacent to the site of injury (Bracken et al., 1992). In addition, inhibition of lipid peroxidation (Anderson et al., 1985; Hall and Braughler, 1981) and inflammatory reactions (Bartholdi and Schwab, 1995; Constantini and Young, 1994; Hsu and Dimitrijevic, 1990) after SCI are thought to contribute to the therapeutic effects of MP. The suppression of inflammation by MP, including the reduction of pro-inflammatory cytokine production (Xu et al., 1998) and microglia-dependent activation of encephalitogenic T cells (Magnus et al., 2001), is believed to be mediated by the glucocorticoid receptor (GR). It has been demonstrated that GR activation results in reduced expression of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1), two major inflammation-related transcription factors (Cato and Wade, 1996; Jonat et al., 1990; Smoak and Cidlowski, 2004; Xu et al., 1998, 2001; Yang-Yen et al., 1990).

The effect of MP on the glial response in terms of ECM expression after SCI is unknown. In our study, we established an *in vitro* reactivated astrocyte model using α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and cyclothiazide induction to mimic the acute phase of CNS injury. We also investigated the effect of MP on astrocyte reactivation and the expression of GFAP and CSPG after MP treatment in adult rats with SCI.

MATERIALS AND METHODS

Materials

The polyclonal antibody against rat GFAP was obtained from Sigma (St. Louis, MO). Monoclonal antibodies against neurocan (clones 1F6 and 1D1) and phosphacan (clone 3F8) were obtained from the Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa (Iowa City, IA). The monoclonal antibody against CS (CS56) was purchased from Sigma. Chondroitinase ABC (Chase ABC) from *Proteus vulgaris* was obtained from Sigma. All chemicals were purchased from Sigma unless otherwise stated.

Primary Culture of Rat Astrocytes

The preparation of primary cultured astrocytes was based on the protocol for glial cell isolation described by Levison et al. (Levison and McCarthy, 1991) with some modifications. Brains from Sprague-Dawley rats (National Laboratory Animal Center, Taipei, Taiwan) (1- to 2-days-old) were isolated and homogenized in DMEM/F12 medium with 10% fetal bovine serum (FBS). The homogenate was filtered through 80-μm nylon mesh. The supernatant, which contained intact, dispersed cells, was incubated in 75-mm flasks (1.5 brains/flask) until cells were confluent. Microglia and oligodendrocytes were removed by shaking at 100 rpm for 8 h and 180 rpm for ~18–20 h, respectively. Immunocytochemistry showed that ~90% of the remaining cells were GFAP-positive astrocytes. The astrocytes were then cultured with DMEM/F12 medium containing 10% FBS in the CO_2 tissue culture incubator at 37°C for use in the following experiments.

Astrocytes Reactivation by Cotreatment with AMPA and Cyclothiazide

To develop an *in vitro* reactivated astrocyte cell model, we used cotreatment with AMPA and cyclothiazide to induce astrocyte reactivation. AMPA is neurotoxic (Deupree et al., 1996; Koh and Choi, 1987), and a high dose of AMPA (500 μM) in the presence of cyclothiazide (100 μM) is rapidly (within 2 h) lethal to astrocytes (David et al., 1996). Therefore, we used a lower dosage of AMPA to induce astrocyte reactivation in our study. Primary cultured astrocytes (1×10^5 cell/well in a 24-well culture plate) at 1 day *in vitro* (DIV) were exposed to 0, 100, 200, and 300 μM AMPA in the presence or absence of 100 μM cyclothiazide, a drug that selectively blocks AMPA receptor desensitization and thus extends AMPA activity. The mRNA levels of GFAP and CSPG neurocan were analyzed by RT-PCR to determine astroglial activation. The viability of the reactivated astrocytes treated with AMPA and cyclothiazide was determined by a MTT assay and by lactate dehydrogenase (LDH) release.

Cell Viability Assay

Cell viability was assessed by the MTT and LDH assays. Viable cells were assessed by the MTT assay, which measures mitochondrial dehydrogenase activity (Mosmann, 1983). Briefly, astrocytes were incubated with the MTT solution (30 μL of 5 mg/mL MTT, added to 220 μL PBS/well) for 4 h at 37°C. Isopropanol (500 μL/well) was added, and cells were incubated for 16 h at RT. Aliquots (150 μL/well) were transferred into a 96-well plate and absorbance was read at 550 nm. Four replicates were performed for each examined group.

In addition, cell death was quantified by measuring LDH, which was released by the reactivated astrocytes, in the culture media (Koh and Choi, 1987). β-NADH, 0.1 mg in 1.2 mL phosphate buffer, 1 mM KH_2PO_4, and 3 mM Na_2HPO_4, pH 7.4, was added to 250 μL of conditioned medium taken from each well, mixed by vortexing, and then incubated for 3 min at room temperature. Following the addition of sodium pyruvate (50 μL/well; 22.7 mmol in phosphate buffer), the incubated solution was transferred to a UV cuvette. The absorbance was read at 340 nm every 30 s for 2 min. The absorbance should decrease over time. The amount of released LDH (units per mL) was determined by averaging the changes in OD over 1 min multiplied by 1,000. Each assay was performed in triplicate.

TABLE 1. Primers Used for the Polymerase Chain Reactions

Gene	Primer Sequence	Fragment Amplified (bp)
GFAP	Forward: 5′-GCCGCT CCT AT GCCTCCTCC GA-3′ Reverse: 5′-TCCAGCGACTCAACC TTCCTCT-3′	548
Neurocan	Forward: 5′-ATCGCAGTTGTCAAAGCCAT-3′ Reverse: 5′-CAGAGCCAATGCTACACTTCTGT-3′	265
Phosphacan	Forward: 5′-TTGACAAGTGATGAAGAGAGTGG-3′ Reverse: 5′-AATCAGCACATCTCGTTCTATCC-3′	256
GAPDH	Forward: 5′-AACTCCCTCAAGATTGTCAGCA-3′ Reverse: 5′-TCCACCACCCTGTTGCTGTA-3′	603

MP Treatment of Reactivated Astrocytes

To study the effect of MP on reactivated astrocytes, astrocytes (1×10^5 cell/well) in a 24-well plate were preincubated with 0, 0.1, 1, 10, and 50 μM MP for 30 min prior to AMPA/cyclothiazide treatment for 48 h. To study the regulatory mechanism of MP, an antagonist of glucocorticoid receptors, RU486 (100 nM), was added to the reactivated astrocytes along with MP and incubated for 48 h. Each experiment was performed at least in duplicate and was carried out using at least three different astrocyte cultures.

Spinal Cord Injury in Rats

The protocol to generate a rat SCI model was developed by Xu et al. (2001). Female Long Evans rats (National Laboratory Animal Center, Taipei, Taiwan) were used in these experiments. Rats were anesthetized by an intra-peritoneal injection of a ketamine and xylazine mixture (50 mg/10 mg per kg body weight, respectively) before surgery. For SCI surgery, spinal clamps were attached to the spinous processes of T8 and T11. Laminectomy was performed at T9 and T10 using the NYU impactor device with a 10-mg rod that was lifted 12.5 mm above the T10 and dropped directly onto the center of T10. For sham surgery, the procedures were the same as those described for SCI surgery, except that the spinal cord was not damaged. The surgical area was closed with wound clips. SCI postoperative care was performed every 8 h until sacrifice, and included regular bladder expression, supplemental administration of Ringers solution and food, cleaning of the paralyzed lower body, and general inspection of the animals' health at the animal core facility at Taipei Medical University.

MP Treatment of Rats with SCI

MP was administered intravenously (30 mg/kg body weight) to rats immediately after SCI surgery (Xu et al., 2001). Animals were sacrificed 8 and 24 h following MP treatment. The experimental group included SCI animals treated with vehicle, SCI animals treated with MP, and sham controls treated with vehicle. Each group contained three rats to fulfill the minimal data requirement for statistical analysis. Approval for the animal studies was obtained from the Institutional Animal Care and Use Committee at Taipei Medical University.

Reverse-Transcription Polymerase Chain Reaction

Total RNA was prepared from cultured astrocytes and from the tissue surrounding the injury site in SCI rats using the RNeasy Mini kit (Qiagen, Valencia, CA). mRNA was then reverse-transcribed into cDNA using reverse transcriptase (OneStep RT-PCR Kit, Qiagen). PCR analyses were performed using the appropriate primers in an Applied Biosystems GeneAmp PCR 2400 (Foster City, CA). The primers used in the PCR reactions are listed in Table 1. The PCR for GFAP was 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 cycles; for neurocan and phosphacan, the PCR conditions were 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min for 30 cycles. GAPDH was used as the loading control. Reaction products were analyzed on 1.5% agarose-TAE gels, and the resolved bands were measured quantitatively using Scion image analysis software (Frederick, MD).

Immuno-Dot Blot

The expression of CS by reactivated astrocytes was analyzed using immuno-dot blots. Total protein (100 μg) from the conditioned media from astrocyte cultures was absorbed onto a PVDF membrane (Millipore) by mild vacuum pumping. Following blocking with 5% skim milk in tris-buffered saline (TBS, 50 mM Tris and 0.5 M NaCl, pH 8.0), the membrane was incubated with a monoclonal antibody against CS (CS56, 1:1,000) in TBS with 0.1% skim milk for at least 1 h at 4°C. After three 10-min washes with TBST (TBS with 0.1% Tween-20), the blot was soaked in a solution containing alkaline phosphatase-conjugated rabbit anti-mouse IgG/M (Pierce, Rockford, IL) secondary antibody (1:3,000) in TBS with 0.1% skim milk for 1 h at room temperature. After extensive washing with TBST, the blot was incubated with NBT/BCIP for colorimetric development. The resulting dots were scanned for quantitative measurement of the intensity of each immunoreactive dot using image analysis software (Scion).

Isolation of Adult Rat DRG Neurons

DRG neurons were isolated from the spinal columns of adult Sprague–Dawley rats weighing ~240–260 g after the rats were sacrificed by pentobarbital anesthesia (80 mg/kg, IP). After incubation with collagenase and

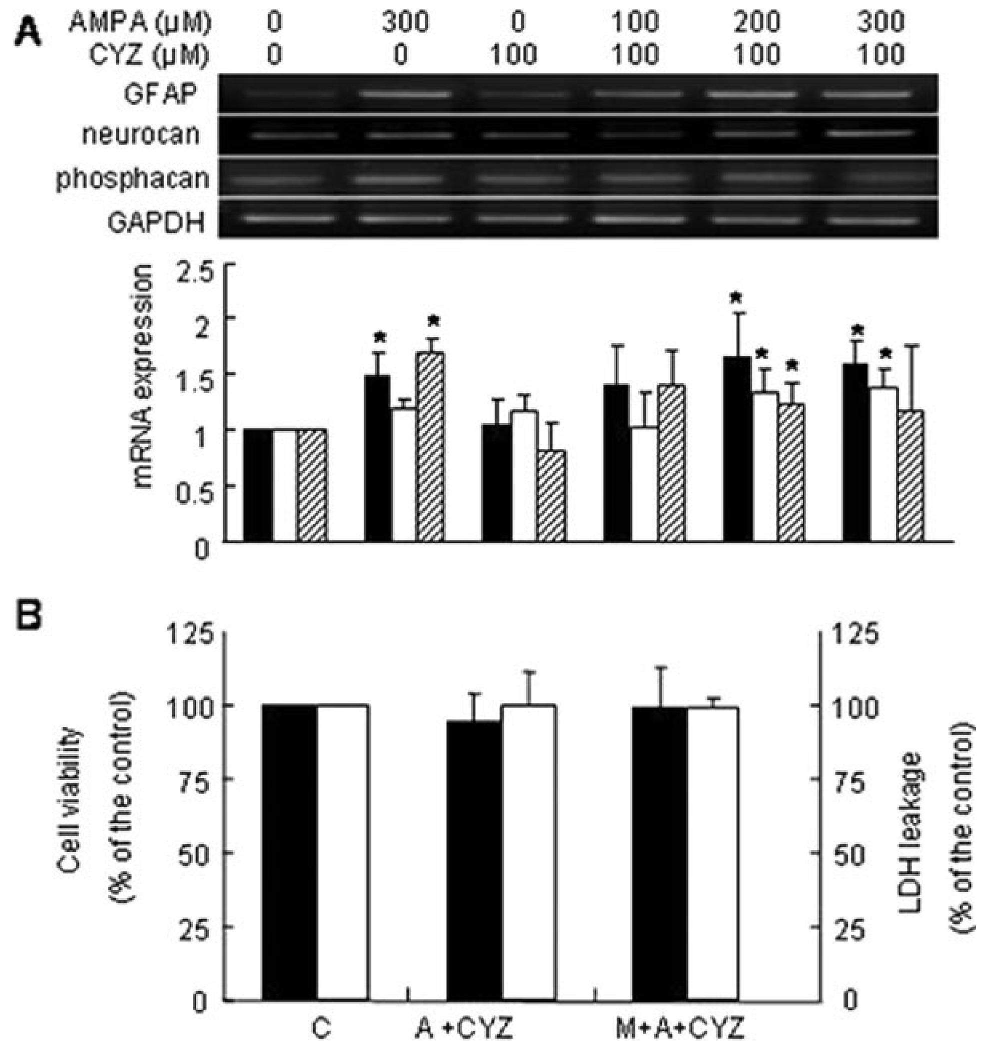

Fig. 1. Treatment of primary cultured astrocytes with AMPA and cyclothiazide (CYZ). **A**: mRNA levels of glial fibrillary acidic protein (GFAP) (black bars), chondroitin sulfate proteoglycan (CSPG) neurocan (clear bars), and phosphacan (hatched bars) were determined by RT-PCR as indicators of astrocyte reactivation by different dosages of AMPA (0–300 μM) in the absence or presence of CYZ (100 μM). GAPDH expression was used as a loading control. *Significantly different ($P < 0.05$) compared with control cells that received no treatment. **B**: Cell viability of cultured astrocytes assessed by measuring MTT (black bars) and LDH release (clear bars) as described in the Materials and methods section. C, control cells; A+CYZ, cells treated with 200 μM AMPA and 100 μM cyclothiazide; M+A+CYZ, cells treated with MP (10 μM) prior to AMPA (200 μM) and cyclothiazide (100 μM). The data represent the mean ± SD of three independent experiments performed in triplicate.

trypsin, the ganglia were subjected to density gradient centrifugation with 30% Percoll (Fluka, St. Gallen, Switzerland) to eliminate the myelin sheaths. The dissociated DRG neurons were then suspended in serum-containing medium (F12 supplemented with 5% FBS), and aliquots of the DRG neurons were seeded onto polylysine-coated wells for 24 h to allow for adhesion.

Assays for DRG Neurite Extension

For the neurite extension experiments, the seeded DRG neurons were incubated with astrocyte-conditioned media for 24 h in a CO_2 incubator at 37°C. The DRG neurites were then observed by immunofluorescence staining. Cells were fixed with 4% paraformaldehyde in PBS for 15 min. After two washes with PBS, ice-cold 95% ethanol and 5% methanol (1 mL/well) were added to fixed cells for 15 min for permeabilization. Following two washes with PBS, cells were blocked with 5% BSA in TBS for 30 min at room temperature and then incubated with a monoclonal anti-β tubulin antibody (clone SDL.3D10, 1:200 in 1% skim milk in TBS, Sigma) for 24 h at 4°C. After two washes with PBS, cells were incubated with FITC-conjugated AffiniPure Goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA), 1:200 in 1% skim milk in TBS for 24 h at 4°C. After two washes with PBS, cells were air-dried and incubated with one drop of mounting medium (Vector Laboratories, Burlingame, CA) for 5 min at 80°C, and then observed under a fluorescence microscope. The number of neurite-bearing cells was counted; such cells were defined as neurons with neurite length that was longer than the diameter of the cell body. The average of the longest neurite length of each neuron, defined as the neurite length, was also measured under the microscope with the help of a measuring eyepiece.

Western Blot Analysis

GFAP expression was analyzed in astrocyte cell lysate and CSPG expression was analyzed in astrocyte culture medium. GAPDH expression was used as the loading control. To analyze CSPG expression, conditioned me-

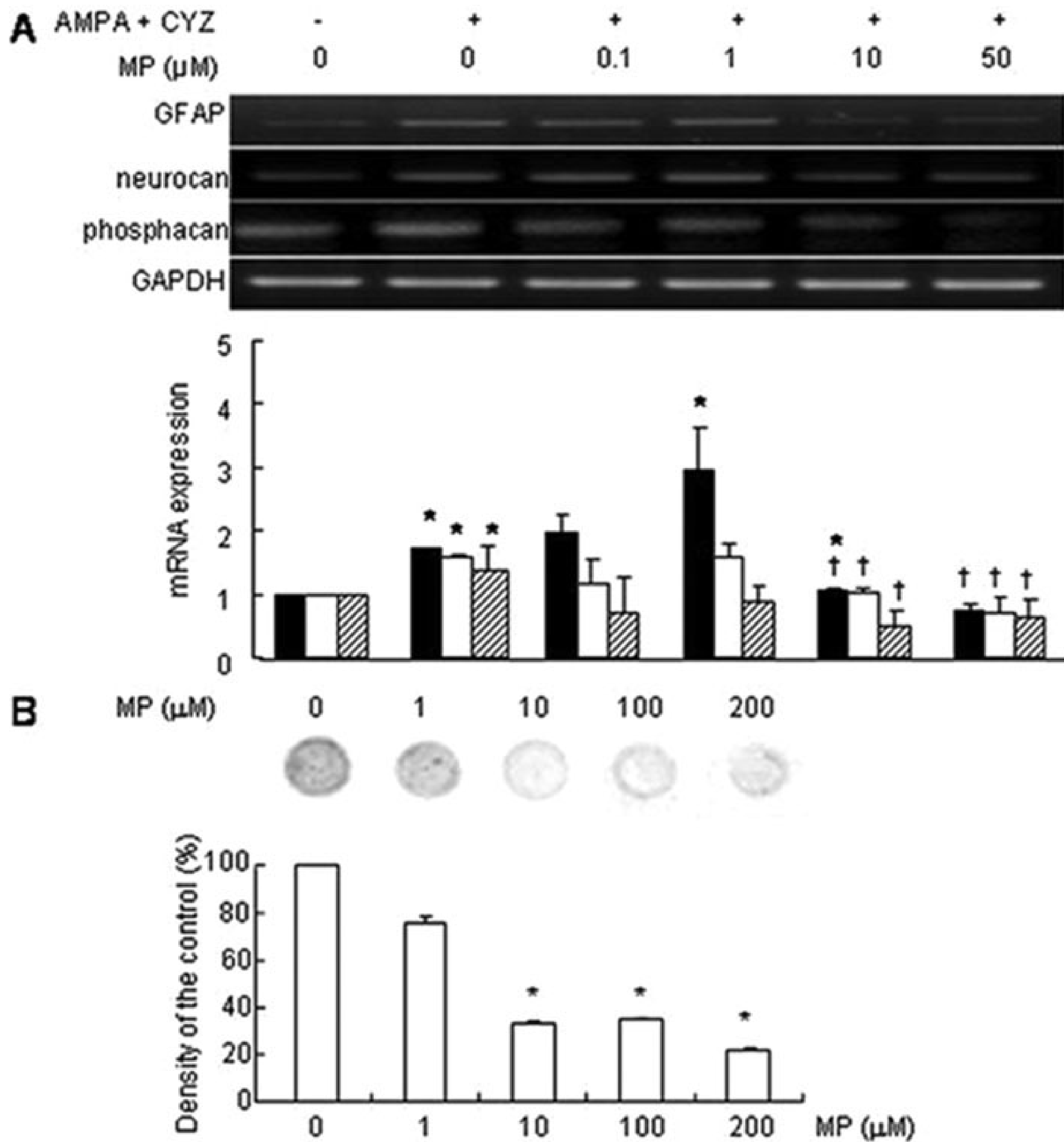

Fig. 2. Treatment of primary cultured astrocytes with methylprednisolone (MP). **A**: mRNA levels of glial fibrillary acidic protein (GFAP) (black bars), chondroitin sulfate proteoglycan (CSPG) neurocan (clear bars), and phosphacan (hatched bars) were determined by RT-PCR in AMPA (200 μM) plus cyclothiazide (CYZ) (100 μM)-induced reactivated astrocytes pretreated with different dosages of MP (0–50 μM). * Significantly different ($P < 0.05$) compared with control cells that received no treatment. $^{+}$Significantly different ($P < 0.05$) compared with cells treated with AMPA+CYZ. **B**: Immuno-dot analysis of the expression of total chondroitin sulfate (CS) secreted by cultured primary astrocytes treated with different dosages of MP (0–200 μM). The inhibition of CS expression by MP was dose-dependent, and the addition of MP >10 μM showed the most potent inhibition. *Significantly different ($P < 0.05$) compared with control cells that received no treatment. The data represent the mean ± SD of three independent experiments performed in triplicate.

dium (500 μL) was preincubated with 0.1 unit Chase ABC at 37°C for 3 h and then concentrated by Centricon-100 (Millipore, Bedford, MA) for Western blot analysis. Whole-cell lysate, or an equal amount of total protein from the conditioned media as determined by a protein assay kit (BCA protein assay, Pierce, Rockford, IL), was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting onto a PVDF membrane (Millipore). After blocking with 5% skim milk in TBS for 1 h, the blot was incubated with a monoclonal antibody against rat GFAP, neurocan (1F6 or 1D1), or phosphacan (3F8) (1:1,000) in TBS with 0.1% skim milk for at least 1 h at 4°C. After three 10-min washes with TBST, the blot was soaked in a solution containing an alkaline phosphatase-conjugated goat anti-rat IgG secondary antibody (1:3,000) in TBS with 0.1% skim milk for 1 h at room temperature. After extensive washing with TBST, the blot was incubated with NBT/BCIP for colorimetric development. The developed membrane image was scanned, and the inten-

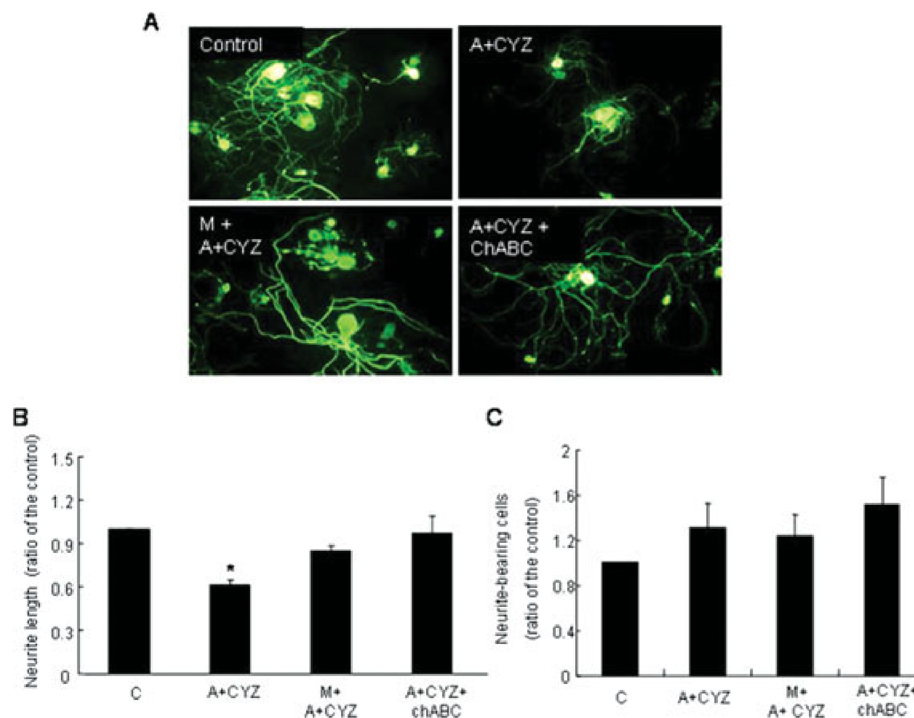

Fig. 3. Neurite outgrowth of rat dorsal root ganglia (DRG) neurons cultured in conditioned medium from reactivated astrocytes. **A**: Immunofluorescence staining of DRG neurons. C, control DRG neurons cultured in media from cultured astrocytes with no treatment. A, DRG neurons cultured in conditioned media from AMPA plus cyclothiazide (CYZ)-induced reactivated astrocytes. M+A +CYZ, DRG neurons cultured in conditioned media from AMPA+ CYZ-induced reactivated astrocytes preincubated with MP (10 μM). A+CYZ+ChABC, DRG neurons cultured in chondroitinase ABC (ChABC)-digested conditioned media from AMPA+ CYZ-induced reactivated astrocytes. **B**: Quantitative measurements of the neurite length of DRG neurons cultured in different conditioned media and **C**: the number of neurite-bearing cell is shown in the bottom panel. *Significantly different ($P < 0.05$) compared with control. The data represent the mean ± SD of three independent experiments performed in duplicate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

sity of the immunoreactive band was quantitatively measured by image analysis software (Scion).

Transfection of GR siRNA

The primary cultures of rat astrocytes (5×10^5 cells/well in a 24-well plate) were transfected with either control siRNA (a scrambled siRNA from Ambion) or siRNA against rat GR (siGR, sense, 5′-GCUACAGUCAAGGUUUCUGTT-3′, and anti-sense, 5′-CAGAAACCUUGACUGUAGCTC-3′ from Ambion) (10 nM) using Siport reagent (Ambion) in accordance with the manufacturer's instructions. Forty-eight hours after transfection, cells were stimulated by AMPA+cyclothiazide, with or without MP pretreatment for another 48 h. The cells were then collected for RT-PCR analysis of GFAP and neurocan expression.

Statistical Analysis

Data are presented as the mean ± SD. Statistical analysis was performed by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Treatment with AMPA+Cyclothiazide Induces Astrocyte Reactivation

To establish an *in vitro* model of reactivated astrocytes that mimicked the conditions following CNS injury *in vivo*, we added the excitotoxic agent AMPA plus cyclothiazide, a potential blocker for AMPA receptor desensitization, to primary cultured astrocytes. The dose-response of isolated rat astrocytes to treatment with 100, 200, and 300 μM AMPA with and without cyclothiazide (100 μM) was assessed by analyzing the expression of GFAP mRNA, a marker of reactivated astrocytes, using RT-PCR analysis. We found that treatment with at least 200 μM AMPA in the presence of cyclothiazide significantly induced increased expression of GFAP compared with the control group that received vehicle treatment ($P < 0.05$; Fig. 1A). In addition, we examined CSPG expression in the AMPA-treated astrocytes. Expression of neurocan and phosphacan, two major CSPGs expressed by astrocytes, were significantly increased in astrocytes treated with either AMPA at 300 μM or lower concentrations (200 μM) of AMPA combined with 100 μM of cyclothiazide ($P < 0.05$; Fig. 1A). Furthermore, the cell viability, as assessed by the MTT and LDH release assays, demonstrated that treating astrocytes with 200 μM AMPA and 100 μM cyclothiazide for 48 h had no effect on viability as compared with the control group (Fig. 1B). This indicates that AMPA treatment is not cytotoxic to astrocytes. We therefore used the dosages of 200 μM AMPA plus 100 μM cyclothiazide to induce astrocyte reactivation in primary cultures for further experimentation.

Methylprednisolone Reduces Astrocyte Reactivation

To study the effects of MP on reactivated astrocytes, pretreatment with MP (0-50 μM) was performed prior to

AMPA+cyclothiazide treatment, and the expression of GFAP and CSPG neurocan and phosphacan were assessed by RT-PCR. At concentrations of 10 and 50 μM, MP significantly downregulated the expression of GFAP, neurocan, and phosphacan in AMPA+cyclothiazide-induced reactivated astrocytes (Fig. 2A). In addition, treatment of AMPA+cyclothiazide-induced reactivated astrocytes with 10 μM MP did not affect cell viability (Fig. 1B). Immuno-dot blot analysis was performed using the CS56 monoclonal antibody raised against the disaccharide repeat composed of both C-4-S and C-6-S, which are found in most side chains of CSPGs (Sorrell et al., 1993). This analysis revealed that the total amount of CSPG secreted in the culture medium of reactivated astrocytes was reduced in a dose-dependent manner with MP treatment, and that treatment with MP ≥10 μM significantly suppressed the effect of AMPA+ cyclothiazide treatment on CSPG expression ($P < 0.05$; Fig. 2B). These results suggest that MP reduces the AMPA-induced expression of GFAP, neurocan, phosphacan, and the secretion of CSPGs by reactivated astrocytes.

Conditioned Medium from Astrocyte Cultures Treated with MP Improves Neurite Outgrowth of Dorsal Root Ganglion Neurons

It is believed that astroglial cells expressing CSPGs impair neurite outgrowth and regeneration after CNS injury. We have demonstrated that MP suppressed the reactivation of astrocytes by reducing the expression of GFAP, neurocan, phosphacan as well as that of extracellular CSPGs. We subsequently investigated whether the conditioned media from the reactivated astrocyte cultures, with and without MP treatment, had distinct effects on neurite outgrowth. Media from astrocytes cultured under different conditions were collected and used to culture rat dorsal root ganglion (DRG) neurons. The neurite outgrowth assay was performed by measuring neurite-bearing cells and neurite lengths (see Fig. 3). The data (see Fig. 3) indicated that the cultured medium from AMPA+cyclothiazide-induced reactivated astrocytes strongly reduced the neurite lengths of DRG neurons. In contrast, media from astrocytes treated with MP prior to AMPA+cyclothiazide treatment greatly reduced the inhibitory effect on DRG neurite outgrowth (Fig. 3B). The effect of MP on DRG neurite outgrowth was similar to the effect of chondroitinase ABC, which eliminates CS side chains of CSPGs in the astrocyte-conditioned medium (Fig. 3B). However, the number of neurite-bearing cells of DRG neurons did not differ among the groups (Fig. 3C).

MP Suppression of GFAP and CSPG Expression is GR-Dependent

To investigate the regulatory mechanism of MP in reactivated astrocytes, the GR-antagonist RU486 was

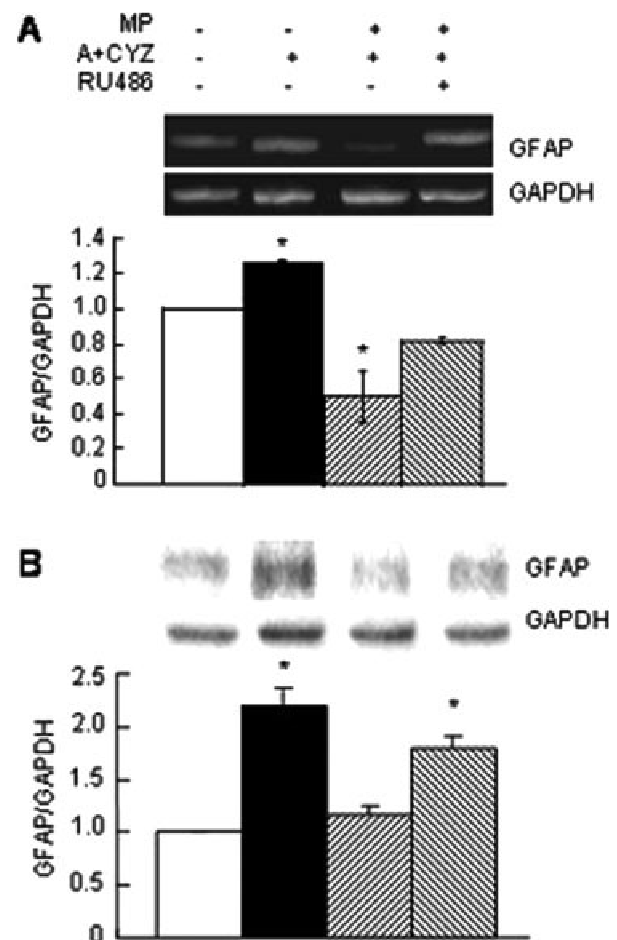

Fig. 4. Effects of AMPA plus cyclothiazide (CYZ), methylprednisolone (MP), and RU486 treatment on the expression of glial fibrillary acidic protein (GFAP) in cultured astrocytes at **A**: the mRNA level by RT-PCR and **B**: the protein level by Western blot analysis. The relative amounts of GFAP mRNA and protein in each group were normalized against GAPDH. *Significantly different ($P < 0.05$) compared with control cells that received no treatment. Quantitatively measured data represent the mean ± SD of three independent experiments performed in triplicate.

added to MP-treated reactivated astrocytes and the expression of GFAP, neurocan, and extracellular CSPG was examined. MP-treatment suppressed mRNA (Fig. 4A) and protein (Fig. 4B) levels of GFAP, and this suppression was largely reversed by RU486. This suggests that the effect of MP on GFAP expression is mediated by GR. In addition, reduced expression of extracellular CSPG by MP treatment in reactivated astrocytes was also reversed by RU486, as revealed by immuno-dot blot analysis (Fig. 5A). Furthermore, MP suppressed expression of neurocan (Fig. 5B) and phosphacan (Fig. 5C) at both the mRNA and protein level, and this effect was reversed by RU486. This suggests that the inhibitory effect of MP on extracellular CSPG expression might also be mediated by GR.

It was possible that RU486 was having an effect by interacting with steroid hormone receptors other than the GR (Honer et al., 2003) To determine whether this was the case, we suppressed GR expression by introduc-

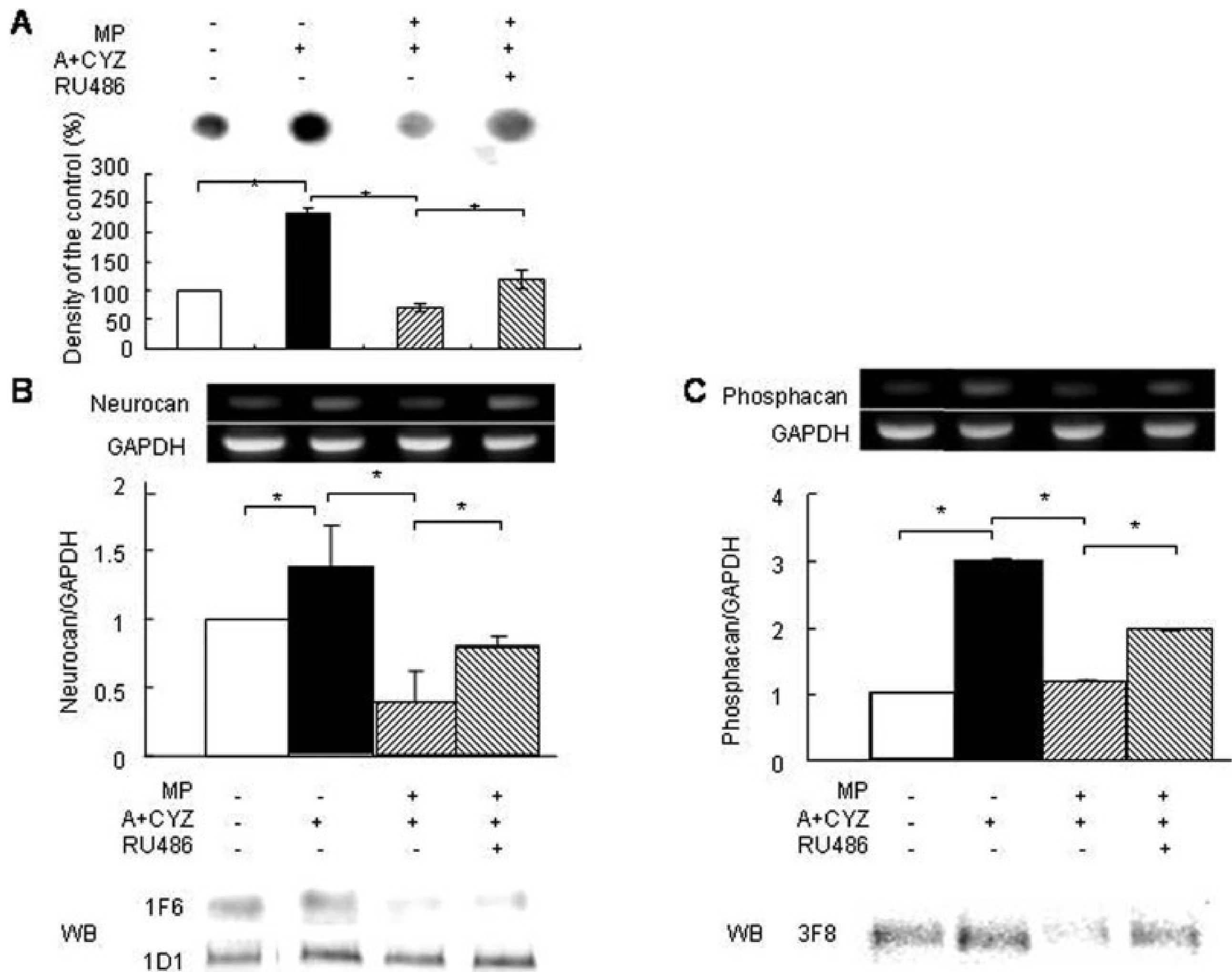

Fig. 5. Effects of AMPA plus cyclothiazide (CYZ), methylprednisolone (MP), and RU486 treatment on the expression of extracellular chondroitin sulfate proteoglycans (CSPGs), neurocan, and phosphacan. **A**: the total amount of secreted CSPGs in the culture medium were assayed by immuno-dot blot analysis, with quantitative data shown at the bottom. *Significantly different ($P < 0.05$) compared with control cells that received no treatment. **B**: neurocan and **C**: phosphacan expression was determined at both the mRNA and protein levels in reactivated astrocytes cultured using the indicated conditions. The mRNA level was quantitatively measured by RT-PCR. Quantitative data are presented as the mean ± SD. *Significantly different ($P < 0.05$) compared with the control. The protein levels of neurocan and phosphacan were analyzed by Western blotting (WB) using the monoclonal antibodies 1F6 (against the neurocan N-terminus), 1D1 (against the neurocan C-terminus), and 3F8 (against phosphacan).

ing siRNA specific to the GR (siGR) into reactivated astrocyte cultures. We found that MP suppression of GFAP and neurocan expression was reversed by siGR transfection (see Fig. 6). This confirmed that the effect of MP on GFAP and neurocan expression was mediated by the GR.

MP Regulates the Expression of GFAP and CSPG Neurocan in Rats with Spinal Cord Injury

To determine whether MP also regulated the expression of GFAP and CSPGs in response to injury *in vivo*, we determined the mRNA levels of GFAP and the CSPG neurocan in adult rats with SCI at the acute phase with and without MP treatment. Eight hours after SCI, the expression of GFAP was significantly increased; it then decreased to the control level 24 h after injury (Fig. 7A). Significantly increased expression of neurocan was not detected until 24 h after SCI (Fig. 7B). Treatment with MP downregulated both GFAP (Fig. 7A) and neurocan (Fig. 7B) expression. The finding that treatment with MP suppressed expression of GFAP and neurocan in rats with SCI is consistent with our observations in the AMPA-treated reactivated astrocytes.

DISCUSSION

Reactive astrogliosis, the characteristic CNS response to almost all neurological insult and disease, may impair axonal regeneration and functional neuronal recovery. Treatment that controls reactive astrogliosis may therefore improve neuronal tissue repair after trauma. This

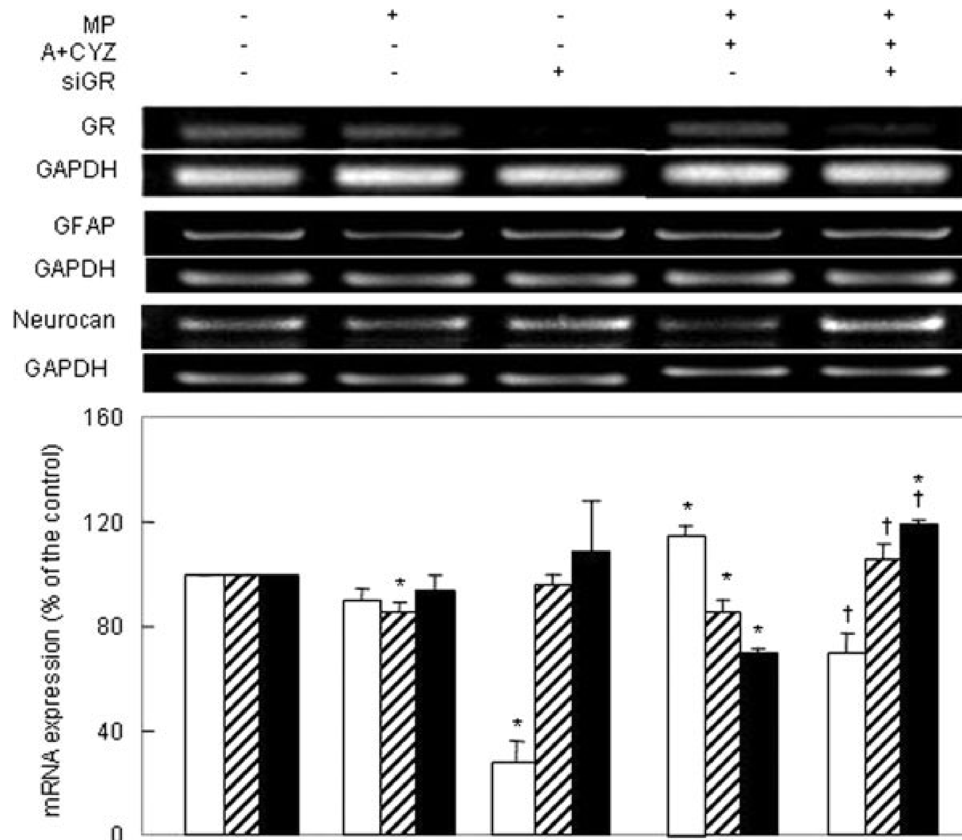

Fig. 6. Glucocorticoid receptor (GR) knockdown blocked the inhibitory effect of methylprednisolone (MP) on glial fibrillary acidic protein (GFAP) and neurocan mRNA expression in reactive astrocytes. The inhibitory activity of MP on GFAP and neurocan expression was significantly blocked by the introduction of siRNA against GR (siGR) in AMPA plus cyclothiazide (CYZ)-induced reactivated astrocytes. Quantitative data are shown in the lower panel. Cultured astrocytes treated with scrambled siRNA served as the control. Open bars, ratio of GR/GAPDH mRNA levels; cross-hatched bars, ratio of GFAP/GAPDH mRNA levels; black bars, ratio of neurocan/GAPDH mRNA levels. *Significantly different ($P < 0.05$) compared with the control. $^{+}$Significantly different ($P < 0.05$) compared with AMPA+CYZ-induced cells.

study established an *in vitro* injured astrocyte cell model by applying excitotoxic AMPA plus cyclothiazide to induce astrocyte reactivation. Specifically, downregulation of astrocyte reactivation by glucocorticoid MP was demonstrated. The AMPA+cyclothiazide-reactivated astrocytes expressed increased levels of GFAP, similar to what occurs during astrogliosis in response to acute-phase CNS injury. Cotreatment with AMPA+ cyclothiazide can enhance AMPA receptor activation, but over-activation of AMPA receptors by exposure to a high dose of AMPA (500 μM) plus cyclothiazide (100 μM) causes rapid lethality to astrocytes (within 2 h) (David et al., 1996). Our data indicated that application of AMPA at a lower dose (200 μM) in the presence of cyclothiazide (100 μM) induced reactivation of astrocytes without affecting cell viability. Hence, this experimental model is a useful *in vitro* model for studying the reactivation of astrocytes upon injury, astrocyte-neuron interactions, and treatment of reactive astrogliosis.

Increased CSPG expression was also observed in AMPA+cyclothiazide-reactivated astrocytes. This is similar to *in vivo* studies that indicate that GFAP-positive astrocytes show increased expression of CSPGs after SCI (Jones et al., 2003; Lemons et al., 1999). Because there is often an acute inflammatory response to traumatic injury in the CNS (Schnell et al., 1999a,b), reactive astrogliosis resulting in increased expression of GFAP (Balasingam et al., 1994; Fitch and Silver, 1997a) and CSPGs (Fitch and Silver, 1997a) might be also caused by inflammation-induced immune cell activation resulting in CNS injury. In addition, it should be aware of that CSPG upregulation in astrocytes can occur independently to inflammatory cells or cytokines (Canning et al., 1993).

MP treatment has been used to effectively reduce post-traumatic inflammation (Xu et al., 1998), which can cause secondary injury after SCI (Hsu and Dimitrijevic, 1990). In this report, we showed that treatment with MP attenuated the increased expression of GFAP and CSPGs in AMPA+cyclothiazide-reactivated astrocytes, suggesting that the neuroprotective effect of MP may be due not only to general reduction of inflammation, but may also be mediated through direct action on astrocytic cells. The effect of MP on reactivated astrocytes appeared to be mediated by GR, since the addition of the GR antagonist RU486 or GR-specific siRNA reversed MP suppression of GFAP and CSPG expression. The GR-mediated effect of MP may involve transcriptional

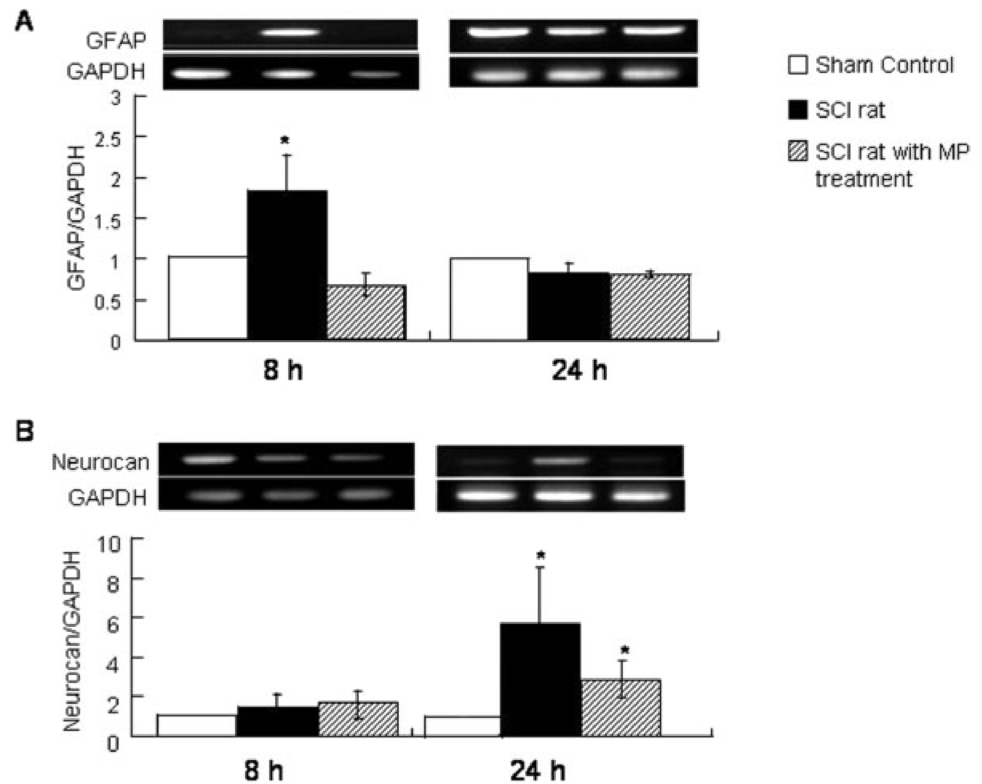

Fig. 7. Methylprednisolone (MP) treatment in adult spinal cord injury (SCI) rats. MP reduced the mRNA expression level of **A:** glial fibrillary acidic protein (GFAP) and **B:** the chondroitin sulfate proteoglycan neurocan. The expression of GFAP was significantly increased 8 h after SCI. MP treatment downregulated GFAP expression to the control level. The expression of neurocan was significantly increased 24 h after SCI. Treatment with MP greatly reduced the expression of neurocan. *Significantly different ($P < 0.05$) compared with the control.

regulation of 5′-upstream sequences of rat GFAP, which contains elements involved in the GR-mediated response to corticosterone (Rozovsky et al., 1995). A putative AP-1 binding site has been identified in the 5′-flanking region of the mouse and human GFAP genes (Masood et al., 1993; Sarid, 1991). MP-induced downregulation of the inflammation-related transcription factor NF-κB and AP-1 has previously been demonstrated (Xu et al., 1998, 2001). In addition, there are also potential AP-1 and NF-κB binding sites in the 5′-upstream region of the CSPG neurocan gene, as predicted by using a search engine that identifies transcription factor binding sites. Therefore, it is possible that MP may regulate the expression of neurocan through its actions on AP-1 and NF-κB signaling cascades. However, GR-independent regulation of the suppression of GFAP and CSPG expression by MP in AMPA+cyclothiazide-induced reactivated astrocytes cannot be excluded. The effect of MP might also be mediated by its attenuation of excitotoxic activity of AMPA with AMPA receptors. Astrocytes have functional AMPA receptors (Hosli and Hosli, 1993). Glucocorticoids can attenuate AMPA cytotoxicity by inhibiting AMPA receptor-associated channels in rat cortical neurons (Shirakawa et al., 2005) and in the nucleus of the solitary tract (Shank and Scheuer, 2003). Therefore, the MP-mediated attenuation of AMPA activity that inhibits AMPA+cyclothiazide-induced astrocytes activation in astrocytes may occur. In addition, it should be noted that not all of the CSPG expression has similar response as the expression of neurocan and phosphacan to MP. Although the total CSPG expression is suppressed by MP treatment in AMPA+cyclothiazide- reactivated astrocytes, the expressions of some CSPGs are not reduced by MP (data not shown). This suggests that MP treatment compared with nonselective removal of CS by chondroitinase ABC may preserve the better ECM structure that improves neuronal regeneration and functional recovery after injury.

In summary, this study demonstrates that the glucocorticoid MP suppresses the reactivation of astrocytes by downregulating GFAP and CSPG (neurocan and phosphacan) expression and consequently improving DRG neurite outgrowth. This adds to our understanding of the effect of MP on the plasticity of astrocytes in response to CNS injury, and may lead to the development of innovative treatments for reactive astrogliosis and for neuronal functional recovery after neurotrauma.

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