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Accumulation of Acrolein–Protein Adducts after Traumatic Spinal Cord Injury

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Reactive oxygen species and resultant lipid peroxidation (LPO) have been associated with central nervous system trauma. Acrolein (2-propenal) and 4-hydroxynonenal (HNE) are the most toxic byproducts of LPO, with detrimental effects in various types of cells. In this study, we used immunoblotting techniques to detect the accumulation of protein-bound acrolein and HNE. We report that protein-bound acrolein and HNE were significantly increased in guinea pig spinal cord following a controlled compression injury. The acrolein and HNE protein-adducts increased in the damaged spinal cord as early as 4 h after injury, reached a peak at 24 h after injury, and remained at a significantly high level up to 7 days after injury. Such increase of protein adducts was also observed in the adjacent segments of the injury site beginning at 24 h post injury. These results suggest that products of lipid peroxidation, especially acrolein, may play a critical role in the secondary neuronal degeneration, which follows mechanical insults.

KEY WORDS: 2-Propenal, 4-hydroxynonenal, lipid peroxidation, spinal cord injury, immunoblotting, densitometry.

INTRODUCTION

Oxidative stress has been implicated in central nervous system (CNS) injury and neurodegenerative diseases (1–3). Free radical-induced lipid peroxidation serves to propagate and amplify oxidant-mediated damage. It is currently believed that the byproducts of lipid peroxidation reactions, such as α, β-unsaturated aldehydes, which include 4-hydroxynonenal (HNE) and 2-propenal (acrolein), mediate many detrimental effects associated with oxidative stress and likely contribute to the pathogenesis of oxidative tissue damage (4–6). Therefore, these byproducts of lipid peroxidation have been postulated to be involved in the pathogenesis of spinal cord injury (5,7). Such hypothesis was supported by the findings that tissue levels of HNE have also been shown to be increased after spinal cord injury (SCI) (8,9). However, similar measurements of acrolein in SCI have not accomplished.

Among all α, β-unsaturated aldehydes, including HNE, acrolein is the most active toward nucleophiles such as lysine (4). This suggests that acrolein may be more important than other α, β-unsaturated aldehydes in the pathological events that lipid peroxidation is involved with. Such speculation is highlighted by the fact that acrolein inflicts severe functional and structural deficits in isolated spinal cord tissue (7,10,11). While acrolein has been shown to be elevated in

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neurodegenerative diseases, such as Alzheimer's disease (12–14), acrolein has not been implicated directly in spinal cord injury.

In this study, we investigated the accumulation of acrolein modified protein following traumatic spinal cord injury using an antibody raised against the acrolein-modified protein (15,16). The results shown here provide strong evidence that acrolein was indeed increased and may contribute to secondary injury after SCI.

EXPERIMENTAL PROCEDURES

Animals and Spinal Cord Injury

All animals used in this study were handled in strict accordance with the NIH guide for the *Care and Use of Laboratory Animals* and the experimental protocol approved by the Purdue Animal Care and Use Committee. Adult female guinea pigs (350–450 g) were anesthetized with an intramuscular injection of 100 mg/kg ketamine HCl and 20 mg/kg xylazine, and the spinal cord was exposed by dorsal laminectomy aseptically at T10–T11. A compression injury was then induced by a constant-displacement 15 s compression of the spinal cord, using a modified forceps possessing a spacer (17,18). The injury site covered T10–T11 spinal cord, which was about 10 mm long. Such lesioning procedure had previously been calibrated to produce an immediate and total loss of compound action potential conduction through the injury and a complete paralysis of the hind limbs (17,18). The wounds were closed and animals were kept warm until awakening with heat lamps. Guinea pigs were housed individually and fed *ad libidum*.

Spinal cord samples were isolated at 4, 24, 72 and 7 days after surgery. Guinea pigs were transcardially perfused with 500 ml oxygenated, cold Krebs' solutions (15 °C). The vertebral column was excised rapidly and a complete laminectomy was performed. The spinal cord was removed from the vertebrae. Three spinal cord segments (about 1 cm long each) including T8–T9, T10–T11, and T12–T13 were dissected and collected.

Detection of Acrolein–Protein Adducts by Western Blotting

The collected spinal cord segments were homogenized in a l-ml solution of PBS containing 10 μl each of antiprotease cocktail (pepstatin 1 mg/ml, leupeptin 2.5 mg/ml, aprotinin 2 mg/ml, phenylmethylsulfonyl fluoride 0.2 M, and EDTA 0.5 M, pH 8.0) and 200 μl of a 1% solution of Triton X-100. After incubation on ice for 1 h, the samples were centrifuged at 12,000*g* for 30 min in an Eppendorf microcentrifuge. The supernatant was collected, and protein content was determined by BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL), with bovine serum albumin as the standard. Samples were aliquoted into 100-μg samples and frozen at −80 °C. Equal amounts of protein (40 μg) were diluted in 5 × ImmunoPure Sample Buffer (Pierce) and heated to 100 °C for 5 min. Samples were then loaded on 10% Precise Protein Gels (Pierce) and electrophoresed at 150 V. The proteins were then transferred to PVDF membrane (Bio-Rad) at 100 V. After rinsing with TBS containing 0.1% Tween-20 (TTBS), the blots were blocked in Blocker Blotto (Pierce) for 1 h at room temperature, and incubated overnight with the primary antibody (1:1000) made up in Blotto. After rinsing in TTBS, blots were incubated for 1 h at room temperature with appropriate secondary antibodies from Enhanced Alkaline Phosphatase Western Blotting kits (Oxford Biomedical Research, Oxford, MI). The color development was achieved by following the procedure recommended by the Kit provider. Specific acrolein/HNE signal was controlled by omitting the primary antibody in blots containing equal amounts of spinal cord protein. The HNE antibody (anti-HNE-modified protein polyclonal antibody) and acrolein antibody (anti-acrolein-lysine monoclonal antibody, mAb5F6) were prepared using HNE-modified KLH and MDA-modified KLH, respectively, as previously described (15,16,19). All other non-mentioned reagents were purchased from Sigma-Aldrich.

Densitometric Analysis

The experiments were performed multiple times (4–5) and quantified by densitometry, and the data were then subjected to statistical analysis. Blots were scanned on an Epson Perfection Scanner (model 1660). All densitometric analyses were performed with 1Dscan Ex software (Fairfax, VA). Area density of each band was obtained from the software and was normalized to the appropriate controls. The data were expressed as a mean ± SD and were analyzed by one-way ANOVA using the statistics software package SPSS (version 12, SPSS, Chicago, IL, USA). Results showing overall significance were subjected to post-hoc least-significance difference test; $p < 0.05$ was considered statistically significant.

Immunohistochemistry

The spinal cord segments extracted at different time points after injury were fixed with 6% paraformaldehyde in phosphate buffer (pH 7.4) for 2 h. Spinal cord segments were sectioned transversely through the injury site using vibratome at 30 μM. Sections were rinsed for 15 min in Tris-buffered saline (TBS) containing 0.1% Triton X 100, and then endogenous peroxidase activity was inhibited with hydrogen peroxide (in TBS). Sections were rinsed three times (5 min each) with TBS, and were blocked for 60 min in Blocker Blotto (Pierce) containing Triton X-100. Sections were incubated overnight at 4 °C with the acrolein antibody (1:200). The sections were then washed four times (10 min each) with TBS and incubated for 1 h at room temperature in a biotinylated horse antimouse secondary antibody (Vector; 1:250 dilution). Sections were rinsed in TBS four times each for 10 min, and the reaction was visualized with 3,3v′-diaminobenzidine (DAB) (Sigma) as the peroxidase substrate.

RESULTS

The HNE antibody used in this experiment recognizes HNE-modified proteins; the acrolein antibody recognizes acrolein-bound lysine. Their specificity was established in previous studies (15,16,19). In the current study, blots performed in the absence of the primary antibody did not exhibit any immunoreactivity (data not shown).

Protein-bound acrolein could not be detected at any time point following surgery in animals that received laminectomy alone (Fig. 1). However, in the injured animals, as early as 4 h following injury, proteins containing cross-linked acrolein could be detected over a broad molecular weight range (Fig. 1a). The immunoblot signal for protein-bound acrolein reached a peak at 24 h following injury and lasted up to seven days after injury (Fig. 1a). Mean density of individual bands was objectively analyzed by densitometry and the differences between bands were plotted in Fig. 1a top panel. Statistically significant differences emerged by 4 h following injury and immunoreactivity remained elevated for the duration of these experiments, reaching a peak at 24 h after injury. Post hoc comparisons (least-significance difference) revealed that immunolabeling in the injured group was significantly greater than in the control group at all time points examined. Meanwhile, the samples obtained from the segments adjacent to the injury site (T8–T9 and T12–T13) also showed an increase of protein-bound acrolein. As an example, the result of 24 h after injury is shown in Fig. 1b. Densitometric analysis and subsequent statistical assessment revealed that the mean density in both T8–T9 and T12–T13 was also significantly higher than that of control ($p < 0.01$) (Fig. 1b top panel). Similar phenomena were also observed at 72 h and 7 days after (data not shown).

The accumulation of protein-bound HNE, which was demonstrated by previous reports in rat spinal cord injury (8,9), was also evident in this guinea pig SCI model, in both the injury site (T10–T11) and adjacent segments (T8–T9 and T12–T13) (Fig. 2). First, similar to acrolein, the protein-bound HNE began to increase at 4 h after injury and remained at a high level during the experiments (up to 7 days after injury). Densitometric analysis showed the protein-bound HNE signal was significantly higher than that of control at all time points (Fig. 2a top panel). Second, protein-bound HNE immunoreactivity was also increased in the segments adjacent to the injury site. As shown in Fig. 2b, at 24 h after injury, T8–T9 and T12–T13 also showed strong protein-bound HNE signals. Densitometric analysis revealed that the density of protein-bound HNE in the adjacent segments (T8–9 and T12–13) was significantly higher than that of controls ($p < 0.01$) (Fig. 2b top panel). We also observed such increase of HNE in the adjacent segments at 72 h and 7 days post injury (data not shown).

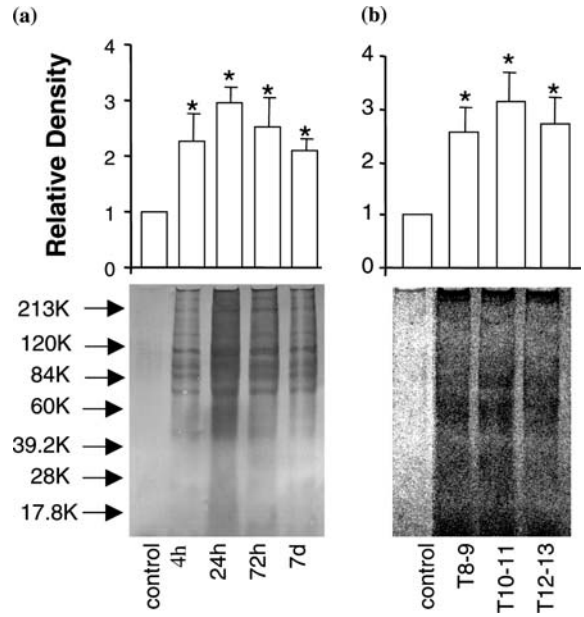

Fig. 1. Accumulation of protein-bound acrolein in the spinal cord tissue after traumatic injury. (a) Western blotting analysis of protein-bound acrolein. The immunoreactivity of protein-bound acrolein at the injury site (T10–T11) was significantly increased at 4 h after injury, and remained at a high level up to 7 days after injury. Five independent experiments were quantified by densitometry and the density was normalized to control (sham-injury). Statistical analysis using ANOVA showed significant differences between control and all the time points after injury. (b) Twenty-four hrs after injury, the accumulation of protein-bound acrolein was evident in both the injury site (T10–T11) and in the adjacent segments (T8–T9 and T12–T13). Densitometric analysis showed that the protein-bound acrolein signals in all the segments (T8–T9, T10–T11 and T12–T13) were significantly higher than that of control (top panel). *$p < 0.01$ when compared with control.

To confirm the data of western blotting, we also performed immunohistochemical staining using the same antibody to visualize the accumulation of acrolein-modified protein. Similar to the results of western blotting, the acrolein-KLH immunoreactivity was significantly increased in the spinal cord tissue after compression injury. An example of 24 h after injury was shown in Fig. 3. Although light, diffuse staining was observed in the control animals, especially in the grey matter area (Fig. 3a), the signal of acrolein-KLH immunoreactivity in the injured spinal cord tissue was significantly stronger in both grey and white matter area (Fig. 3b).

DISCUSSION

The results of the present study provide direct evidence that the lipid peroxidation products acrolein

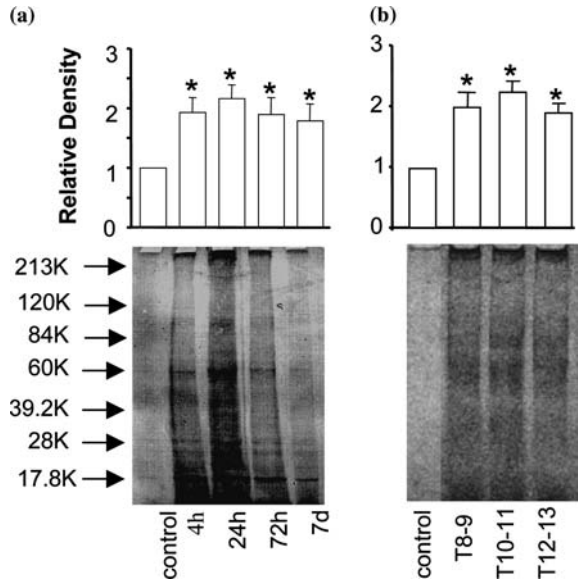

Fig. 2. Accumulation of protein-bound HNE in the spinal cord tissue after traumatic injury. (a) Western blotting analysis of protein-bound HNE. The immunoreactivity of protein-bound HNE began to increase at 4 h after injury, reaching a peak at 24 h after injury and maintained at a high level up to 7 days after injury. Densitometric analysis of 4 independent experiments showed that protein-bound HNE at all the time points was significantly higher than that of control. (b) Twenty-four hrs after injury, protein-bound HNE was increased in both injury site (T10–T11) and in the adjacent segments (T8–T9 and T12–T13). Densitometric analysis showed the protein-bound HNE in all the segments (T8–T9, T10–T11 and T12–T13) was significantly higher than that of control (top panel). $*p < 0.01$ when compared with control.

and HNE rapidly accumulate following spinal cord injury and bind to numerous proteins of varying molecular weight. The accumulation of acrolein and HNE spread up to two segments outside of the original injury site, which is roughly 10 mm away from the injury epicenter. Furthermore, this spreading lasted at least 7 days post injury. The finding that the accumulation of lipid peroxidation products is long lasting and diffusive is consistent with several previous reports. Using a rat spinal cord contusion model, Baldwin and colleagues found a significant increase of HNE in a region two segments outside of the compression zone (8). Similar diffusive accumulation of HNE was also observed by Springer and colleagues in a rat spinal cord injury model (9). These findings, along with the results of the current study, reveal that secondary oxidative stress resulting from the primary insult is not limited to the original impact zone but is more widely distributed. It has been suggested that LPO products may in general play more important detrimental roles than conventional reactive oxygen species due to their stability. For example, it has been estimated that LPO products could have a half life in the order of several days (20) while the half-life of most other known reactive oxygen species are in the order of 10^{-12} sec (21). Given the endotoxic character and stability of many of these chemical intermediates and products (4), biochemical abnormalities likely lead to the long-term tissue destruction and widespread cell death observed after severe spinal cord injury.

Acrolein is the most active product of lipid peroxidation known so far (4). Recently, we have shown that acrolein can inflict structural and functional damage to isolated spinal cord tissue (7,10,11), as well as to PC 12 cells in culture (Luo and Shi, unpublished observations). Furthermore, we have shown that acrolein can directly induce mitochondrial oxidative stress and functional impairment (Luo and Shi, unpublished observations). Therefore, acrolein may play a particularly important role in the pathogenesis of neurodegenerative disease and CNS trauma, mainly due to its prominent toxicity and stable lifetime. An observation we found to be intriguing is that there are some differences between the bands in Figs. 1 and 2. Whether acrolein and HNE bind and affect different proteins is not known. However, it is known that acrolein is more active

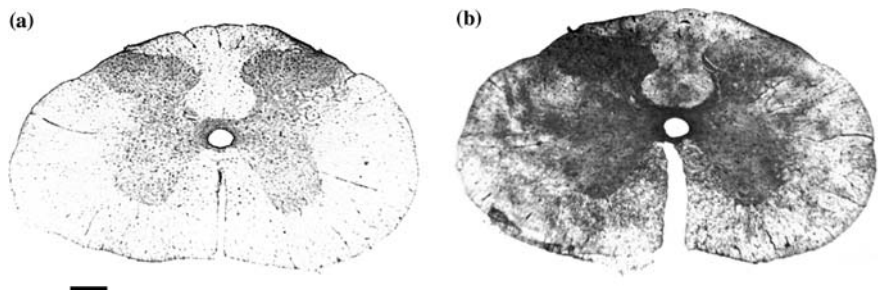

Fig. 3. Immunohistochemistry demonstrates strong acrolein-KLH immunoreactivity in the spinal cord tissue after traumatic injury. Light acrolein-KLH immunostaining occured in control animals, especially in the grey matter area (a). Significantly stronger acrolein-KLH immunoreactivity occured in the spinal cord tissue at 24 h after injury, both in the white and grey matter (b). Scale bar = 200 μm.

than HNE; for example, acrolein is estimated to be approximately 100-fold more reactive than HNE towards nucleophiles (4,5). Therefore, it is possible that acrolein and HNE may act differently when they attack proteins to form protein-adducts.

One particular pathological scenario that may link acrolein to neurodenegartion is the axonal membrane damage, a critical factor that is known to lead to axonal degeneration and cell death. First, as we mentioned earlier, acrolein inflicts significant membrane damage to isolated spinal cord axons (7,10). Second, significantly elevated levels of acrolein have been measured not only in the original compression site, but also at 10 mm from the injury site as well at 24 h post-injury (this study). Interestingly, in the same injury model, severe axonal membrane damage in the same region was also noticed, but was not apparent until 3 days post-injury (22). These data are consistent with the hypothesis that diffusive elevation of acrolein preceeds and leads to membrane disruption and subsequent cell death in *in vivo* spinal cord trauma. These results, along with our previous findings that acrolein directly causes neuronal membrane damage and oxidative stress (7,10), suggest that acrolein may play an important role in the pathogenesis of secondary injury following spinal cord injury. Ongoing experiments are examining whether acrolein or HNE can be inhibited by antioxidants that have been demonstrated to effectively inhibit LPO. It would also be very interesting to test whether there are any beneficial effects when acrolein or HNE is specifically reduced following spinal cord injury.

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