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Letter to Neuroscience

ACROLEIN INFLICTS AXONAL MEMBRANE DISRUPTION AND CONDUCTION LOSS IN ISOLATED GUINEA-PIG SPINAL CORD

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We have examined the effect of acrolein, an aldehyde product of lipid peroxidation, on axons in isolated guinea-pig spinal cord white matter. We found that 200 μM acrolein, but not 50 μM, induced a time-dependent loss of compound action potential conduction. Such conduction loss was irreversible within 1 h after acrolein perfusion. Parallel anatomical assessment indicates membrane integrity breakdown based on a horseradish peroxidase-exclusion assay. This is the first report to suggest that acrolein inflicts severe axonal damage. Since axonal damage within white matter plays a key role in the pathology of traumatic spinal cord injury, we suggest that acrolein may be a critical factor in mediating secondary functional loss.

As one of the most toxic aldehyde products of lipid peroxidation (LPO), acrolein has been implicated in chronic neurodegenerative disorders such as Alzheimer's disease (Lovell et al., 2001). However, the role of acrolein in acute CNS traumatic injury has not been examined. 4-Hydroxynonenal (HNE), another aldehyde product of LPO with a structure similar to acrolein, has been linked to the pathology of spinal cord trauma (Baldwin et al., 1998; Springer et al., 1997). With a greater formation rate and higher reactivity, acrolein may be a much more important factor than HNE in inflicting injuries secondary to initial insults in spinal cord trauma. In the current study, we examined the effect of acrolein on membrane integrity and axonal conduction of mammalian spinal cord axons in an *in vitro* tissue preparation. This study forms a basis upon which the critical role of acrolein in *in vivo* spinal cord injury, and perhaps in other neurodegenerative diseases, can be revealed.

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Abbreviations: CAP, compound action potential; HNE, 4-hydroxynonenal; HRP, horseradish peroxidase; LPO, lipid peroxidation.

Using a guinea-pig spinal cord white matter preparation (Shi and Blight, 1996, 1997; Shi and Borgens, 1999; Shi and Pryor, 2000, 2002), we first examined the loss of compound action potential (CAP) as a result of acrolein exposure. Acrolein, at a concentration of 200 μM, was applied to the spinal cord through the perfusion input (Fig. 1B). After a delay of about 30 min (34 ± 2 min, $n = 5$), the CAP amplitude began to steadily decline (Fig. 1C). By the end of 60 min of continuous exposure, the amplitude of CAP was reduced significantly to 67 ± 12% of pre-exposure value ($P < 0.001$, $n = 7$). A 60 min period of wash did not lead to recovery of CAP amplitude. Instead, the CAP continued to worsen, falling to 54 ± 11% of control ($n = 5$), which is significantly lower than the values at the end of 60 min exposure ($P < 0.05$). The slope of deterioration was not affected by the wash (Fig. 1). When the cord was exposed to acrolein continuously for 120 min, the CAP was reduced to 46 ± 9% of control ($n = 4$). This was not significantly different than CAPs recorded from cords that were subjected to 60 min acrolein followed by 60 min wash ($P > 0.05$). Perfusion of 50 μM of acrolein for 2 h resulted in little action potential conduction loss (97 ± 5% of control, $n = 4$). A comparison of the response amplitude before and after 60 min of acrolein exposure (200 μM) to a set of identical stimulus currents shows no significant change in the relative decrease in amplitude at different stimulus intensities over a wide range (data not shown). This indicates there was no consistent selectivity of loss of conduction in fibers of low (large fibers) or high threshold (small fibers) in response to acrolein.

The membrane permeability change of the spinal cord white matter axons was evaluated using horseradish peroxidase (HRP)-exclusion assay (Shi and Borgens, 2000; Shi and Pryor, 2000). Since HRP molecules (44 kDa) are too large to pass through any known channels, the uptake of HRP was interpreted as an indication of membrane disruption. As shown in Fig. 2A, D, little HRP labeling was found in uninjured cord strips (29axons/mm^2, $n = 5$). After 60 min exposure, acrolein at a con-

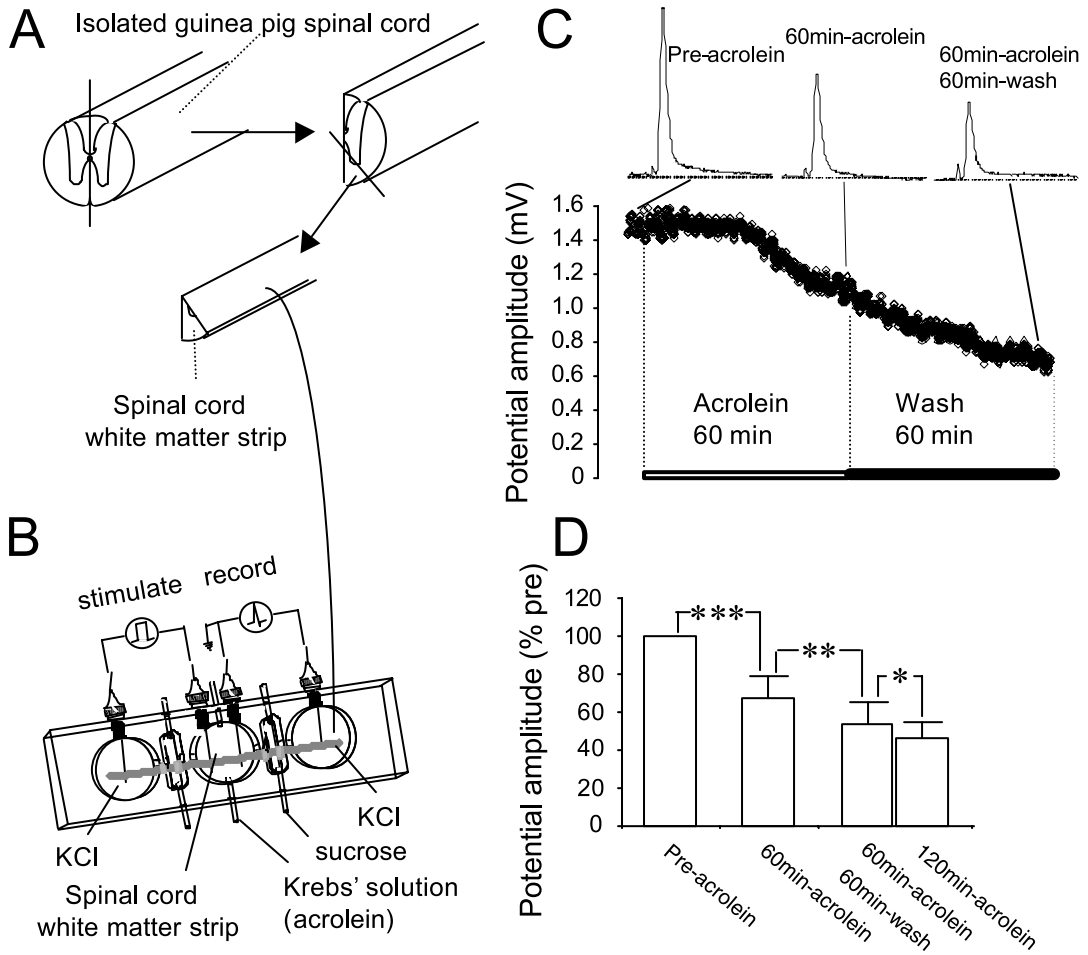

Fig. 1. Diagram showing spinal cord tissue extraction, recording apparatus, and axonal conduction changes in response to acrolein exposure. (A) Drawing showing the steps to isolate white mater strips from a spinal cord extracted from an adult guinea-pig. (B) Recording arrangement. The isolated spinal cord tract is shown mounted in the apparatus. The central well is continuously perfused with Krebs' solution (where acrolein was perfused). The two ends of the tract were placed in separate wells filled with 120 mM KCl, divided from the central well by narrow channels filled with flowing isotonic sucrose solution. Ag–AgCl electrodes for recording and stimulation were placed at the locations shown. Action potentials were stimulated at the left sucrose gap and recorded at the right gap. (C) An example of the reduction of CAP amplitude recorded from a strip of white matter in response to 60 min acrolein (200 μM) followed by 60 min wash by normal Krebs' solution. Each symbol (open diamond) represents the amplitude of a CAP recorded every 3 s. Note the decrease of CAP amplitude that began 30 min after the onset of acrolein perfusion and continued through the end of the washing period. The individual CAPs (pre-acrolein; 60 min acrolein; 60 min acrolein and 60 min wash) shown here were taken at the time of experiment indicated in the graph. (D) The average of the % of CAP amplitude loss after 60 min acrolein exposure ($n = 5$), 60 min acrolein exposure followed by 60 min wash ($n = 5$), and 120 min continuous acrolein exposure ($n = 4$). Asterisks indicate significance of difference between two groups specified in the graph. $*P > 0.05$, $**P < 0.05$, $***P < 0.001$. The number of experiments in each condition was either four and five.

centration of 200 μM significantly increased the number of axons labeled with HRP (184 ± 42 axons/mm^2, $n = 5$, $P < 0.005$; Fig. 2B). HRP labeling was even more pronounced after 2 h of acrolein exposure (289 ± 18 axons/mm^2, $n = 8$). Although 50 μM acrolein resulted in little membrane damage after 2 h exposure, 100 μM acrolein rendered membrane damage (214 ± 20 axons/mm^2, $n = 6$) that is significantly higher than that in control ($P < 0.0005$). These findings indicate membrane disruption as the anatomical basis for the CAP conduction loss as a result of acrolein exposure. Furthermore, the threshold for acrolein-mediated membrane disruption is between 50 and 100 μM.

LPO, which generates acrolein, is known to increase in spinal cord injury secondarily to an initial insult (Hall, 1996). Free polyunsaturated fatty acids, a known reactive oxygen species source, increase as early as 1 min and reach 20 times the control value following primary injury to spinal cord (Demediuk et al., 1985; Murphy et al., 1994). Among different potential products of fatty acid peroxidation, acrolein may play the most damaging role due to its relatively long half-life and high reactivity compared to other LPO products and reactive oxygen species (Esterbauer et al., 1991; Halliwell, 1992). Acrolein is estimated to be approximately 100-fold more reactive than that of HNE inside the cell (Esterbauer et al., 1991). Therefore, acrolein is likely to be a key factor in secondary pathogenesis following initial trauma.

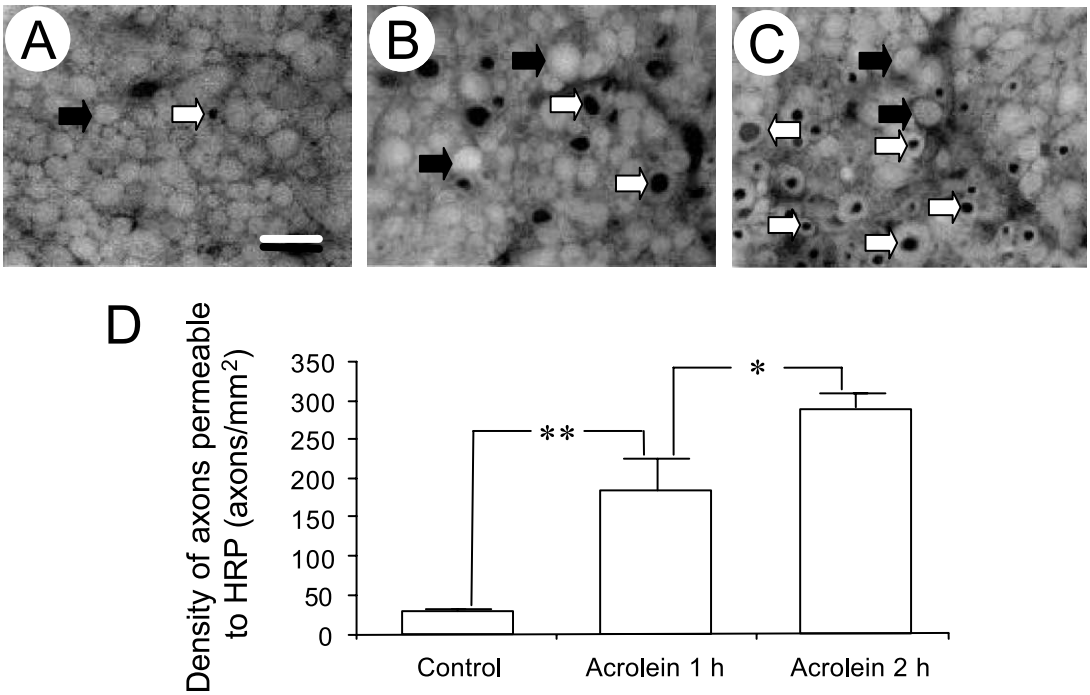

Fig. 2. HRP labeling following acrolein exposure under different conditions. HRP labeling of a representative cross-section of spinal cord in the control group (A), 1 h (B) and 2 h (C) after 200 μM acrolein exposure. The cord strips were immersed in a solution of HRP and subsequently fixed and developed using a diaminobenzidine technique. Vibratome sections were cut in the middle of the cord segment, which was exposed to acrolein, or Krebs' solution (control group). The open arrows indicate axons that were labeled with HRP and filled arrows denote axons that excluded HRP. In A, scale bar = 10 μm, and is also applicable to B and C. Note the lack of axonal labeling in A and increased HRP labeling in B and C. (D) The density of axons permeable to HRP (axons/mm^2) as a result of acrolein (200 μM) exposure. Note HRP labeling following 1 h exposure of acrolein was significantly higher than that of pre injury values. Furthermore, HRP labeling following 2 h of acrolein was significantly higher than that after 1 h of exposure. Asterisks indicate significance of difference between two groups specified in the graph. *$P < 0.05$, **$P < 0.01$. The number of experiments in each condition was between four and nine.

Our study demonstrated for the first time that acrolein, at a concentration of 200 μM, is capable of inflicting membrane damage which caused the loss of membrane integrity and CAP conduction after 1–2 h of continuous exposure. We have also shown that the threshold for this detrimental effect is between 50 and 200 μM. It is known that the formation of acrolein is 40-fold greater than that of HNE (Dennis and Shibamoto, 1990). It is also known that HNE can accumulate in cellular membranes and its concentration can reach up to 1 mM in pathological conditions (Toyokuni et al., 1994). The level of acrolein in the sera of a normal human was estimated to reach 50 μM (3-formyl-3,4-dehydropiperidino equivalent concentration; Satoh et al., 1999). Moreover, acrolein is estimated to reach 80 μM in respiratory tract lining fluids as a result of smoking (Nardini et al., 2002). Hence, it is more than likely that the concentration of acrolein we used here (50–200 μM) occurs in *in vivo* spinal cord injury.

Future study of this topic will be to measure the dynamics of acroelin accumulation following traumatic spinal cord injury and correlate it to functional loss. The goal will be to inhibit the activity of acrolein, thereby reducing the functional loss and enhancing the overall level of recovery after spinal cord injury. Such intervention may benefit chronic nervous system degenerative diseases as well.

EXPERIMENTAL PROCEDURE

The technique for isolation of the cord has been described previously and also illustrated in Fig. 1A (Shi et al., 2000; Shi and Blight, 1996). Guinea-pigs were anesthetized and perfused with oxygenated, cold Krebs' solution to remove the blood and to lower core temperature. The vertebral column was excised rapidly and the spinal cord was removed from the vertebrae and immersed in cold Krebs' solution. The cord was then further subdivided to produce ventral strips of white matter that were subsequently incubated in fresh Krebs' solution at room temperature, bubbled continuously with 95% oxygen, 5% carbon dioxide. The composition of the Krebs' solution was described in our previous publications (Shi and Blight, 1996).

The isolated strips of ventral white matter were then placed in a sucrose-gap recording chamber. The construction of the recording chamber has been described in our previous publications (Shi et al., 2000; Shi and Blight, 1996) and again illustrated in Fig. 1B. The central compartment of the chamber was continuously superfused with oxygenated Krebs' solution through which acrolein was introduced (ca. 2 ml/min). The temperature of the chamber was maintained at 37°C. The axons were stimulated in the form of constant-current unipolar pulses of 0.1 ms at intensities corresponding to maximal CAP response and CAPs recorded by silver–silver chloride wire electrodes.

HRP uptake was measured as described in previous studies (Shi et al., 2000; Shi and Pryor, 2000, 2002). In brief, segments of spinal cord strips were transferred at targeted times after acrolein exposure to oxygenated Krebs' solution containing 0.015% HRP. After incubation for 1 h, the tissue was fixed by immersion in 2.5% glutaraldehyde in phosphate buffer. Trans-

verse sections of the tissue were cut at 30 μm on a Vibratome and stained with diaminobenzidine reaction to reveal the extent of HRP uptake into damaged axons. The number of unsealed axons was counted and normalized by dividing this number by the unit area of the sample and expressed as a density (axons/mm^2).

Student's *t*-test was used in all the data analysis, with a significant level chosen at $P < 0.05$.

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