Post-traumatic inflammatory reaction may contribute to progressive tissue damage after spinal cord injury (SCI). Two key transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), are activated in inflammation. An increase in NF-κB binding activity has been shown in the injured spinal cord. We report activation of AP-1 after SCI. Electrophoretic mobility shift assay showed that AP-1 binding activity increased after SCI, starting at 1 hr, peaking at 8 hr, and declining to basal levels by 7 d. Methylprednisolone (MP) is the only therapeutic agent approved by the Food and Drug Administration for treating patients with acute traumatic SCI. MP reduced post-traumatic inflammatory genes such as MMP-1 and MMP-9 that are transactivated jointly by AP-1 and NF-κB may not be suppressed by inhibiting only AP-1 activity.

Key words: inflammation; methylprednisolone; NF-κB; protease; RU486; transcription factor

Methylprednisolone (MP), a synthetic glucocorticoid (GC), is the only therapeutic agent approved by the Food and Drug Administration (FDA) for the treatment of acute traumatic spinal cord injury (SCI) in humans (Bracken, 1990). However, the effect of MP in SCI is modest (Nesathurai, 1998), and its mechanism of action remains to be fully delineated. GCs including MP are anti-inflammatory agents with a wide range of useful clinical applications (Barnes, 1998). A post-traumatic inflammatory reaction has been extensively documented in animal SCI models (Balentine, 1978a,b; Means and Anderson, 1983; Xu et al., 1990; Blight, 1992; Dusart and Schwab, 1994; Bartholdi and Schwab, 1995; Hamada et al., 1996; Popovich et al., 1996, 1997; Zhang et al., 1997). Inhibition of lipid peroxidation (Hall and Braughler, 1981) and inflammatory reaction (Hsu and Dimitrijevic, 1990; Bartholdi and Schwab, 1995) are thought to contribute to the therapeutic effects of MP in SCI. GC suppression of inflammation is mediated by a glucocorticoid receptor (GR) mechanism. GCs, functioning as ligands, bind to the cytosolic GR to form activated GR (aGR) (Barnes, 1998). aGR is an anti-inflammatory transcription factor that inhibits the activation of two major proinflammatory transcription factors, nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990; Ray and Prefontaine, 1994; Caldenhoven et al., 1995; Scheinman et al., 1995).

NF-κB activation, known to transactivate proinflammatory genes, including those encoding cytokines, adhesion molecules, inducible nitric oxide synthase, and others in immune and inflammatory processes, has been demonstrated in the injured cord (Bethea et al., 1998; Xu et al., 1998). NF-κB activation after SCI was suppressed by MP (Xu et al., 1998). AP-1 activation after SCI, however, has not been studied previously. AP-1 is a dimer composed of various Fos and Jun family proteins (Chiu et al., 1988; Halazonetis et al., 1988). Immediate early genes of the fos and jun families are upregulated after ischemic and traumatic CNS injury (An et al., 1993; Yang et al., 1994). AP-1, functioning as a proinflammatory transcription factor, transactivates a number of genes that are expressed in inflammation (Karin et al., 1997; Wisdom, 1999). Among these are genes encoding matrix metalloproteinases (MMPs) (Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990; Sato et al., 1993; Yokoo and Kitamura, 1996). Excessive MMP expression leads to increased capillary permeability in numerous neurological disorders such as multiple sclerosis, infection, and ischemia (Yang et al., 1998). Alteration of vascular permeability, a key feature of inflammation, has been noted after SCI (Hsu et al., 1985).

In this study we examined the impact of SCI on AP-1 transactivation of downstream genes MMP-1 and MMP-9, as gauged by MMP-1 and MMP-9 protein expression. Because GR activation mediates the inhibition of NF-κB and AP-1 (Barnes, 1998), and
GR expression is increased after SCI (Yan et al., 1999), we also explored whether the activation of the AP-1 cascade after SCI could be modulated by a GR mechanism.

MATERIALS AND METHODS

Spinal cord injury model. A total of 91 female Long-Evans rats (240 ± 40 g; Simonsen Laboratories, Gilroy, CA) were used for the present study. All surgical procedures and animal experimentation protocols followed the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and the Guidelines and Institutional Animal Care and Use Committee protocols provided by the Animal Studies Committee of Washington University School of Medicine. The method for inducing SCI followed the Multicenter Animal Spinal Cord Injury Study (MASCIS) protocol (Basso et al., 1996) as reported previously (Liu et al., 1997). Briefly, rats were anesthetized with pentobarbital (35–45 mg/kg), and a laminectomy was made at the T-10 segment. Using a New York University Impactor, SCI was induced by dropping a 10 g weight at a height of 12.5 mm (Gruner, 1992). Animals subjected to identical surgical procedures without injury served as sham-operated controls. In addition, animals that received no surgery were also included as controls. Postoperative care, including bladder management, was detailed previously (Liu et al., 1997; Yan et al., 1999).

Treatment protocols. Animals were treated with MP intravenously at a dose of 30 mg/kg, 15 min after injury. Rats given the vehicle only served as controls. In selected group of MP-treated rats, RU486 (15 mg/kg; Simonsen Laboratories, Gilroy, CA) were used for the present study. In addition, animals that received no surgery were also included as controls. In selected group of MP-treated rats, RU486 (15 mg/kg; Simonsen Laboratories, Gilroy, CA) were used for the present study.

RESULTS

AP-1 activation after spinal cord injury A low basal level of AP-1 binding activity could be detected in the normal control and sham-operated cords. SCI resulted in a substantial increase in AP-1 activity in a time-dependent manner starting at 1 hr and peaking at 8 hr after SCI. Increase in AP-1 activity was still evident up to 3 d after SCI but returned to the basal level by 7 d (Fig. 1A). The criteria for establishing the validity of AP-1 binding activity has been detailed elsewhere (An et al., 1993; Liu et al., 1994). The specificity of AP-1 binding activity is demonstrated by complete abolishment of the AP-1 band in the presence of 200-fold excess of cold AP-1 oligonucleotide (data not shown) (An et al., 1993) or by a c-fos antisense strategy (see below).

Effect of MP and RU486 on AP-1 activation after SCI GC suppression of inflammation is mediated by a GR mechanism acting mainly through transrepression of proinflammatory genes driven by two key transcription factors, NF-κB and AP-1 (Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990; Ray and Prefontaine, 1994; Caldenhoven et al., 1995; Scheinman et al., 1995; Barnes, 1998). NF-κB activation has been shown in SCI (Bethea et al., 1998; Xu et al., 1998) and could be inhibited by MP treatment (Xu et al., 1998). In the present study, MP (30 mg/kg) also inhibited AP-1 activation after SCI (Fig. 1B). RU486, a potent GR antagonist, reversed anti-inflammatory effects of GCs by blocking a GR-mediated actions (Laue et al., 1988; Jewell et al., 1988).
with similar results. Free probe; 1, 4, 4 hr; 5, 8 hr; 6, 1 d; 7, 3 d; and 8, 7 d after SCI. Note progressive increase in AP-1 binding activity starting at 1 hr and peaking at 8 hr. AP-1 activation was still evident 3 d after injury. B, Effect of MP and RU486 on AP-1 binding activity after SCI. Rats were treated with or without MP (30 mg/kg, i.v.; 15 min after injury). In some MP-treated animals, pretreatment with RU486 (15 mg/kg, i.p.; 30 min before injury) was given. Lanes 1, Free probe; 2, sham-operated control; 3, normal control; 4, SCI; 5, SCI + MP; and 6, SCI + RU486 + MP. Note MP inhibition of post-traumatic AP-1 activation. This MP effect was reversed by RU486 pretreatment. Data shown in A and B are representative of three separate experiments with similar results.

To further confirm the specificity of AP-1 binding activity, we used an antisense strategy directed at c-fos to decrease AP-1 activation as has been successfully shown in a brain injury model (Liu et al., 1994; Cui et al., 1999). The same strategy, entailing an in vivo transfection of a c-fos antisense ODN, reduced post-traumatic activation of AP-1 (Fig. 3A). To reduce nonspecific ODN effects, a c-fos sense ODN was also tested and showed no effect on AP-1 activation after SCI (Fig. 3A). The specificity of the c-fos antisense ODN on AP-1 activity was supported by the observation that it did not affect NF-κB activation after SCI (Fig. 3B).

Figure 1. A, Time-dependent changes in AP-1 binding activity after SCI based on EMSA. Lanes 1, Normal control; 2, sham-operated control; 3, 1 hr; 4, 4 hr; 5, 8 hr; 6, 1 d; 7, 3 d; and 8, 7 d after SCI. Note progressive increase in AP-1 binding activity starting at 1 hr and peaking at 8 hr. AP-1 activation was still evident 3 d after injury. B, Effect of MP and RU486 on AP-1 binding activity after SCI. Rats were treated with or without MP (30 mg/kg, i.v.; 15 min after injury). In some MP-treated animals, pretreatment with RU486 (15 mg/kg, i.p.; 30 min before injury) was given. Lanes 1, Free probe; 2, sham-operated control; 3, normal control; 4, SCI; 5, SCI + MP; and 6, SCI + RU486 + MP. Note MP inhibition of post-traumatic AP-1 activation. This MP effect was reversed by RU486 pretreatment. Data shown in A and B are representative of three separate experiments with similar results.

Figure 2. Increase in Fos-B and c-Jun immunoreactivity after SCI. A, In a sham-operated cord segment, little Fos-B immunoreactivity was detected. B, Fos-B immunoreactivity was intense in a section of the injured cord 7 mm rostral to the epicenter; 8 hr after SCI. C, Preabsorption with Fos-B in a section adjacent to the section shown in B resulted in the disappearance of Fos-B immunoreactivity. D, E, Fos-B immunoreactivity was detected in both the gray (D) and white (E) matter in the injured cord, 5 mm distal to the epicenter. F, G, c-Jun expression was also detected in the gray (F) and white (G) matter, in areas corresponding to D and E. Note that Fos-B and c-Jun immunoreactivity could be localized in the cytoplasm and more intensely in the nucleus of cells with morphology suggestive of neurons in the gray matter (D, F) and glial cells in the white matter (F, G). Results shown are representative of two separate experiments with similar results. Scale bars: A–C, 150 μm; D, F, 75 μm; E, G, 100 μm.

Figure 3. Effect of c-fos antisense and sense ODN on AP-1 and NF-κB binding activity. Rats were treated with c-fos antisense or sense ODN or vehicle (lipofectin only) 16 hr before SCI. The injured cord segment was sampled for AP-1 or NF-κB binding activity by EMSA 8 hr after SCI. Lanes 1, Lipofectin; 2, c-fos sense ODN; and 3, c-fos antisense ODN. Note c-fos antisense ODN treatment blocked post-traumatic increase in AP-1 (top panel) but not NF-κB (bottom panel) binding activity. Data shown are representative of three separate experiments with similar results.

MMP-1 and MMP-9 expression after SCI
MMP-1 and MMP-9 are expressed in inflammation (Brenner et al., 1989; Vu and Werb, 1998). Genes encoding these two pro-
teases are transactivated jointly by AP-1 and NF-κB (Yokoo and Kitamura, 1996; Bond et al., 1998, 1999; Vincenti et al., 1998). With the demonstration of AP-1 activation in the present study and previous report of NF-κB activation after SCI (Bethea et al., 1998; Xu et al., 1998), we expected transactivation of MMP-1 and MMP-9 genes by AP-1 and NF-κB after SCI. Immunohistochemical studies showed increased expression of MMP-1 (Fig. 4) and MMP-9 (Fig. 5). During their peak expression, both MMP-1 and MMP-9 were expressed with high intensity in neurons and glial cells after SCI. Immunohistochemical demonstration of MMP-1 and MMP-9 expression was confirmed by Western blotting (Fig. 6).

Effects of MP and RU486 on MMP-1 and MMP-9 expression after SCI

GCs including MP have been shown to suppress the activation of both AP-1 and NF-κB in inflammatory disorders (Barnes, 1998). MP has also been shown to inhibit NF-κB (Xu et al., 1998) and AP-1 activation (Fig. 1B). Thus, MP was anticipated to repress post-traumatic expression of MMP-1 and MMP-9. Our results are in accordance with this prediction. MP inhibited post-traumatic increase in MMP-1 and MMP-9 expression as shown by immunohistochemistry (Figs. 4, 5) and Western blotting (Fig. 6). RU486 pretreatment reversed this MP effect (Figs. 4–6). The antisense c-fos ODN, which selectively blocked AP-1, but not NF-κB activation failed to repress MMP-1 and MMP9 expression after SCI (Fig. 7).

Figure 4. MMP-1 expression after SCI. A, Sham-operated control showing little MMP-1 immunoreactivity. B, Intense MMP-1 expression 1 d after SCI in a section 5 mm rostral to the epicenter. C, MP suppression of MMP-1 expression in a cord section corresponding to B from an animal treated with MP. D, RU486 reversal of MP inhibition of MMP-1 expression after SCI in a section corresponding to B in an animal pretreated with RU486 and post-treated with MP. Results shown are representative of three separate experiments with similar results. Scale bar, 100 μm for all panels.

Figure 5. MMP-9 expression after SCI. A, Sham-operated control. B, Increased expression of MMP-9 1 d after SCI in a cord section 5 mm rostral to the epicenter. C, MP suppression of MMP-9 expression in a cord section corresponding to B from an animal treated with MP. D, RU486 reversal of MP inhibition of MMP-9 expression after SCI in a section corresponding to B in an animal pretreated with RU486 and post-treated with MP. Results shown are representative of three separate experiments with similar results. Scale bar, 100 μm for all panels.

Figure 6. Western blot analysis showing effects of MP and RU486 on MMP-1 and MMP-9 expression after SCI. A, MMP-1. B, MMP-9. The injured cord segment was sampled for Western blot analysis 1 d after SCI. For both A and B, Lanes 1, Normal control; 2, sham-operated control; 3, SCI; 4, SCI + MP; and 5, RU486 + SCI + MP. Note MP suppression of MMP-1 and MMP-9 expression after SCI and RU486 reversal of the MP effect. Each lane represents a sample obtained from one individual rat. Data shown are representative of three separate experiments with similar results.

Figure 7. The effect of c-fos antisense and sense ODNs on MMP-1 and MMP-9 expression after SCI. Rats were treated with vehicle (lipofectin), c-fos sense, or antisense ODN 16 hr before SCI. The injured cord segment was sampled for Western blotting 1 d after SCI. A, MMP-1. B, MMP-9. For both A and B, Lanes 1, Lipofectin vehicle; 2, sense ODN; and 3, antisense ODN. Note no difference in intensity of MMP-1 or MMP-9 expression among the three groups. Each lane represents a sample obtained from one individual rat. Data shown are representative of three separate experiments with similar results.
DISCUSSION

An inflammatory reaction has been extensively documented in animal models of SCI (Balentine, 1978a,b; Means and Anderson, 1983; Xu et al., 1990; Blight, 1992; Dusart and Schwab, 1994; Bartholdi and Schwab, 1995; Hamada et al., 1996; Popovich et al., 1996, 1997; Zhang et al., 1997). NF-κB and AP-1 are two major proinflammatory transcription factors that are activated in inflammation (Barnes and Karin, 1997; Karin et al., 1997). We (Xu et al., 1998) and others (Bethea et al., 1998) have shown earlier that NF-κB was activated after SCI in a rat model. In the present study, we noted that AP-1 was also activated after SCI starting as early as 1 hr and peaking at 8 hr after injury. Elevated AP-1 binding activity was noted for at least 3 d after injury. Immuno-cytological studies revealed the increased expression of Fos and Jun family proteins. These constituent components of AP-1 were not only localized to the cytosol but also in nuclei, suggesting their nuclear translocation to form the AP-1 transcription factor. The specificity of the observed AP-1 activation was confirmed by an antisense strategy directed at c-fos that blocked AP-1 activation after SCI. To our knowledge, the present study is the first to report AP-1 activation after SCI. AP-1 transactivates a large set of genes (Sharp, 1994), including MMP-1 (Brenner et al., 1989; Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990) and MMP-9 (Sato et al., 1993; Yokoo and Kitamura, 1996). Recent studies indicate that activation of both AP-1 and NF-κB are required for the transactivation of MMP-1 (Vincenti et al., 1998; Bond et al., 1999) and MMP-9 genes (Yokoo and Kitamura, 1996; Bond et al., 1998). Because both AP-1 (Fig. 1A) and NF-κB (Bethea et al., 1998; Xu et al., 1998) were activated after SCI, it was expected that MMP-1 and MMP-9 should also be transactivated after SCI. This contention was supported by immunohistochemical studies and Western blot analysis. MMP-1 and MMP-9 immunoreactivity was localized to neurons and glial cells. MMP-1 plays a major role in tissue destruction in inflammation (Davis et al., 1984; Postlethwaite et al., 1993). However, as inflammation is resolving, MMP-1 may also contribute to tissue repair and remodeling (Henson and Johnston, 1987; Alexander and Werb, 1989). MMP-9, another inflammatory gene downstream of AP-1, degrades the extracellular matrix component of basement membrane leading to the loss of vascular integrity (Liotta et al., 1980; Gijbels et al., 1994). An increase in vascular permeability causing extravasation of macromolecules is a prominent feature of inflammation and has been shown after SCI (Hsu et al., 1985).

The activation of two key proinflammatory transcription factors, NF-κB and AP-1, after SCI provides an underlying molecular mechanism for a post-traumatic inflammatory reaction. MP, a synthetic GC and the only therapeutic agent approved by FDA for treating acute traumatic SCI in humans, shares the potent anti-inflammatory action of GCs. Moreover, that this MP action in SCI is mediated by a GR mechanism comes from the finding that RU486, a potent GR antagonist, was able to reverse MP inhibition of AP-1 and NF-κB activation, it has to bind to GR. We have previously shown that GR expression was increased after SCI, suggesting the availability of GR in the injured cord for MP action. Further support for the hypothesis that MP-induced inhibition of AP-1 is mediated by a GR mechanism comes from the finding that RU486, a potent GR antagonist, was able to reverse MP inhibition of AP-1 (Fig. 1B) and NF-κB (our unpublished observation) activation. Furthermore, RU486 also reversed MP inhibition of MMP-1 and MMP-9 expression after SCI. These findings are again consistent with the contention that AP-1 and NF-κB are required to transactivate MMP-1 and MMP-9 and therefore are subjected to the inhibitory action of MP via a GR mechanism.

In summary, we have demonstrated post-traumatic activation of AP-1 and the induction of two inflammatory genes downstream of this transcription factor. The activation of AP-1 and NF-κB, two key proinflammatory transcription factors, is likely to play a major role in transactivating inflammatory genes after SCI. The inhibitory effects of MP on post-traumatic activation of NF-κB and AP-1 and the respective downstream genes are consistent with the well documented anti-inflammatory action of GCs. Moreover, that this MP action in SCI is mediated by a GR mechanism is supported by the effectiveness of RU486, a GR antagonist, in blocking MP inhibition of AP-1 activation and MMP-1 and MMP-9 expression after SCI. Further studies are needed to causally link the anti-inflammatory action of MP to its therapeutic effects in acute SCI.

REFERENCES


Xu et al. • AP-1 Activation after Spinal Cord Injury


