

Resistance of isolated mammalian spinal cord white matter to oxygen-glucose deprivation

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Peasley, Melissa A., and Riya Shi. Resistance of isolated mammalian spinal cord white matter to oxygen-glucose deprivation. *Am J Physiol Cell Physiol* 283: C980–C989, 2002. First published May 22, 2002; 10.1152/ajpcell.00591.2001.— We found that isolated guinea pig spinal cord white matter is resistant to acute oxygen-glucose deprivation. Sixty minutes of oxygen-glucose deprivation resulted in a 60% reduction of compound action potential (CAP) conductance, and there was a near complete recovery after 60 min reperfusion. Corresponding horseradish peroxidase-exclusion assay showed little axonal membrane damage. To further deprive the axons of metabolic substrate, we added 2 mM sodium cyanide or 2 mM sodium azide, both mitochondrial suppressors, to the ischemic medium, which completely abolished CAP and resulted in a 15 to ~30% recovery postreperfusion. Both compounds preferentially reduced the conductance of large diameter axons. We suggest the residual ATP in our ischemic model can protect anatomic integrity and physiological functioning of spinal axons following ischemic insult. This further suggests that oxygen-glucose deprivation alone cannot be solely responsible for short-term functional and anatomic damage. The damaging effects of ischemia *in vivo* may be mediated by factors originating from the gray matter of the cord or other systemic factors; both were largely eliminated in our *in vitro* white matter preparation.

axons; neurotrauma; reperfusion; mitochondria; membrane

OXYGEN-GLUCOSE DEPRIVATION, or ischemia, can produce or exacerbate many devastating insults to the central nervous system (CNS), such as stroke and paralysis (1, 21, 29, 31, 35, 36). In the case of spinal cord injury, paralysis can be a consequence of even brief ischemic episodes such as result from clamping of the aorta during life-saving surgeries (1, 7, 12, 13, 19). Furthermore, ischemia can arise secondary to physical insults when the integrity of blood vessels is compromised (20, 35, 36). Altogether, it is widely recognized that ischemia plays a critical role in the neuropathology of the CNS in general and the spinal cord in particular (20, 29, 31).

Although there is little doubt that ischemia causes cellular damage, the mechanism of just how ischemia can produce such damage at the cell and tissues level of organization is not fully understood. This is because

ischemia has been shown to activate many physiological and/or chemical pathways, such as prolonging neurotransmitter release, elevation of reactive oxygen species, and the increase of calcium influx due to membrane depolarization (6, 16, 18, 35). It is possible that these secondary effectors, not oxygen-glucose deprivation alone, are the real culprits in causing cellular damage. It is also possible that there is a hierarchy of importance when each of these mechanisms acts in concert to cause cell damage, though some mechanisms may be more damaging to the cell than others. In our opinion, it is difficult to investigate these possibilities in the absence of a model where the overall condition of the cord, the environment to which it is exposed, and the various secondary insults can be both individually varied and effectively controlled.

Separating ischemia from the secondary events that are associated with it in live animals has proven to be an extremely difficult task. This is because many injury mechanisms can be activated concomitantly in response to ischemic insults, and the control of these many and variable pathways is difficult, if not impossible, in live animals (35). It was the purpose of this study to develop an *in vitro* ischemic model that would mimic *in vivo* situations and, at the same time, provide control over numerous experimental conditions. The goal of this ischemia *in vitro* model was to determine the most critical factors causing irreversible damage following oxygen-glucose deprivation. We chose to use spinal cord white matter as being representative of CNS tissue because it can be easily separated from adjacent gray matter, a difficult task in the brain. Moreover, the catastrophic loss of behavioral function following spinal cord injury is mainly associated with damage to spinal cord white matter, not the gray matter. This also helps to focus the purpose of the study even further. We have learned that it is possible to create an isolated spinal cord white matter ischemic injury model where oxygen-glucose can be lowered significantly *in vitro*. Combined with sensitive functional and anatomic measurements, this is a significant step toward an effective method to unravel the mechanism of ischemic spinal cord injury.

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EXPERIMENTAL PROCEDURES

Isolation of Spinal Cord

Adult female Hartley guinea pigs of 350- to 500-g body wt 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, immersed in cold Krebs solution, and then immediately subdivided, first along the sagittal midline and then by cutting each half of the cord radially, to isolate the ventral white matter. Each white matter strip was subsequently incubated in fresh Krebs solution at room temperature, bubbled continuously with 95% O₂-5% CO₂ (Fig. 1A). The composition of the Krebs solution was as follows (in mM): 124 NaCl, 5 KCl, 1.2 K₂HPO₄, 1.3 MgSO₄, 2 CaCl₂, 20 glucose, 10 sodium ascorbate, and 26 NaHCO₃, equilibrated with 95% O₂-5% CO₂ to produce a pH of 7.2–7.4.

Electrophysiological Recording of Isolated Spinal Cord White Matter

Recording chamber. The construction of the recording chamber is illustrated in Fig. 1B. A strip of isolated spinal cord white matter, ~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) KCl. The white matter strip was sealed on either side of the sucrose gap channels by 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 (CAPs) were recorded at opposite ends of the strip of white matter by Ag-AgCl 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 by using a bridge amplifier and Neurocoder (both from Neurodata Instruments). Subsequent analysis was performed by using custom Labview software (National Instruments) on a Compaq personal computer. Further details and description of the chamber can be found in our previous publications (23–26).

CAP 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.

Activation threshold. Spinal cord CAPs 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 because of 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.

Refractory period. The refractory period was examined by stimulating the cord with a series of twin pulses with various interstimulus intervals, ranging from 0.5 to 13 ms. A supra-

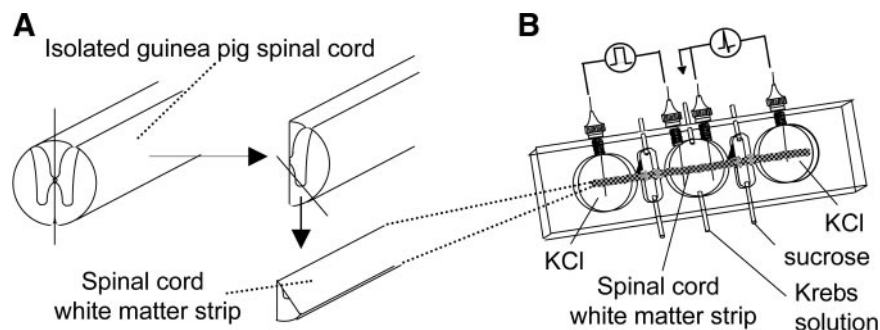


Fig. 1. Diagram showing the isolation of spinal cord white matter strips and recording apparatus. *A*: steps to isolate white matter strips from a spinal cord extracted from an adult guinea pig. *B*: double-sucrose gap recording chamber. *Left to right*, the 1st large compartment contains 120 mM KCl, the central large compartment contains the physiological test solutions, and the 3rd compartment also contains 120 mM KCl. The small chambers on either side of the central compartment contain 230 mM sucrose. Sucrose and the physiological test media are perfused continuously by capillary pumping into the chambers on one side and removed at the same rate on the opposite side. Seals fashioned from coverslips are secured in place with high-vacuum silicon grease at the locations shown to inhibit the exchange of the various media from one compartment to the next. Ag-AgCl electrodes for recording and stimulation are in series with socket connectors at the locations shown. Note that the central chamber is at a ground potential for recording. Spinal cord strips (~35 mm long) were placed in the chamber along its axis and immersed in the various media in all compartments before the placement of the coverslip seals. Action potentials were stimulated at the left side of the spinal cord strip, as shown, conducted through the spinal cord in the central compartment (which also includes the ischemia site), and recorded at the right side of the spinal cord strip, as shown.

maximal stimulus (110% of the maximal stimulus) intensity was chosen for the refractory test. The amplitude of the first responding action potential remained the same for each pair of stimuli. The period immediately after the first stimulus when no action potential could be elicited was defined as the absolute refractory period. The time when the second responding CAP was the same height as the first one was defined as the relative refractory period. For the analysis, the amplitude of the second CAP was expressed as a percentage of the first one.

Ability to follow train stimuli in response to ischemia. A supramaximal stimulus intensity (110% of the maximal stimulus) was chosen for this test. The train of repetitive stimuli were delivered to the spinal cord in the chamber at both 500 and 1,000 Hz for 100 ms. For the 500-Hz and 100-ms train stimuli, a total of 50 action potentials were elicited. For the 1,000-Hz and 100-ms train stimuli, a total of 100 action potentials were elicited. The last three CAPs were averaged and expressed as a percentage of the first peak.

Ischemic Insult and Reperfusion

Once a stable CAP 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 in which glucose was omitted and that was bubbled with a 95% N₂-5% CO₂ gas mixture (see composition of normal Krebs solution in *Isolation of Spinal Cord*). Reperfusion was accomplished by switching back to perfusion of the cord with normal Krebs medium, well oxygenated as described in EXPERIMENTAL PROCEDURES. The solutions took ~1 min to enter the chamber. In other experiments, we used sodium cyanide or sodium azide, dissolved in oxygen-glucose-deprived solution, to further suppress mitochondrial function.

Percent Oxygen Tension

A dissolved oxygen meter (World Precision Instruments) was used during the experiments to monitor the percentage of oxygen in the central chamber as the cord was perfused with different solutions. The probe was calibrated before the experiment and was suspended into the central chamber adjacent to the spinal cord strip. This enabled immediate and accurate O₂ readings to be recorded throughout the experiment. For the oxygen-glucose deprivation situation used in our experiment, the oxygen level can be reduced by ~80% (Fig. 2A).

Horseradish Peroxidase Histochemistry

To examine the extent of anatomic damage caused by ischemic injury, we transferred the white matter strips to an oxygenated 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% glutaraldehyde in phosphate buffer. The ventral white matter preparations were transversely sectioned at 30 μm with 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 (3, 24–26).

Two-Dimensional Morphometry

The number of unsealed axons were counted from HRP-stained Vibratome sections. The images were first digitized

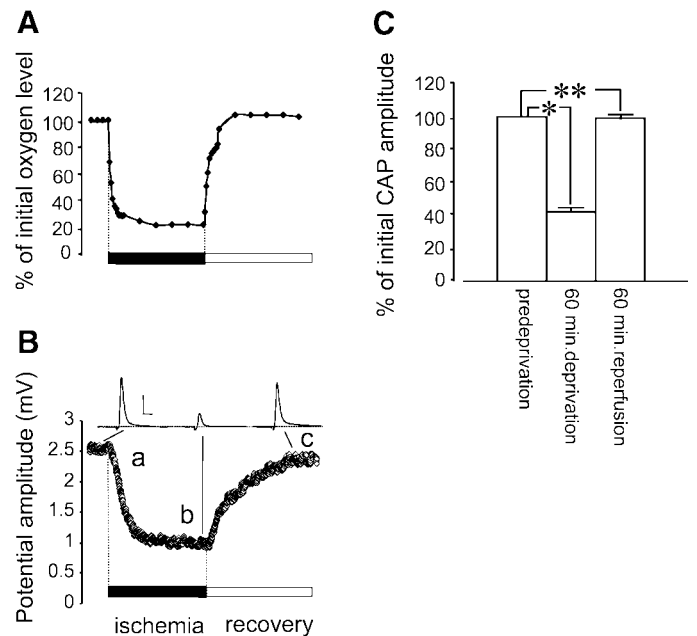


Fig. 2. **A:** example of the reduction and complete recovery of oxygen tension in response to oxygen-glucose deprivation (filled horizontal bar). Changes in amplitude of oxygen tension are plotted against time. Note that oxygen tension decreased rapidly over the course of 5 min from the oxygen-glucose deprivation. Also note the swift recovery of oxygen tension in response to reperfusion (open horizontal bar). **B:** experimental protocol of in vitro ischemic insult and recovery. Each symbol represents the amplitude of a compound action potential (CAP). Data were recorded every 3 s. In this particular case, the length of ischemia (oxygen-glucose deprivation) and reperfusion are 60 min each. Note the decrease of CAP amplitude immediately after the onset of ischemic insult and the plateau reached 30 min into the ischemic period. The individual action potential at *a* (CAP taken prior to ischemia), *b* (CAP recorded at the end of ischemia), and *c* (CAP recorded after a 60-min recovery period) are shown (inset). Note the near complete recovery of the CAP conductance following reperfusion. **C:** average of the percentage of CAP amplitude loss following oxygen-glucose deprivation (60 min) and recovery following reperfusion (60 min) ($n = 8$). * $P > 0.05$; ** $P < 0.01$, significance of difference between groups specified.

and captured to a Macintosh Quadra 800 computer by using a Leitz Ortoplan microscope and a JVC video camera. Representative cross sections were then selected from each strip by using a $\times 6.3$ 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. With the use of a $\times 16$ 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/mm²).

Statistical Treatment

Throughout the paper, Student's *t*-test was used to compare electrophysiological and histological measurements between different groups. Linear correlation between some electrophysiological measurements was expressed by the Pearson correlation coefficient (*r*). Statistical significance was attributed to values of $P < 0.05$. Averages were expressed as means \pm SE.

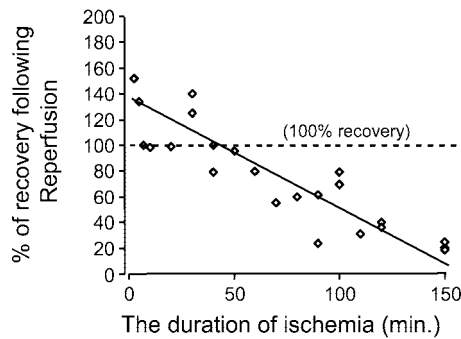


Fig. 3. Percent recovery of the CAP following various periods of ischemia. All data were taken following a period of ischemia plus 60 min of reperfusion. Note that when the periods of ischemia were 5 to ~30 min long, there was at least 100% CAP amplitude recovery following reperfusion. The recovery was ~90% when the period of ischemia was ~60 min. Note that even after an ischemic insult of 150 min, there was still 20% recovery of CAP.

RESULTS

Electrophysiological Properties of Spinal Cords Following Ischemia and Reperfusion

The characteristics of CAPs recorded from isolated guinea pig spinal cords by using the double-sucrose gap recording chamber have been described previously (22–26). Briefly, after a period of ~30–60 min of stabilization in the recording chamber, the amplitude of the CAP would plateau. The spinal cord strips were then subjected to a period of oxygen-glucose deprivation followed by 60 min of reperfusion (Fig. 2, A and B). The oxygen level and the CAP were monitored continuously. A series of tests designed to examine the properties of CAP conduction were performed both before and after ischemia and reperfusion.

Changes in CAP amplitude in response to ischemia. We first subjected white matter strips to a 60-min period of oxygen-glucose deprivation (Fig. 2, A and B). Surprisingly, the deprivation reduced the CAP amplitude to $40 \pm 3\%$ of preischemic levels, a 60% reduction, even after 60 min of elapsed time. This value is significantly lower than control ($P < 0.01$, Fig. 2, B and C). After a 60-min period of reperfusion with normal medium, the cord regained $98 \pm 2\%$ of the conductance, which is not significantly different from that of the preischemic insult ($P > 0.05$, Fig. 2C). The profile of a typical ischemic insult and reperfusion is shown in Fig. 2B, which shows the change of CAP amplitude in response to a 60-min period of oxygen-glucose deprivation followed by a same period of reperfusion. The individual action potentials of points *a*, *b*, and *c* in Fig. 2B are shown in the *inset* and represent the potentials at predeprivation (*a*), the end of deprivation (*b*), and the end of reperfusion (*c*), respectively. It is obvious that in addition to a near complete recovery of the action potential amplitude resulting from reperfusion, no appreciable difference is evident in the overall shape of the single action potential between preischemic insult (*a*) and postreperfusion (*c*).

We further explored the relationship between the duration of exposure and the action potential conduc-

tance recovery. A similar protocol was followed. We subjected the white matter strips to ischemic insults for periods ranging from 5 to 150 min (Fig. 3). We found that even with an extended period of oxygen-glucose deprivation (150 min), which reduced CAP to only $5.8 \pm 0.6\%$ of preischemic level, the white matter still recovered $21.5 \pm 2.9\%$ of its preinjury CAP amplitude after 60 min of reperfusion ($n = 3$). Interestingly, 5–30 min of deprivation were followed by a recovery of CAP amplitudes ranging from 100 to 150% of preinjury level.

Changes in activation threshold, refractory period, and the ability to follow train stimuli in response to ischemia. To detect any changes in electrophysiological properties, such as activation threshold, refractory period, and the ability to follow train stimuli, we performed an additional series of tests designed to detect differences that cannot be seen with our normal protocol. To detect changes in activation threshold for the CAP, we performed voltage tests. The spinal cord CAPs were recorded for increasing stimulus intensities before oxygen-glucose deprivation and after reperfusion (Fig. 4). The amplitudes of responding CAP were plot-

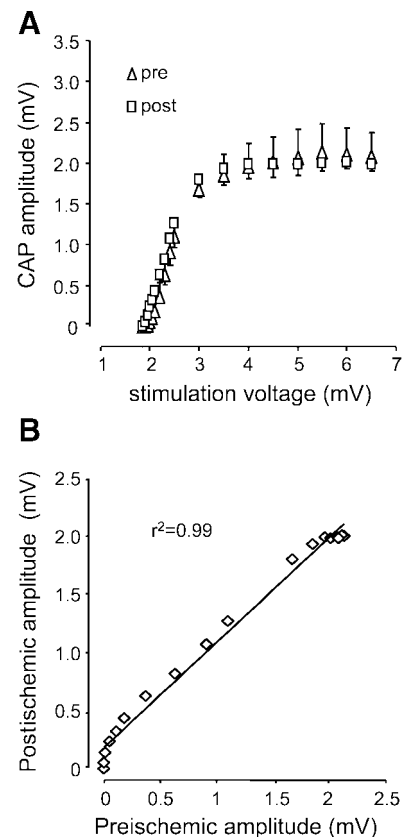


Fig. 4. Examination of the relationship between stimulus intensity and response amplitude at preischemia and 1 h postischemia. A: CAP of the spinal cord strips in response to increasing stimulus intensities both preischemia and 60 min postischemia. Both trends are almost superimposable. B: CAP amplitude at 1 h postischemia plotted against preischemic amplitude at the same stimulus intensity for 6 different spinal cord strips. The error bars are omitted for clarification. The linear relation is apparent, and the least-squares linear regression line is not significantly different from 1:1 linearity, indicating that there is little difference in the stimulus thresholds profile of the spinal cord strips.

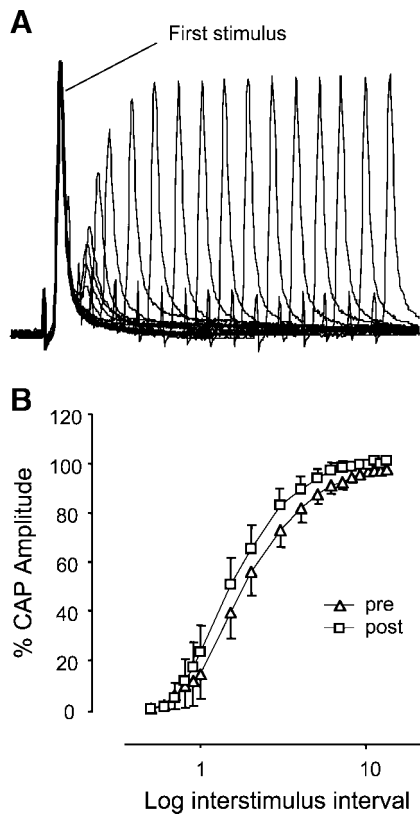


Fig. 5. Refractory period changes preischemia and 1 h postischemia. A: 20 individual records of CAP responses to twin pulse stimuli are superimposed. The 1st waveform represents the initial CAP, with the other waves occurring after the 2nd stimuli. Because this 1st CAP is always produced by a stimulus of the same intensity, each of the superimposed individual recordings was identical. *Left to right*, CAP produced by the 2nd stimulus is shown. Note the typical dampened amplitude of the 2nd CAP when triggered during the relative refractory period followed by the typical plateau in amplitude produced when the 2nd stimulus is applied subsequent to the relative refractory period. B: response to the 2nd stimulus (as a percentage of the 1st CAP amplitude) vs. the interstimulus interval is plotted both before ischemia and 1 h postischemia. Note that a sigmoidal period of relative refractory is apparent and that the %CAP is slightly higher in postischemic measurement, although no significance was found.

ted in the same graph and compared (Fig. 4). In Fig. 4A, the activation curve of pre- and postischemic injury almost overlapped for 60-min ischemic insults, indicating not only a similarity in CAP amplitude but also the activation threshold pattern before and after ischemic insults. This phenomenon can also be seen in Fig. 4B, where the linear regression line is not significantly different from 1:1 linearity, indicating that there is little difference in the activation threshold before and after 60 min of ischemic insults ($n = 6$).

Next, we evaluated the changes in refractory period of the cords following oxygen-glucose deprivation. Shown in Fig. 5, paired stimuli demonstrated typical dampening of the CAP amplitude soon after the absolute refractory period. To illustrate how such a test was conducted, we superimposed all of the waveforms for a normal cord (stimulus interval ranging from 0.5 to 13.0 ms) to produce Fig. 5A. The pre- and postischemia

results were plotted together for the 60-min ischemic insults shown in Fig. 5B. The y-axis indicates the magnitude of the second CAP waveform as a percentage of the magnitude of the initial CAPs; the x-axis represents a log interstimulus range of 0.5–13 ms. Note that pre- and postischemia plots seem to have the same absolute and relative refractory periods. There was a trend for the postischemic CAP to be slightly higher than those recorded from preischemic spinal cord, although these differences were not significant ($P > 0.05$).

Changes in the ability to follow a train of stimuli were also tested for both before and after a 60-min ischemic insult. For the 60-min ischemic insult ($n = 7$), the results for the preischemic train test of 500 Hz and 100 ms were $55.7 \pm 4.7\%$. The postischemia/recovery results were $71.3 \pm 4.6\%$ ($P < 0.05$). The results for the 1,000-Hz, 100-ms train test of preischemia were $32.2 \pm 2.7\%$, and the postischemia/recovery results were $47.1 \pm 4.2\%$ ($P < 0.01$, Fig. 6B). These data indicate that the ability of spinal cord to follow the train stimuli is actually enhanced after 60 min of ischemia.

Amplitude of CAP: oxygen-glucose deprivation plus mitochondrial inhibitor. Because 60 min of oxygen-glucose deprivation failed to significantly attenuate action potential conductance, we decided to examine the conduction of the ventral white matter in the presence of 2 mM sodium cyanide or 2 mM sodium azide

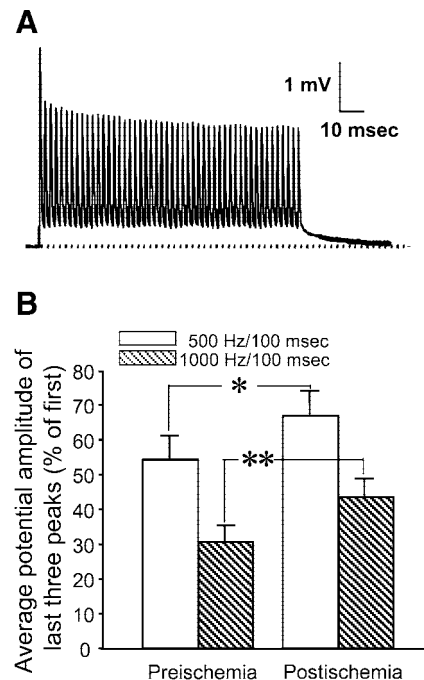


Fig. 6. Response of spinal cord strips to train stimuli at frequencies of 500 and 1,000 Hz for 100 ms. A: series of CAP from an uninjured cord strip in response to a train stimuli of 1,000 Hz and 100-ms duration. B: plot of the potential amplitude of the last peak as a percentage of the 1st peak for the response both preischemia and 60-min postischemia. Note that the postischemic amplitudes are higher than the corresponding amplitudes preceding the ischemic insult. * $P < 0.05$; ** $P < 0.01$, significance of difference between groups specified.

(inhibitors of mitochondrial respiration) in addition to ischemic conditions. The results of the changes in CAP amplitude can be seen in Fig. 7A. The bar graphs represent the amplitude of the pre- and postischemia-reperfusion CAPs, following a 30- or 60-min period of ischemia, expressed as a percentage of the preinjury CAP. Specifically, for ischemia plus sodium azide, the 30-min insult resulted in a CAP that was $72.7 \pm 18.9\%$ of the original CAP ($n = 5$), and the 60-min insult was $15.1 \pm .6.1\%$ ($n = 5$). The CAP following an ischemic insult plus sodium cyanide was $52.7 \pm 12.2\%$ of the original CAP for a period of 30 min ($n = 8$) and $31.6 \pm 6.2\%$ of the original for a period of 60 min ($n = 3$).

Changes of activation pattern: oxygen-glucose deprivation plus mitochondrial inhibitor. Similar to that in the oxygen-glucose deprivation experiments, we wanted to detect if there are differences in susceptibility of axons to mitochondrial inhibitor on the basis of their diameter. Such information can be deduced on the basis of changes in activation patterns and threshold by using voltage tests (23, 24). Figure 7B presents a hypothetical scenario where more larger axons are responsible for CAP (*line a*) or more smaller caliber axons are responsible for CAP (*line c*) following deprivation and mitochondrial inhibition. Figure 7C shows results of the preischemic CAPs with different stimulating strength plotted against the postischemic CAPs

(after 60 min of reperfusion) in the presence of sodium azide. It is obvious from the data that there is a relative delay of axonal activation following ischemic insults during lower strength stimulation, indicating a reduced number of functional, lower threshold, fast-activating, large-diameter axons (similar to *line c* in Fig. 7B). This result is quite different from those conducted with oxygen-glucose deprivation without chemical mitochondrial inhibitors, in which there were no differences in the activation pattern before and after insult (Fig. 4). This finding suggests that there is a selective vulnerability of the larger diameter axons to mitochondrial inhibition. A similar result using the same voltage protocol was obtained for sodium cyanide, which can be seen in Fig. 7D, indicating a similar selective vulnerability of large axons to sodium cyanide.

Membrane Damage Detected by HRP Uptake

HRP histochemistry was used to determine whether there was any loss of axolemmal integrity as a result of ischemia. A breach in the membrane is indicated if the HRP gains entry to the axon, which strongly correlates to membrane potential changes in our previous studies (3, 24–26). Consistent with our previous findings, the preischemic cords had little HRP labeling, indicating the intactness of the axonal membrane (25, 26) (Fig.

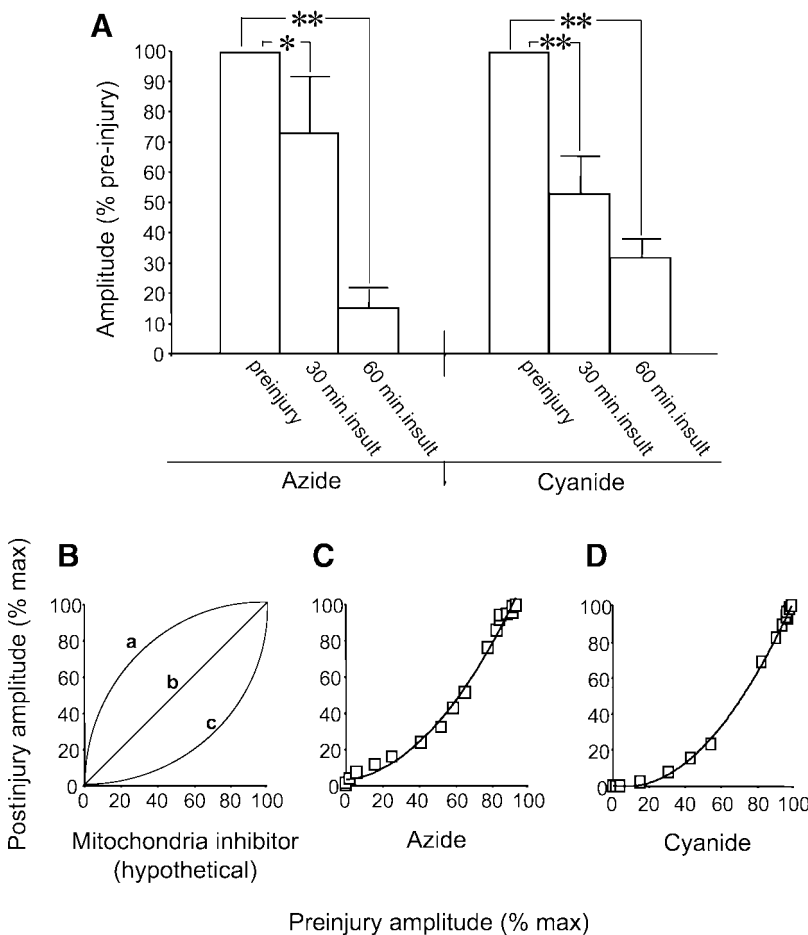
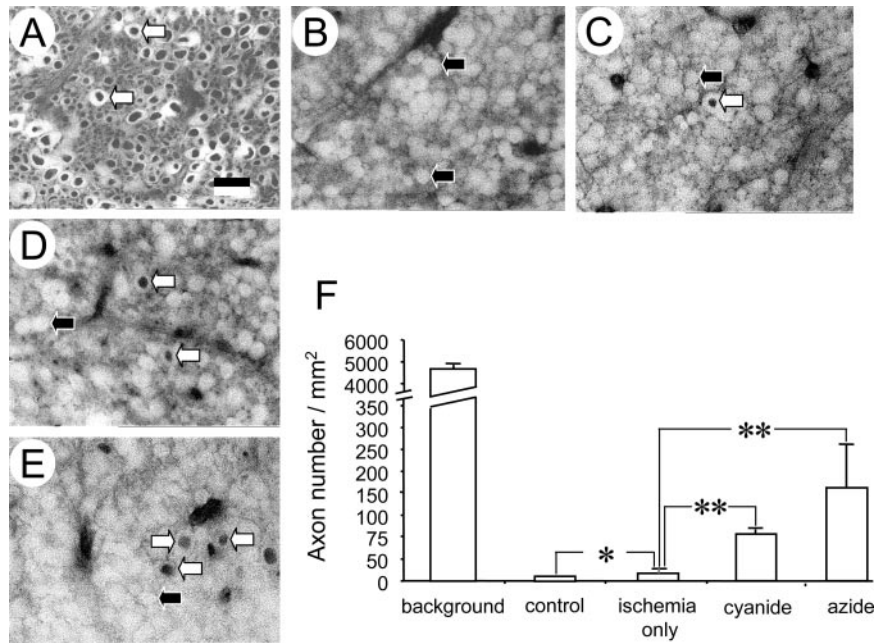


Fig. 7. Characteristics of impulse conductivity in response to sodium azide and sodium cyanide, in addition to ischemia. A: %CAP amplitude recovery measured 60 min postischemia. Data from both 30- and 60-min ischemic insults are presented. * $P > 0.05$; ** $P < 0.005$, significance of difference between groups specified. B–D: examination of the relationship between stimulus and response amplitude before and after mitochondrial inhibition and ischemia. B: hypothetical plotting of data is shown where more larger caliber fibers (with a lower stimulus threshold) are responsible for the CAP (*a*) or more smaller caliber fibers are recruited to produce the recovered CAP (*c*) after injury compared with that preischemia. *Line b* indicates that the stimulus-response pattern remains the same following injury. C: average amplitude ($n = 6$) of CAP at 1 h following sodium azide exposure, plotted against the preischemic amplitude at the same stimulus intensity. A linear relationship, such as that shown in Fig. 4B or by *line b* in B, is not exhibited. Instead, the trend of the data indicates that the larger diameter axons were less likely to be activated after the combination of ischemia and sodium azide. This suggests that the large diameter axons were more affected by such treatment. D: amplitude of the CAP at 1 h following sodium cyanide exposure, plotted against the preischemic amplitude at the same stimulus intensity for 6 different spinal cord strips. Once again, these results closely resemble those for sodium azide, indicating that larger diameter axons were affected by the combination of ischemia and sodium cyanide.

Fig. 8. Photographs of Vibratome sections showing horseradish peroxidase (HRP) labeling and the quantification under different conditions. The micrographs from 5 groups represent background (transection) (A), controls (uninjured) (B), ischemia alone (60 min) (C), ischemia with sodium cyanide (D), and ischemia with sodium azide (E). Open arrows indicate axons labeled with HRP; filled arrows denote axons that exclude HRP. F: quantification of HRP-labeled axons in different conditions. Note the significant increase of HRP labeling in the cyanide and azide groups. Also note the relatively small numbers of axons showing HRP uptake of all the groups compared with the background group. In the background group, the cords were transected and immediately (<2 min) immersed in the HRP solution. Such a method was used to establish the maximal level of HRP uptake that could be expected during the evaluation of the ischemic cords. Values represents means \pm SE of 5–8 experiments. * $P > 0.05$; ** $P < 0.05$, significance of difference between groups specified. Scale bar = 10 μ m (applies to A–E).



8B). After a 60-min ischemic insult and 60 min of reperfusion, the HRP labeling remained low (Fig. 8C), suggesting little membrane damage. However, addition of sodium cyanide or sodium azide induced more axons labeled with HRP (Fig. 8, D and E). Quantitative measurements have shown that the control, uninjured cords had <0.5% of the axon labeled with the HRP molecule (10.3 ± 2 axons/mm², Fig. 8). After a 60-min ischemic insult, the proportion of axons that were labeled represented <1% of the total number of axons in a cross-sectional area (ischemia only: 20.5 ± 8.5 axons/mm², Fig. 8), which is not significantly different from those in control, uninjured cords ($P > 0.05$). Interestingly, even with an extended 150 min of deprivation, the HRP-labeled axons still remained low, at 21.7 ± 6.3 axons/mm² ($n = 3$). This value is not significantly different from those after a 60-min ischemic insult ($P > 0.05$). There were 1.5% of the axons in the sodium cyanide group (68.8 ± 8 axons/mm²) and about 3% of the axons in the sodium azide group (155.5 ± 7.4 axons/mm²) labeled with HRP; both results were significantly higher than those in the ischemia-only group ($P < 0.05$). In summary, the results using HRP-labeling were in agreement with the electrophysiological results.

DISCUSSION

Isolated Guinea Pig Spinal Cord Ischemic Model

We have previously shown that there is little secondary injury in the first 1–2 h following mechanical insult in isolated spinal cord white matter (23, 24, 26, 27). This interpretation is based on the fact that there is a stable action potential conductance and membrane potential between 5 and 120 min posttrauma. We suggest that the lack of significant secondary damage in this study of oxygen-glucose deprivation is due to the con-

tinuous perfusion of the spinal cord with fresh Krebs solution constantly 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 note that fluorescent labeling of O₂ showed little increase secondary to oxygen-glucose deprivation (M. A. Peasley and R. Shi, unpublished observations). This observation is also consistent with the fact that action potential conductance and membrane potential both recover completely following oxygen-glucose deprivation. This conclusion is further supported by the fact that lipid peroxidation products, when they are present at an in vivo relevant concentration, inflict significant functional and anatomical damage (J. Luo, M. A. Peasley, and R. Shi, unpublished observations).

Thus we conclude that the lack of oxygen-glucose alone does not cause irreversible short-term damage in isolated white matter. We suggest that the irreversible functional and anatomic damage seen in in vivo and other in vitro ischemic injuries is mainly due to secondary injury mechanisms or is influenced by adjacent gray matter (15, 16, 29, 32, 35).

Mechanism of Resistance to Oxygen and Glucose Deprivation: Role of Residual ATP

It is likely that the resistance of isolated spinal cord to oxygen-glucose deprivation is due in part to the residual ATP in the tissue. Both sodium cyanide and sodium azide, two effective suppressors of mitochondrial functions (9), can significantly inhibit action potential recovery. In addition, the oxygen level in the perfusion media was measured to be reduced by 80%; thus oxygen was not completely eliminated from the system. Perhaps this residual oxygen is sufficient to support the production of sufficient levels of ATP,

which sustain low levels of CAP conduction during the period of deprivation and, in turn, allow a full recovery subsequent to reperfusion. Because glucose was completely removed from the perfusion solutions, it is unlikely that the cells depended on glucose for energy production. Because the conditions in an *in vivo* ischemic insult are usually a reduction of blood flow but not a complete absence of blood (28), a low level of oxygen is indeed likely to persist during ischemia. Therefore, we believe that the significantly reduced oxygen level in our studies is relevant to *in vivo* ischemic injury.

Mechanisms of CAP Conduction Failure Due to Oxygen and Glucose Deprivation

The normal generation and propagation of action potentials rely on the functioning of voltage-gated sodium and, to a lesser extent, potassium channels (11). The proper functioning of these channels is very sensitive to membrane potential. Likewise, the membrane potential is, in turn, dependent on the functioning of the energy-dependent sodium and potassium exchangers. These ATP-dependent pumps are largely responsible for maintaining proper extracellular ion gradients across the membrane, which is necessary for action potential propagation. Almost 50% of the total cellular energy required by the nervous system is used in the maintenance of these gradients (2, 8, 10). In the absence of a constant energy supply, there is a rapid dissolution of ion gradients, leading to ionic derangement inside and outside the cell (14, 17). This contributes to conduction failure.

The concentration of intracellular calcium is also critical for the maintenance of proper function of the cellular organelles. Prolonged elevation of intracellular calcium causes irreversible deterioration of cellular structures and functional loss. It is reasonable to suggest, however, that the conduction failure during the ischemic periods in our study would not be associated with a substantial increase in intracellular calcium levels in this study, because no irreversible structural and functional loss was noted. The calcium influx through voltage-dependent calcium channels was likely counterbalanced by the calcium efflux through specific calcium-sodium pumps (29, 31). However, when the residual amount of ATP sufficient to support energy-dependent pumps was further reduced through the use of mitochondrial suppressors, it is likely that the calcium influx increased due to membrane depolarization (M. Peasley and R. Shi, unpublished observations). The irreversible CAP loss, as a result of intracellular calcium accumulation, can also be induced by the reverse operation of the calcium-sodium pumps in response to intracellular sodium overloading. This was clearly demonstrated by Stys and colleagues (33, 34) in rat optic nerves. The increased intracellular calcium likely triggered irreversible deterioration, which caused sustained CAP conduction loss and, to a lesser extent, membrane damage sufficient enough to allow HRP to enter the axons.

It is interesting to point out that the addition of mitochondrial inhibitors reduced CAP conduction significantly (by 70–85%, Fig. 7A), whereas the parallel morphological measurements have shown that HRP-labeled axons remained relatively low in the presence of mitochondrial inhibitors, despite the significant increase compared with those in ischemia only (Fig. 8). Overall, only 1.5–3% of the axons were labeled with HRP in the presence of mitochondrial inhibitors. This may simply imply that membrane integrity is necessary but not sufficient to ensure the impulse conduction of the spinal axons. It is known that a prolonged depolarization in an otherwise intact axon can block CAP conduction. In the current study, the loss of CAP conduction in response to mitochondrial inhibition is likely a result of membrane depolarization and not the direct result of membrane breakdown seen in our previous studies, where the mechanical insult was the primary injury (23–26).

Previous Studies of In Vitro Ischemic Insult

Stys and colleagues (30, 32) have shown that rat optic nerve lost half of its CAP in 2–4 min and all of its CAP conductance in 8–10 min in anoxia solution. An ~30% conduction recovery was observed after 60 min of anoxia followed by 60 min of reperfusion (30, 32, 33). In the current study, the average time for the guinea pig spinal cord to lose half of its CAP conduction was 9.3 ± 0.6 min ($n = 21$). At 60 min into oxygen-glucose deprivation, the cord lost 60% of its original CAP. At an extensive 150 min of deprivation, the cord still retained ~6% of the CAP ($5.8 \pm 0.6\%$). Therefore, our data indicate that guinea pig spinal cord axon is more resistant than rat optic nerves to short-term ischemic conditions. The mechanism underlying the difference between our studies and the studies by Stys and colleagues is not clear at this point. One likely difference is the anatomy between naked white matter and nerve tissue. Optic nerve is protected by peri nerve sheath, limiting exchange of media, whereas spinal cord white matter is naked and exposed. Because the conditions for induction of an ischemic insult and the length of ischemia were similar in both studies, we believe the oxygen in bathing solutions should not be significantly different. Another reason may be that rat optic nerves are intrinsically more sensitive to oxygen deprivation and have greater amounts of calcium-dependent proteases, a hypothesis that has yet to be confirmed.

Vulnerability of Large Axons to Oxygen and Glucose Deprivation and Mitochondrial Inhibition

It has been shown that there is a selective loss of large caliber axons in an *in vivo* spinal cord injury (4, 5). However, results from our laboratory have clearly shown that mechanical insults, such as compression and stretch, do not selectively injure a greater number of large-diameter axons compared with small-diameter axons *in vitro* (23, 24, 26). These observations have led us to hypothesize that the vulnerability of large axons

in in vivo spinal cord injury may result from true progressive secondary pathological mechanisms and perhaps a selective metabolic susceptibility (23, 26). The data from our current study have indicated that this hypothesis may be correct. We have shown that when the cords were subjected to oxygen-glucose deprivation and mitochondrial inhibition, there indeed was a selective loss of large-diameter axons, based on voltage tests (Fig. 7).

Hypersensitivity Following Oxygen and Glucose Deprivation

It is interesting to note that the excitability of spinal cord axons is slightly increased following oxygen-glucose deprivation, whereas refractoriness is slightly decreased. This was observed in the following ways. 1) When the cord was subjected to oxygen-glucose deprivation for a period of 5–30 min, the recovered conductance was better than that preinjury, with recovered amplitude ranging from 100 to 180% of the preinjury value (Fig. 3). 2) During the refractory period, the amplitude postischemia was slightly higher than that of the preischemia period (Fig. 5). 3) The ability to follow a train of repetitive stimuli is more pronounced postinjury than preinjury (Fig. 6). The mechanism of such a phenomenon is not entirely clear. It is possible that the threshold for axonal activation may be reduced, producing higher CAP amplitude postischemic insults. Therefore, there may be a group of otherwise silent axons that can be activated after an ischemic episode due to a reduction in their threshold of activation.

Future Study Using This Model

The double-sucrose gap recording chamber allows us to control numerous experimental conditions and can separate the primary injury from the secondary. Thus we can modify a number of conditions to create an “artificial” secondary injury. This may make it possible to identify the unique and perhaps subtle factors causing irreversible functional and anatomic damage secondary to the ischemic condition. As a result, this model may serve as an effective screening tool for possible treatments for spinal cord injury.

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