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Targeting Enolase in Reducing Secondary Damage in Acute Spinal Cord Injury in Rats

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Abstract

Spinal cord injury (SCI) is a complex debilitating condition leading to permanent life-long neurological deficits. The complexity of SCI suggests that a concerted multi-targeted therapeutic approach is warranted to optimally improve function. Damage to spinal cord is complicated by an increased detrimental response from secondary injury factors mediated by activated glial cells and infiltrating macrophages. While elevation of enolase especially Neuron Specific Enolase (NSE) in glial and neuronal cells is believed to trigger inflammatory cascades in acute SCI, alteration of NSE and its subsequent effects in acute SCI remains unknown. This study measured NSE expression levels and key inflammatory mediators after acute SCI and investigated the role of ENOblock, a novel small molecule inhibitor of enolase, in a male Sprague-Dawley (SD) rat SCI model. Serum NSE levels as well as cytokines/chemokines and metabolic factors were evaluated in injured animals following treatment with vehicle alone or ENOblock using Discovery assay. Spinal cord samples were also analyzed for NSE and MMPs 2 and 9 as well as glial markers by Western blotting. The results indicated a significant decrease in serum inflammatory cytokines/chemokines and NSE, alterations of metabolic factors and expression of MMPs in spinal cord tissues after treatment with ENOblock (100µg/kg, twice). These results support the hypothesis that activation of glial cells and inflammation status can be modulated by regulation of NSE expression and activity. Analysis of SCI tissue samples by immunohistochemistry confirmed that ENOblock decreased gliosis which may have occurred through reduction of elevated NSE in rats. Overall, elevation of NSE is deleterious as it promotes extracellular degradation and production of inflammatory cytokines/chemokines and metabolic factors which activates glia and damages neurons. Thus, reduction of NSE by ENOblock may have potential therapeutic implications in acute SCI.

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Disclosures

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Keywords

Spinal cord injury; Enolase; Glia; Cytokines and chemokines; Matrix metalloproteinases

Introduction

Enolase is a multifunctional glycolytic enzyme that acts as a plasminogen binding protein and is found to be involved in hypoxia and ischemia (1–3). Enolase plays multiple roles in growth control, immune activation, inflammation, and allergic responses (4–8). Cell-surface expression of enolase is often detected on activated monocytes/macrophages, microglia, and astrocytes, which promotes degradation of extracellular matrix (ECM), production of pro-inflammatory cytokines/chemokines, and invasion of inflammatory cells in the sites of injury (2, 9–11). Upon activation, enolase can also act as a heat-shock protein by binding to cytoskeletal and chromatin structures, and may play crucial roles in a variety of injury, and pathophysiological processes (9, 12, 13). While enolase expression is detected throughout the body, distinct isoforms are found to be tissue specific. A switch from enolase-1 (α -enolase) to enolase-2 (γ -enolase) occurs in neuronal cells. Similarly, a switch from enolase-1 (α) to enolase-3 (β -enolase) occurs in muscle tissue during development in rodents. Neuron-specific enolase (NSE), the neuronal form of the glycolytic enzyme enolase (γ -enolase), is found almost exclusively in neurons and cells of neuroendocrine origin and is measurable in blood and cerebrospinal fluid (14, 15). NSE is likely to concentrate plasminogen on the cell surface and promotes pericellular plasminogen activation, ECM degradation, proliferation of inflammatory glial cells, and invasion and metastasis of tumor cells (16, 17). NSE also participates in actin remodeling, which may enhance migration of activated macrophages/microglia into the injury site, promoting neuronal cell death.

Spinal cord injury (SCI) is a devastating debilitating condition with progressive pathological changes that include complex and evolving molecular cascades (18–22). Insights into the role of enolase in inflammatory conditions have not been thoroughly investigated. Although NSE is implicated in ischemia, hypoxia, and diverse metabolic, proliferative and inflammatory autoimmune events (23–27), less attention has been given to the pathogenic role of NSE in secondary injury of SCI. While NSE is thought to be an important molecule that directly assesses functional damage to neurons, it remains unknown whether pharmacological inhibition of NSE is beneficial in the setting of SCI. Neuroprotective agents (e.g., methylprednisolone, gacyclidine, thyrotropin releasing hormone, nimodipine, etc.) against secondary SCI, together with neuroregenerative agents (e.g., gangliosides, Rho antagonist, anti-Nogo antibodies, acidic fibroblast growth factor) have been used in SCI (Table I) with mixed outcomes (28–39). Recent studies also evaluated the potential beneficial effects of estrogen, which could be a neuroprotective agent in the treatment of SCI and TBI (40–43). Using a novel strategy of nanoparticle delivery of estrogen showed promising results with induction of anti-inflammatory effects after SCI in rats (44). In spite of these studies, there is currently no FDA approved pharmacotherapeutic available for the effective treatment of acute SCI. Thus, the underlying pathophysiology of SCI with special emphasis on multimodal therapy targeting novel neuroprotective as well as neuroregenerative agents should be explored.

Interactions of NSE with many other nuclear, cytoplasmic, or membrane molecules in the CNS (18, 19, 45) also raise the possibility of interactions with a number of inflammatory cascades that are associated with neuronal damage. A recent study examined the expression levels of enolase in different tissues following SCI in rats (46), which showed that enolase expression is significantly increased after acute SCI. However, it remains unknown whether regulation of enolase activity modulates inflammatory events in the host influencing secondary damages in SCI. Here, we tested the hypothesis that increased NSE expression after SCI promotes inflammatory events in spinal cord and activates degradative pathways leading to activation of inflammatory cytokines/chemokines, which aggravate secondary damages of SCI. Thus, the expression levels of NSE and key inflammatory mediators were examined in vehicle treated SCI in rats and compared those with SCI animals treated with a novel small molecule inhibitor of enolase ENOblock (C31H43FN8O3). ENOblock is a novel small molecule which is the first, non-substrate analogue that directly binds to enolase and inhibits its expression and activity (47). It is the first reported enolase inhibitor that is suitable for biological assays, and ENOblock treatment has been shown to target cell-surface-bound enolase to modulate cell growth through downregulation of AKT and BCL-xL. ENOblock also reduces intracellular enolase expression and activity, and decreases the ability of cells to adapt to the hypoxic inflammatory condition. Our data show that NSE is markedly elevated after acute SCI, and ENOblock treatment attenuated NSE levels in serum and spinal cord tissues, decreased inflammatory cytokines/chemokines in serum, reduced MMP levels, and diminished glial activation. Thus, reduction of enolase expression and activity may have therapeutic potential in attenuating inflammatory mediators in acute SCI.

Materials and Methods

Induction of Spinal Cord Injury and Treatment with ENOblock

NSE expression/activity, as well as glial and immune activation after SCI was assessed in male Sprague-Dawley rats (200–250 gm). Rats were anesthetized with ketamine (80mg/kg)/xylazine (10mg/kg), and laminectomy was performed at T10. The spine was immobilized with a spinal stereotactic device, and SCI was induced using a modified method by Perot of dropping a constant weight (5 gm) from a height of 8 cm onto an impounder (0.3 cm in diameter) gently placed on the spinal cord (48, 49). ENOblock was purchased from Bioscience (Catalog No. A11840), dissolved in vehicle (0.01% DMSO in saline), and was administered (100µg/kg, 100µl in volume) at 15 min and 24 h post-injury via intravenous injection. Vehicle-treated animals received the same volume of vehicle (100µl). Sham animals received T10 laminectomy. Blood and tissue samples were collected 48 h post-injury.

ELISA

NSE levels in serum samples were measured by using a double-sandwich ELISA (Rat NSE ELISA Kit, BioSource) and the manufacturer's standardized protocol. Briefly, pre-coated rat NSE monoclonal antibody and the detecting biotin labeled antibody were used. Samples and biotin labeled antibody were added into ELISA plate wells. After washing with TBS-T, avidin-peroxidase conjugates were added to ELISA wells. Wells were again thoroughly washed with TBS-T and developed by TMB substrate for coloring. TMB turns blue in

peroxidase catalytic and finally turns into yellow under the action of acid. Absorbance was measured at 450 nm, and the NSE concentrations were measured using a standard curve and expressed as ng/ml. Data are representative of two separate experiments.

Cytokine, chemokine and metabolic arrays

Serum samples from sham operated, SCI-vehicle treated, and SCI-ENO treated rats were analyzed by Eve Tech's Discovery Assays (cytokine/chemokine and metabolic arrays) for determining inflammatory cytokines/chemokines and alteration of metabolic enzymes (<https://www.evetechtechnologies.com/discoveryassays.php>), and expressed as pg/ml.

Immunoblotting

Western blot analyses of spinal cord samples for the expression of NSE and MMPs were performed as described previously (50–53). Briefly, spinal cords were homogenized in a standard lysis buffer (10mM Trizma base, 150 mM NaCl, 1% Triton-X 100) plus protease inhibitor as described (54–56). Equal protein concentrations from designated tissue lysates were separated on a 4–12% Bis/Tris NuPage gel (Invitrogen, Grand Island, NY). Proteins were transferred onto a nitrocellulose membrane (Pierce) and probed with antibodies (Santa Cruz) for the expression of NSE, MMP-2, and MMP-9. The secondary antibodies used were horseradish peroxidase conjugated anti-mouse (Pierce), anti-rabbit, or anti-goat IgG (Santa Cruz). A monoclonal antibody for β -actin (Santa Cruz) was used as a protein loading control. Relative protein expression was assessed using a using Image J software (National Institutes of Health, Bethesda, MD) and expressed as relative density for each sample (53, 57, 58).

H&E Staining

Frozen spinal cord tissues were sliced into 8 μ m sections. Immune cell infiltration into the injured spinal cord and perivascular cuffing were examined following H&E staining of the tissue sections, as we described previously (59). Photomicrographs were taken at 200 $\times$ magnification.

Immunohistochemistry

Spinal cord tissues (injury, 1 cm) were sectioned, fixed with 4% methanol-free formaldehyde in PBS overnight. Cell marker or protein specific immunohistochemical labeling was performed as previously described (60). Briefly, prior to primary antibody staining, non-specific binding sites were blocked with the same serum as the secondary antibody for 1 hour at room temperature then incubated with NSE (1:100, Santa Cruz), MMP-9 (1:200, Abcam), or monoclonal glial fibrillary acidic protein (GFAP; 1:500, Abcam) overnight at 4°C. Sections were incubated with a (1:100) secondary antibody conjugated with either Texas Red (NSE) (Vector Laboratories, Burlingame, CA) or Nova Red (MMP-9), and with a (1:500) secondary antibody conjugated with either Texas Red (GFAP) for 1 hour in the dark. The slides were mounted with 1 drop of Vectashield Mounting Medium (Vector Laboratories) and coverslipped. The sections were viewed under a fluorescence microscope. NSE, MMP-9, and Iba-1 stained sections were viewed at 20 $\times$ magnification.

Statistical analyses

Statistical analyses were performed using StatView software. Data expressed as mean $\pm$ standard error of the mean (SEM) of three separate experiments, and compared by one-way analysis of variance (ANOVA) followed by Fisher's post-hoc test. Student's t-test was also used in some cases. Differences between each treatment group were expressed as $p < 0.05$.

Results

A novel small molecule inhibitor of enolase, ENOblock, attenuates serum and tissue NSE in SCI rats

To date, only few studies have focused on the role of NSE in spinal cord protein and the changes induced after SCI. Nonetheless, insight into the role of NSE in SCI-mediated dysfunctions remains unclear. While recent studies in a rat model of SCI (48, 49) suggest elevated enolase/NSE expression in spinal cord tissues, the effects of enolase/NSE inhibition in SCI have never been investigated. Pooled serum samples from naïve, sham-operated/vehicle treated, sham-operated/ENOblock (C31H43FN8O3) treated, SCI/vehicle, and SCI/ENOblock treated animals were tested for NSE levels by ELISA. Data obtained from this study showed that NSE levels were elevated in SCI rat sera and spinal cord tissues as compared to sham operated animals (Fig. 1). Treatment of rats with ENOblock significantly inhibited NSE levels (8 vs 4.5 ng/ml), as compared to vehicle treated animals (Fig. 1A). These data showed that ENOblock treatment markedly inhibited NSE levels in SCI serum samples as compared to SCI/vehicle control animals.

NSE expression levels were also tested in spinal cord tissues from sham-operated/vehicle, SCI/vehicle, and SCI/ENOblock treated rats, with higher levels of NSE expression detected in SCI/vehicle animals as compared to sham controls (Fig. 1B). Treatment of rats with ENOblock markedly reduced NSE expression in SCI tissues as compared to SCI/vehicle.

Inhibition of enolase attenuates the production of inflammatory cytokines and chemokines in SCI rats

Studies have shown that after SCI the production of TNF- α is markedly increased, which may serve as an external signal, initiating apoptosis in neurons and oligodendrocytes (61). Similarly, cytokine IL-1 β , a major mediator of inflammation, and chemokines MIP1 α and IP-10 can play important roles in the propagation of secondary injury spread in SCI and traumatic brain injury (62–64). Because ENOblock decreased NSE in sera and tissues of SCI rats, analysis of serum cytokines and chemokines after SCI and ENOblock treatment was performed to assess a relationship between alteration of NSE and key cytokines/chemokines involved in the secondary injury of SCI. Serum cytokines and chemokines were analyzed by using Discovery Rat Cytokine Array/Chemokine Arrays. Interestingly, expression levels of inflammatory cytokines (TNF- α , IL-1 β , and IL-6) and chemokines (MIP-1 α , IP-10) were increased in vehicle treated SCI rats, which were markedly decreased in ENOblock treated animals after 48 h post-SCI as compared to SCI/vehicle control (Table 1). Data also showed that MIP-1 α was slightly increased in sham operated rats following ENOblock treatment, but it was not statistically significant as compared to sham vehicle. By contrast, an increased level of IP-10 was detected in sham operated rats after ENOblock

treatment, and it was statistically significant as compared to that of sham vehicle. IP-10 can be amplified by all three types (α , β , and γ) of IFN (65–67). Thus, it remains unclear if ENOblock differentially regulates IFN signaling and influences IP-10 production after injury. Taken together, these data suggest that inhibition of enolase attenuates different cellular pathways involved in the production of inflammatory cytokines/chemokines in SCI rats.

Inhibition of enolase modulates metabolic factors following SCI in rats

The connecting peptide called C-peptide can exert insulin-independent biological effects on cells, and is known to act as a bioactive peptide with anti-inflammatory properties (68). Data from metabolic arrays showed that serum C-peptide levels were significantly elevated in SCI-ENO rats as compared to SCI-vehicle rats (Fig. 2A). Leptin, the so-called king of hormones, has been implicated in the pathogenesis of chronic inflammation, and the elevated circulating leptin levels increase the risk of developing cardiovascular diseases, type II diabetes, or degenerative disease including autoimmunity and cancer (69). A recent study also suggests that the peptide hormone, amylin, is strongly associated with inflammatory markers in a variety of injury conditions (70). Results showed that leptin and amylin levels were significantly elevated in SCI-vehicle rats as compared to sham operated rats (Fig. 2, B&C). Interestingly, ENOblock treatment significantly reduced leptin and amylin levels in SCI-ENO rats as compared to SCI-vehicle rats, suggesting that enolase may be a target to design novel therapeutics for SCI.

Inhibition of enolase decreases MMP-9 protein expression in injured spinal cord tissues

Primary injury to spinal cord leads to secondary injury, causing gliosis, inflammation, and neurodegeneration, but treatment of animals with ENOblock may inhibit these inflammatory events after SCI, promoting neuroprotection. Data obtained showed that ENOblock treatment slightly altered MMP2 expression in injured tissue which was not statistically significant, as determined by Western blotting and densitometric analysis (Fig. 3). However, NSE and MMP-9 levels were significantly upregulated in injured tissues, and ENOblock treatment markedly decreased NSE and MMP-9 protein expression in SCI tissues (Fig. 3). These results support the hypothesis that activation of glial cells following SCI may promote inflammatory cascades, which can be modulated by regulation of enolase and/or NSE. Immunohistochemical analyses of spinal cord tissues from ENOblock treated animals suggest that enolase inhibition attenuates inflammatory events after acute SCI, and favors neuroprotection.

MMP-9 plays an important role in blood-spinal cord barrier dysfunction and induction of inflammation (71, 72). MMP-9 is increased rapidly after a moderate contusion SCI, reaching a maximum at 24h. Results obtained MMP-9 expression in spinal cord section is downregulated by ENOblock treatment (Fig. 4, upper panel), suggesting that inhibition of MMP-9 by ENOblock may be a new strategy for blocking inflammatory events in acute SCI.

Inhibition of enolase decreases gliosis after SCI in rats

Recovery after SCI is in part attributed to secondary events that govern both detrimental and reparative processes. Elevation of MMP-9 in SCI rats could possibly promote microglial/

astroglial activation and release of inflammatory cytokines/chemokines, leading to neuronal cell death. Studies using immunohistochemistry showed that microglia/macrophage-specific calcium-binding protein Iba1, which is an activation marker of microglia, was markedly upregulated in injured tissues in acute SCI (Fig. 4, middle panel). Like microglial marker Iba1, astroglial marker GFAP was also increased in SCI/vehicle tissues (Fig. 4, lower panel). Both Iba1 and GFAP expression levels were decreased by ENOblock, suggesting that inhibition of enolase attenuates gliosis following SCI. The observation that inhibition of MMP-9 and decreased gliosis in injured spinal cord tissues suggest that reduction of NSE by ENOblock may have potential therapeutic effects in acute SCI.

Discussion

The importance of this study is that for the first time inhibition of enolase or NSE expression and activity has been shown to influence inflammatory events in SCI. NSE expression is believed to be an important marker that directly assesses functional damage to neurons. The expression of NSE has been evaluated in SCI (46), TBI (73, 74), stroke (75), and anoxic encephalopathy after cardiac arrest (76, 77), but its influence in activation of glial cells in spinal cord, infiltration of inflammatory cells, and production of inflammatory cytokines/chemokines after SCI remains unclear. Studies here suggest that NSE concentrations are elevated in rat serum after SCI, as compared to those in sham operated animals. Data also indicate that several inflammatory cytokines and chemokines are markedly elevated in rat sera after SCI, suggesting that elevation of NSE may activate a number of cellular pathways leading to neuronal injury. The remarkable finding here is that blocking enolase by a novel small molecule inhibitor ENOblock attenuates NSE, inflammatory cytokines and chemokines, and gliosis in acute SCI.

The pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) that are elevated after SCI are believed to play crucial roles in the secondary damage of SCI, as reported by other groups (78–81). Anti-inflammatory cytokines IL-4 and IL-10 could minimize this damage by suppressing the inflammatory functions of CNS macrophage and microglia. IL-4 may contribute to conversion of inflammatory type 1 microglia to type 2 microglia (82), which could attenuate inflammation after SCI. IL-10 may also reduce the production of TNF- α by activated astrocytes as well as presentation of CNS antigens by both astrocytes and microglia. IL-10 may also block the activation of NF-kB and attenuate the synthesis of various other inflammatory cytokines/chemokines, and matrix metalloproteases (MMP) 2 and 9. Our recent study showed that elevation of enolase activates antigen presentation via MHC class II pathway (83), suggesting that increased NSE expression may promote antigen presentation after SCI. Upon injury, microglia and astrocytes express higher levels of enolase which may help enhanced presentation of neuronal antigens to T cells, producing inflammatory cytokines/chemokines which are harmful to neurons.

Our study suggests that ENOblock treatment modulates metabolic hormones in SCI rats. The metabolic factor C-peptide was found to be elevated in SCI rats following inhibition of enolase or NSE by treatment with ENOblock. This finding has an important implication for recovery of injury because several other metabolic hormones (leptin and amylin) which are important players in injuries were downregulated by ENOblock. A recent study suggests that

an addition of the C-peptide may reduce secretion of inflammatory cytokines (e.g., IL-6, IL-8), and chemokines (MIP-1 α , MIP-1 β) in supernatants from activated monocytes and macrophages (84). On the other hand, leptin is thought to be a mediator of the inflammatory response in injuries (85), and amylin has been shown to promote IL-1 β -mediated activation of astrocytes (86). Thus, inhibition of enolase in SCI triggers distinct cellular and metabolic pathways leading to reduction of inflammatory mediators and possible recovery after acute SCI.

Members of the MMP family regulate physiological and pathophysiological events in development, injury and repair. MMPs are also thought to be responsible for the turnover and degradation of connective-tissue proteins in injury. ENOblock treatment inhibited the activation of MMP-9 which may lower the development of inflammatory cascades in local injury. Since enolases are metalloenzymes and can function as plasminogen receptors (87), surface expression of NSE upon injury may initiate MMP activation cascade by cleaving pro-MMP. NSE levels are often elevated in patients with CNS disorders associated with serious secondary brain injury (88, 89). Thus, NSE could be considered as a biomarker of CNS injury (11), and has only recently been evaluated in ischemic brain damage (90), spinal cord injury (SCI) (46), traumatic brain injury (TBI) (73, 74), stroke (75), and cardiac arrest (76, 77). SCI's progressive pathological changes include complex and evolving molecular cascades (29, 30, 91), and insights into the alterations of these dysfunctions remains to be fully investigated. The acute SCI is a two-step process involving primary and secondary injury mechanisms. While primary injury results from external mechanical forces due to irreversible tissue detrition and necrosis, secondary injury involves multiple cellular and molecular events such as ischemia, edema, excitotoxicity, inflammatory cytokines/chemokines, electrolyte imbalance, free radical damage, and apoptosis (21, 92), which may be pharmacologically targeted.

Although ENOblock treatment downregulated several inflammatory cytokines and chemokines, it remains unclear whether the treatment also alters anti-inflammatory cytokines/chemokines after acute and chronic SCI. Treatment of SCI animals with ENOblock may result in a decrease in infiltration of activated neutrophils, macrophages and T cells, which could help reduce activation of glial cells in spinal cord, attenuating inflammation and neuronal death. ENOblock may also decrease other inflammatory factors (Cox-2, iNOS, etc.) produced by activated glial cells, improving neuronal survival and recovery after chronic SCI. Studies are underway to test these parameters in acute SCI with or without ENOblock treatment. The primary insult to the spinal cord followed by secondary biochemical events lead to cellular death possibly via necrosis and apoptosis. While necrosis predominates immediately following primary insult, apoptosis occurs at a slower pace as delayed neuronal and oligodendroglial cell death are observed. In this scenario, ENOblock may act as an efficient modulator of the glial cell proliferation and death, protecting neurons after SCI.

Since this study was primarily focused on acute SCI, functional recovery was not monitored in SCI rats following ENOblock treatment. In order to understand the mechanisms how ENOblock works in attenuating inflammatory mediators in SCI, further elaborate studies are needed to assess the recovery of function following chronic SCI. Our current findings

suggest that downregulation of NSE expression and activity by ENOblock may be a new avenue for suppressing neuroinflammation in secondary injury of SCI as well as other CNS injury. Systemic administration of ENOblock is not likely to completely abolish NSE, but will significantly lower NSE as well as other enolase isoform levels. Thus, the so-called protective role of NSE in neuronal growth may not be obstructed. To block secondary injury and preserve functions, it is important to focus on better protection of nerve cells from further damage, enhancement of axonal growth to support regeneration, replacement of damaged cells if possible, and an improved CNS plasticity to restore functions. Overall, this study suggests that elevation of NSE is detrimental, and inhibition of NSE may provide greater attenuation of secondary damage following SCI, thereby inducing enhanced protection of neurons.

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Figure 1. ENOblock treatment attenuates NSE levels in serum and spinal cord tissues after acute SCI in rats

Spinal cord injured rats were treated with vehicle (DMSO, 0.01%) alone or ENOblock (100µg/kg, twice, iv), and blood and tissue samples were obtained 48h post-injury. **(A)** NSE levels in naïve, sham operated and SCI rat sera were measured by ELISA as described in the methods. Representative data from three experiments (n=3) were presented. Statistical analyses were performed by Student's t-test. Serum NSE levels in SCI/vehicle rats were compared with sham surgery, sham/ENOblock or naïve animals. * $p < 0.01$, SCI/vehicle vs SCI/ENOblock. **(B)** Immunohistochemical analysis was performed for NSE (red) protein expression (arrows) in SCI (T10) tissues as described in the methods. Sham operated (T10) animals were used as a negative control. Arrows indicate NSE expression in injured spinal cord. Representative results from 2–3 rats were shown. Magnification, 10×.

Figure 2. ENOblock treatment differentially influences metabolic enzymes after SCI in rats
 SCI rats were treated with vehicle alone or ENOblock (100µg/kg, twice, intravenously), and then blood samples were obtained 48h post-injury. Sham operated rats (T10 laminectomy) were used as controls. Serum c-peptide, leptin, and amylin levels were analyzed by using Discovery rat metabolic arrays. Results obtained from serum samples (duplicate analyses) of three rats suggested that ENOblock treatment significantly downregulated leptin and amylin levels while elevated c-peptide as compared with vehicle treated SCI rats. Statistical analyses were performed using Student's t-test.

Figure 3.

ENOblock treatment decreases the expression of NSE and MMPs 2 and 9 in acute SCI in rats. Spinal cord injured rats were treated with vehicle alone or ENOblock (100µg/kg, twice, iv) and compared with sham surgery alone. **(A)** Spinal cord samples (T10, lesion, S-3, 1 cm) from different groups (naïve, sham/vehicle, Sham/ENOblock, SCI/vehicle and SCI/ENOblock) were analyzed by Western blotting for NSE, MMP-2 and MMP-9 proteins. Actin was used a loading control. Representative blots showing protein levels in treated and untreated groups consisting of 2–3 animals.

Figure 4.

ENOblock treatment attenuates MMP-9 and gliosis (Iba1 and GFAP, glial markers) in acute SCI. Spinal cord injured (T10) rats were treated with vehicle (DMSO, 0.01% in saline) alone or ENOblock (100µg/kg, twice, iv), and spinal cord samples were obtained 48h post-injury. Immunohistochemical analysis was performed for MMP-9 (magnification 10×), Iba1 (microglia, magnification=10×) and GFAP (astrocytes, magnification=20×). Representative figures from three rats show that MMP9 and Iba1/GFAP protein expression (arrows) in SCI rat spinal cords (T10) were markedly upregulated, which were downregulated by ENOblock treatment as compared to vehicle controls. Sham operated (T10) animals (n=2) were used as negative controls.

Table I

Current therapeutic options for SCI

Treatment	Treatment strategy and target	Outcome	References
Methylprednisolone	Anti-inflammatory drug	Improved functions	28, 29, 30
Nimodipine	Calcium channel blockers	Improved recovery	28, 31, 32
Fampridine	Potassium channel blocker	Improved recovery	31,33
Gacyclidine	NMDA receptor antagonist	Improved recovery	28, 30, 31
GM1 ganglioside	Neuronal plasticity and repair	Improved recovery	28, 31
Minocycline	Broad-spectrum antibiotic	Improved recovery	28, 30, 31
Cethrin	Rho antagonist, extracellular matrix	Improved recovery	28, 31
Nogo	Neuro-regeneration	Improved functions	28, 31
Cellular transplantation	Immunomodulatory, regeneration	Improved functions	31, 34
Neurotrophins and other growth factors	Angiogenesis, neural repair, regeneration	Improved functions	29, 35, 36, 37
Cellular therapy with growth factors	Electrophysiological recovery, regeneration	Improved functions	30, 35, 36
Removal of inhibitory factors	Angiogenesis, regeneration	Improved recovery	37, 38, 39
Stem cell therapy	Neural repair, regeneration	Improved functions	31, 34, 38

Table II

Effects of ENOblock on the alteration of inflammatory cytokines and chemokines in rats after SCI

	TNF- α (pg/ml)	IL-1 β (pg/ml)	IL-6 (pg/ml)	MIP-1 α (pg/ml)	IP-10 (pg/ml)
Naïve	23.16 $\pm$ 0.56	3.36 $\pm$ 0.14	11.41 $\pm$ 0.91	86.95 $\pm$ 4.6	457.38 $\pm$ 5.5
Sham/vehicle	36.82 $\pm$ 0.89	8.05 $\pm$ 0.37	18.27 $\pm$ 0.65	98.57 $\pm$ 2.5	516.51 $\pm$ 6.9
Sham/ENOblock	27.14 $\pm$ 1.23	5.67 $\pm$ 0.15	17.38 $\pm$ 0.74	109.34 $\pm$ 3.8	628.96 $\pm$ 3.1
SCI/vehicle	41.69 $\pm$ 1.54	16.83 $\pm$ 0.92	24.87 $\pm$ 0.86	152.27 $\pm$ 3.2	959.21 $\pm$ 4.8
SCI/ENOWock	27.94 $\pm$ 1.96	10.19 $\pm$ 0.67	19.26 $\pm$ 0.58	121.43 $\pm$ 4.5	623.85 $\pm$ 7.9

SCI animals were treated with vehicle alone or ENOblock (100 μ g/kg, twice, iv), and blood samples were obtained 48h post-injury. Serum cytokines and chemokines were analyzed by using Discovery rat cytokine/chemokine arrays. Results from three rats showed ENOblock treatment significantly attenuated TNF- α , IL-1 β , IL-6, MIP-1 α , IP-10.