Evidence for the Role of Mitogen-Activated Protein Kinase Signaling Pathways in the Development of Spinal Cord Injury

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ABSTRACT

Mitogen-activated protein kinase (MAPK) signaling pathways involve two closely related MAPKs, known as extracellular signal-regulated kinase (ERK1) and ERK2. The aim of the present study was to evaluate the contribution of MAPK3/MAPK1 in the secondary damage in experimental spinal cord injury (SCI) in mice. To this purpose, we used 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), which is an inhibitor of MAPK3/MAPK1. Spinal cord trauma was induced by the application of vascular clips (force of 24 g) to the dura via a four-level T5-T8 laminectomy. SCI in mice resulted in severe trauma characterized by edema, neutrophil infiltration, and production of inflammatory mediators, tissue damage, and apoptosis. PD98059 treatment (10 mg/kg i.p.) at 1 and 6 h after the SCI significantly reduced 1) the degree of spinal cord inflammation and tissue injury (histological score), 2) neutrophil infiltration (myeloperoxidase activity), 3) nitrotyrosine formation, 4) proinflammatory cytokines expression, 5) nuclear factor-κB activation, 6) phospho-ERK1/2 expression, and 6) apoptosis (terminal deoxynucleotidyl transferase dUTP nick-end labeling staining, Fas ligand, Bax, and Bcl-2 expression). Moreover, PD98059 significantly ameliorated the recovery of limb function (evaluated by motor recovery score) in a dose-dependent manner. Taken together, our results clearly demonstrate that PD98059 treatment reduces the development of inflammation and tissue injury associated with spinal cord trauma.

Spinal cord injury (SCI) is a highly debilitating pathology (Maegle et al., 2005). Although innovative medical care has improved patient outcome, advances in pharmacotherapy for the purpose of limiting neuronal injury and promoting regeneration have been limited. The complex pathophysiology of SCI may explain the difficulty in finding a suitable therapy. The primary traumatic mechanical injury to the spinal cord causes the death of a number of neurons that cannot be recovered and regenerated. Studies indicate that neurons continue to die for hours following traumatic SCI (Profyris et al., 2004). The events that characterize this successive phase to mechanical injury are called “secondary damage.” Secondary damage is determined by a large number of cellular, molecular, and biochemical cascades. Considerable recent data suggest the presence of a local inflammatory response, which amplifies secondary damage.

Moreover, various evidence has suggested that resident microglia and macrophages originating from blood are two key cell types related to the occurrence of neuronal degeneration in CNS after traumatic injury. In particular, when SCI occurs, microglia in parenchyma is activated, and macrophages in circulation can cross the blood-brain barrier to act as intrinsic spinal phagocytes. Therefore, these cells can release various neurotrophic peptides such as brain-derived neurotrophic factor (Batchelor et al., 2002), and laminin, which are excellent substrates for growing neuritis. Concomitantly, different proinflammatory mediators, such as proinflammatory cytokines (Genovese et al., 2006a) and reactive oxygen species (Park et al., 2004), are also produced. In
addition, various studies have clearly demonstrated that NO is closely involved in the development of pathological processes in vivo, such as post-traumatic spinal cord cavitation (Matsuyama et al., 1998). It is confirmed in vitro that NO-induced cell injury is mediated via either the necrotic or apoptotic pathway, depending upon the severity of the cellular damage. A recent study clearly demonstrates that NO produced by inducible nitric-oxide synthase (iNOS) modulates the secondary inflammatory response following traumatic SCI (Genovese et al., 2006b).

Other studies have also implicated peroxynitrite (ONOO -), a cytotoxic molecule generated when NO and superoxide combine, in the secondary neuronal damage of SCI (Liu et al., 2000, 2005; Xu et al., 2001; Bao and Liu, 2002; Bao et al., 2003; Scott et al., 2004; Xiong et al., 2007). Not only was ONOO - detected in spinal cord tissues from rats following traumatic injury (Liu et al., 2000; Xu et al., 2001; Xiong et al., 2007) but also ONOO - donor administration directly into the rat spinal cord has been shown to cause neuronal cell death and neurological deficits. More recently, a three-step cascade of events related to the occurrence of neuronal damage after SCI has been indicated by different observations from in vitro and in vivo study: 1) activation of mitogen-activated protein kinases (MAPKs), 2) initiation of inflammatory responses, and 3) degeneration of neurons. Furthermore, it has been demonstrated that phosphorylation of ERK1/2 and p38 MAPK results in expression of genes mediating the inflammatory responses, such as tumor necrosis factor (TNF-α) and NO (Bhat et al., 1998; Wang et al., 2004a). In contrast, administration of p38 and c-Jun NH2-terminal kinase (JNK) inhibitors partially reduced neurons from death in the lipopolysaccharide-treated microglia-neuron coculture (Xie et al., 2004), and inhibition of ERK1/2 activation may also reduce IL-1-induced cortical neuron damage. These findings suggest that initiation of inflammatory responses in CNS is related to activation of MAPKs, especially ERK1/2 and p38 MAPK, and that their activation would be a determinant for neuronal death or survival on certain occasions. In this regard, Xu et al. (2006) have clearly demonstrated that the ERK1/2 and p38 MAPK signaling pathway play an important role in NO-mediated degeneration of neuron in the spinal cord following SCI. However, in vivo evidence of strategies directed to blocking the initiation of this cascade linking MAPK activation in post-traumatic pathophysiological process of SCI is not fully evaluated.

In this study, the use of PD98059, a specific inhibitor of the activation of mitogen-activated protein kinase kinase (MAPK3/MAPK1) (Alessi et al., 1995), allowed us to demonstrate that MAPK activation plays a key role in the modulation of secondary injury in the spinal cord. In particular, we have determined the following endpoints of the inflammatory response: 1) histological damage, 2) motor recovery, 3) neutrophil infiltration, 4) NF-κB expression, 5) nitrotyrosine formation, 6) proinflammatory cytokine production, 7) apoptosis as terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, and 8) Bax and Bcl-2 expression.

**Materials and Methods**

**Animals.** Male Adult CD1 mice (25–30 g; Harlan Nossan, Milan, Italy) were housed in a controlled environment, and they were provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) and with the EEC regulations (O.J. of E.C. L 355/1 12/18/1986).

**Spinal Cord Injury.** Mice were anesthetized using chloral hydrate (400 mg/kg b.w.). We used the clip compression model described by Rivlin and Tator (1978), and we produced SCI by extradural compression of a section of the spinal cord, exposed via a four-level T5-T8 laminectomy, in which the prominent spino- neural process of T5 was used as a surgical guide. A six-level laminectomy was chosen to expedite timely harvest and to obtain enough s.c. tissue for biochemical examination. With the aneurysm clip applicator oriented in the bilateral direction, an aneurysm clip with a closing force of 24 g was applied extradurally at T5-T8 level. The clip was then rapidly released with the clip applicator, which caused s.c. compression. In the injured groups, the cord was compressed for 1 min. After surgery, 1.0 ml of saline was administered s.c. to replace the blood volume lost during the surgery. During recovery from anesthesia, the mice were placed on a warm heating pad, and they were covered with a warm towel. The mice were singly housed in a temperature-controlled room at 27°C for a survival period of 10 days. Food and water were provided to the mice ad libitum. During this time, the animals’ bladders were manually voided twice a day until the mice were able to regain normal bladder function. Sham-injured animals were only subjected to laminectomy.

**Experimental Design.** Mice were randomized into four groups (n = 40 animals/group). Sham animals were subjected to the surgical procedure except that the aneurysm clip was not applied, and they were treated i.p. with vehicle (10% dimethyl sulfoxide) or 10 mg/kg PD98059 at 1 and 6 h after the surgical procedure. The remaining mice were subjected to SCI as described above, and then they were treated with an i.p. bolus of vehicle (saline) or 10 mg/kg PD98059 at 1 and 6 h (early treatment) or at 6 and 12 h (late treatment) after SCI or with U0126 (16 μg/mouse) 30 min before and 1 and 6 h after SCI. The doses of PD98059 (10 mg/kg) used here were based on a previous in vivo study (Minutoli et al., 2005), and the dose of U0126 (16 μg/mouse) was chosen in agreement with a previous study in vivo (Wang et al., 2004b). To investigate the motor score, in another set of experiments, the animals were treated with PD98059 at 1 and 6 h (early treatment) or at 6 and 12 h (late treatment) after SCI and daily until day 9 or with U0126 (16 μg/mouse) 30 min before and 1 and 6 h after SCI and daily until day 9. Ten mice from each group were sacrificed at different time points to collect samples for the evaluation of the parameters as described below.

**Myeloperoxidase Activity.** Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was determined in the spinal cord tissues as described previously (Mullane, 1989) at 24 h after SCI. At the specified time following SCI, spinal cord tissues were obtained and weighed, and each piece was homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl ammonium bromide dissolved in 10 mM potassium phosphate buffer, pH 7.0, and centrifuged for 30 min at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H2O2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37°C, and it was expressed as units of MPO per milligram of proteins.

**Immunohistochemical Localization of TNF-α, Nitrotyrosine, Fas Ligand, and Phosphorylated JNK, Bax, and Bcl-2.** Twenty-four hours after SCI, nitrotyrosine, a specific marker of nitrosative stress, was measured by immunohistochemical analysis in the spinal cord sections to determine the localization of “peroxynitrite formation” and/or other nitrogen derivatives during SCI. At 24 h after SCI, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde, and 8-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100, and the sections were blocked with 3% (w/v) nonfat milk in PBS for 1 h before incubation with antibodies against TNF-α, nitrotyrosine, Fas Ligand, phosphorylated JNK, Bax, and Bcl-2.
HRP kit; DBA). In brief, sections were incubated with 15 detection kit according to the manufacturer’s instruction (Apotag, 2010 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, the cytosolic fraction. The pellets, containing enriched nuclei, were centrifuged at 1000 positive in all the experiments carried out. was found in the sections, indicating that the immunoreactions were and Bcl-2, some sections were also incubated with only the primary antibody (no secondary antibody) or with only the secondary antibody (no primary antibody). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all the experiments carried out.

**TUNEL Assay.** TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer’s instruction (Apotag, HRP kit; DBA). In brief, sections were incubated with 15 µg/ml proteinase K for 15 min at room temperature, and then they were washed with PBS. Endogenous peroxidase was inactivated by 3% H2O2 for 5 min at room temperature, and then it was washed with PBS. Sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, washed with PBS, and then they were incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA). To verify the binding specificity for nitrotyrosine, TUNEL-α, FasL, phosphorylated JNK, Bax, and Bcl-2, some sections were also incubated with only the primary antibody (no secondary antibody) and with only the secondary antibody (no primary antibody). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all the experiments carried out.

**Light Microscopy.** Spinal cord tissues were taken at 24 h after trauma. Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin embedded and cut into 5-µm-thick sections. Tissue sections were deparaffinized with xylene, stained with hematoxylin and eosin or methyl green pyronin (used to simultaneously detect DNA and RNA), and studied using light microscopy (Dialux 22; Leitz, Wetzlar, Germany). The segments of each spinal cord were evaluated by an experienced histopathologist. Damaged neurons were counted, and the histopathological changes of the gray matter were scored on a 6-point scale (Sirin et al., 2002): 0, no lesion observed; 1, gray matter contained one to five eosinophilic neurons; 2, gray matter contained 5 to 10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the gray matter area); 5, moderate infarction (one third to one half of the gray matter area); and 6, large infarction (more than half of the gray matter area). The scores from all the sections from each spinal cord were averaged to give a final score for individual mice. All the histological studies were performed in a blinded manner.

**Measurement of Spinal Cord TNF-α and IL-1β Levels.** Portions of spinal cord tissues, collected at 24 h after SCI, were homogenized as described previously in PBS containing 2 mM PMSF (Sigma-Aldrich), and tissue TNF-α and IL-1β levels were evaluated. The assay was carried out by using a colorimetric commercial kit (Calbiochem-Novabiochem, San Diego, CA), according to the manufacturer’s instructions. All TNF-α and IL-1β determinations were performed in duplicate serial dilutions.

**Western Blot Analysis for IκB-α, Phospho-NF-κB p65 (Ser536), NF-κB p65, Bax, Bcl-2, pERK1/2, ERK2, TNF-α, FasL, Phospho-p38 (Thr180/Tyr182), and Phospho-SAPK/JNK (Thr183/Tyr185).** Cytosolic and nuclear extracts were prepared as described previously (Bethea et al., 1998), with slight modifications. In brief, spinal cord tissues from each mouse were suspended in extraction buffer A containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 g/ml sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 0.15 mM MgCl2, 1 mM sodium orthovanadate; homogenized at the highest setting for 2 min; and centrifuged at 10000 g for 10 min at 4°C. Supernatants were processed for Western blot analysis. The pellets, containing enriched nuclei, were resuspended in buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 20 µM leupeptin, and 0.2 mM sodium orthovanadate. After centrifugation at 15,000 g for 30 min at 4°C, the supernatants containing the nuclear protein were stored at −80°C for further analysis. The levels of IκB-α, phospho-NF-κB p65 (Ser536), phospho-SAPK/JNK, phospho-p38 MAP kinase, Bax, and Bcl-2 were quantified in cytosolic fraction from spinal cord tissue collected after 24 h after SCI, whereas NF-κB p65 levels were quantified in nuclear fraction. The filters were blocked with 1× PBS, 5% (w/v) nonfat dried milk for 40 min at room temperature, and they were subsequently probed with specific antibodies IκB-α (1:1000; Santa Cruz Biotechnology, Inc.), phospho-NF-κB p65 (Ser536) (1:1000; Cell Signaling Technology Inc., Danvers, MA), anti-Bax (1:500; Santa Cruz Biotechnology, Inc.), anti-Bcl-2 (1:500; Santa Cruz Biotechnology, Inc.), anti-ERK2 (1:1000; Cell Signaling Technology Inc., Danvers, MA), anti-ERK2 (1:1000; Santa Cruz Biotechnology, Inc.), anti-phospho-p38 (1:200; Santa Cruz Biotechnology, Inc.), anti-FasL (1:200; Santa Cruz Biotechnology, Inc.), or anti-phospho-p38 MAP Kinase (Thr180/Tyr182) (1:1000, C-178, IgG; Cell Signaling), or anti-phospho-SAPK/JNK (Thr185/Tyr185) (1:1000; Cell Signaling, Technology Inc.) in 1× PBS, 5% (w/v) nonfat dried milk, and 0.1% Tween 20 at 4°C overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against α-tubulin protein (1:10,000; Sigma-Aldrich, Milan, Italy). The relative expression of the protein bands of IκB-α (37 kDa), phospho NF-κB (65 kDa), NF-κB p65 (75 kDa), Bax (23 kDa), Bcl-2 (29 kDa) TNF-α (26 kDa), Fasl (40 kDa), phospho-p38 MAP kinase (43 kDa), and phospho-SAPK/JNK (54 and 46 kDa) was quantified by densiometric scanning of the X-ray films with GS-700 imaging densitometer (GS-700; Bio-Rad, Milan, Italy) and a computer program (Molecular Analysis; IBM Corp., Armonk, New York), and expression was standardized for densitometric analysis to α-tubulin levels.

The dual-phosphorylated form of ERK (pERK) antibody identified two bands of approximately 44 and 42 kDa (corresponding to pERK1 and pERK2, respectively). The anti-ERK2 antibody detects total ERK2 (i.e., detects both phosphorylated and nonphosphorylated forms of ERK2).

**Grading of Motor Disturbance.** The motor function of mice subjected to compression trauma was assessed once a day for 10 days after injury. Recovery from motor disturbance was graded using the modified murine Basso, Beattie, and Bresnahan (BBB) (Basso et al., 1995) hind limb locomotor rating scale (Joshi and Fehlings, 2002a,b).
The following criteria were considered: 0, no hind limb movement; 1, slight (<50% range of motion) movement of one to two joints; 2, extensive (>50% range of motion) movement of one joint and slight movement of one other joint; 3, extensive movement of two joints; 4, slight movement in all three joints; 5, slight movement of two joints and extensive movement of one joint; 6, extensive movement of two joints and slight movement of one joint; 7, extensive movement of all three joints; 8, sweeping without weight support or plantar placement and no weight support; 9, plantar placement with weight support in stance only or dorsal stepping with weight support; 10, occasional (0–50% of the time) weight-supported plantar steps and no coordination (front/hind limb coordination); 11, frequent (50–94% of the time) to consistent (95–100% of the time) weight-supported plantar steps and no coordination; 12, frequent to consistent weight-supported plantar steps and occasional coordination; 13, frequent to consistent weight-supported plantar steps and frequent coordination; 14, consistent weight-supported plantar steps, consistent coordination and predominant paw position is rotated during locomotion (lift off and contact) or frequent plantar stepping; consistent coordination and occasional dorsal stepping; 15, consistent plantar stepping and coordination, no/occasional toe clearance, paw position is parallel at initial contact; 16, consistent plantar stepping and coordination (front/hind limb coordination) and frequent toe clearance and predominant paw position is parallel at initial contact and rotated at lift off; 17, consistent plantar stepping and coordination and frequent toe clearance and predominant paw position is parallel at initial contact and lift off; 18, consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and rotated at lift off; 19, consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and lift off; 20, consistent plantar stepping, coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off; 21, consistent plantar stepping, coordinated gait, consistent coordination and predominant paw position is rotated during locomotion; 22, consistent weight-supported plantar steps and frequent coordination (front/hind limb coordination) and frequent toe clearance and predominant paw position is rotated during locomotion; 23, consistent weight-supported plantar steps and frequent coordination and consistent toe clearance and predominant paw position is rotated during locomotion; 24, consistent weight-supported plantar steps and occasional coordination; 25, consistent weight-supported plantar steps and occasional toe clearance and predominant paw position is rotated during locomotion; 26, consistent weight-supported plantar steps and occasional toe clearance and predominant paw position is rotated during locomotion; 27, consistent weight-supported plantar steps and frequent coordination; 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98, consistent weight-supported plantar steps and occasional toe clearance and predominant paw position is rotated during locomotion; 99, consistent weight-supported plantar steps and occasional coordination; 100, consistent weight-supported plantar steps and occasional toe clearance and predominant paw position is rotated during locomotion.

Results

PD98059 Reduces the Severity of Spinal Cord Trauma. The severity of the trauma at the level of the perilesional area, assessed the presence of edema and alteration of the white matter (Fig. 1, A and E; histological score), was evaluated at 24 h after injury. A significant damage to the spinal cord was observed in the spinal cord tissue from SCI mice compared with sham-operated mice (data not shown). It is noteworthy that a significant protection against the spinal cord injury was observed in PD98059 (early treatment)-treated mice (Fig. 1, B and E; histological score) and in PD98059 (late treatment)-treated mice (Fig. 1, C and E; histological score). To evaluate whether histological damage to the spinal cord was associated with a loss of motor function, the modified BBB hind limb locomotor rating scale score was evaluated. Although motor function was only slightly impaired in sham mice, mice subjected to SCI had significant deficits in hind limb movement (Fig. 2). PD98059 early or late treatment ameliorated the functional deficits induced by SCI (Fig. 2). Please note that no significant difference was found in the ability to reduce spinal cord injury by PD98059 administered as early or late treatment (Figs. 1 and 2).

In addition, to confirm that the protective effects of PD98059 on the severity of spinal cord trauma are related to the inhibition of ERK1/2, we also investigated whether U0126, another structural unrelated ERK1/2 inhibitor, attenuates the tissue injury and the motor dysfunction induced by SCI. The treatment with U0126 (16 μg/mouse) significantly reduced the histological damage to the spinal cord (Fig. 1, D and E; histological score) and leads to an amelioration of hind limb motor disturbances (Fig. 2). Please note that no significant difference was found between the PD98059 and U0126 treatments (Figs. 1 and 2).

Effects of PD98059 on Neutrophil Infiltration. The above-mentioned histological pattern of spinal cord injury
seemed to be correlated with the influx of leukocytes into the spinal cord. Therefore, we investigated the effect of PD98059 on the neutrophil infiltration by measuring tissue MPO activity. MPO activity was significantly elevated in the spinal cord at 24 h after injury in mice subjected to SCI compared with sham-operated mice (Fig. 3A). Treatment with PD98059 attenuated neutrophil infiltration into the spinal cord at 24 h after injury (Fig. 3A).

**PD98059 Modulates the Expression of TNF-α and IL-1β after SCI.** To test whether PD98059 may modulate the inflammatory process through the regulation of the secretion of proinflammatory cytokines, we analyzed the spinal cord tissue levels of TNF-α and IL-1β. A substantial increase in TNF-α and IL-1β production was found in spinal cord tissues samples collected from SCI mice 24 h after SCI (Fig. 3, B and C, respectively). Spinal cord levels of TNF-α and IL-1β were significantly attenuated by the i.p. injection of PD98059 (Fig. 3, B and C, respectively). Likewise, at 24 h after SCI, the expression of TNF-α in the spinal cord homogenates was investigated by Western blot. A significant increase of TNF-α (Fig. 4, A and A1) levels were observed in the spinal cord from mice subjected to SCI. On the contrary, PD98059 treatment prevented the SCI-induced TNF-α (Fig. 4, A and A1) expression. In addition, spinal cord sections were also taken at 24 h after SCI to determine the immunohistochemical staining for TNF-α expression. There was no staining for TNF-α in spinal cord obtained from the sham mice (data not shown). A substantial increase in TNF-α (Fig. 4, B and D; densitometry analysis) expression was found in inflammatory cells and in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues collected from SCI mice 24 h after SCI (Fig. 4B1). Spinal cord expression of TNF-α (Fig. 4, C and D; densitometry analysis) was significantly attenuated in PD98059 (early treatment) SCI mice compared with SCI animals.

**Effects of PD98059 on Nitrotyrosine Formation and Lipid Peroxidation after SCI.** Spinal cord sections from sham-operated mice did not stain for nitrotyrosine (data not shown), whereas spinal cord sections obtained from SCI mice exhibited positive staining for nitrotyrosine (Fig. 5, A and C; densitometry analysis). The positive staining was mainly localized in inflammatory cells and in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues (Fig. 5A1). PD98059 treatment reduced the degree of positive staining for nitrotyrosine (Fig. 5, B and C; densitometry analysis) in the spinal cord. In addition, at 24 h after SCI, thiobarbituric acid-reactant substance levels were also measured in the spinal cord tissue as an indicator of lipid peroxidation. A significant increase in thiobarbituric acid-reactant substance levels was observed in SCI mice compared with sham-operated mice (data not shown).
tant substances (Fig. 5D) was observed in the spinal cord collected at 24 h from mice subjected to SCI compared with sham-operated mice. Thiobarbituric acid-reactant substances (Fig. 5D) were significantly attenuated by the i.p. injection of PD98059 (early treatment).

**Effects of PD98059 Treatment on IκB-α Degradation, Phosphorylation of Ser536 on the NF-κB Subunit p65, Nuclear NF-κB p65.** We evaluated IκB-α degradation and phosphorylation of Ser536 on the NF-κB subunit p65 nuclear NF-κB p65 by Western blot analysis to investigate the cellular mechanisms by which treatment with PD98059 may attenuate the development of SCI.

A basal level of IκB-α was detected in the spinal cord from sham-operated animals, whereas IκB-α levels were substantially reduced in SCI mice. PD98059 (early treatment) administration prevented the SCI-induced IκB-α degradation (Fig. 6, A and A1). In addition, SCI caused a significant increase in the phosphorylation of Ser536 at 24 h (Fig. 6, B and B1). The early treatment with PD98059 significantly reduced the phosphorylation of p65 on Ser536 (Fig. 6, B and 5D) were significantly attenuated by the i.p. injection of PD98059 (early treatment).

**Effects of PD98059 Treatment on IκB-α Degradation, Phosphorylation of Ser536 on the NF-κB Subunit p65, Nuclear NF-κB p65.** We evaluated IκB-α degradation and phosphorylation of Ser536 on the NF-κB subunit p65 nuclear NF-κB p65 by Western blot analysis to investigate the cellular mechanisms by which treatment with PD98059 may attenuate the development of SCI.

A basal level of IκB-α was detected in the spinal cord from sham-operated animals, whereas IκB-α levels were substantially reduced in SCI mice. PD98059 (early treatment) administration prevented the SCI-induced IκB-α degradation (Fig. 6, A and A1). In addition, SCI caused a significant increase in the phosphorylation of Ser536 at 24 h (Fig. 6, B and B1). The early treatment with PD98059 significantly reduced the phosphorylation of p65 on Ser536 (Fig. 6, B and
Moreover, NF-κB p65 levels in the nuclear fractions from spinal cord tissue were also significantly increased at 24 h after SCI compared with the sham-operated mice (Fig. 6, C and C1). PD98059 (early treatment) treatment significantly reduced the levels of NF-κB p65 as shown in C. α-Tubulin was used as internal control. A representative blot of lysates obtained from each group is shown, and densitometric analysis of all animals is reported (n = 5 rats from each group). The relative expression of the protein bands from three separated experiments was standardized for densitometric analysis to α-tubulin levels, and data are reported in A1, B1, and C1. *p < 0.01 versus sham; °, p < 0.01 versus SCI.

PD98059 Modulates the Activation of MAPK Pathways and the Expression of Phospho-SAPK/JNK after SCI. To investigate the cellular mechanisms by which treatment with PD98059 may attenuate the development of SCI, we also evaluated the phosphorylation of ERK1/2, which results in expression of proinflammatory genes mediating the inflammatory responses characteristic of SCI and phosphorylation of phospho-SAPK/JNK. The activation of MAPK pathways, in particular, the phosphorylation of ERK1/2 expression, was investigated by Western blot in spinal cord homogenates at 24 h after SCI. A significant increase in pERK1/2 levels were observed in SCI mice (Fig. 7, A and A1).
Treatment of mice with PD98059 significantly reduced the level of pERK1/2 (Fig. 7, A and A1). Likewise, treatment with U0126 (16 μg/mouse) significantly reduced the SCI-induced increase of the level of pERK1/2 (Fig. 7, B and B1). In addition, a marked positive immunostaining for the phosphorylated JNK was found mainly localized in inflammatory cells and in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues collected from SCI mice at 24 h after SCI (Fig. 8, A1 and C; densitometry analysis). PD98059 (early treatment) reduced the degree of the expression of JNK (Fig. 8, B and C; densitometry analysis). Sections of spinal cord obtained from the sham mice did not reveal any immunoreactivity for phosphorylated JNK (data not shown). Likewise, at 24 h after SCI, the expression of phospho-SAPK/JNK in spinal cord homogenates was investigated by Western blot. A significant increase in phospho-SAPK/JNK (Fig. 8, D and D1) levels were observed in the spinal cord from mice subjected to SCI. On the contrary, PD98059 treatment prevented the SCI-induced (Fig. 8, D and D1) expression of these kinases. Moreover, we evaluated the phospho-p38 expression by Western blot analysis to further investigate the cellular mechanisms by which treatment with PD98059 may attenuate the development of SCI. SCI caused a significant increase in the phospho-p38 expression at 24 h after SCI (Fig. 8, E and E1). The early treatment with PD98059 significantly reduced the p38 expression (Fig. 8, E and E1).

Effects of PD98059 on Fas-Ligand Expression in Spinal Cord after Injury. Immunohistological staining for Fas ligand in the spinal cord was also determined 24 h after injury. Spinal cord sections from sham-operated mice did not stain for Fas ligand (data not shown), whereas spinal cord sections obtained from SCI mice exhibited positive staining for Fas ligand (Fig. 9, A and C; densitometry analysis) mainly localized in inflammatory cells and in nuclei of Schwann cells (Fig. 9A1). PD98059 (early treatment) reduced the degree of positive staining for Fas ligand in the spinal cord (Fig. 9, B and C; densitometry analysis). There was no staining for Fas ligand in spinal cord tissues obtained from the sham group of mice (data not shown). Likewise, at 24 h after SCI, the expression of Fas ligand in the spinal cord homogenates was investigated by Western blot. A significant increase in Fas ligand (Fig. 9, D and D1) levels was observed in the spinal cord from mice subjected to SCI. On the contrary, PD98059 treatment prevented the SCI-induced Fas ligand (Fig. 9, D and D1) expression.

Effects of PD98059 on DNA/RNA Alteration and Apoptosis in Spinal Cord after Injury. The simultaneous presence of DNA and RNA was detected by methyl green pyronin staining. In sham animals (data not shown), the simultaneous presence of DNA and RNA was clearly evident by methyl green pyronin staining in both lateral and dorsal funiculi of the spinal cord. At 24 h after the injury, a significant loss in DNA and RNA presence in lateral and dorsal funiculi was observed in SCI mice (Fig. 10A). In contrast, in PD98059-treated mice, the DNA and RNA degradation was attenuated in the central part of lateral and dorsal funiculi (Fig. 10B). Moreover, to test whether spinal cord damage was associated to cell death by apoptosis, we measured TUNEL-like staining in the perilesional spinal cord tissue. Almost no apoptotic cells were detected in the spinal cord from sham-operated mice (data not shown). At 24 h after the trauma, tissues from SCI mice demonstrated a marked appearance of

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**Fig. 7.** Effect of PD98059 on activated kinases. The spinal cord extract were immunoblotted for the dual-phosphorylated form of ERK or active ERK1/2 (pERK1/2) and total ERK1/2 (ERK2). pERK1/2 is up-regulated in injured mice compared with sham-operated mice (A and A1). Spinal cord levels of pERK1/2 were significantly attenuated in PD98059 (10 mg/kg)-SCI treated mice compared with SCI animals. Likewise, the treatment with U0126 (16 μg/mouse) significantly reduced the SCI-induced increase of the level of pERK1/2 (B and B1). Respective densitometric analysis of protein bands from three separated experiments is reported in A1 and B1. *, p < 0.01 versus sham; °, p < 0.01 versus SCI + vehicle.
dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 10, C and C1; see positive cell count E). In contrast, tissues obtained from mice treated with PD98059 (early treatment) demonstrated no apoptotic cells or fragments (Fig. 10D; see positive cell count E).

**Western Blot Analysis and Immunohistochemistry for Bax and Bcl-2.** At 24 h after SCI, the appearance of proapoptotic protein Bax in spinal cord homogenates was investigated by Western blot. Bax levels were appreciably increased in the spinal cord from mice subjected to SCI (Fig. 11, A). Densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for pJNK (C) from spinal cord tissues was assessed. Likewise, at 24 h after SCI, expression of phospho-SAPK/JNK (Thr183/Tyr185) in the spinal cord was investigated by Western blot. A significant increase in phospho-SAPK/JNK (D and D1) was observed in the spinal cord from mice subjected to SCI. On the contrary, PD98059 treatment prevented the SCI-induced (D and D1) expression of this protein. Moreover, SCI caused a significant increase in phospho-p38 expression at 24 h after trauma (E and E1). The early treatment with PD98059 significantly reduced p38 expression (E and E1). The relative expression of the protein bands was standardized for densitometric analysis to α-tubulin levels, and data are reported in D1 and E1. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days. *p < 0.01 versus sham; **p < 0.01 versus SCI + vehicle.
A and A1). On the contrary, PD98059 treatment prevented the SCI-induced Bax expression (Fig. 11, A and A1).

We also analyzed Bcl-2 expression in homogenates from spinal cord of each mouse by Western blot analysis. The basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (Fig. 11, B and B1). Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in spinal cord from SCI mice (Fig. 11, B and B1). Treatment of mice with PD98059 significantly blunted the SCI-induced inhibition of antiapoptotic protein expression (Fig. 11, B and B1).

Moreover, samples of spinal cord tissue were taken at 24 h after SCI also to determine the immunohistological staining for Fas ligand. A substantial increase in Fas ligand (A) expression was found in inflammatory cells and in nuclei of Schwann cells (A1) in wm and gm of the spinal cord tissues from SCI mice. Spinal cord levels of Fas ligand (B) were significantly attenuated in PD98059-SCI treated mice compared with SCI animals. Densitometry analysis of immunocytochemistry photographs (* = 5 photos from each sample collected from all mice in each experimental group) for Fas ligand (C) from spinal cord tissues was assessed. Likewise, at 24 h after SCI, the expression of Fas ligand in the spinal cord homogenates was investigated by Western blot. A significant increased Fas ligand (D and D1) expression was observed in the spinal cord from mice subjected to SCI. On the contrary, PD98059 treatment prevented the SCI-induced Fas ligand (D and D1) expression. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). The relative expression of the protein bands was standardized for densitometric analysis to α-tubulin levels, and data are reported in D1. This figure is representative of at least three experiments performed on different experimental days. *, p < 0.01 versus sham; **, p < 0.01 versus SCI + vehicle.
positive staining (data not shown), whereas in SCI mice the staining significantly reduced (Fig. 12, D and F; densitometry analysis). PD98059 (early treatment) attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (Fig. 12, E1 and F; densitometry analysis).

Discussion

Primary injury to the adult spinal cord is irreversible, whereas secondary degeneration is delayed and therefore amenable to intervention. Accordingly, several studies have shown that therapies targeting various factors involved in the secondary degeneration cascade lead to tissue sparing and improved behavioral outcomes in spinal cord-injured animals (Bao et al., 2003; Cuzzocrea et al., 2006; Genovese et al., 2006b; Glaser et al., 2006). Much of the damage that occurs in the spinal cord after traumatic injury is due to the secondary effects of glutamate excitotoxicity, Ca\(^{2+}\) overload, and oxidative stress, three mechanisms that take part in a spiraling interactive cascade ending in neuronal dysfunction and death (Tator, 1991; Anderson and Hall, 1993).

MAPK family members, including ERK1/2, c-Jun NH\(_2\)-terminal protein kinases, and p38 kinases, are thought to be important mediators of signal transduction from cell surface to the nucleus. It was reported that neuroprotection of hypoxic preconditioning in cerebellar granular neurons was related to phosphatidylinositol 3-kinase/Akt activation and mitogen-activated protein kinase kinase/ERK phosphorylation (Wick et al., 2002).

The inhibition of MAPK3/MAPK1 is thought to be beneficial in a number of experimental models of neurodegenerative diseases, diabetes type II, bipolar disorders, stroke, cancer, sepsis, and chronic inflammatory disease. Moreover, Xu et al. (2006) have clearly demonstrated in vivo enhanced activation of ERK1/2 and p38 MAPK in microglia/macrophages in the injured spinal cord after traumatic SCI, which are activated within 1 h after injury and the activation persists for at least 24 h after injury (Xu et al., 2006).
spinal cord; characterized by increased IxB-α degradation; enhanced NF-κB activation; amplified expression of proinflamatory mediators, proinflammatoy cytokines, and nitrotyrosine; and increased MPO activity. Our results show that PD98059 reduced 1) the degree of spinal cord damage, 2) neutrophils infiltration, 3) NF-κB activation, 4) IxB-α degradation, 5) nitrotyrosine formation, 6) proinflammatoy cytokines production, 7) apoptosis as TUNEL staining, and 8) Bax and Bcl-2 expression.

The ERK1/2 and p38 MAPK signaling pathways have been found to be involved in microglia/macrophage activation (Bhat et al., 1998; Tikka et al., 2001; Choi et al., 2003). Previous studies show that the expression of activated ERK1/2 and p38 MAPK in microglia/macrophages may play a key role in production of CNS inflammatory cytokines and free radicals, such as NO (Combs et al., 2001; Choi et al., 2003). Recent evidence suggests that the activation of NF-κB may also be under the control of oxidant/antioxidant balance (Haddad, 2002). Moreover, various experimental evidence has clearly suggested that NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in secondary inflammation associated with SCI (La Rosa et al., 2004). NF-κB is normally sequestered in the cytoplasm, bound to regulatory proteins IxBs. In response to a wide range of stimuli, including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, IxB is phosphorylated by the enzyme IxB kinase (Bowie and O’Neill, 2000). The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus. The exact mechanisms by which PD98059 suppress NF-κB activation in inflammation are not known. We report here that SCI caused a significant increase in the phosphorylation of Ser536 on p65 in the spinal cord tissues at 24 h, whereas PD98059 treatment significantly reduced this phosphorylation. Moreover, we also demonstrate that PD98059 inhibited IxB-α degradation and NF-κB translocation. Taken together, the balance between proinflammatory and prosurvival roles of NF-κB may depend on the phosphorylation status of p65, and MAPK play a central role in this process. These observations are in agreement with previous in vitro studies that clearly showed that pretreatment with the ERK1/2 inhibitor PD98059 prevented butylhydroperoxide-induced increases in p65 translocation, NF-κB luciferase activity, and phospho-IKKα/β suggesting that t-butylhydroperoxide induces NF-κB activation through the IKK pathway, which involves ERK activation (Lee et al., 2005). NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation. These include the genes for TNF-α, IL-1β, iNOS and cyclooxygenase-2, to name but a few (Verma, 2004). In this regard, it has been well demonstrated that in SCI the expression of proinflammatory cytokines (TNF-α and IL-1β) at the site of injury regulates the precise cellular events after SCI (Streit et al., 1998; Genovese et al., 2006a). We have clearly confirmed a significant increase in TNF-α and IL-1β in SCI. On the contrary, no significant expression of TNF-α and IL-1β was observed in the spinal cord sections obtained from SCI-operated mice that received PD98059, suggesting that MAPK3/MAPK1 pathway play an important role in the regulation of proinflammatory cytokines. This observation is in agreement with a previous study in which the inhibition of ERK1/2 pathway activation in an experimental mouse model

In this report, we demonstrate that PD98059, an inhibitor of MAPK3/MAPK1, exerts beneficial effects in a mouse model of spinal cord injury. We demonstrate that SCI resulted in edema and loss of myelin in lateral and dorsal funiculi. This histological damage was associated with the loss of motor function. SCI induced an inflammatory response in the
of middle cerebral artery occlusion may also reduce production of proinflammatory cytokine IL-1β mRNA and significantly decrease brain infarct volume (Wang et al., 2004b). As expected, a significant decrease of pERK1/2 levels was observed in the spinal cord sections obtained from SCI-operated mice that received PD98059.

Several studies suggest that glial cells in neurodegenerative diseases (i.e., Alzheimer’s disease) are affected more than neurons by apoptotic cell death (Smale et al., 1995; Beattie et al., 2000). Apoptosis is an important mediator of secondary damage after SCI (Jänicke et al., 1998; Beattie et al., 2002). It incurs its affects through at least two phases: an initial phase, in which apoptosis accompanies necrosis in the degeneration of multiple cell types and a later phase, which is predominantly confined to white matter and involves oligodendrocytes and microglia (Chittenden et al., 1995). Chronologically, apoptosis initially occurs 6 h postinjury at the lesion center and lasts for several days associated with the

Fig. 12. Immunohistochemical expression of Bax and Bcl-2. SCI caused, at 24 h, an increase in Bax expression (A and A1). PD98059 (10 mg/kg) treatment reduced the degree of positive staining for Bax in the spinal cord (B and B1). On the contrary, positive staining for Bcl-2 was observed in the spinal cord tissues from sham-operated mice (data not shown), whereas the staining was significantly reduced in SCI mice (D). PD98059 treatment (10 mg/kg) attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (E). Densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for Bax (C) and for Bcl-2 (F) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percentage of total tissue area. This figure is representative of at least three experiments performed on different experimental days. *p < 0.01 versus sham; °p < 0.01 versus SCI + vehicle.
steadily increased number of apoptotic cells in this region. An important intracellular signal transduction pathway that leads to apoptosis after SCI involves activation of the caspases, in particular, caspase-3 (Chittenden et al., 1995). In an effort to prevent or diminish levels of apoptosis, we have demonstrated that the treatment with PD98059 attenuates the degree of apoptosis, measured by TUNEL detection kit, in the spinal cord after the damage. Moreover, various studies have postulated that preserving Bax, a proapoptotic gene, plays an important role in developmental cell death (Bar-Peled et al., 1999) and in CNS injury (Nesic-Taylor et al., 2005). Likewise, it has been shown that the administration of Bcl-xL fusion protein (Bcl-2 is the most expressed antiapoptotic molecule in adult central nervous system) into injured spinal cords significantly increased neuronal survival, suggesting that SCI-induced changes in Bcl-xL contribute considerably to neuronal death (Casha et al., 2001). Based on such evidence, we have identified in SCI proapoptotic transcriptional changes, including up-regulation of proapoptotic Bax and down-regulation of antiapoptotic Bcl-2, by immunohistochemical staining. We report in the present study that the pharmacological inhibition of MAPK3/MAPK1 pathway by PD98059 in an SCI experimental model documents features of apoptotic cell death after SCI, suggesting that protection from apoptosis may be a prerequisite for regenerative approaches to SCI. In particular, we demonstrated that the treatment with PD98059 reduced Bax expression, whereas on the contrary, Bcl-2 was expressed much more in mice treated with PD98059. Several studies have linked apoptosis to thoracic SCI. Furthermore, some studies have shown that Fas and p75 receptors are expressed on oligodendrocytes, astrocytes, and microglia in the spinal cord following SCI. Fas and p75 colocalize on many TUNEL-positive cells, suggesting that the Fas- and p75-initiated cell death cascade may participate in the demise of some glia following SCI.

Therefore, FasL plays a central role in apoptosis induced by a variety of chemical and physical insults (Dosreis et al., 2004). In a recent study, it has been pointed out that FasL signaling plays a central role in SCI (Ackery et al., 2006). We confirm here that SCI leads to a substantial activation of FasL in the spinal cord tissues that probably contributes in different capacities to the survivability of tissues. In the present study, we found that PD98059 treatment leads to a substantial reduction of FasL activation. However, it is not possible to exclude the fact that the antiapoptotic effect observed after PD98059 treatment may be partially dependent on the attenuation of the inflammatory-induced damage. Further studies are needed to clarify these mechanisms.

Finally, in this study, we demonstrate that PD98059 treatment significantly reduced SCI-induced spinal cord tissue alteration and improved motor function. The results of the present study enhance our understanding of the role of MAPK3/MAPK1 pathway in the pathophysiology of spinal cord cell and tissue injury following trauma, implying that inhibitors of the activity of MAPK3/MAPK1 pathway may be useful in the therapy of spinal cord injury, trauma, and inflammation.

References


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