Posttraumatic hypothermia in the treatment of axonal damage in an animal model of traumatic axonal injury

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Object. Many investigators have demonstrated the protective effects of hypothermia following traumatic brain injury (TBI) in both animals and humans. Typically, this protection has been evaluated in relation to the preservation of neurons and/or the blunting of behavioral abnormalities. However, little consideration has been given to any potential protection afforded in regard to TBI-induced axonal injury, a feature of human TBI. In this study, the authors evaluated the protective effects of hypothermia on axonal injury after TBI in rats.

Methods. Male Sprague–Dawley rats weighing 380 to 400 g were subjected to experimental TBI induced by an impact-acceleration device. These rats were subjected to hypothermia either before or after injury, with their temporalis muscle and rectal temperatures maintained at 32°C for 1 hour. After this 1-hour period of hypothermia, rewarming to normothermic levels was accomplished over a 90-minute period. Twenty-four hours later, the animals were killed and semiserial sagittal sections of the brain were reacted for visualization of the amyloid precursor protein (APP), a marker of axonal injury. The density of APP-marked damaged axons within the corticospinal tract at the pontomedullary junction was calculated for each animal. In all hypothermic animals, a significant reduction in APP-marked damaged axonal density was found. In animals treated with preinjury, immediate postinjury, and delayed hypothermia, the density of damaged axons was dramatically reduced in comparison with the untreated controls (p < 0.05).

Conclusions. The authors infer from these findings that early as well as delayed posttraumatic hypothermia results in substantial protection in TBI, at least in terms of the injured axons.

Key Words • traumatic brain injury • axonal injury • β-amyloid precursor protein immunohistochemistry • rat

Within the last decade, considerable evidence has been found in both laboratory and clinical settings to indicate that posttraumatic hypothermia can afford substantial brain protection, which results in the retention of neurons and improved neurological outcome after traumatic brain injury (TBI).2,6,13,14,19–22,25,27,30,33,41,45 In the laboratory setting, the induction of moderate hypothermia has been shown to provide protective effects in trauma-induced blood-brain barrier alterations43 and nitric oxide synthesis,37 as well as neuronal cell loss,3,9 contusion volume,27 and ventricular enlargement.2 In contrast to other injury states such as ischemic brain injury,4,13,14,16,28 however, some controversy exists as to whether moderate hypothermia used to treat brain/spinal cord injury affords its protective effects by attenuating extracellular levels of excitatory neurotransmitters.10,17,27 In the clinical setting, moderate hypothermia has also been suggested to produce protective effects that translate into improved outcome in patients with TBI.7,21,22,36 In addition to the aforementioned structural and functional protection afforded by hypothermia, it has recently been reported that the use of moderate hypothermia can blunt the generation of traumatically induced axonal injury, with the inference that this may also result in improved outcome.21 Because traumatically induced axonal injury/diffuse axonal injury is a common feature of TBI in animals and humans,5,13,20,30,33,34 this observation is of considerable importance. However, the overall significance of this finding remains debatable because this observation has been made by only one investigatory group,21 who used a model of injury that may not replicate the full repertoire of diffuse axonal change typically seen in humans with TBI.11,24 Moreover, because these authors relied on the use of cytoskeletal markers to detect axonal change rather than more contemporary markers such as antibodies that target anterogradely transported amyloid precursor proteins (APPs),1,12,38,40 the overall relevance of these findings to the pathobiological mechanisms of diffuse axonal injury in animals and humans can also be called into question. Because of this, in our report we have attempted to reevaluate the protective effects of moderate hypothermia on traumatically induced axonal damage by using antibodies that target APPs. Our primary goal was to determine if moderate hypothermia can blunt the genesis of these APP immunoreactive axons,
Whereas a secondary goal was to explore the use of various pre- and postinjury hypothermic paradigms to determine if the timing of hypothermia influences its protective effects, in terms of any observed reduction in the number of APP immunoreactive axons.

Materials and Methods

Twenty male Sprague–Dawley rats weighing 380 to 400 g (mean 389.2 ± 1.9 g, standard error of the mean [SEM]) were used in this experiment. For induction of anesthesia the rats were exposed to 3% isoflurane. Next, the animals were rapidly intubated and ventilated with a mixture of 1% to 2% isoflurane in 30% O2/70% N2O. The femoral artery was exposed and cannulated for blood gas sampling, and then the animals were prepared for the delivery of impact-acceleration brain injury as described by Marmarou and colleagues.11,24,32 Briefly, a midline incision was made to expose the skull between the coronal and lambdoid sutures. A metallic disk 10 mm in diameter was firmly affixed by dental acrylic to this portion of the skull over, because this is an anatomically discrete region that is well delineated from the surrounding brain tissue, its choice seemed optimal for conducting detailed qualitative and quantitative analyses. After harvesting of this 2-mm-wide sagittal block, the tissue section was flat mounted and serially sectioned on the vibratome at a thickness of 50 μm. The sections were processed for the visualization of antibodies targeted to the APP, a marker of impaired axonal transport and axonal damage.14,39-40 In this strategy, the APP was visualized using a new method involving microwave antigen retrieval, wherein the sections were placed in citric acid buffer and transferred to wells in a computer-controlled microwave in which the coils had been mapped, with the additional protection provided by water loads.

After microwave processing, the sections were allowed to cool for 20 minutes, rinsed three times for 10 minutes in phosphate-buffered saline (PBS), preincubated for 1 hour with 2% Triton X in 10% normal horse serum (NHS) in PBS, and then incubated overnight in the monoclonal APP primary antibody diluted 1:100 in 1% NHS in PBS and reincubated in rat-absorbed biotinylated anti–mouse immunoglobulin G (Vector Laboratories, Burlingame, CA) in 1% NHS in PBS. The tissue was rinsed three times for 1 minute in PBS and subsequently incubated with an avidin–biotin complex (ABC elite kit; Vector Laboratories) for 1 hour. After rinsing three times for 10 minutes in PBS and two times for 10 minutes in 0.1 M phosphate buffer, the reaction product was visualized with 0.05% diaminobenzidine, 0.01% hydrogen peroxide, and 0.3% imidazole in 0.1 M phosphate buffer for 10 to 20 minutes. The sections were dehydrated, cleared, and mounted on glass slides with coverslips.

Quantitative Analysis

After preparation of the tissue for APP immunoreactivity at the light microscopic level, the slides were transferred to a microscope interfaced with a computer-assisted image analysis system (Imaging Research, St. Catherine’s, Ontario, Canada). The pontomedullary junction was enlarged to a magnification of 10 and the part of this structure containing damaged and/or immunoreactive APP profiles was outlined and its area calculated. Next, the number of damaged APP immunoreactive profiles larger than 10 μm in diameter within that area were counted. These reactive profiles were seen as grossly swollen and/or truncated axonal segments characteristic of the retraction balls/terminal clubs associated with the traumatic induction of axonal injury.35 Once the counts were obtained in one section, five adjacent semiserial sections from the same tissue block were processed and analyzed in the same fashion. On completion of these counts, the number of damaged axons was expressed as a function of the unit area of tissue analyzed. For statistical analysis, the Kruskal–Wallis test was used to test significance of difference
between groups. In cases of significance (p < 0.05), a Duncan’s post hoc analysis was performed for multiple comparisons.

**Results**

**General Physiological Observations**

Using the experimental paradigms noted earlier and illustrated in Fig. 1, we achieved a controlled temporal pattern of body cooling and gradual warming. As illustrated in Fig. 2, the animals subjected to hypothermia (Groups 2, 3, and 4) required on average 16.5 ± 1.8 minutes (SEM) for the temporalis muscle to reach the target temperature of 32˚C. Once this target temperature was reached, it was maintained over the entire 1-hour period of hypothermic insult. Similarly, as can be seen in Fig. 2, after the completion of hypothermia the animal’s body temperature was slowly and steadily rewarmed over a 90-minute period (mean 91 ± 7.3 SEM) to reach normothermic levels. Importantly, during both the hypothermic and the normothermic periods, the blood gas values did not change overtly. As can be seen in Table 1, these values did not exceed the norms for PaO₂, PCO₂, and pH, clearly indicating that all animals were in physiological balance.

**General Microscopic Findings**

Consistent with previous reports of studies in which this model system was used, the animals that sustained traumatic impact-acceleration injury but that were not subjected to hypothermic intervention showed numerous

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time Before &amp; After Induction of TBI</th>
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<tr>
<td></td>
<td>60 Min</td>
</tr>
<tr>
<td>SBP normothermia</td>
<td>102.5 ± 3.6</td>
</tr>
<tr>
<td>hypothermia</td>
<td>100.6 ± 3.1</td>
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<tr>
<td>DBP normothermia</td>
<td>84.9 ± 6.1</td>
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<td>hypothermia</td>
<td>71.7 ± 4.1</td>
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<tr>
<td>pH normothermia</td>
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</tr>
<tr>
<td>hypothermia</td>
<td>7.45 ± 0.01</td>
</tr>
<tr>
<td>PCO₂ normothermia</td>
<td>45.1 ± 3.3</td>
</tr>
<tr>
<td>hypothermia</td>
<td>37.5 ± 1.1</td>
</tr>
<tr>
<td>PO₂ normothermia</td>
<td>131.2 ± 5.6</td>
</tr>
<tr>
<td>hypothermia</td>
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* All values are expressed as the mean ± SEM. Abbreviations: DBP = diastolic blood pressure; SBP = systolic blood pressure.
APP-immunoreactive axonal profiles within the pontomedullary junction, which was the focus of the current investigation (Fig. 3). However, in addition to this locus, numerous other regions throughout the brain also showed APP-positive axons consistent with findings originally made in the model with the use of antibodies to the neurofilament subunits. In the pontomedullary junction, these immunoreactive profiles appeared predominantly as large reactive axonal end bulbs denoting sites of axonal discontinuity and swelling (Fig. 3 lower). In contrast, the sections harvested from animals subjected to TBI with hypothermic intervention also showed immunoreactive profiles in the pontomedullary junction, yet in these cases, the overall number of immunoreactive axonal profiles appeared significantly reduced in comparison with the non-hypothermia-treated animals (Fig. 4).

Quantitative Findings

The quantitative analyses conducted in this investigation statistically confirmed the impressions derived from the previously described routine light microscopic analysis (Figs. 3 and 4). Specifically in the injured, nonhypothermia-treated animals, the overall density of immunoreactive axonal profiles per square millimeter was 1012.1 ± 70.3 (SEM). In contrast the animals subjected to hypothermia before and immediately after TBI, as well as those subjected to hypothermia 1 hour later, showed a statistically significant reduction in axonal density in the range of 700 ± 100 (SEM) damaged axons/mm² (Group 2, 698.1 ± 107.8; Group 3, 586.5 ± 63.4; Group 4, 701.9 ± 76.7, p < 0.05) (Fig. 5).

Discussion

In this communication, we have shown that the use of both immediate and delayed posttraumatic hypothermia results in a profound reduction in the density of injured axonal profiles within 24 hours of injury. From a clinical perspective these findings are consistent with the suggest-
Axonal protection with hypothermia

ed efficacy of hypothermia recently reported in several clinical trials in patients with TBI.\textsuperscript{7,21,22,36} Furthermore these studies confirm and significantly extend the observations made by Marion and White\textsuperscript{23} wherein they reported the protective effects of hypothermia in rodents in the laboratory setting. What is unique and provocative in our study is the fact that we relied on the use of contemporary immunocytocchemical strategies for identifying reactive axonal profiles, which directly target impaired axoplasmic transport, an occurrence that has long been linked to axonal failure in TBI.\textsuperscript{18} This approach was dissimilar to the studies of Marion and White, which relied on the use of antibodies to target the cytoskeletal constituents. Moreover, and perhaps more important, in contrast to their study, in which rapid rewarming was used (approximately 15 minutes), in this study we used hypothermic paradigms more in keeping with the clinical situation, involving the induction of moderate hypothermia (32°C) followed by a delayed and progressive rewarming to normothermic levels. Of further interest in the current communication is the fact that the use of pretraumatic and delayed posttraumatic hypothermia with gradual warming also resulted in profound axonal protection, in terms of the total immunoreactive axonal profiles seen in the injured animals. The findings of protective effects of immediate posttraumatic hypothermia parallel those described by Marion and White, yet it is important to note that our observations of continued protection even with delayed posttraumatic hypothermia are not consistent with their findings; they did not report comparable protection. The reasons for these different responses are likely many and varied, but we propose that the use of controlled hypothermia followed by gradual warming could constitute a dependent variable that explains these differences. Alternatively, differences in the animal models of injury could also have contributed to the observed differences in outcome. In the study published by Marion and White, the traumatic insult was delivered via cortical impact that evokes overt cortical change/contusion, with damaged axons detected in the internal capsule. In contrast, our model did not cause cortical contusion or other overt central nervous system change, but rather resulted in scattered damaged axons in the pontomedullary junction as well as multiple other brain sites, consistent with the original descriptions of Foda and Marmarou.\textsuperscript{11} Such differences would indicate that our animals sustained less severe focal injury, which may further explain the variation in these two studies. Additionally, we believe that our studies have clinical relevance in that they illustrate that the induction of hypothermia at a relatively delayed posttraumatic time point provides considerable protection in terms of the damaged axonal profiles. One may argue that a 1-hour therapeutic window is much too short a time to be useful in humans with TBI, but we propose that because many of the metabolic changes occurring in rats compared with humans occur at a considerably accelerated rate, this indicates that the comparable therapeutic window in humans could be considerably longer.

From the mechanistic perspective, it is difficult to speculate on the specific potential mechanisms by which hypothermia blunts the progression of the TBI-induced axonal damage. It is well known that the swollen reactive axons or end bulbs require a 6- to 24-hour period to evolve fully and progress to axonal disconnection. Thus, it seems reasonable to assume that early induction of hypothermia may influence one of the many processes involved in delayed pathogenesis. The most likely candidates for early hypothermic protection include stabilization of the axolemma, in which altered permeability change has been linked to subsequent cytoskeletal failure and axonal collapse and disconnection.\textsuperscript{32} Similarly, it is quite possible that the hypothermia itself could also be acting on the axonal cytoskeleton and organelle population, preventing their subsequent breakdown that leads to axonal failure and disconnection. Alternatively, the effects of hypothermia could act on axonal transport, as has been posited by some.\textsuperscript{26,46,47} However, if this is the case, it is difficult to explain how axoplasmic transport could be decreased in one population of axons while continuing to progress in another population to swelling and disconnection.

Whereas the results of the current study clearly indicate that early and delayed posttraumatic hypothermia give considerable protection in terms of the damaged axonal profiles, we must admit that this protection has only been evaluated at the 24-hour posttraumatic time point. In this context, it is unknown if with increased survival, that is, 48 to 72 hours postinjury, increased numbers of damaged axons may reemerge in the hypothermia-treated animals. This will require continued evaluation and will constitute the basis of another investigation focusing on the issue of delayed protection. However, we do not think that with more prolonged survival a reemergence/increase in the number of damaged axons will occur. Such an assumption
is based on indirect evidence from other experimental studies of hypothermia, wherein the early behavioral and motor protection afforded by hypothermia persisted over a relatively long posttraumatic course, indicating no reduction in hypothermia’s efficacy with more prolonged survival.1

Conclusions

We believe that this study has shown that hypothermia can elicit dramatic reductions in the number of damaged axons per unit area in a well-controlled animal model of traumatic axonal injury. Use of both early and delayed hypothermia coupled with gradual warming mimics many of the protocols currently in use in the clinical setting and in our estimation provides further support for the continued clinical use and evaluation of hypothermia as a reasonable tool for the better care and management of patients with TBI.

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References

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