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Ischemic insult exacerbates acrolein-induced conduction loss and axonal membrane disruption in guinea pig spinal cord white matter

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Abstract

Cellular destruction following ischemic insult may be due to secondary injury mechanisms, not the oxygen–glucose deprivation itself. We have examined the effect of acrolein, an aldehyde product of lipid peroxidation (LPO) and oxidative stress, on the axons in isolated guinea pig spinal cord white matter following ischemic insult. We have found that acrolein at 50 μM, which is unharmful to spinal cord when applied alone, causes action potential conduction failure and membrane disruption following 1 to 2 h of exposure when applied during the reperfusion period. Ischemic insult also exacerbates the effect of acrolein at 200 μM, which does inflict functional and anatomical damage when applied alone. Unlike metabolic poisoning, acrolein-mediated damage is not a function of axonal size and does not affect the refractoriness in response to dual and multiple stimuli. These results indicate that spinal cord axons, in addition to experiencing elevated free radicals, are more vulnerable to acrolein attack when the level of oxygen and glucose is low. We conclude that free radicals and lipid peroxidation in general, and acrolein in specific, may play a critical role in cellular destruction and functional loss in such injury.

Keywords: Neurotrauma; Oxygen–glucose deprivation; Reperfusion; Membrane; Metabolism

1. Introduction

Acrolein, or 2-propenal, is the most reactive compound of the α,β-unsaturated aldehydes [1,2]. Compared with other known aldehyde products of lipid peroxidation (LPO), such as 4-hydroxynonenal (HNE), which is structurally similar, acrolein is estimated to be approximately 100-fold more reactive [1] and is formed at a 40-fold greater rate than that of HNE [3]. What makes acrolein a potentially deadly killer is that its half-life, in a range of several hours, is more than 100 billion times longer than the much studied oxygen radicals [1,4]. Therefore, acrolein is likely to play an important role in free radical and LPO related pathogenesis following various traumas. Indeed, acrolein has already been implicated in chronic neurodegenerative disorders such as Alzheimer's disease [5]. However, the role of acrolein in acute central nervous system (CNS) trauma has not been examined in detail.

It is widely recognized that ischemia plays a critical role in the neuropathology of the CNS in general, and the spinal cord in particular. In the case of spinal cord injury, paralysis can be a consequence of even brief ischemic episodes, such as those that result from clamping of the aorta during life-saving surgeries [6–10]. Furthermore, ischemia can arise secondary to physical insults when the integrity of the blood vessels is compromised [11–13]. Although the mechanism of how ischemia can produce damage at the cell and tissue level of organization is not fully understood, there is little doubt that the ischemia–reperfusion injury produces free radicals and consequently increases lipid peroxidation [14]. Therefore, as an LPO byproduct, acrolein is likely to play an important role in ischemic related damage.

In a previous study using an isolated guinea pig preparation similar to the one used in this study, we found that mammalian spinal cord ventral white matter is resistant to an acute ischemic insult when the secondary injuries are largely eliminated [15]. It was this finding that led us to speculate that lack of oxygen and glucose itself cannot be solely responsible for the cellular damage seen in in vivo ischemic injuries. We further hypothesized that secondary injuries, such as free radical attack and lipid peroxidation,

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are responsible for the damage. It was the purpose of this study to examine the role of acrolein, a lipid peroxidation product, in inflicting tissue damage in an ischemic (oxygen and glucose deprivation)–reperfusion injury.

Using electrophysiological and anatomical analysis, we have found that acrolein, at a concentration that is otherwise unharmful to healthy tissue, following 2 h of exposure, causes significant functional and structural damage to isolated guinea pig ventral white matter during ischemia–reperfusion injury. At a higher concentration, acrolein caused more damage when combined with ischemia. This is a significant step towards revealing the role of free radicals in general, and acrolein in particular, in ischemia–reperfusion injuries. These results may also direct us towards effective methods for the reversal of the cellular damage that occurs following ischemic spinal cord injury.

2. Materials and methods

2.1. Isolation of spinal cord

Adult female Hartley guinea pigs of 350–500 g body weights were used. They were deeply anesthetized with a combination of ketamine (80 mg/kg) and xylazine (12 mg/kg). They were then perfused through the heart with oxygenated, cold, Krebs' solution, to remove the blood and lower core temperature. The entire vertebral column was excised rapidly and a complete laminectomy was performed. The spinal cord was removed from the vertebrae and immersed in cold Krebs' solution, then immediately subdivided, first along the sagittal midline and then by cutting each half of the cord radially, to isolate the ventral white matter (Fig. 1A). Each white matter strip was subsequently incubated for 1 h in fresh Krebs' solution at room temperature, bubbled continuously with 95% oxygen–5% carbon dioxide (Fig. 1A). The composition of the Krebs' solution was as follows (in mM): 124 NaCl, 5 KCl, 1.2 K_2HPO_4, 1.3 $MgSO_4$, 2 $CaCl_2$, 20 glucose, 10 sodium ascorbate, and 26 $NaHCO_3$, equilibrated with 95% O_2–5% CO_2 to produce a pH of 7.2–7.4.

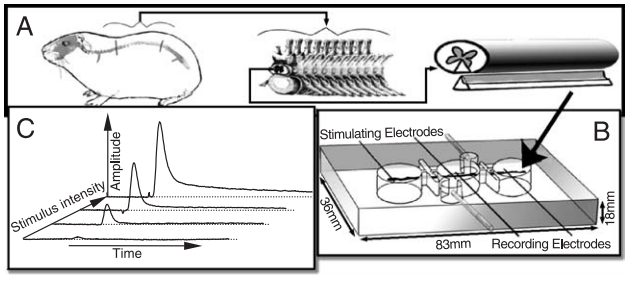

Fig. 1. Diagram showing experimental model and electrophysiological apparatus. (A) Drawing of the guinea pig, the vertebral column and the isolated spinal cord after extraction from the spinal column. The ventral white matter is then excised from the spinal cord and used for the electrophysiological recordings. (B) Diagram of the double sucrose gap recording chamber showing the various compartments, placement of the ventral white matter strips, and the position of the stimulating and recording electrodes. (C) An example of CAP recordings showing an increase in wave amplitude with increasing stimulus intensity. Note the high signal to noise ratio, as indicated by the small stimulus artifact and stable CAP trend.

2.2. Electrophysiological recording of isolated spinal cord white matter

2.2.1. Recording chamber

The construction of the recording chamber is illustrated in Fig. 1B. A strip of isolated spinal cord white matter, approximately 35 mm in length, was supported in the central compartment and continuously perfused with oxygenated Krebs' solution (2 ml/min) by means of a peristaltic pump. The free ends of the spinal cord strip were placed across the sucrose gap channels to side compartments filled with isotonic (120 mM) potassium chloride. The white matter strip was sealed on either side of the sucrose gap channels using fragments of plastic coverslip and a small amount of silicone grease to attach the coverslip to the walls of the channel. Isotonic sucrose solution was continuously run through the gap channels at a rate of 2 ml/min. The temperature of the solution was maintained at 37 °C with an inline heater (Warner). The axons were stimulated and compound action potentials were recorded at opposite ends of the strip of white matter by silver/silver-chloride wire electrodes positioned within the side chambers and the central bath. The central bath was connected to an instrument ground. Stimuli were delivered through a stimulus isolation unit and were usually in the form of 0.1 ms constant current unipolar pulses. Recordings were made using a bridge amplifier and Neurocorder (both from Neurodata Instruments). Examples of a series of CAP waveforms in response to increasing stimuli are illustrated in Fig. 1C. Notice the stable recording as indicated by the high signal to noise ratio. Subsequent analysis was performed using custom Labview® software (National Instruments™) on a Compaq PC™. Further details and description of the chamber can be found in our previous publications [16–19].

2.2.2. Compound action potential amplitude

For the recording of CAP amplitude, stimuli were delivered at a frequency of one stimulus for every 3 s. A supramaximal stimulus (110% of the maximal stimulus) intensity was chosen for this test. The digitized profile of each responding CAP was recorded continuously and stored in the computer for later analysis. In addition, a real time plot of CAP amplitude was also displayed during the experiment.

2.2.3. Activation threshold

Spinal cord compound action potentials consist of many single unit action potentials fired by each individual axon. Axons with different diameters have different thresholds to fire an action potential in response to a stimulation. Voltage tests, which consist of a series of stimuli with increasing

intensity, can gradually stimulate axons of different groups to fire action potentials. The larger diameter axons will be activated first due to the lower threshold. This test was used to detect changes in activation threshold (probability) before and after ischemic insult. The test protocol was programmed in the software and was performed by the computer automatically. The stimulus intensities ranged from 0.015 to 2 mA. At each stimulus intensity level, five stimuli were repeated and an average value was used. Throughout the test, the stimulus was always delivered at a frequency of one stimulus every 3 s.

2.3. Ischemic insult and reperfusion with acrolein

Once a stable compound action potential and membrane potential were obtained, the Krebs' solution that was pumped through the central chamber was switched to a solution modified to produce tissue ischemia. This solution was a Krebs' solution where the glucose was omitted and it was gas bubbled with a 95%N_2–5% CO_2 gas mixture (see composition of normal Krebs' solution in Section 2.1). Reperfusion was accomplished by switching back to perfusion of the cord with the original, well oxygenated, Krebs' media, with or without either 50 or 200 μM acrolein. The solutions took approximately 1 min to enter the chamber, and the reperfusion was carried out for 120 min. After the 120-min period of reperfusion, the spinal cord segments were then subjected to various tests.

2.4. Horseradish peroxidase histochemistry

To examine the extent of anatomical damage caused by ischemic injury, a portion of the white matter strips (the portion of the strip in the middle chamber, which is approximately 15 mm long) was cut and transferred to an oxygenated normal Krebs' solution containing 0.015% horseradish peroxidase (HRP) (Sigma) after various periods of ischemic insult. After incubation for 1 h at room temperature the tissue was fixed by immersion in 2.5% gluteraldehyde in phosphate buffer. The ventral white matter preparations were transversely sectioned at 30 μm using a vibratome (Electronic Microscopy Science). These sections were stained with a diaminobenzidine reaction to reveal the extent of HRP uptake into damaged axons. Sections were viewed with a light microscope. Those axons labeled with HRP showed dark reaction product inside the axon [15,21,22]. The axons labeled with HRP are defined as unsealed axons and those not labeled with HRP are defined as sealed axons. This dye exclusion test [15,21,22] is dependent on the uptake of HRP from the bathing media by injured axons; that is, HRP gains access to the cytoplasm through a breach (or breaches) in the axolemma. It is possible that HRP molecules are capable of entering axons through cut ends. However, based on our experience, HRP can only travel 1–2 mm along the longitudinal axis of axons through the cut end with 1 h incubation time. In our experimental protocol, the segments immersed in HRP solution were about 15 mm long. Therefore, the HRP uptake in the middle of the segment, where the HRP labeling was observed and quantified, can only come from the damaged axonal membrane and not from the cut end.

2.5. Two-dimensional morphometry

The number of unsealed axons were counted from HRP-stained Vibratome sections. The images were first digitized and captured to a Macintosh Quadra 800 computer using a Leitz Orthoplan® microscope and a JVC® video camera. Then, representative cross sections were selected from each strip, using a 6.3 × objective. Representative area samples were chosen from peak regions of dye uptake in transverse sections to quantify axonal sealing. The size of the region varied depending on the thickness of the white matter. Using a 16 × objective, axons were measured within the region. Images were first color-transformed and binarized using IP Lab Spectrum. Counts of individual axons were normalized per unit area and expressed as a density (axons/mm^2) [22].

2.6. Chemicals

All chemicals, including acrolein (Product Number: 48501, *Neat*) and HRP (Type IV, product Number: P-8375), were purchased from Sigma (St. Louis, MO).

2.7. Statistical treatment

ANOVA and Tukey–Kramer test were used to compare the data related to the changes of the amplitude of compound action potential where multiple comparisons were made (Fig. 4). Student's *t*-test was used in all other analyses where a single comparison between two groups was made. Linear correlation between some electrophysiological measurements was expressed by Pearson correlation coefficient (r). Statistical significance was attributed to values $P < 0.05$. Averages were expressed as mean ± standard deviation (S.D.).

3. Results

3.1. The change of CAP amplitude in response to acrolein

The characteristics of compound action potentials (CAP) recorded from isolated guinea pig spinal cords using the double sucrose-gap chamber has been described previously [16–18,23]. The acrolein was introduced to the isolated ventral white matter following a 30-min stabilization period. Two concentrations of acrolein, 50 and 200 μM, were used to examine their effect on the CAP amplitude of uninjured cord. The CAP amplitude, as well as other conduction properties, were monitored for a period of 120 min in the presence of acrolein.

At a concentration of 50 μM, the exposure of acrolein for 2 h resulted in little CAP amplitude reduction (Fig. 2). In addition, the shape of individual CAPs before and after the 2-h exposure of acrolein is virtually the same. Fig. 2A shows an example of the profile of a CAP amplitude recorded over time when the spinal cord segment was subjected to 50 μM acrolein for 120 min. It is obvious that the CAP amplitude of this cord was not affected by the perfusion of 50 μM acrolein for 2 h. The insets illustrate the similarity of the CAP recording before and after exposure to 50 μM acrolein. The average CAP following the 120 min acrolein exposure was 103.8 ± 4.6% of the initial CAP recorded prior to the addition of acrolein ($n = 7$, Fig. 2C). This change is not significant (Fig. 2C, $P > 0.05$).

In contrast, 200 μM of acrolein led to a significant decrease of the CAP amplitude (Fig. 2B and C). Fig. 2B shows an example of conduction changes over time when the spinal cord segments were subjected to 200 μM acrolein for 120 min. As shown, it takes about 30 min for acrolein at this level to begin to affect the conduction of the cord (Fig. 2B). After 2 h of exposure to acrolein, the amplitude of the CAP was reduced by almost half, falling to an average of 55.6 ± 3.5% of the pre-acrolein value ($n = 7$) (Fig. 2B and C). The insets of Fig. 2B show an example of a CAP both before and after exposure to 200 μM acrolein. It is obvious that amplitude of the CAP was significantly reduced, while the overall shape remained relatively the same.

3.2. The change of electrophysiological properties in response to acrolein and oxygen–glucose deprivation

The protocol of oxygen–glucose deprivation is similar to that reported in our previous publication [15]. We slightly modified the protocol in the current study by introducing acrolein during the reperfusion period. CAP amplitude, as well as a series of tests designed to examine the properties of CAP conduction, were performed both before and after oxygen–glucose deprivation and reperfusion with acrolein. The following are our findings.

3.2.1. The changes in CAP amplitude in response to ischemia/acrolein reperfusion

In a previous study, we showed that guinea pig spinal cord ventral white matter could recover almost all of its initial CAP when deprived of oxygen and glucose for 60 min and then reperfused with normal oxygenated Krebs' solution for an additional 60 min [15]. As well, based on our experience using this model, the amplitude of the compound action potential from a typical uninjured cord strip decays little with 4 to 5 h of observation [20]. In the current study, we have confirmed that virtually the same result can be obtained when the reperfusion period is extended to 120 min (Fig. 3A). An example of a CAP trend over time can be seen in Fig. 3A, which shows a typical profile of CAP conduction recorded over time with 60 min of oxygen–glucose deprivation and 120 min reperfusion of normal

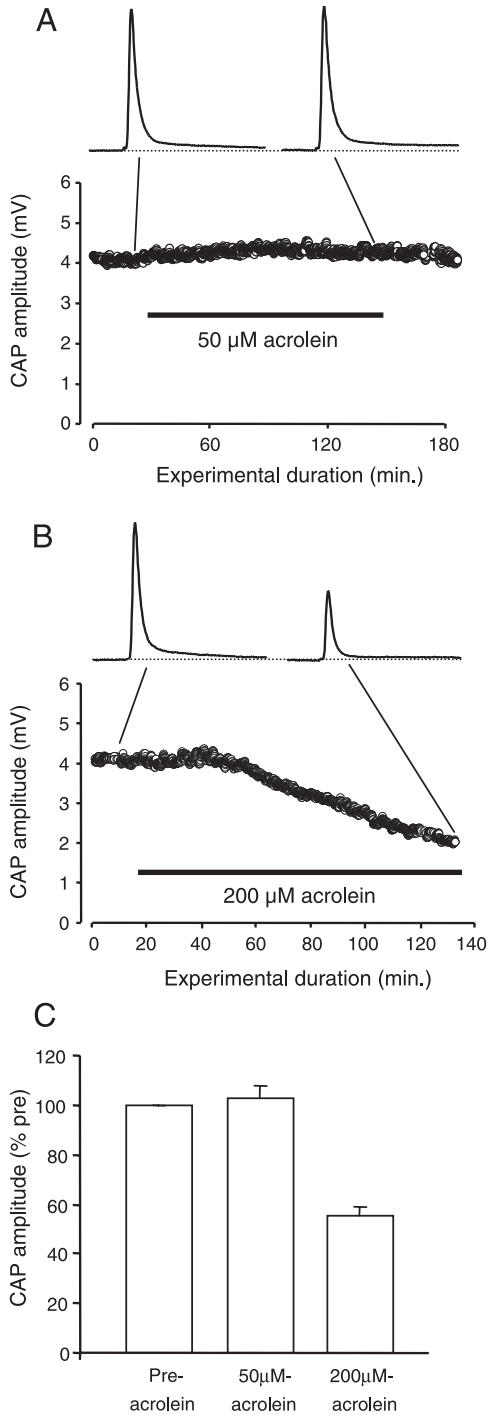

Fig. 2. The effects of 50 and 200 μM acrolein on healthy ventral white matter. (A) The trendline is a CAP recording over a period of time plotted against the CAP amplitude. The 120-min acrolein exposure is indicated by the bar. Note that there was no significant change in the CAP amplitude following 120 min exposure to 50 μM acrolein. The two waveforms set above the graph represent a CAP recording before and after 120 min of 50 μM acrolein exposure. (B) This figure is similar to (A), with the exception that the acrolein concentration was increased to 200 μM. Note that the CAP trend decreases significantly over time. Shown in the inset are examples of the CAP waveforms recorded at the time points indicated in the graph. (C) This is a graphical representation of the previous data. This bar graph shows the final CAP recording, after acrolein exposure, as a percentage of the initial CAP recording.

oxygenated solution. The inset in Fig. 3A illustrates that there is little change in the characteristics of the individual CAP before the deprivation and after reperfusion.

Acrolein at a concentration of 50 μM is not harmful to normal spinal cord (Fig. 2). However, when 50 μM acrolein was introduced to the perfusion media during the 120-min reperfusion period, the cord only partially recovered its pre-ischemic amplitude. Fig. 3B shows a typical cord subjected to such treatment where the resultant amplitude is about 70% of the pre-ischemic level (Fig. 3B). Overall, the

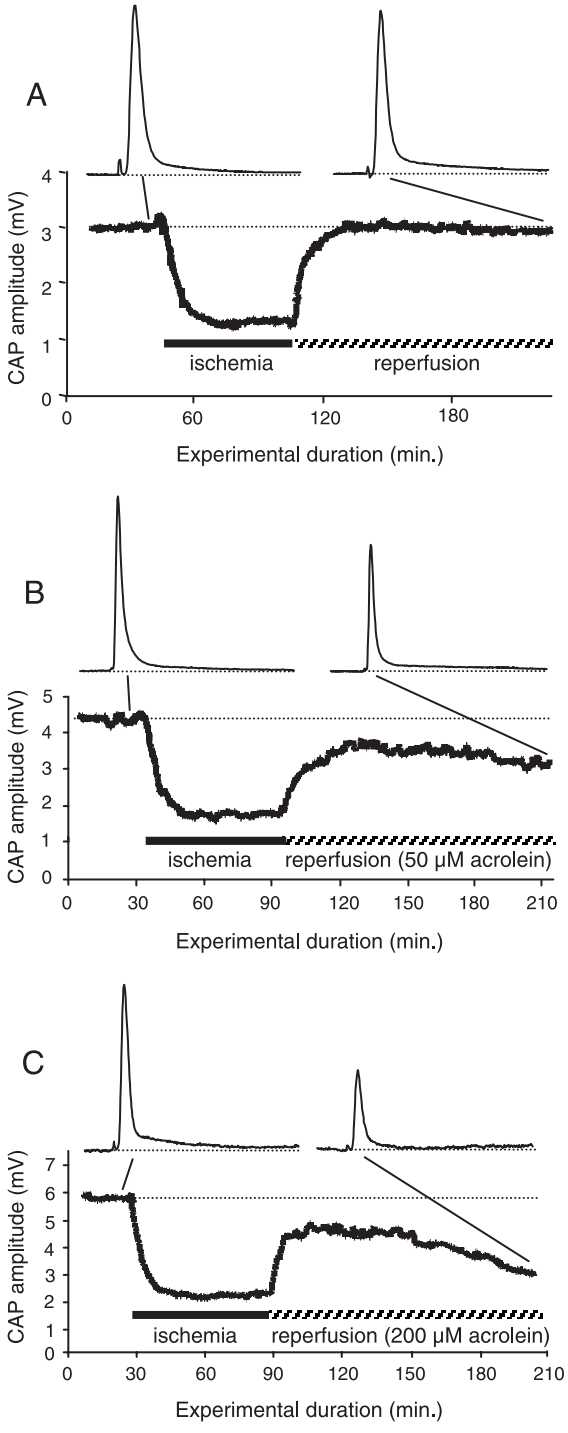

Fig. 3. The effects of 50 and 200 μM acrolein reperfusion on CAP conduction following 60 min ischemia. The trendlines represent CAP recordings over a period of time plotted against the CAP amplitude. (A) The first trend shows the CAP amplitude during an initial stabilization period, and then followed by a 60-min period of ischemia, and then a 120-min recovery. Note the decrease in CAP amplitude during ischemia and subsequent complete recovery of the CAP when the tissue was reperfused with normal Krebs'. The waveforms are shown as an inset for the period before ischemia and after recovery from ischemia. Note that there is little difference from the initial waves and the one recorded following recovery from ischemia. (B) This graph shows the CAP trend when the spinal cord segment was reperfused with 50 μM acrolein following ischemia. Note the decrease in CAP amplitude after 120 min reperfusion with 50 μM acrolein as compared to part A when there was no acrolein in the reperfusion solution. The insets are waveform taken before the ischemic insult and then after reperfusion with 50 μM acrolein. (C) This graph shows the effects of 200 μM on spinal cord segments following ischemia. Note that the final CAP is more dramatically reduced when compared with the 50 μM acrolein (Fig. 1A).

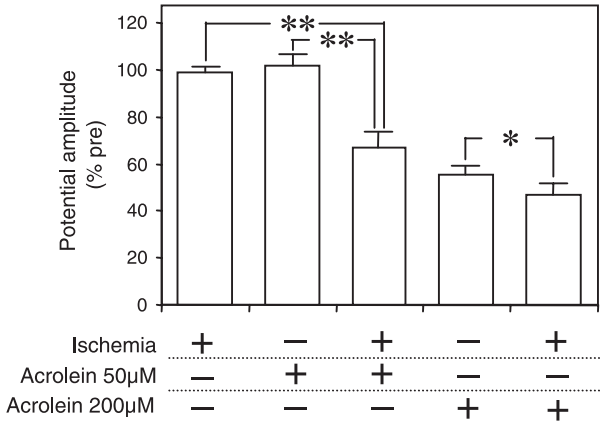

Fig. 4. Graphical representation of the effects of 50 and 200 μM acrolein reperfusion on CAP conduction following 60 min ischemia. The bar graph shows the final CAP as a percentage of the initial CAP for ischemia alone in addition to both 50 and 200 μM acrolein with and without a prior ischemic insult (as indicated by + and − under the graph). Note that 50 μM acrolein does not significantly affect CAP conduction, but after 60 min ischemia, 50 μM acrolein reperfusion leads to conduction failure. Also note the significant decrease in the final CAP for the 200 μM acrolein under control conditions and after ischemia. Asterisks indicate significance of difference between the groups specified in the graph. $^{**}P<0.001$; $^{*}P<0.05$, ANOVA, Tukey–Kramer test.

average CAP amplitude following 60 min oxygen–glucose deprivation and 120 min reperfusion with 50 μM acrolein is $67 \pm 6.5\%$ of the initial CAP ($n=7$) (Fig. 4), which is significantly lower than that following oxygen–glucose deprivation and reperfusion without acrolein (Fig. 4, $P<0.001$). In addition, the resultant conductance following acrolein (50 μM) and ischemic reperfusion injury is significantly lower than that of acrolein exposure (50 μM) alone (Fig. 4, $P<0.001$).

In addition to 50 μM acrolein, we also examined the effects of 200 μM acrolein, which does inflict damage to normal cord. We repeated the above mentioned testing protocol except using the higher concentration of acrolein (200 μM) in the reperfusion solution. A typical cord sub-

jected to such treatment is shown in Fig. 3C, which demonstrates the reduction of conductance when reperfused with 200 μM acrolein. The insets show examples of CAP waveforms before ischemia and following 120 min reperfusion with 200 μM acrolein, demonstrating a significant reduction of CAP amplitude (see inset of Fig. 3C). Overall, the resultant CAP amplitude is 46.5 ± 5.3% of the pre-ischemic level following such treatment ($n = 6$). The conduction failure in the cords subjected to both ischemia and acrolein is more severe than that obtained in normal cord subjected to 200 μM acrolein alone (46.5 ± 5.3% vs. 55.6 ± 3.5%, Fig. 4, $P < 0.05$).

Since the perfusion of 50 μM acrolein inflicted significantly more conduction loss of CAP conductance when combined with ischemic–reperfusion injury, we wanted to know whether such CAP loss is reversible. For this purpose, we modified the testing protocol. Following a period of 60 min oxygen–glucose deprivation, the cord was reperfused with 50 μM acrolein for 60 min and followed by normal Krebs' for another 60 min. The final resultant CAP amplitude following such protocol is 69.1 ± 7.0% of pre-ischemic conductance ($n = 7$), which is not significantly different than those following 60 min oxygen–glucose deprivation and 120 min 50 μM acrolein reperfusion (69.1 ± 7.0 vs. 67 ± 6.5%, $P > 0.05$). Similar testing was carried out with 200 μM acrolein and the results indicate that the conduction loss was similar with or without wash, indicating the conduction loss was irreversible (data not shown).

3.2.2. Changes in activation threshold and refractory period in response to ischemia/acrolein reperfusion

Since ischemia–reperfusion significantly increases the vulnerability of spinal cord segments to 50 μM acrolein, we wanted to determine if there were additional changes in electrophysiological properties as a response to ischemia/acrolein reperfusion. Additional tests were performed to detect differences that cannot be seen using the normal protocol, which was designed to mainly monitor CAP amplitude.

In order to detect changes in activation threshold for the CAP, voltage tests were performed. The spinal cord CAPs were recorded for increasing stimulus intensities before oxygen–glucose deprivation and after reperfusion with or without acrolein. In a previous study, we found that there was no significant change in activation threshold after a 60-min ischemic insult and subsequent 60 min recovery period when compared to the initial voltage test recorded prior to the ischemia [15]. The testing results from those cords subjected to 50 μM acrolein alone were similar, also indicative of little change in activation pattern (data not shown). For this study, we examined whether or not ischemia/50 μM acrolein reperfusion would alter the activation threshold. Fig. 5A shows an example of a series of CAPs recorded both before the ischemic insult and after 50 μM acrolein reperfusion. Although the CAP amplitude was significantly lower following ischemia/acrolein reperfusion

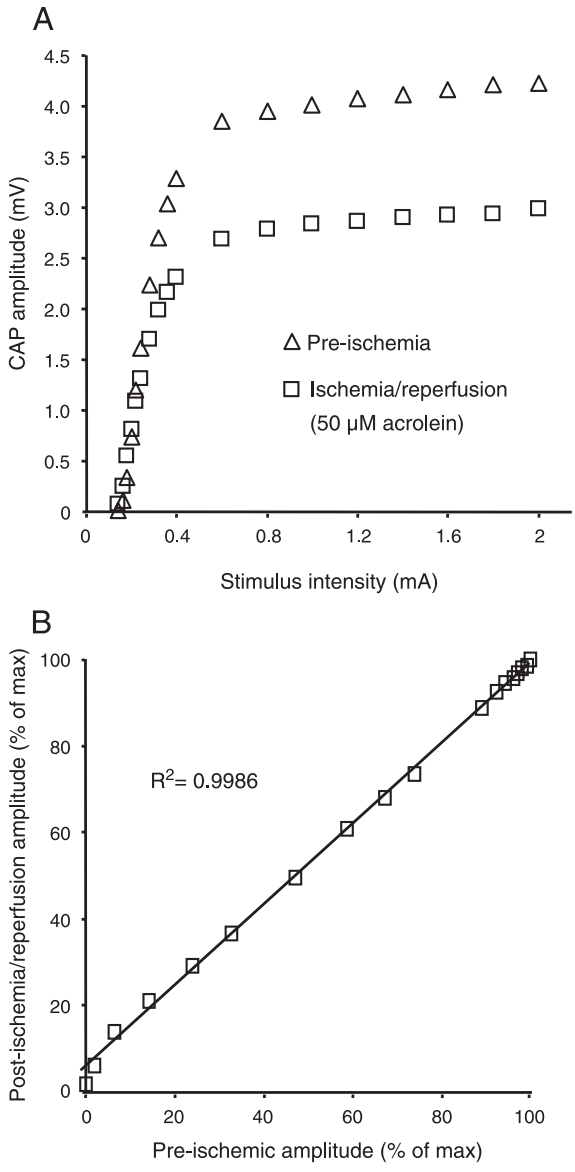

Fig. 5. Examination of the relation between the stimulus intensity and response amplitude before and after ischemia/acrolein reperfusion. (A) This graph shows the CAP in response to increasing stimulus intensities both pre- and post-ischemia/50 μM acrolein reperfusion. Note the decrease in CAP response to increasing stimulus intensities following reperfusion with 50 μM acrolein. (B) Amplitude of the CAP after 120 min ischemia/acrolein reperfusion plotted against the initial CAP amplitude recordings at the same stimulus intensity. Note the linear relationship with the least-square linear regression line not significantly different than 1:1 linearity, indicating that there is little difference in the stimulus threshold profile of the spinal cord segments.

(Fig. 5A), the activation patterns were similar, indicating that there was no significant change in activation thresholds (Fig. 5A). This phenomenon is further displayed in Fig. 5B, a plot of the pre-ischemia/acrolein reperfusion CAP amplitude vs. the post-ischemic/acrolein reperfusion amplitude. The slope of the linear trend is not significantly different than 1, indicating little change in activation patterns. This further suggests that there is no difference in susceptibility

to ischemia/acrolein insult of either large or small diameter axons.

We also evaluated the changes in refractory period of the cords following oxygen–glucose deprivation and 50 μM acrolein reperfusion. In our previous studies, ischemia alone did not significantly change the refractory period [15]. In the current study, we performed the same test as those used in our previous studies, except combining ischemia with acrolein reperfusion. Our data indicates that there was little change in the absolute and relative refractory periods following 50 μM acrolein reperfusion (data not shown). Furthermore, the changes in the ability to follow a train of stimuli, before and after ischemia/acrolein reperfusion, were also not significant (data not shown).

3.3. Membrane damage detected by horseradish peroxidase uptake

HRP histochemistry was used to determine if there was any loss of axolemmal integrity as a result of ischemia and reperfusion with acrolein. A breach in the membrane is indicated if the HRP gained entry to the axon, which strongly correlated to membrane potential changes in our previous studies [16,19,21,22,24]. Consistent with our previous findings, the pre-ischemic cords have little HRP labeling, with a value of HRP-labeling at 10.3 ± 2 axons/mm^2, which is less than 0.5% of the total axons detectable with such method. This indicates little axonal membrane damage in control-uninjured cords (Fig. 6). After a 60-min ischemic insult and reperfusion, the HRP labeling remains low, at a level of 20.5 ± 8.2 axons/mm^2, which is not significantly different than those of control-uninjured cords (P>0.05) (Fig. 6), suggesting little compromise in membrane intactness. Perfusion of a healthy cord with 50 μM acrolein induced little HRP-labeling (4.3 ± 2.2 axons/mm^2), while 200 μM acrolein exposure resulted in significantly more HRP-labeling (87.0 ± 9.7 axons/mm^2) (Fig. 6).

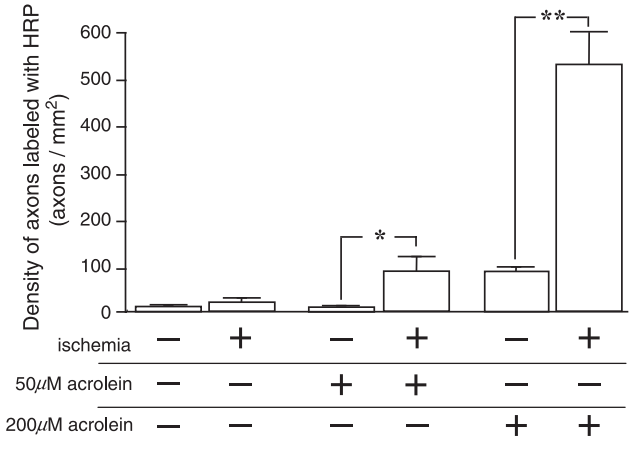

Fig. 6. HRP labeling under different conditions. The bar graph shows the quantification of HRP labeled axons as a density (axons/mm^2) under various conditions. Similar to Fig. 4, the conditions are marked with a + or − below the graph. Uninjured cords were used as normal controls. Note the significant increase in labeled axons between the groups exposed to acrolein alone, and the groups that was reperfused with 50 μM or 200 μm acrolein, following an ischemic insult. Each bar represents the average axonal density ± S.D. *P<0.005, **P<0.001. Student's t-test.

When the cord was subjected to ischemic–reperfusion with acrolein, either at 50 or 200 μM, the HRP-labeling increased significantly when compared to acrolein exposure alone (Fig. 6). Specifically, after 60 min ischemia and 120 min reperfusion with 50 or 200 μM acrolein, the HRP labeling was 86.8 ± 29.1 and 528.0 ± 69.9 axons/mm^2, respectively; both being significantly higher than those subjected to acrolein perfusion alone (Fig. 6) (*P<0.005 and **P<0.001, respectively). In summary, the results using HRP-labeling were in agreement with the electrophysiological results. It appears that for both 50 and 200 μM acrolein, ischemic insult exacerbates the effect of acrolein in inflicting membrane damage.

4. Discussion

4.1. Isolated guinea pig spinal cord injury model

Using an isolated guinea pig spinal cord injury model, we have shown that there is little secondary injury in the first 2 h following mechanical insult as action potential conductance and membrane potential remained stable between 5 and 120 min post-trauma [17,19]. In another study, where oxygen–glucose was deprived for 60 min and then reperfused for 60 min, the cord recovered completely without any acute secondary injury [15]. The detailed discussion of this phenomenon is presented previously, and we refer the interested reader to our previous relevant publication [15]. Briefly, the lack of significant secondary damage in those studies is likely to due to the continuous perfusion of the spinal cord with fresh Krebs' solution at a rate of 2 ml/min. This would be expected to prevent or reduce the accumulation of metabolic toxins that may cause progressive damage. Consistent with this, we noted that fluorescent labeling of O_2^-, one of the most abundant members of reactive oxygen species and the hallmark of secondary injury, showed little increase secondary to oxygen–glucose deprivation when the cord was perfused continuously (Peasley and Shi, unpublished observations). However, when the injured cord was bathed in steady, non-perfused, Krebs' solution, there is a significant increase of labeling of O_2^- following a similar type of injury (Luo and Shi, unpublished observations). These observations highlighted the ability of this model to control multiple experimental conditions and therefore give us the ability to separate the primary injury from the secondary. Furthermore, it also presents the possibility that we can modify the condition to create an "artificial" secondary injury by introducing factors individually or in combination. The current study is an example of the versatility of this model. Using this technique, we were

able to examine the role of acrolein in a form of artificial secondary injury in our in vitro system and demonstrate its heightened detrimental role in ischemic–reperfusion insult.

In our previous studies using the isolated guinea pig spinal cord ventral white matter, ascorbate was always a part of perfusion solution [16–18,23]. Aiming to be consistent with the accumulated data, we have also included ascorbate in the current study. However, we believe ascorbate, a known antioxidant, did not have a significant effect on the experimental condition of this study. This consideration is based on the above mentioned observation that free radicals, such as O_2^-, showed little increase secondary to oxygen–glucose deprivation when the cord was perfused continuously (Peasley and Shi, unpublished observations). Furthermore, there is no established evidence to suggest that ascorbate directly interacts with acrolein. Therefore, we believe that it is unlikely that ascorbate significantly influenced the result of the current study, or more specifically, underestimated the toxicity of acrolein.

4.2. Role of acrolein in ischemic–reperfusion injury

In a previous study we found that when the spinal cord ventral white matter was subjected to a 60-min ischemic insult, it was able to recover almost 100% of its initial CAP upon reperfusion [15]. This finding led us to believe that there were some factors other than lack of oxygen and glucose, that were eliminated in our in vitro system, that are responsible for the function deficits seen in vivo ischemic injury. Based on our studies and many others, we had proposed that free radical and lipid peroxidation may play a significant role in mediating the structural and functional damage seen in ischemic injury. In agreement with such hypotheses, we have found that (1) when the free radicals were allowed to accumulate in our in vitro system by bathing the cord in a steady non-perfused media, significant structural and anatomical damage resulted from ischemic insult (Luo and Shi, unpublished observations). (2) When acrolein, an LPO product was reintroduced into the system during the reperfusion stage, damage occurred (current study) [21]. We believe that what made the current study particularly interesting is that acrolein, at a concentration that is otherwise unharmful to normal cord, inflicted significant damage when exposed during reperfusion. This indicates that free radicals may play a detrimental role in ischemic injury, not only because its concentration increases significantly, but also because the tissue is more sensitive, or more vulnerable, to free radical and LPO attack under such conditions. In summary, the abovementioned data all point to an important role of free radicals and LPO in ischemic injury. Since most of the mechanical injuries are accompanied by ischemia due to the damage of blood vessels, the role of free radical damage as a critical secondary injury mechanism may exist in various injury types.

4.3. The relevance of the concentration of acrolein used in current study

The concentration of acrolein used in the current study was chosen based on its estimations in in vivo studies and in vitro initial testing for the current study. In our initial stage of this in vitro study, we demonstrated for the first time that acrolein, at a concentration of 200 μM, is capable of inflicting membrane disruption and CAP conduction loss after 1–2 h of continuous exposure [21] (current study). We have also showed that at 50 μM, acrolein causes little damage when incubated alone for 2 h. Therefore the concentration threshold for the detrimental effects, we believe, lies between 50 and 200 μM when exposed for up to 2 h. Hence, these two concentrations provide us with excellent reference points at which the effects of acrolein in ischemic–reperfusion injury can be examined.

As well, these two concentrations are relevant to in vivo estimations. For example, it is known that the formation of acrolein is 40-fold greater than that of HNE, another LPO byproduct that has a structure similar to that of acrolein [3]. HNE has been shown to rise significantly in mammalian spinal cord following mechanical injury [25,26]. It is also known that HNE can accumulate in cellular membranes and its concentration can reach up to 1 mM in pathological conditions [27]. Furthermore, the level of acrolein in the sera of a normal human has been estimated to reach 50 μM [28]; and acrolein is estimated to reach 80 μM in respiratory tract lining fluids as a result of smoking [29]. With this information, we believe that the assumption that the concentrations of acrolein we used here (50–200 μM) occur in in vivo spinal cord injury is not invalid.

4.4. The mechanism of acrolein-mediated damage and its exacerbation by ischemia

The mechanism in terms of how acrolein damages neuronal tissue is not clear at this point. One possibility is that acrolein may damage tissue by producing superoxide. It is known that acrolein can generate superoxide in the presence of xanthine oxidase [2,30]. Furthermore, our preliminary studies show that acrolein-mediated axonal damage can be attenuated by free radical scavengers (Luo and Shi, unpublished observations), which supports the role of free radicals in acrolein mediated injury. However, it is still not clear at this point whether acrolein can also directly harm neuronal tissue.

The mechanisms that make neuronal tissue more sensitive to acrolein and free radical attack following ischemic insult may lie in the fact that the lower energy level may affect the intrinsic defense system which scavenges free radicals. However, the relation of energy and free radical scavengers has yet to be established. Should this hypothesis be correct, then the underlying mechanism of free radical mediated damage in post-ischemic injury is not only due to the depletion of the antioxidant system (e.g., SOD), but also

due to the poor function of such system as a result of the low energy level.

4.5. The non-selectivity of axons based on their size by acrolein

In our previous report, metabolic poisoner, such as cyanide and azide, preferentially damages large diameter axons, an indication of a particular vulnerability of large axons to energy depletion [15]. In the current study, it is clear that acrolein-mediated axonal damage is not a function of axonal diameter (Fig. 5). This study indicates that acrolein-mediated axonal damage is targeting the cellular structure, such as axonal membrane, rather than directly affecting energy level.

4.6. The implication of current study in in vivo therapeutic intervention of ischemia

Based on our study, a few points could be used for suggesting effective therapies in ischemic injury. Specifically, in addition to providing exogenous antioxidants such as methyprednisoline, it is also recommended to boost the function of the intrinsic antioxidant system, which is likely impaired in such situations. One strategy is to boost the overall energy level soon after the ischemic insult.

Our study also indicates that acrolein may play a particularly important role due to its high toxicity and long half-life [2]. Therefore, specific acrolein-detoxifying agents, such as glutathione [31], which can bind to acrolein, may also be recommended as effective in such treatment. Indeed, our preliminary testing suggests that glutathione can significantly reduce acrolein toxicity following ischemia (Peasley and Shi, unpublished observations). In summary, our study provided some evidence that may be useful in suggesting more effective therapies, and perhaps optimizing the current existing therapy.

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