Calpain Inhibitor Inhibits p35-p25-Cdk5 Activation, Decreases Tau Hyperphosphorylation, and Improves Neurological Function after Spinal Cord Hemisection in Rats

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Abstract

Aberrant calpain activation is a key mediator of neuron death. We examined the cell-permeable calpain inhibitor MDL28170 in the pathophysiological processes after spinal cord injury (SCI) including p35-p25-cyclin-dependent kinase-5 (Cdk5) activation, tau hyperphosphorylation, neuron cell death, calpain I activation, astroglial, and microglia activation. Our study showed that intrathecal administration of MDL28170 improved neurologic dysfunction, prevented neuron loss, decreased the number of apoptotic cells, and abated astrogliosis and microgliosis 7 days after spinal cord hemisection in rats. Reverse transcription polymerase chain reaction demonstrated calpain inhibition significantly attenuated the ratio of pro-apoptotic Bax/anti-apoptotic Bcl-2 mRNA in the lesion and penumbra after SCI. Calpain, the calcium-activated proteolytic enzyme, was found to digest p35 to its truncated product, p25. Moreover, abnormal Cdk5 activation by p25 and subsequent tau hyperphosphorylation triggers pathologic events leading to neurodegeneration and neurofibillary tangles. We found p35-p25-Cdk5 activation and tau hyperphosphorylation in SCI, and then we showed that intrathecal MDL28170 treatment could diminish p35 truncation, and abrogate aberrant tau phosphorylation. Double labeling of calpain I and phosphorylated tau (AT8) in the same cells of spinal cord lesion further implicated pathogenesis of SCI. In conclusion, MDL28170 abated p35-p25-Cdk5 activation by p25 and subsequent tau hyperphosphorylation in SCI, and then we showed that intrathecal MDL28170 treatment could diminish p35 truncation, and abrogate aberrant tau phosphorylation. Double labeling of calpain I and phosphorylated tau (AT8) in the same cells of spinal cord lesion further implicated pathogenesis of SCI. In conclusion, MDL28170 abated calpain I activation, inhibited apoptosis and neuron loss, quenched microgliosis and astrocyte activation, and significantly improved neurologic deficit one week after spinal cord hemisection. The neuroprotective mechanisms of calpain inhibitor in SCI could be attenuating upregulation of Bax/Bcl-2 ratio, preventing p35 truncation in the lesion and penumbra, and abrogating tau hyperphosphorylation.

Key Words: Apoptosis, Calpain inhibitor, Hemisection, p35, Spinal cord injury, Tau.

INTRODUCTION

Spinal cord injury (SCI) interrupts conduction of nerve impulses, leading to neurologic dysfunction (1). Primary injury to the spinal cord immediately disrupts cell membranes, destroys myelin and axons in the longitudinal tracts, damages microvessels, and thus triggers devastating secondary injury process with the release of various deleterious factors (2). Multiple cellular and molecular mechanisms of the secondary injury process cause neurodegeneration through complex cascades to spread neurodegeneration beyond the site of primary injury (3).

Secondary injury cascades are active biologic processes and thus provide a window of opportunity for the treatment of SCI using selective inhibitors. Appropriate treatment strategies targeted to active secondary injury mediators of SCI may protect neuronal cells, and augment axonal regeneration and reconnection (4).

Spinal cord injury leads to an inflammatory response by activation of microglia, blood-born macrophages and astrocytes (5). Microglia and astrocytes become activated right after injury, undergoing remarkable changes in cell shape and functionality. Those cells proliferate and increase in size. Astrocytes undergo a process known as reactive astrogliosis in response to a central nervous system (CNS) injury (6, 7). Among the hallmarks of this process is an increase in immunoreactivity to glial fibrillary acidic protein (GFAP), indicating hypertrophy and proliferation of astrocytes. Reactive astrocytes comprise the most abundant component of the “glial scar,” which eventually forms within the CNS following injury. This glial scar tissue inhibits regeneration in the CNS by forming a physical barrier impeding axon ingrowth. Microglia, immune cells in the CNS, have been reported to proliferate and become activated after spinal cord injury (8, 9). This proliferation may accelerate neuronal damage by releasing toxic substances, including super oxide, nitric oxide,
and tumor necrosis factor-alpha (8, 10). These activated astrocytes and microglia increase calpain expression (11). Since calpain degrades many myelin and axonal structural proteins, the increased calpain activity and expression of this enzyme may be responsible for destruction of myelinated axons adjacent to the lesion site following SCI (12, 13).

Apoptosis is now accepted as an important mechanism in the pathogenesis of the secondary injury process following SCI (14, 15). Apoptosis is an active gene-directed death process mediated by activation of a number of cysteine proteases including calpain (16); therefore, it may be preventable with selective inhibitors. Furthermore, recent studies have exhibited a pivotal role for calpain, a cytosolic Ca"+-dependent neutral proteinase, in various types of cell death (17, 18). Two major isoforms of calpain have been identified: calpain I (μ-calpain) and calpain II (m-calpain), which respectively require low (μM) and high (mM) intracellular Ca"+ concentrations for activation (19). Excess calpain activation after insult subsequently causes membrane bleb formation by degrading cytoskeletal proteins, such as talin and α-actinin (20). Increased intracellular calpain protease activity results in the loss of mitochondrial membrane potential and increased membrane permeability that leads to cell death (21). Therefore, calpain plays an important role in the mediation of apoptosis in SCI.

Cyclin-dependent kinase 5 (Cdk5) and its neuron-specific activator p35 are required for neurite outgrowth and cortical lamination (22). Proteolytic cleavage of p35 produces p25, which accumulates in the brains of patients with Alzheimer disease (23). Conversion of p35 to p25 by calpain causes prolonged activation and mislocalization of Cdk5. Furthermore, the Cdk5-p25 complex hyperphosphorylates tau (microtubule-associated protein), causing disruption of microtubule integrity and inevitable neuron death (24). However, the mechanisms of p35-p25-Cdk5 activation and subsequent tau hyperphosphorylation in SCI have never been well studied.

Calpain modulated key processes governing the pathogenesis of neurodegeneration. Therefore, calpain inhibitors are presumed as therapeutic agents in a variety of disorders, such as stroke, spinal cord injury, Parkinson disease, and Alzheimer disease (25). MDL28170 is a potent and cell-permeable inhibitor of calpain I and II (Ki = 8 nM), and it also exhibits protective effects against thioacetamide-induced acute liver failure, global cerebral ischemia and contusive spinal cord injury (26–28). In this study, we examined MDL28170 as a neuroprotective agent after spinal cord hemisection in rats. Apoptosis and inflammatory response such as astrocytes and microglia activation after SCI were investigated. Furthermore, we hypothesized that aberrant calpain upregulation could result in p35-p25-Cdk5 activation, and subsequent tau hyperphosphorylation after spinal cord hemisection. Finally, we tested whether calpain inhibitor could inhibit both p35-p25-Cdk5 activation and tau hyperphosphorylation in SCI.

**MATERIALS AND METHODS**

**Animal Care and Intrathecal Catheterization**

Male Sprague-Dawley rats (Academia Sinica, Taiwan), weighing 280–330 g, were kept two per cage for at least 5 days after their arrival. The rats had access to food and water ad libitum and were housed within a room with a 12:12 hour dark–light cycle. This study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee in Kaohsiung Chang Gung Memorial Hospital.

Intrathecal catheters were implanted under isoflurane inhalation anesthesia 3 days before spinal cord hemisection injury. Briefly, a polyethylene (PE-5) catheter, filled with 0.9% saline, was advanced 8 cm caudally through an incision in the atlanto-occipital membrane, and its tip positioned at the level of the lumbar enlargement. The rostral tip of the catheter was passed subcutaneously, and connected to an Alzet® microosmotic pump model 1003D with rate of 1 μL per hour (DURECT, Cupertino, CA). Pump function was confirmed by weighing the pump before placement and after the experiment. Rats showing neurologic deficits after implantation were excluded.

**Induction of Spinal Cord Hemisection Injury in Rats**

For hemisection, the rats received isoflurane inhalation anesthesia and were placed in a spinal cord unit of a stereotoxic apparatus (David Kopf Instruments, Tujunga, CA). By means of an adjustable wire knife, the rats had their spinal cord lesioned in the left side (n = 32) or were submitted to sham operation (n = 6). Laminectomy was performed at 11th thoracic vertebrae with delicate diamond drills and the guide of the wire knife was placed in a vertical plane close to the lateral surface of the low thoracic level of the spinal cord. This level was chosen so that cranial and caudal unlesioned segments of the spinal cord could be analyzed. The knife, that was previously turned medially, was then extended 1.5 mm and the guide was lifted 4.0 mm to hemitranssect the spinal cord. Indocyanine green was used to ensure the completeness of the hemisection. The fascia and skin was closed with sutures by layer, and animals were allowed to recover on a 36.5°C heating pad. Postoperative treatments included saline (1.0 mL subcutaneously) for rehydration. Following surgery, animals were maintained under the same preoperative conditions, and were eating and drinking within 3 hours after surgery. Weight loss was minimal, occurred acutely over the first 2 postoperative days, and was not greater than 5% of the total body weight. The extent of the hemisection lesion, assessed histologically, was confined unilaterally and included the dorsal column, Lissauer’s tract, lateral and ventral column systems, and gray matter. Locomotor function was observed and recorded using the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (29) to ensure that a motor deficit of the ipsilateral limb occurred and did not impair the somatosensory behavioral tests. Animals that demonstrated loss of locomotion in both hindlimbs, indicating bilateral corticospinal tract transection, were excluded from the study. Sham operation consisted of only laminectomy.

**Treatment of SCI**

Rats were randomly divided into two groups (each group n = 16). MDL group received intrathecal administration MDL28170 100 μg 30 minutes before hemisection surgery.
and continuous infusion (10 μg/hour) for 3 days. Vehicle group received dimethyl sulfoxide (DMSO) 10 μl 30 minutes before surgery and continuous infusion (1 μl/hour) for 3 days. The sham group received intrathecal catheterization only (n = 6). Upon recovery from anesthesia, hemitransected animals were evaluated neurologically with affected hindlimb paralyzed initially. BBB locomotor rating scales (29) were recorded 1 and 7 days after injury. MDL28170 was purchased from Calbiochem (San Diego, CA) and dissolved in DMSO. DMSO was obtained from Sigma-Aldrich (St. Louis, MO).

Histology, Immunohistochemistry and Cell Count

On the seventh day after hemisection spinal cord injury, animals were deeply anesthetized by isoflurane and perfused through the left ventricle with phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde in 0.15 M sodium phosphate buffer, pH 7.4. The spinal cord was removed immediately, postfixed for 8 hours in the same fixative at 4°C, and cryoprotected for 2–3 days in 15% and 30% sucrose. The spinal cord was frozen in powdered dry ice and stored at −80°C until needed. Five-micrometer sections were cut with a freezing and sliding microtome at the center of spinal cord hemisection. The sections were prepared for either immunostaining or apoptosis staining. For immunohistochemistry, sections were washed in PBS and incubated in 3% normal goat serum with 0.3% Triton X-100 in PBS for 1 hour. The sections were incubated free-floating at 4°C with anti-NeuN (neuron-nuclear specific protein) (Chemicon, Temecula, CA), anti-calpain I (H-65) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GFAP (DakoCytomation, Glostrup, Denmark), anti-CD11b (OX-42) (Chemicon), anti-p35 (C-19) (Santa Cruz

![Graph](image)

**FIGURE 1.** (A) Calpain inhibitor improves neurologic deficit after spinal cord hemisection. Treatment with MDL28170 resulted in significant improvement of Basso, Beattie, and Bresnahan (BBB) scales of affected hindlimb versus vehicle-treated after spinal cord injury (SCI). A normal limb would be 21 on BBB scale (Day 1: **, p < 0.01; Day 7: ***, p < 0.001, Mann-Whitney U test, n = 16 in each group). Representative photographs of NeuN-stained sections of the spinal cord from sham controls (B, E), hemisection with MDL28170 treatment (C, F), and hemisection with vehicle treatment (D, G). Note that there are normal neurons in sham group (E), and relative preservation for NeuN-stained neurons in MDL group (F), compared with severe neuron loss in vehicle group (G) after SCI. Arrows indicate NeuN positive cells (Original magnification: B–D, 12×; E–G, 400×). (H) Vertical bars indicate the mean (±SEM) number of neurons per tissue section for the sham controls (sham), hemisection with MDL28170 treatment (MDL), and hemisection with vehicle treatment (vehicle). (Sham vs vehicle: ***, p < 0.001, MDL vs vehicle: ###, p < 0.001, n = 5 in each group).
Biotechnology), or anti-p-Tau (AT8) (Pierce Biotechnology, Rockford, IL) antibodies. Immunoreactivity was visualized using the Vectastain Elite ABC Peroxidase method (Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB) as the chromagen. Dako EnVision Doublestain System (DakoCytomation) was performed for the double labeling of calpain I (fast red) and p-tau (DAB). Furthermore, apoptosis after hemisection was detected by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) using the apoptosis detection kit (Oncogene Research Products, Cambridge, MA). TUNEL staining was performed according to the manufacturer’s instructions. A negative control of TUNEL staining was generated by omission of Klenow enzyme, while negative control sections of other immunohistochemical studies were incubated as above without primary antibodies. Cell counting was performed on every sixth section at the center of spinal cord hemisection stained with the above antibodies at a magnification of 400×. Only cells with clearly visible stain were counted. All data are presented as means ± SEM of 5 consecutive cell quantifications.

Reverse Transcription-Polymerase Chain Reaction Analysis of Bax and Bcl-2 mRNA

For mRNA analysis, animals were anesthetized and decapitated 7 days after SCI. A 4.5-cm-long spinal cord section with the lesion at the epicenter was collected and divided equally into three 1.5-cm segments (rostral [R], injury [I], and caudal [C]). The expression of Bax and Bcl-2 mRNA was investigated by reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from harvested spinal cord with chloroform and TRIzol reagent (Molecular Research Center, Cincinnati, OH). RNA was then reverse transcribed into cDNA. A 20-μl reverse transcription reaction mixture containing 1 μg of total RNA, 1× PCR buffer (10 M Tris-HCl, pH 8.3, 0.05 M KCl), 300 μM deoxynucleotide triphosphates, 1 unit of RNase inhibitor, 2.5 μM oligo(dT)16

Figure 2. Representative photographs of triphosphate nick-end labeling (TUNEL)-stained sections of the spinal cord from sham controls (A), hemisection with MDL28170 treatment (B), hemisection with vehicle treatment (C), and negative control (without Klenow enzyme) of hemisection with vehicle treatment (D) (Original magnification: 400×). Arrows indicate TUNEL positive cells. (E) Vertical bars indicate the mean (±SEM) number of TUNEL-stained cells per tissue section for the sham controls (sham), hemisection with MDL28170 treatment (MDL), and hemisection with vehicle treatment (vehicle) (sham vs vehicle: ***, p < 0.001, MDL vs vehicle: ###, p < 0.001, MDL vs sham: $$$, p < 0.001, n = 5 in each group).
and 10 units of M-MLV reverse transcriptase was incubated at 42°C for 1 hour, heated to 95°C for 5 minutes, and then quickly chilled to 5°C for 5 minutes. PCR was performed at a final concentration of 1 × PCR buffer, 1.0 μM each of 3’ and 5’ primers, and 10 units of Advan-Taq Plus DNA polymerase (Clontech, Palo Alto, CA) in a total volume of 50 μl. The cDNA of Bax and Bcl-2 were semiquantified by Apoptosis PCR Bax/Bcl-2 multiplex primer sets (APOPCR, Sigma-Aldrich). The mixture was amplified with a thermal cycler (Stratagene, La Jolla, CA). The amplification profile involved initial denaturation at 95°C for 2 minutes, then denaturation at 94°C for 45 seconds, annealing at 53°C for 45 seconds, and extension at 72°C for 1.5 minutes for 30 cycles with final extension at 72°C for 7 minutes according to the user manual. The expected product length of Bax, GAPDH, and Bcl-2 were 487, 349, and 127 bp, respectively. Amplification products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The gel was scanned at a NucleoVision imaging workstation (NucleoTech, San Mateo, CA), and quantified using GelExpert release 3.5.

**Immunoblotting of Cdk5 Activator, p35 and p25**

For immunoblotting, spinal cord samples were homogenized in lysis buffer (20 mM Tris pH 7.6, 150 mM NaCl, 1 mM EGTA, 5 mM NaF, and 1 mM dTT) supplemented with complete protease inhibitor cocktail tablets (Roche, Mannheim, Germany). For analysis of Cdk5 activator, p35 and p25 expression after SCI, 25 μg protein extracts were electrophoresed on a 12% acrylamide SDS-PAGE gel and immunoblotted onto PVDF membranes. The membranes were blocked for 1 hour at room temperature and incubated overnight with anti-p35 antibody (C-19) (Santa Cruz Biotechnology) (1:1000) and α-tubulin antibody (Santa Cruz Biotechnology) (1:1000). α-Tubulin was used as internal control and verification of equal loading. Antibody binding

**FIGURE 3.** Representative photographs of calpain I-stained sections of the spinal cord from sham controls (A, D), hemisection with MDL28170 treatment (B, E), and hemisection with vehicle treatment (C, F). Intense specific stainings of calpain 1 were demonstrated in the vehicle group (C, F). This activation was significantly inhibited in the MDL group (B, E) (Original magnification: A–C, 12×; D–F, 50×). (G) Vertical bars indicate the mean (±SEM) number of calpain I-stained cells per tissue section for the sham controls (sham), hemisection with MDL28170 treatment (MDL), and hemisection with vehicle treatment (vehicle) (sham vs vehicle: ***, p < 0.001, MDL vs vehicle: ###, p < 0.001, n = 5 in each group).
was detected using a goat anti-rabbit horseradish peroxidase-linked IgG. The bands were visualized by an ECL detection system (Amersham-Pharmacia Biotech, Buckinghamshire, England). Band intensities were quantified by using an image analyzer (Densitograph AE-6900M, Atto, Tokyo, Japan).

**Statistical Analysis**

All data are presented as means ± SEM (at least 3 separate experiments). Statistical analysis was performed using a one-way ANOVA. p values less than 0.05 (p < 0.05) were considered significant. For BBB scale analysis, Mann-Whitney U test was used.

**RESULTS**

**Neurological Deficit and Apoptosis after Spinal Cord Hemisection**

For evaluating neurologic impairment after SCI, BBB locomotor scales were recorded. BBB scales of the affected hindlimb after hemisection were significantly improved in the MDL group as compared to the vehicle group (Day 1: p < 0.05; Day 7: p < 0.001, Mann-Whitney U test) (Fig. 1A). Immunohistochemistry of NeuN, neuron-specific marker, disclosed severe neuron loss in the vehicle group, while greatly preserved in the MDL group (Fig. 1B–H). Furthermore, TUNEL-positive cells were easily demonstrated in the vehicle group, while apoptosis was hardly detected in the MDL group (Fig. 2). These data suggested that calpain inhibitor could prevent neurologic deficit, neuronal loss and apoptosis after spinal cord hemisection, and thus provided the rationale of improved BBB scales after calpain inhibitor treatment.

**Calpain I Activation and Inflammatory Response after SCI**

There are two major isoforms of calpain, calpain I and calpain II, which require low and high intracellular Ca^{2+} concentrations for activation. Rapid calpain I activation with cytoskeletal degradation following CNS injury had been
demonstrated (30). We further studied expression of calpain I after hemitranssection. Intense specific staining of calpain I was noticed in the vehicle group. This activation was significantly inhibited in the MDL group (Fig. 3). Astrocyte and microglia activation are the major inflammatory responses after SCI. The expression of GFAP and CD11b, markers of activated astrocytes and microglia, respectively, were investigated. Strong immunostaining of GFAP and CD11b were demonstrated in the spinal cord lesion. However, these activations were greatly attenuated in the MDL group (Figs. 4, 5). These results showed MDL28170, a potent calpain inhibitor, abated activation of calpain I, astrocytes, and microglia after SCI.

**Bax to Bcl-2 mRNA Ratio after SCI**

Pro-apoptotic Bax and anti-apoptotic Bcl-2 serve to measure the balance between cell survival and death (31). Bax resides in mitochondria and is known to be activated by calpain I (32). Because calpain activities increase in SCI, we further examined the relative expression of Bax and Bcl-2 levels in lesion and penumbra following SCI with calpain activation by Apoptosis PCR Bax/Bcl-2 multiplex primer sets. Bax to Bcl-2 mRNA ratio increased 7 days after hemisec- tion. Meanwhile, MDL28170 treatment could attenuate this upregulation effectively (Fig. 6). There were statistical significances of reduction of Bax to Bcl-2 mRNA ratio in all of the 3 cord segments of the MDL group as compared to the vehicle group (rostral segment [R]: *p < 0.05; injury segment [I]: **p < 0.01; caudal segment [C]: *p < 0.05). These findings, together with prominent anti-apoptotic effects on MDL group (Fig. 2), suggested that calpain inhibitor could prevent apoptosis after spinal cord hemisection by altering levels of Bcl-2 family members in the cell.

**Alteration of Cdk5 Activators, p35 and p25, after Spinal Cord Hemisection**

It is known that neurotoxicity induces cleavage of p35 to p25 by calpain (33). For further exploring relationship between the protective mechanism of MDL28170 and the Cdk5 activators p35 and p25, immunohistochemical and immuno-blotting analysis of p35 were investigated. Specific staining of p35 was upregulated in the vehicle group, and MDL28170 administration prevented this activation significantly (Fig. 7).
Chemistry disclosed phosphorylated tau (AT8) expression was decisive role in the pathogenesis of neurodegenerative diseases. Immunohistochemistry disclosed phosphorylated tau in the same cytoplasm after hemisection, and the colocalization diminished in the MDL group after intrathecal calpain inhibitor treatment (Fig. 8F–G). These data suggested calpain inhibitor could prevent abnormal calpain activation and inhibit tau hyperphosphorylation after SCI.

**DISCUSSION**

Delineating the cellular and molecular pathophysiological mechanism of spinal cord injury can provide valuable clues for the development of new therapeutic strategies (39). The primary injury immediately causes cell death or necrosis at the site of the lesion and then initiates a secondary injury process that leads to an extension of the lesion into rostral and caudal areas of the spinal cord. Apoptosis or programmed cell death appears to play an important role in the progression of secondary injury after SCI. Apoptosis occurs in response to appropriate death signals and requires activation of cysteine proteases, including calpain (40). Excessive activation of calpain has been proposed as a contributing factor in ischemic neurodegeneration and neurotrauma. Calpains, particularly calpain I, are clearly activated during insults resulting in necrotic death. Calpain inhibition is neuroprotective in some studies, but not in others (41–43). In this study, neurologic deficit in terms of BBB scales after SCI significantly improved in the MDL group (Fig. 1A). Upregulation of calpain activity was prominent in the spinal cord after hemisection. Calpain inhibitor attenuated severe neuron loss, programmed cell death and aberrant calpain I activation after SCI (Fig. 1B, Figs. 2, 3).

Neuronal and nonneuronal cells in the vicinity of a spinal cord lesion undergo morphologic and functional changes which are related to the local inflammatory events that mediate primarily the normal nervous tissue repair (44). It is postulated that the size of a secondary neurodegeneration after spinal cord injury depends on the magnitude of the inflammatory events. At the immediate vicinity of injury, reactive astrocytes interweave their processes to form a barrier termed anisomorphic gliosis. This glial scar can be an impediment to regenerating axons. Increased glial fibrillary acidic protein (GFAP) expression is a hallmark of reactive astrocytes and this cytoskeletal protein contributes to a barrier effect of the glial scar for axonal extension. This response is fortified by the migration of microglia and macrophages to the damaged area. Astrocytes and microglia deserve special attention regarding their roles in promoting the glial scar formation during and after the inflammatory process in the spinal cord lesion (5, 45). Our study demonstrated marked activation of astrocytes and microglia after SCI by immunostaining of GFAP and CD11b, and MDL28170 treatment effectively abolished these inflammatory responses (Figs. 4, 5).

The “commitment” step of apoptosis is regulated by the expression of genes of the Bcl-2 family (46), which includes both pro-apoptotic and anti-apoptotic members. In the nervous system, Bax and Bcl-2 genes dominate in regulating apoptosis. Bax/Bcl-2 mRNA ratio determines the survival or death following an apoptotic insult (47, 48). An elevation of Bax/Bcl-2 ratio...
affects mitochondria permeability, releases cytochrome c, and finally directs the cells to die by apoptosis (31). In this experiment, we demonstrated increased Bax to Bcl-2 ratio in the spinal cord lesion and penumbra after hemisection, and this elevated ratio could be effectively inhibited by MDL28170 treatment (Fig. 6). This finding provides the molecular evidence of anti-apoptotic effect of calpain inhibitor in SCI.

Hyperphosphorylated tau is a major component of neurofibrillary tangles, one of the hallmarks of Alzheimer disease. Cdk5 is a kinase that phosphorylates the tau protein, and its endogenous activators, p35 and p25, regulate its activity (49). Calpain was found to digest p35 to its truncated product, p25, which has more powerful kinase activity than p35. Recent studies showed that aberrant Cdk5 activation by p25 triggers pathologic events leading to neurodegeneration and neurofibrillary tangles (34, 50). However, the mechanism of p35-p25-Cdk5 activation and tau hyperphosphorylation in SCI has never been well studied. In the present study, immunohistochemistry and immunoblotting disclosed upregulation of both p35 and its truncated product p25 expressions in the spinal cord lesion and its penumbra. MDL28170 treatment could abate this conversion indicating its neuroprotection effect in SCI might mediate through inhibition of p35-p25-Cdk5 activation.

**FIGURE 7.** Representative photographs of p35(C-19)-stained sections of the spinal cord from sham controls (A), MDL group (B), vehicle group (C), and negative control (omission of primary antibody) of vehicle group (D) 7 days after spinal cord injury (SCI). Specific staining of p35 were upregulated in the vehicle group (C), while MDL28170 administration prevented this activation (B) (Original magnification: 400×). (E) Vertical bars indicate the mean (± SEM) number of p35-stained cells per tissue section for the sham controls (sham), hemisection with MDL28170 treatment (MDL), and hemisection with vehicle treatment (vehicle) (sham vs vehicle: ***, p < 0.001, MDL vs vehicle: ###, p < 0.001, n = 5 in each group). (F) Representative photographs of proteolytic cleavage of p35 to p25 by immunoblotting. There was no p35 cleavage in the sham group. Truncation of p35 with p25 accumulation was exhibited in the spinal cord lesion and penumbra of vehicle group following SCI. This truncation was inhibited in the MDL group (R, rostral segment; I, injury segment; C, caudal segment). (G) α-Tubulin was used as internal control and verification of equal loading (3 separate experiments).
Alzheimer disease is pathologically characterized by the occurrence of neurofibrillary tangles composed primarily of hyperphosphorylated tau protein. Hyperphosphorylated tau lacks normal tau's activities of binding microtubules and stimulating their assembly. Instead, it sequesters normal tau and other microtubule-associated proteins and finally disorganizes the cytoskeleton (38, 51). The phosphorylation-dependent anti-tau antibodies have distinct epitopes. AT8 reacts with tau only when multiple sites around Ser202, including Ser199, Ser202, and Thr205, are phosphorylated. Single phosphorylation of any of the residues is not enough for AT8 reactivity (52, 53). Thus, AT8 is useful and quite specific in detecting phosphorylation of Ser202/Thr205 for proline-directed kinases, including Cdk5 complexes. It has been shown that cleavage of p35 to p25 by calpain regulates not only the overall kinase activity of Cdk5, but also the sequential phosphorylation of Ser202 and Thr205 in tau (37). Most of the tau studies focus on neurodegenerative Alzheimer disease, and we could not find such investigations of tau hyperphosphorylation in SCI models yet. In this experiment, we demonstrated colocalization of calpain I and phosphorylated tau double stained sections of the spinal cord from hemisection with MDL28170 treatment (F), and hemisection with vehicle treatment (G), while most of AT8 staining was inhibited in the MDL group (F). Arrows indicate double labeling positive cells (Original magnification: 400×).
apoptosis and neuron loss, quenched microglia and astrocyte activation, and significantly improved neurologic deficit 1 week after spinal cord hemisection. Furthermore, calpain inhibition attenuated upregulation of Bax/Bcl-2 ratio, prevented p35 truncation in the lesion and penumbra, and abrogated tau hyperphosphorylation following SCI. These findings suggest that calpain inhibition is a promising neuroprotective approach after SCI. Further studies involving alternative dosages, routes and postinjury treatment paradigms are necessary to determine the potential clinical application of MDL28170 as a treatment for SCI.

REFERENCES


43. Adamec E, Beermann ML, Nixon RA. Calpain I activation in rat hippocampal neurons in culture is NMDA receptor selective and not essential for excitotoxic cell death. Brain Res Mol Brain Res 1998;54:35–48
44. Giulian D, Vaca K. Inflammatory glia mediate delayed neuronal damage after ischemia in the central nervous system. Stroke 1993;24:184–90