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NEUROPROTECTION BY DIARYLPROPIONITRILE IN MICE WITH SPINAL CORD INJURY

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ABSTRACT

The initial impact of spinal cord injury (SCI) often results in inflammation leading to irreversible damage with consequent loss of locomotor function. Minimal recovery is achieved once permanent damage has occurred. Using a mouse model of SCI we observed a transitory increase followed by a rapid decline in gene expression and protein levels of nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of cellular anti-oxidative genes. Immediate treatment with diarylpropionitrile (DPN), a non-steroidal selective estrogen receptor β ligand, resulted in a significant increase in Nrf2 levels, and reduction of inflammation and apoptosis compared to untreated SCI animals. Furthermore, DPN-treatment improved locomotor function within 7 days after induction of SCI. DPN acted through activation of PI3K/Akt pathway, known to be involved in down-regulation of apoptosis and up-regulation of cell survival in injured tissues. These findings suggest that immediate activation of cellular anti-oxidative stress mechanisms should provide protection against irreversible tissue damage and its profound detrimental effect on locomotor function associated with SCI.

Keywords: Spinal cord injury, nuclear factor erythroid 2-related factor 2, diarylpropionitrile, antioxidant, apoptosis, anti-inflammation

INTRODUCTION

Pathogenesis of spinal cord injury (SCI) is characterized as being biphasic, consisting of primary injury which causes the initial spinal cord trauma and is considered irreversible, and secondary injury which refers to the molecular and biochemical events that occur following the primary injury (Rowland et al., 2008). Earlier studies suggested that local inflammatory response and oxidative stress following SCI are the major contributors to further damage (David et al., 2012). Thus resident microglia in the central nervous system as well as infiltrating macrophages from the periphery may further contribute to inflammation by releasing pro-inflammatory cytokines, such as interleukin-1β (IL-1β), as well as building up reactive oxygen species (ROS) (Pajoohesh-Ganji and Byrnes, 2011). Inflammatory response resulting from astrogliosis around the injury site inhibits the tissue repair process (Rolls et al., 2009), and the primary function of neuroprotection after SCI is to counteract the
mechanisms of inflammation and oxidative
stress to minimize their pathological effects.
One mechanism of neural repair and preven-
tion of cell death is via the estrogen signaling
pathway through binding to estrogen recep-
tors ERα or ERβ for the activation of down-
stream kinases, such as PI3K/Akt. Treatment
of SCI with 17β-estradiol, which binds to
both ERα and ERβ, attenuates the inflamma-
tory response and reduces neuronal cell
death (Tiwari-Woodruff et al., 2007).

Diarylpropionitrile (DPN) (2,3-bis(4-hy-
droxyphenyl)-propionitrile), a non-steroidal
selective high affinity ligand of ERβ (80-300
folds over ERα) (Carroll et al., 2012), has
neuroprotective effects in a number of neuro-
logical diseases (Kumar et al., 2013). DPN
has been shown to exert anti-inflammatory
role in neuroprotection by attenuating in-
flammatory cytokines IL-1β and IL-6 ex-
pression in mouse brain during neuroin-
flammation (Brown et al., 2010). DPN also
increases the expression of a panel of bioen-
ergetic enzymes and antioxidant proteins in
primary rat cultured hippocampal neurons
(Irwin et al., 2012). Nuclear factor erythroid
2-related factor 2 (Nrf2) has been recognized
for its role in detoxification during the build-
up of ROS and in the activation of phase II
anti-oxidative genes in a number of CNS
diseases and disorders (Zhang et al., 2013),
as well as in traumatic brain injury (Jin et al.,
2008, 2009) and SCI (Mao et al., 2010;
Wang et al., 2012). In this study, we exam-
ined the ameliorating effects of DPN in mice
from the early stage of SCI up to 7 days by
measuring the mRNA expressions and pro-
tein levels of Nrf2 and of proteins involved
in inflammation, apoptosis, and the pathway
related to DPN neuroprotection for SCI.

MATERIALS AND METHODS

Animals and preparation of SCI
A total of 114 C57Bl/6 female 3-5
month-old mice (30-35 g) were supplied by
the Department of Laboratory Animal Medi-
cine, UCLA. These mice were housed at 20-
22 °C, in relative humidity of 40-60 %, and
with illumination from 6 a.m. to 6 p.m. and
allowed ad libitum access to food and water. The standard environmental enrichment for
mice included social housing (3 animals/cage) in traditional wire-topped cages (800 cm²) with 1-cm layer of wood chip
bedding. Animal care, handling and treat-
ments were carried out according to the rules
and regulations of UCLA Animal Research
Committee and Department of Laboratory
Animal Medicine. Mice were anesthetized
with inhalation of isoﬂurane in oxygen-rich
air. After dorsal midline incision and expo-
sure of the lumbar vertebrae, laminectomy
was performed at the L1/L2 level to expose
the spinal cord. Moderate crush SCI was
made using No.5 Dumont forceps (Fine Sci-
ence Tools) ground down to a tip width of
0.4 mm and modiﬁed with a spacer so that at
maximal closure a 0.4 mm space remained
(Faulkner et al., 2004). The behavior was
tested before and every 24 h after performing
SCI.

Drug administration
After induction of SCI, animals were
immediately subcutaneously injected latera-
ly to the injury site with 8 mg DPN (Tocris
Bioscience, Ellisville, MO)/kg of body
weight (Tiwari-Woodruff et al., 2007), or
with vehicle (Miglyol 812 N liquid oil; gift
from Sasol North America, Houston, TX)
and this treatment was repeated every 24 h
until tissue collection. Normal mice were
used as controls for comparison with DPN-
treated and -untreated SCI animals. Tissue
samples from three mice for each experiment
were collected at 6, 12, 24, 48, 72 h and 7
days after induction of SCI.

Nrf2 and IL-1β mRNA expression in the
injured spinal cord of mice as detected by
Real-Time PCR
The region of injured spinal cord tissue
was dissected, immediately frozen in liquid
nitrogen and stored at -80 °C until processed.
Total tissue RNA was prepared using RNA-
easy Mini kit (Qiagen, CA) and cDNA syn-
thesis was performed using RETROscript®
reverse transcription kit (Ambion, TX) as
Quantitative (q)-PCR was conducted in SsoAdvanced™ SYBR® Green Supermix (Bio-Rad, CA) containing 300 nM of each of the following primers; Cyclophilin A (PPIA) forward: 5'-CGAGCTGTTTGCAGACAAA G-3' and reverse 5'-TCTGTGAAAGGAGG AACCCTTA-3' primers; Nrf2 forward 5'-TGGAGAACATTTGCAGCTG-3' and reverse 5'-CCGCTTTCAGTATGAGCA-3' primers; or IL-1β forward 5'-GTGAAATGCCACCTTTTGACA-3'; and reverse 5'CAA AGTTTGGAAAGCAGCC-3' primers (Kumar et al., 2009). Thermocycling (CFX96, Bio-Rad, CA) of triplicate samples was performed as follows: 5 min at 95 °C; 45 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C; followed by a melting curve analysis of 1 min at 95 °C, 1 min at 55 °C, 60 cycles of 5 s with temperature increase of 0.5 °C/cycle starting from 65 °C to 95 °C. The expression level of the gene of interest was normalized to that of PPIA using the formula \(2^{-\Delta\Delta CT}\). The results were analyzed using Bio-Rad CFX manager software (Bio-Rad, CA).

Nrf2, IL-1β, activated caspase-3, PI3K and Akt protein expression in the injured spinal cord of mice as detected by Western blot analysis

Equal amounts of protein from tissue lysate were separated on 10 % SDS-PAGE under reducing condition, and transferred onto polyvinylidene fluoride membranes. The membranes were incubated with 1:1000 rabbit anti-Nrf2 (GeneTex), 1:200 rabbit anti-IL-1β (Santa Cruz), 1:500 rabbit anti-PI3K, -p-PI3K, -Akt, and -p-Akt (Cell Signaling), 1:200 rabbit anti-activated caspase-3 (Chemicon), or 1:20000 mouse anti-GAPDH (Ambion), and then incubated with a HRP-conjugated secondary antibody (Zymed). The signal was detected with ECL Western blotting substrate (Bio-Rad) and captured on Hyperfilm™ (Amersham Pharmacia Biotech).

Score of locomotor function of mouse hind limbs

Recovery of locomotor function was evaluated to assess the gross voluntary use of the hind limbs using open field locomotor test every 24 h after SCI induction. The behavior of animals was observed for 5 min by three individuals blinded to the experimental conditions. A simple six-point scale was used, which is found to be more appropriate for the model of upper lumbar SCI (Herrmann et al., 2008), and animals received a score for voluntary movement of each hind limb. In brief, the scale involves closely monitoring limb movement, weight-bearing capability, coordinated and proper gait, movement in all joints of the hind limb, full weight support and appropriate limb position. Left and right hind limbs were scored separately and averaged.

Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keul’s post hoc test. \(P < 0.05\) is considered statistically significant different among control, SCI, and DPN-treated groups.

RESULTS

Gene expression of Nrf2, involved in detoxifying ROS, was detected in mouse spinal cord tissues within 12 h after SCI, then continuously declined after 24 h until day 7 (Figure 1A) and treatment with DPN resulted in enhancing Nrf2 gene expression, starting from 6 h up to day 7. There is a concomitant significant elevation of Nrf2 protein levels from 24 h to day 7 compared to non DPN-treated SCI group (Figure 1B). After 6 h post SCI, increased mRNA and protein levels of IL-1β were detected in spinal cord tissues compared with normal controls. With DPN treatment, IL-1β mRNA (Figure 1C) and protein (Figure 1D) levels significantly declined after 6 h and 12 h respectively. Similarly, levels of the apoptosis protein, activated caspase-3, show a significant increase in SCI
group compared to normal control, starting from 6 h to day 7, which was attenuated by DPN treatment after 24 h (Figure 2). After 12 h following SCI, phosphorylated-PI3K (p-PI3K) and phosphorylated-Akt (p-Akt) levels are significantly higher in DPN-treated mice compared to untreated SCI group (Figure 3). As for the open field locomotor test, DPN-treated SCI mice show significant elevation of locomotor score after 48 h compared to untreated SCI group and continuously improved till the end of the test period (Figure 4).

Figure 1: Neuroprotective effects of DPN in activation of Nrf2 following SCI. DPN can significantly elevate Nrf2 mRNA (A) and protein (B) levels. DPN treatment also attenuates inflammatory cytokine, IL-1β, mRNA (C) and protein (D) levels induced by SCI. Results are expressed as mean ± SEM of 3 independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to normal control; # p < 0.05, ## p < 0.01, ### p <0.001 compared to SCI group.

Figure 2: DPN protects spinal cord tissue against SCI-induced apoptosis. Treatment of DPN significantly reduces activated caspase-3 level after 24 h following SCI induction. The results are expressed as mean ± SEM of 3 independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to normal control; # p < 0.05, ## p < 0.01 compared to SCI group.

Figure 3: DPN treatment significantly activates phosphorylation of PI3K (A) and Akt (B) after 12 h following SCI. The results are expressed as mean ± SEM of 3 independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to normal control; # p < 0.05, ## p < 0.01, ### p < 0.001 compared to SCI group.
DISCUSSION

In this study, we demonstrate that oxidative stress and inflammation (as evidenced by transient induction of anti-oxidative Nrf2 gene expression and a more sustained stimulation of inflammatory cytokine IL-1β mRNA and protein levels) occurred within 6-12 hours from the induction of SCI, which induced neuronal apoptotic death and dysfunction in open field locomotor activity. Treatment with DPN, a ligand of ERβ, immediately following SCI resulted in elevation of Nrf2 level, attenuation of IL-1β and activated caspase-3 levels, and improvement of locomotor function. Wang et al. (2012) have demonstrated that Nrf2-deficient mice are more susceptible to oxidative stress and show more severe neurological dysfunctions and spinal cord edema after SCI. Moreover, administration of Nrf2-activator sulforaphane provides a neuroprotective effect in SCI rats (Benedict et al., 2012). Sribnick et al. (2005) demonstrated that estrogen treatment in SCI rats attenuated inflammatory response by reducing the levels of IL-1β and NF-κB. Kumar et al. (2013) showed a decrease in activated caspase-3 after DPN treatment in experimental autoimmune encephalomyelitis mice and that DPN increases oligodendrocyte progenitor cell numbers and improves myelination. The improvement in locomotor function in DPN-treated SCI mice in our study was similar to that reported with estrogen (Sribnick et al., 2010).

DPN-induced neuroprotection is dependent on kinase activities, including the PI3K/Akt signaling pathway (Mannella and Brinton, 2006). DPN treatment of SCI mice elevated levels of p-PI3K and p-Akt compared to untreated SCI animals. Activation of PI3K/Akt pathway mediates cell survival in a multiple sclerosis animal model (Kumar et al., 2013). In addition, PI3K/Akt pathway plays a key role in the regulation of Nrf2-dependent neuroprotection by inducing Nrf2 to translocate into the nucleus and activate anti-oxidative genes (Zhang et al., 2013), and inhibition of PI3K with wortmannin attenuates Nrf2 expression (Zou et al., 2013). Besides its anti-oxidative effects through the activation of PI3K/Akt pathway, DPN likely mediates neuroprotection against SCI through its direct interaction with ERβ. To clarify whether ERβ plays a significant role in the neuroprotective effects of DPN, Zhao et al. (2011) implemented (R,R)-THC, a specific ERβ inhibitor, to confirm that the neuroprotective effects of DPN against Aβ1-42 neurotoxicity are mediated through ERβ pathway in rat cultured hippocampal neurons. The findings from this study indicated that the oxidative stress, apoptosis and inflammatory processes accompanying SCI can, in part, be ameliorated by the immediate DPN administration, leading to partial restoration of locomotor function.

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