Inhibition of the nuclear factor–κB activation with pyrrolidine dithiocarbamate attenuating inflammation and oxidative stress after experimental spinal cord trauma in rats

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Object. The nuclear factor–κB (NF–κB) is a transcription factor that plays a pivotal role in the induction of genes involved in physiological processes and in the response to inflammation. The authors of recent studies have demonstrated that NF–κB and oxidative stress contribute to secondary injury after impact-induced spinal cord injury (SCI) in the rat. Dithiocarbamates are antioxidants that are potent inhibitors of NF–κB. The authors postulated that pyrrolidine dithiocarbamate (PDTC) would attenuate NF–κB–related inflammatory and oxidative events that occur after SCI.

Methods. Spinal cord injury was induced by the application of vascular clips (force of 50 g) to the dura mater after a four-level T5–8 laminectomy. The authors investigated the effects of PDTC (30 mg/kg administered 30 minutes before SCI and 6 hours after SCI) on the development of the inflammatory response associated with SCI in rats. Levels of myeloperoxidase activity were measured as an indicator of polymorphonuclear infiltration; malondialdehyde levels in the spinal cord tissue were determined as an indicator of lipid peroxidation. The following studies were performed: immunohistochemical analysis to assess levels of inducible nitric oxide synthase (iNOS), nitrotyrosine formation, poly[adenosine diphosphate]-ribose polymerase (PARP) activity; Western blot analysis to determine cytoplasmic levels of inhibitor–κB–α (IκB–α); and electrophoretic mobility-shift assay to measure the level of DNA/NF–κB binding.

The PDTC treatment exerted potent antiinflammatory effects with significant reduction of polymorphonuclear cell infiltration, lipid peroxidation, and iNOS activity. Additionally, administration of PDTC reduced immunohistochemical evidence of formation of nitrotyrosine and PARP activation in the spinal cord section obtained in the SCI-treated rats. Additionally, PDTC treatment significantly prevented the activation of NF–κB (electrophoretic mobility-shift assay and immunoblot analysis).

Conclusions. Overall, the results clearly demonstrate that PDTC-related prevention of the activation of NF–κB reduces the development of some secondary injury events after SCI. Therefore, inhibition of NF–κB may represent a novel approach in the treatment of SCIs.

Key Words • spinal cord injury • secondary injury • nuclear factor–κB • pyrrolidine dithiocarbamate • inducible nitric oxide synthase • rat

POSTTRAUMATIC inflammatory reaction may play an important role in the secondary injury processes after SCI. The primary traumatic mechanical injury to the spinal cord causes the death of various neurons that have not, to date, been shown to be recoverable; however, neurons continue to die hours after SCI—a potentially avoidable event. Secondary neuronal death is predicated on numerous cellular, molecular, and biochemical cascades. One such cascade, which has been implicated in the evolution of the secondary damage, is the local inflammatory response in the injured spinal cord. Although the neuroaxis is considered immunologically privileged as well as poorly influenced by the inflammatory processes, there is a large body of recent data to indicate that a local inflammatory response occurs and that aspects of this response to injury amplify the secondary damage. The cardinal features of inflammation—namely, infiltration of inflammatory cells (polymorphonuclear neutrophils, macrophage, and lymphocytes), release of inflammatory mediators, and activation of endothelial cells leading to increased vascular permeability, edema formation, and tissue destruction—have been well and extensively characterized in animal models of SCI.
The NF-κB family (c-Rel/p75, RelA/p65, RelB/p68, p50, and p52) of transcription factors are key regulators of inflammatory gene expression.5 Nuclear factor–κB has been shown to activate, via transcription, the genes encoding proinflammatory cytokines (TNFα, IL-1β, and IL-12), cell adhesion molecules (vascular cell adhesion molecule–1 and intercellular cell adhesion molecule–1), iNOS, and COX-2.11,34,48,72 These, together with NO derived from iNOS and COX–2–produced prostaglandin E2, play important roles in the pathogenesis of inflammation, neurodegenerative disease, and posttraumatic inflammatory reaction after SCI.2,7,23,26,70 The production of ROS such as H2O2, superoxide, and hydroxyl radicals, as well as peroxynitrite, also contribute to the tissue injury observed during inflammation, neurodegenerative disease, and posttraumatic inflammatory reaction after SCI.21,36,75 Reactive oxygen species and peroxynitrite also cause DNA damage,14,42 properties which results in the activation of the nuclear enzyme PARP, depletion of nicotinamide adenine dinucleotide and ATP, and ultimately cell death.14,24,66,67

Traditionally, oxidants have been considered to exert their effects through a direct toxic action on target cells; however, recent investigators have also suggested that oxidants contribute to gene induction. Nuclear factor–κB is a pleiotropic transcription factor activated by low levels of ROS and is inhibited by antioxidants.62 Consensus-binding sequences for NF-κB have been identified in the promoter regions of several genes implicated in the pathogenesis of acute and chronic inflammation.14 Furthermore, increased NF-κB–binding activity has been reported in rat SCI models.77,79 Evaluation of these data indicates that local oxidative stress may play a role in the posttraumatic inflammatory reaction through gene induction. Conversely, antioxidants may, in part, mediate their salutary effects by preventing induction of the cytokine cascade and upregulation of adhesion molecules.77,10,36

The diithiocarbamates represent a class of antioxidants reported to be potent inhibitors of NF-κB in vitro.62 The metal-chelating properties of the diethyl derivative of diethiocarbamate have been exploited for decades for the treatment of metal poisoning in humans.62 More recently, diethyldithiocarbamate has been used to retard the onset of acquired immune deficiency syndrome in human immunodeficiency virus–infected individuals,60 a phenomenon thought to be related to its effect on NF-κB activation.60,69 In this regard, the most effective NF-κB inhibitor appears to be the pyrrolidine derivative of dithiocarbamate (that is, PDTC) as a result of its ability to traverse the cell membrane and its prolonged stability in solution at physiological pH.69 The potential for modulating both cell activation and the effects of oxidants with the diithiocarbamates indicates that these agents may offer therapeutic benefit in acute and chronic inflammatory conditions in which activation of NF-κB plays a major role.

The present studies were designed to evaluate the effects of PDTC in animal models of SCI. In particular, we investigated the effects of PDTC on the posttraumatic inflammatory reaction associated with SCI. To gain a better insight into the mechanism(s) of action of PDTC, we also investigated the effects of PDTC on NF-κB activation, the expression of iNOS, the nitration of cellular proteins by peroxynitrite, and the activation of the nuclear enzyme PARP.

G. La Rosa, et al.

Materials and Methods

Animal Model

Male Sprague–Dawley rats (Harlan Nossan, Milan, Italy) weighing 300 to 350 g were housed in a controlled environment and provided with standard food and water. Care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes and with other official regulations.

Induction of SCI

Anesthesia was induced using chloral hydrate (400 mg/kg). We used the clip compression model described by Rivlin and Tator,53 producing SCI by extradural compression of a section of the spinal cord exposed via a four-level T5–8 laminectomy, in which the prominent spinous process of T-5 was used as a surgical guide. A six-level laminectomy was chosen to expedite timely removal of the specimens and to obtain enough spinal cord tissue for biochemical examination. With the applicator oriented in the bilateral direction, an aneurysm clip (closing force 50 g) was quickly applied extradurally at the T5–8 level. The clip was then rapidly released with the applicator, which caused spinal cord compression. In the injured groups, the cord was compressed for 13 seconds. The same clip was used in all the animals in which SCI was created.

Experimental Groups

Rats were randomly allocated into the following groups: Group 1, SCI and saline administration (50 rats); Group 2, the PDTC group (SCI plus saline administration and a 30-mg/kg dose of PDTC administered 30 minutes before SCI and 6 hours after SCI [50 rats]); Group 3, sham and saline group (same surgical procedures except no aneurysm clip was applied [50 rats]); and Group 4, sham and PDTC group (identical to Group 3 except a 30-mg/kg dose of PDTC was administered 30 minutes before and 6 hours after SCI [50 rats]). At different time points (Fig. 1) 10 animals from each group for each time point were killed to evaluate the various parameters.

Preparation of Whole Extracts

All the extraction procedures were performed on ice using cold reagents. Tissues obtained in each rat were suspended in 6 ml of a high-salt extraction buffer (20 mM pH 7.9 HEPES, 420 mM NaCl, 1.5 μM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulphonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg ml−1 pepstatin A, 5 mg ml−1 leupeptin, 0.1 mM benzamidine, and 0.5 mM dithiothreitol) and homogenized at the highest setting for 2 minutes in a tissue homogenizer. The homogenates were chilled on ice for 15 minutes and then vigorously shaken for a few minutes in the presence of 20 μl 10% Nonidet P-40. After centrifugation at 13,000 g at 4°C for 5 minutes, the protein concentration in the supernatant was determined using a protein assay kit (Bio-Rad Laboratories, Milan, Italy) and then it was placed in aliquots and stored at −80°C.

Electrophoretic Mobility-Shift Assay

Double-stranded oligonucleotides containing the NF-κB recognition sequence (5′-GAT CGA GGG GAC TTT CCC TAG-3′) were endlabeled with γ[32P]ATP (ICN Biomedicals, Milan, Italy). Aliquots of whole extracts collected 2 hours after SCI (20 μg of protein for each sample) were incubated for 30 minutes with radiolabeled oligonucleotides (2.5–5 × 104 cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1mM EDTA, 1 mM DL-dithiothreitol, 1 mg/ml bovine serum albumin, and 1% glycerol. The specificity of the DNA/protein binding was determined for NF-κB by competition reaction in which a 50-fold molar excess of unlabeled wild-type mutant or Sp1 oligonucleotide was added to the binding reaction 10 minutes before adding the radiolabeled probe. Protein–nucleic acid complexes were resolved by electrophoresis on 4% nondenaturing polyacrylamide gel in 0.5 × Tris borate EDTA buffer at 150 V for 2 hours at 4°C. The gel was dried and submitted to autoradiography with intensifying screen at −80°C for 20 hours. Subsequently, the
Pyrralidine dithiocarbamate in posttraumatic spinal cord injury

**Western Blot Analysis for iIkB-α**

The levels of iIkB-α were quantified in whole extracts 2 hours after SCI; this was done by immunoprecipitation followed by Western blot analysis according to the manufacturer’s instructions (Cellbio, Milan, Italy). Briefly, proteins were then transferred onto nitrocellulose membranes, according to the manufacturer’s instructions. The membranes were saturated by incubation at 4°C overnight with 10% BSA (Pierce, Rockford, Illinois). Briefly, proteins were then transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK). Subsequently, the relative expression of the proteins was quantified by densitometric scanning of the x-ray films with the densitometer and computer program.

**Immunohistochemical Localization of Nitrotyrosine, PAR, and iNOS**

Evidence of tyrosine nitration (an index of the nitrosylation of proteins by peroxynitrite and/or oxygen-derived free radicals), PAR formation (an indicator of PARP activation), and iNOS was sought using immunohistochemistry as previously described.14,19,20,21,24 Twelve hours after SCI, tissues were fixed in 10% (w/v) formaldehyde, and 10-µm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) H2O2 in 60% (v/v) methanol for 30 minutes. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 minutes. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 30 minutes. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 minutes with avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with primary antibodies: anti-nitrotyrosine antibody (1:1000 dilution), primary anti-PAR or -iNOS antibodies (1:500 dilution), or with control solutions including buffer alone or nonspecific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (DBA). The counterstain was developed with DAB (brown) and nuclear fast red (red background).

**Malondialdehyde Measurement**

Malondialdehyde levels in the spinal cord tissue were determined as an indicator of lipid peroxidation7 1 hour after SCI. The 1-hour post-SCI time point was chosen in agreement with other studies.9 Spinal cord tissue collected at the specified time was homogenized in 1.15% (w/v) KCl solution. A 100-µl aliquot of the homogenate was added to a reaction mixture containing 200 µl of 1.6 mM tetramethylbenzidine and 0.1 mM H2O2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. The MPO activity was defined as the quantity of enzyme degrading 1 µmol of peroxide min⁻¹ at 37°C and was expressed in milliunits g⁻¹ of wet tissue.

**Grading of Motor Disturbance**

The motor function of rats subjected to traumatic compression was assessed once a day for 7 days after injury. Recovery from motor disturbance was graded using the Tarlov criteria¹ as follows: 0, no voluntary movement; 1, perceptible joint movement; 2, good joint movement but unable to stand; 3, able to stand and walk; and 4, complete recovery.

**Other Materials**

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). The PDTC and primary anti-PAR antibody were obtained from Alexis (Milan, Italy). Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, primary anti-nitrotyrosine, anti-iNOS, anti-PAR antibodies and avidin–biotin peroxidase complex were obtained from DBA. Antibodies against iIkB-α and iIkB-β were purchased from Santa Cruz Bio-

**FIG. 1. Chart demonstrating different time points at which rats were killed to evaluate the various parameters. Ten rats in each group were studied at each time point. H = hour.**
The influences of PDTC on the extent of SCI-induced oxidative stress were investigated at 1 hour after injury. Spinal cord injury is associated with a significant increase in the MDA levels, indicative of lipid peroxidation in the injured tissue (Fig. 2). The PDTC treatment (30 minutes before SCI indution) caused a significant reduction of the MDA levels in the injured tissue (Fig. 2). No significant lipid peroxidation was observed in the sham-operated rats (Fig. 2).

Effect of PDTC on IkB-α Degradation and NF-κB Translocation

The appearance of IkB-α in homogenate spinal cord tissues was investigated by immunoblot analysis at 2 hours after SCI. A basal level of IkB-α was detected in the homogenated spinal cord tissues obtained in sham-operated animals (Fig. 3). The IkB-α levels were substantially reduced in the spinal cord–injured rats (Fig. 3). The PDTC treatment (administered 30 minutes prior to SCI) prevented such SCI-mediated IkB-α degradation and the spinal cord IkB-α band remained unchanged 2 hours after injury in PDTC-treated rats (Fig. 3).

To detect NF-κB/DNA binding activity, whole extracts from spinal cord tissue of each rat underwent EMSA. A low basal level of NF-κB/DNA binding activity was detected in nuclear proteins of tissue obtained in sham-operated rats (Fig. 4). The DNA binding activity significantly increased in whole extracts obtained in spinal cord tissues of vehicle-treated rats 2 hours after insult (Fig. 4). The PDTC treatment (30 minutes before SCI) caused a significant inhibition of SCI-induced NF-κB/DNA binding activity, as revealed by specific EMSA (Fig. 4).

The specificity of the NF-κB/DNA binding complex was demonstrated by its complete displacement in the presence of a 50-fold molar excess of unlabeled NF-κB probe in the competition reaction. In contrast, a 50-fold molar excess of unlabeled mutated NF-κB probe, or Sp-1 oligonucleotide had no effect on this DNA-binding activity (data not shown).

Effects of PDTC on Neutrophil Infiltration in the Spinal Cord

Infiltration of leukocytes into the spinal cord has been suggested to contribute significantly to SCI-related tissue injury and dysfunction, because activated PMNs release large amounts of free radicals. The increase in malondialdehyde levels in the spinal cord correlated positively with the increase in tissue levels of MPO activity at 4 hours after SCI, indicating an important neutrophil infiltration (Fig. 5). In rats in which surgery was performed, administration of PDTC 30 minutes prior to SCI significantly reduced the neutrophil infiltration (increase in tissue MPO activity) (Fig. 5).

Effects of PDTC on iNOS Expression

At 12 hours after SCI, 10-μm spinal cord sections were acquired to perform immunohistochemical staining for iNOS. Although staining in the spinal cord sections of sham-operated animals was negligible (Figs. 6A and 7), immunohistochemical analysis, involving a specific anti-iNOS antibody, revealed a positive staining, primarily localized in various cells in the gray matter (Figs. 6B and 7). The PDTC treatment reduced the numbers of iNOS positive cells in surgically treated rats (Figs. 6C and 7).

Effects of PDTC on Nitrotyrosine Formation and PARP Activity

To determine the localization of peroxynitrite formation and/or other nitrogen derivatives produced during SCI, nitrotyrosine, a specific marker of nitrative stress, was measured using immunohistochemical analysis of 10-μm spinal cord sections obtained at 12 hours after injury. Sections of the spinal cord were acquired at the same hour after SCI to determine the immunohistochemical results for PAR. Sections of spinal cord obtained in sham-operated rats stained for neither nitrotyrosine nor PAR (Figs. 7, 8A,
Pyrrolidine dithiocarbamate in posttraumatic spinal cord injury

Fig. 3. A: Western blot showing the effect of PDTC on degradation of IκB-α in spinal cord tissue collected at 2 hours after injury. Immunoblotting is representative of one spinal cord of 10 analyzed. B: Bar graph representing the results of densometric analysis. Values are expressed as the means ± SEMs based on 10 blots. *p < 0.01 compared with sham; °p < 0.01 compared with SCI. CON = basal level of IκB-α band was present in the tissue obtained in sham-operated rats; O.D. = optical densometer; SCI = IκB-α band has disappeared in the tissue in spinal cord–injured rats. SCI PDTC = IκB-α band remained unchanged in the tissue in spinal cord–injured rats receiving PDTC.

Fig. 4. Results of EMSA demonstrating the effect of PDTC on NF-κB/DNA binding activity in the rat spinal cord. Whole extracts from injured (SCI) or noninflamed (sham) rat spinal cord were prepared and incubated with 32P-NF-κB probe. Representative EMSA of NF-κB shows the effect of PDTC (PDTC + SCI) on NF-κB/DNA binding activity evaluated in spinal cord tissue 2 hours after the injury. The illustrated data are derived from a single experiment and are representative of three separate experiments.

Fig. 5. Bar graph demonstrating the effect of PDTC on MPO activity in the spinal cord 4 hours after injury. The MPO activity was significantly increased 4 hours postinjury. Treatment of PDTC reduced the SCI-induced increase in MPO activity. Values represent the means ± SEMs measured in 10 rats for each group. *p < 0.01 compared with sham; °p < 0.01 compared with SCI.

and 9A). Spinal cord sections obtained from vehicle-treated surgically treated rats with SCI exhibited positive staining for nitrotyrosine and PAR (Figs. 7, 8B, and 9B) localized in various cells in the gray matter. Administration of PDTC 30 minutes before and 6 hours after injury reduced the extent of positive staining for nitrotyrosine and PAR in surgically treated rats with SCI (Figs. 7, 8C, and 9C).
Effect of PDTC on Recovery of Hind Limb Motor Disturbances

Based on Tarlov Scale criteria no motor deficit was observed at any time during the recovery period in sham-operated rats (data not shown). In contrast, significant motor damage was clearly demonstrated by significant increase of the mean Tarlov Scale score (Fig. 10). Significant resolution of hind-limb motor disturbances was observed in the SCI/surgery-treated rats in which PDTC was administered 30 minutes before and 6 hours after injury (Fig 10).

Discussion

It is now widely accepted that the formation of proinflammatory cytokines (for example, TNFα, IL-1β, IL-6, or IL-8), the expression on endothelium and neutrophils of...
adhesion molecules (that is, vascular cell adhesion molecule–1 or intercellular cell adhesion molecule–1), and the overproduction of vasoactive mediators (for example, NO by iNOS or eicosanoids via COX-2) play important roles in the pathophysiology of inflammation and post-SCI inflammatory reaction.2,7,23,28,70,75

This study provides the first evidence that PDTC attenuates the following: 1) the degree of lipid peroxidation in the injured spinal cord; 2) IκB-α degradation; 3) NF-κB activation; 4) infiltration of the injured spinal cord with PMNs; and 5) iNOS expression, nitrotyrosine formation, and PARP activation. These findings support the view that PDTC attenuates the extent of damage associated with SCI in rats.

What, then, is the mechanism by which PDTC protects against SCI? Both PDTC and other dithiocarbamates inhibit the activation of NF-κB and possess antioxidative properties.37,58,62,77 Recent evidence has been provided to

![Fig. 8. Photomicrographs showing localization of nitrotyrosine. Administration of PDTC to spinal cord–injured rats produced a marked reduction in the immunostaining for nitrotyrosine (A) in spinal cord tissue compared with positive nitrotyrosine staining (B) observed in rat spinal cord tissue 12 hours after injury. No positive staining was observed in the tissue section from sham-operated rats (C). These images are representative of at least three experiments performed on different experimental days. DAB and nuclear fast red, original magnification × 175.](image)

![Fig. 9. Photomicrographs showing localization of PARP activation. Administration of PDTC to spinal cord–injured rats produced a marked reduction in the immunostaining for PAR (indicative of PARP activation) in spinal cord tissue (A) when compared with positive PAR staining (B) demonstrated in the spinal cord tissue obtained in rats 12 hours after the injury. No positive staining was observed in the tissue section from sham-operated rats (C). This figure is representative of at least 3 experiments performed on different experimental days. DAB and nuclear fast red, original magnification × 175.](image)
indicate that NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation; additionally, it indicates that the activation of NF-κB may also be controlled by oxidant/antioxidant balance. This hypothesis is based primarily on the observation that low doses of peroxides, including \( \text{H}_2\text{O}_2 \) and tert-butylhydroperoxide, can induce NF-κB activation, whereas certain antioxidants prevent it.\(^5\) The exact mechanisms by which PDTC suppresses NF-κB activation in inflammation and posttraumatic injury is not known; however, the results of this study demonstrate that PDTC inhibits the degradation of IκB-α as well as NF-κB activation.

Although PDTC is an antioxidant, recent evidence indicates that this property may not be responsible for its ability to inhibit NF-κB in tubular epithelial cells.\(^2\) Paradoxically, the prooxidant and metal-chelating properties of PDTC could also be involved in its ability to inhibit NF-κB.\(^4\) In this regard, PDTC, at micromolar concentrations, appears to act catalytically to cause the oxidation of several hundred molar equivalents of intracellular glutathione.\(^4\) The latter may explain the steep concentration gradient of PDTC-mediated NF-κB inhibition. Moreover, in this study, the transient loss of IκB-α, which occurred in the injured spinal cord of rats, was prevented by PDTC treatment and well correlated with the inhibition of NF-κB activation, indicating that PDTC may also inhibit NF-κB activation through stabilization of IκB-α. This hypothesis is in agreement with a recent study in which investigators found that PDTC prevents lipopolysaccharide- or carrageenan-induced IκB-α degradation and thus inhibited NF-κB activation in vivo.\(^1\)\(^5\)

Numerous NF-κB–related binding sequences on vari-
Pyrrolidine dithiocarbamate in posttraumatic spinal cord injury

ous genes with important immunological functions characterize this transcription factor as a pluripotent factor in the inflammatory response. In addition to the effects of PDTC on NF-κB inhibition, other mechanisms may contribute to the antiinflammatory property. Further biological effects of PDTC that have been considered include the interference with reactive oxygen metabolism, the chelation of bivalent metal ions, and its influence on intracellular thiol levels. Therefore, the improvement in organ injury after acute and chronic inflammation could be also due to a strong antioxidant effect, which has previously been demonstrated. In particular, inhibition of reactive oxygen intermediates and superoxide anions generated by xanthine oxidase are possibly involved in PDTC’s protective effect. In general, antioxidants are known to exhibit beneficial effects during inflammation, neurodegenerative disease, and post-SCI inflammatory reaction processes. It is unlikely that metal chelation is the principal component of PDTC action because it is impossible to overcome PDTC-mediated blockade of NF-κB activation by using Cu²⁺, Mn²⁺, or Fe³⁺ as has been previously shown. Likewise, PDTC’s influence on intracellular thiol levels does not appear to contribute significantly to the observed effects because other substances interfering with thiol binding, such as N-acetyl-l-cysteine, inhibit NF-κB only at higher and subtoxic concentrations. Thus, the reduction of iNOS expression and/or the antioxidant properties of PDTC may have contributed to its attenuation of nitrotyrosine in the spinal cord–injured rats in this study. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the endogenous formation “footprint” of peroxynitrite. There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; for example, the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine. Thus, increased nitrotyrosine staining is considered an indication of increased nitrosative stress rather than a specific marker for peroxynitrite generation. Both ROS and peroxynitrite produce cellular injury and necrosis through several mechanisms including peroxidation of membrane lipids, protein denaturation, and DNA damage. Reactive oxygen species produce strand breaks in DNA that trigger energy-consuming DNA repair mechanisms and activate the nuclear enzyme PARP, resulting in the depletion of its substrate NAD⁺ in vitro and a reduction in the rate of glycolysis. Because NAD⁺ functions as a cofactor in glycolysis and the tricarboxylic acid cycle, its depletion leads to a rapid fall in intracellular ATP. This process has been termed “the PARP Suicide Hypothesis.” There is recent evidence that the activation of PARP may also play an important role in inflammation and neurodegenerative disease. We found in our experiments that PDTC attenuated the increase in PARP activation in the spinal cord in injured rats. Although concerns may arise concerning the PDTC pretreatment in our study, it is worth noting that such a protocol is commonly used in the experimental setting of this agent.

Furthermore, this study was focused on the molecular mechanisms on which PDTC acts; such mechanisms turned out to be a possible therapeutic target for reducing some secondary injury events after SCI. Thus, we propose (Fig. 11) the following timeline for pathophysiological events following injury: early ROS production → NF-κB activation → PMN infiltration → more ROS production and PARP activation → tissue injury. Treatment with PDTC would intervene in this timeline in the early phase (NF-κB activation).

Conclusions

Analysis of our results indicates that PDTC has strong antiinflammatory properties resulting in the following effects: 1) inhibition of NF-κB; 2) reduction of PMN infiltration; and 3) reduced expression of iNOS and, ultimately, the extent of peroxynitrite formation and tissue injury. Our findings highlight the role of inflammation, oxidative stress, and NF-κB activation after SCI, and we provide a novel therapeutic strategy. Because PDTC is a well-tolerated substance in vivo at concentrations lower than 50 mg/kg, further studies should be undertaken to investigate other possible mechanisms.

References


G. La Rosa, et al.
Pyrrolidine dithiocarbamate in posttraumatic spinal cord injury


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