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The Combination of Either Tempol or FK506 with Delayed Hypothermia: Implications for Traumatically Induced Microvascular and Axonal Protection

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Abstract

Following traumatic brain injury (TBI), inhibition of reactive oxygen species and/or calcineurin can exert axonal and vascular protection. This protection proves optimal when these strategies are used early post-injury. Recent work has shown that the combination of delayed drug administration and delayed hypothermia extends this protection. Here we revisit this issue in TBI using the nitroxide antioxidant Tempol, or the immunophilin ligand FK506, together with delayed hypothermia, to determine their effects upon cerebral vascular reactivity and axonal damage. Animals were subjected to TBI and treated with Tempol at 30 or 90 min post-injury, or 90 min post-injury with concomitant mild hypothermia (33°C). Another group of animals were treated in the same fashion with the exception that they received FK506. Cranial windows were placed to assess vascular reactivity over 6 h post-injury, when the animals were assessed for traumatically induced axonal damage. Vasoreactivity was preserved by early Tempol administration; however, this benefit declined with time. The coupling of hypothermia and delayed Tempol, however, exerted significant vascular protection. The use of early and delayed FK506 provided significant vascular protection which was not augmented by hypothermia. The early administration of Tempol provided dramatic axonal protection that was not enhanced with hypothermia. Early and delayed FK506 provided significant axonal protection, although this protection was not enhanced by delayed hypothermia. The current investigation supports the premise that Tempol coupled with hypothermia extends its benefits. While FK506 proved efficacious with early and delayed administration, it did not provide either increased vascular or axonal benefit with hypothermia. These studies illustrate the potential benefits of Tempol coupled to delayed hypothermia. However, these findings do not transfer to the use of FK506, which in previous studies proved beneficial when coupled with hypothermia. These divergent results may be a reflection of the different animal models used and/or their associated injury severity.

Key words: axonal injury; pial vessels; vascular reactivity; traumatic brain injury

Introduction

It is well known that the treatment of traumatic brain injury (TBI) has not proved successful in the clinical setting. Although preclinical studies have suggested the utility of multiple agents including, but not limited to, oxygen radical scavengers, immunophilin ligands, and hypothermia, these have proved useful only in the early post-traumatic period, and thus have not proved to be transferrable to the human clinical situation, in which very early post-traumatic intervention is not feasible. Recently, we have observed that the use of combinations of therapeutic interventions coupling hypothermia with other neuroprotective agents extended their therapeutic window and resulted in improved efficacy, even when they were given in a delayed post-traumatic administration schedule (Baranova et al., 2008; Oda et al., 2011). In this context, we have recently demonstrated that the use of delayed hypothermia, coupled with the use of delayed topically-applied superoxide dismutase (SOD) exerted significantly enhanced vascular protection over the use of either therapy alone (Baranova et al., 2008). Similarly, the use of delayed hypothermia coupled with the delayed administration of FK506 imparted enhanced protection, not only in terms of the vascular responses to fluid percussion injury (FPI), but also of the overall burden of traumatically induced axonal damage (Oda et al., 2011). Despite the provocative nature of

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these findings, their overall applicability to the clinical setting is limited by the fact that SOD proved efficacious only when applied topically. Further, there was no information whether this radical scavenger exerted axonal protection. Additionally, in both experimental studies utilizing either SOD or FK506, concerns existed as to whether these targeted therapies coupled with hypothermia would prove efficacious in other traumatically brain-injured animal model systems. Recently, Deng-Bryant and colleagues (Deng-Bryant et al., 2008) have shown in a convincing fashion that systemic Tempol could exert widespread neuroprotective actions when given early post-injury. The benefits of Tempol were linked to the fact that it served as a nitroxide antioxidant that decomposes the superoxide radical as well as peroxynitrite-derived free radicals, with excellent blood–brain barrier penetrance. Unfortunately, however, with delayed administration of the Tempol, some of its protective effects were lost (Deng-Bryant et al., 2008). In light of these findings, it appeared reasonable and rational to revisit the protective effects of this agent, now coupling delayed Tempol administration with delayed hypothermia to determine if this combinational approach could exert continued protection on both the axonal and microvascular fronts. Additionally, to obviate concerns regarding model specificity, it also appeared reasonable and rational to revisit these studies in a different model of TBI, now incorporating impact acceleration injury (IAI) with the use of Tempol and delayed hypothermia. Lastly, to provide a comparative base, it also seemed rational to revisit the protective effects of the combination of delayed hypothermia with FK506 in the same model system to allow for a more comprehensive appreciation of the potential usefulness of these combinational approaches.

Methods

General preparation

All experimental procedures were performed with a protocol approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. Thirty-five adult male Sprague-Dawley rats weighing 350–500 g were used in the current study. The animals were housed in individual cages on a 12-h light/dark cycle with free access to water and food. The animals were initially anesthetized intraperitoneally with sodium pentobarbital at 60 mg/kg. The femoral artery was cannulated with a PE50 catheter (Becton Dickinson, Sparks, MD) for continuous monitoring of arterial blood pressure (PowerLab; AD Instruments, Colorado Springs, CO) and periodic collection of blood samples for determination of arterial oxygen tension (Pao_2), arterial carbon dioxide pressure ($Paco_2$), and pH values (Stat Profile® pHOx; Nova Biomedical, Waltham, MA). Arterial blood gas samples (100 μL) were taken 10 min prior to injury and every hour post-injury. The femoral vein was cannulated with a PE50 catheter for administration of medication as needed as well as the FK506 and Tempol. After completion of the tracheotomy, the animals were mechanically ventilated (Harvard Apparatus, Holliston, MA) on room air. Post-injury pancuronium bromide (3 mg/kg) was administrated intravenously to produce neuromuscular blockade. The resting $Paco_2$ was maintained at a constant level, between 35 and 40 mm Hg, by adjusting the rate and/or volume of the respirator. Body temperature was maintained at 37°C with a heat lamp and/or a heating pad throughout the experiment except during the hypothermic period.

The induction of hypothermic procedure and drug delivery

Hypothermia was accomplished by whole body cooling achieved through the use of ice packs. Body and brain temperatures were measured via a thermometer in the rectum (Yellow Spring Instruments, Yellow Spring, OH) and a thermistor in the temporalis muscle (Physitemp Instruments, Clifton, NJ), respectively. The temporalis muscle temperature was employed based on the fact that the readings obtained therein closely parallel brain temperature (Jiang et al., 1991). Typically, 13–15 min of ice pack use was required to achieve the target rectal temperature of 33°C. Hypothermia was initiated 1 h post-injury and maintained at 33°C for 60 min, followed by rewarming over a span of 90 min. This rewarming was accomplished using a protocol detailed previously (Suehiro et al., 2003). Animals also received either FK506 (Astellas Pharma US, Deerfield, IL) or Tempol (Alexis® Biochemicals Corporation, San Diego, CA) delivered intravenously 30 or 90 min post-injury. A single 10-mg/kg dose of Tempol or 3 mg/kg of FK506 diluted with 0.9% sterile saline to a total volume of 1.0 mL was administrated intravenously over a 20-min period. These dosages were based on previous work from our laboratory and others on Tempol (Deng-Bryant et al., 2008; Kato et al., 2003; Kwon et al., 2003; Rak et al., 2000) and FK506 (Marmarou and Povlishock, 2006; Oda et al., 2011; Reeves et al., 2007).

Visualization and assessment of the cerebral microcirculation

Visualization and assessment of the cerebral microcirculation was performed as described in our previous studies (Ellis et al., 1983; Gao et al., 2010; Levasseur et al., 1975; Oda et al., 2011). Briefly, rats were placed in a stereotaxic frame; a middle sagittal incision was performed to expose the skull bone. A 2×4-mm rectangular craniotomy was made in the skull over the left parietal cortex and the underlying dura mater was incised and removed. Next, a cranial window was installed over the exposed brain surface, and fixed in place by bone wax and dental acrylic. The cranial window consisted of a stainless steel ring with three outlets and a circular glass plate inside a ring. Two of the outlets served as inflow and outflow paths for the perfusion and clearance of selected vasoactive agents, while the free end of the other outlet was set at a predetermined height to achieve an intracranial pressure (ICP) of 5 mm Hg. The space under the cranial window and the three outlets was then filled with sterile artificial cerebrospinal fluid (CSF), with the pH adjusted to 7.35 by equilibration with a gas mixture containing 6% O_2 and 6% CO_2 balanced with N_2. The underlying pial microcirculation was visualized with a microscope and pial arteriolar diameters were measured with a Vickers image-splitting device (Vickers Instruments Inc., Maiden, MA). Typically, in each window preparation a minimum of 4 arteriolar segments were evaluated. In the current study, acetylcholine (ACh), which was well known to elicit endothelial-dependent vasodilation, was used in two different concentrations to assess vascular dilation following its topical application via the cranial window (Kontos et al., 1988; Wei et al., 1992). ACh (Sigma-Aldrich, St Louis, MO) was

dissolved in artificial CSF to achieve the final concentrations of 10^{-7} M and 10^{-5} M, and then applied via the cranial window to induce a vasodilatory response. After each application of ACh in the space under the window for 2–4 min, vessel diameter was measured. The vascular reactivity to ACh was expressed as a percent change from the baseline diameter at each measurement time point.

Experimental traumatic brain injury

After the baseline vessel diameter and ACh-induced vessel diameter were measured, the cranial window was gently removed. The exposed brain was then covered with a piece of wet gauze. Next, a 10-mm circular stainless steel helmet was installed over the sagittal suture between the bregma and the lambda and secured with dental acrylic. The respiratory tube was disconnected from the tracheal tube, and the animal was rapidly placed prone on a foam pad and subjected to IAI, as described in detail in previous publications (Marmarou et al., 1994). Briefly, a weight of 450 g was dropped from a height of 2 m through an acrylic glass tube on the metal helmet, which was centered immediately under the lower end of the tube. The animal was returned to the respirator immediately after the injury with the continuation of blood gas and blood pressure monitoring. The helmet was then removed and the cranial window reinstalled.

Experimental design

In this study, both vascular function and the burden of axonal damage were assessed after IAI followed by hypothermia and/or the administration of Tempol or FK506. To this end, the animals were arbitrarily divided into four groups in the Tempol series (groups 1, 2, 3, and 4) and FK506 series (groups 1, 2′, 3′, and 4′). Each group contained 5 animals.

Group 1: IAI with no treatment. These animals were subjected to IAI. The body temperature was maintained at normothermic levels during the entire experimental period. Vascular reactivity to the two concentrations of ACh was assessed prior to IAI and at 4, 5, and 6 h following injury (Fig. 1).

Group 2 or 2′: IAI followed by early Tempol or FK506 administration. These animals were injured and then Tempol or FK506 was injected 30 min post-injury. The vascular responses to ACh were assessed prior to IAI and at 4, 5, and 6 h post-injury (Fig. 1).

Group 3 or 3′: IAI followed by delayed Tempol or FK506 administration. These animals were injured and then Tempol or FK506 was injected 90 min post-injury. The vascular responses to ACh were assessed prior to IAI and at 4, 5, and 6 h post-injury (Fig. 1).

Group 4 or 4′: IAI followed by hypothermic intervention and delayed Tempol or FK506 administration. In this group, at 1 h following injury, a 60-min period of hypothermia was induced, followed by a 90-min period of rewarming. These animals also received Tempol or FK506 administration 90 min post-injury, with vascular reactivity to ACh assessed prior to IAI and at 4, 5, and 6 h post-injury (Fig. 1).

Tissue preparation

For evaluating the burden of axonal damage following TBI with hypothermia and/or Tempol or FK506, we analyzed axonal damage in brainstem projection axons using strategies previously utilized in our laboratory (Gao et al., 2010; Koizumi and Povlishock, 1998; Suehiro and Povlishock, 2001). In this study, we used the same animals evaluated in groups 1–4 for the conduct of detailed axonal analyses. At 6 h post-injury after the measurement of vascular reactivity to ACh (in groups 1–4), the rats were sacrificed with an overdose of euthanasia solution under general anesthesia and then transcardially perfused with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M Millonig's phosphate buffer. After perfusion, the brains were removed, transferred to fixative, and stored overnight. They were placed in a sagittal brain-blocking device with 2 mm of the brain cut from the brain's midsection, followed by further sagittal blocking to include the medulla, pons, and midbrain. This blocking strategy was based on the fact that in this model system, numerous damaged axons occur in the pyramids at the medullospinal junction in the descending corticospinal tracts

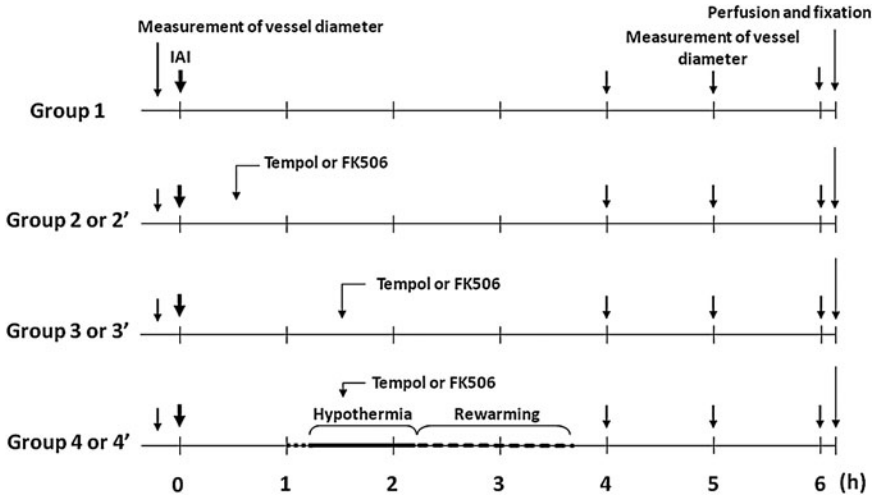

FIG. 1. This chart shows the time course for each experimental group. Tempol was administered in groups 2, 3, and 4. FK506 was administered in groups 2′, 3′, and 4′.

(Povlishock et al., 1997). After harvesting the 2-mm-wide sagittal block, the tissue section was flat-mounted and serially sectioned on the vibratome at a thickness of 40 μm. Sagittal sections ($n=60$) were serially collected in alternating wells, with each well containing adjacent sections. Systematic uniform sampling of sagittal sections was initiated from a random starting well, with every fourth section collected for a total of 15 sections per animal.

Immunocytochemistry for axonal damage

These sections were processed for visualization of an antibody targeting amyloid precursor protein (APP), a marker of impaired axonal transport and axonal damage, using a protocol adapted in our laboratory (Stone et al., 2000). Briefly, the sections were reacted with 0.3% H_2O_2 in phosphate-buffered saline (PBS) for 30 min to block endogenous peroxidase activity and microwaved twice in citric acid buffer while maintaining a 45°C maximum temperature for 5 min (Stone et al., 1999). After microwave processing, the sections were allowed to cool for 20 min each time. The sections were pre-incubated for 1 h in 10% normal goat serum (NGS) with 0.2% Triton X in PBS, and then incubated for 18 h with the rabbit anti-β-APP (Invitrogen, Carlsbad, CA) diluted 1:1500 in 1% NGS in PBS. On the following day, the sections were incubated for 1 h with biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Inc., Burlingame, CA) diluted 1:1200 in 1% NGS in PBS. Then, sections were visualized via incubation for 1 hour in avidin biotinylated enzyme complex (Vectastain® ABC kit; Vector Laboratories), followed by 0.05% diaminobenzidene, 0.01% H_2O_2, and 0.3% imidazole in 0.1% mol/L sodium phosphate buffer for 15 min. The sections were mounted on 0.5% gelatin-coated glass slides, serially dehydrated, and cover-slipped.

Quantitative analysis of the axonal damage

After completion of the APP immunocytochemical procedures, the slides were transferred to an Eclipse 800 microscope (Nikon, Tokyo, Japan) interfaced with a computer-assisted imaging system DP Controller, Version 3.2 (Olympus Corporation, Tokyo, Japan). Consistent regions of the medulla at the medullospinal junction were enlarged to a magnification of 10× and saved as a tagged image file format (TIFF). Based on our previous experience (Gao et al., 2010), the image was viewed on a monitor using image analysis software IPLab, version 3.7 (BD Biosciences Bioimaging, Rockville, MD) and changed to grey scale. The APP-immunoreactive axonal profiles were outlined and overlaid with cyan color, a strategy used to suppress background immunoreactivity. The sampling area in the medullospinal junction was delineated by a rectangle measuring 500×200 μm that was superimposed over the specified region. The number of damaged APP-immunoreactive axonal profiles within these rectangles that exceeded 20 μm^2 was then counted. This number was expressed as the density of damaged axons per unit area. For the corticospinal tract, eight alternate serial sections from the same tissue block were analyzed in this fashion, together with the use of investigator blinding.

Statistical analysis

Statistical analysis was performed using the statistical software PASW Statistics 17.0 (SPSS Inc., Chicago, IL). All data were presented as mean ± standard error of the mean (SEM). The physiological parameters, laboratory data, and vascular reactivity to ACh, which were normally distributed, were analyzed by one-way analysis of variance (ANOVA). When a significant difference was found, multiple comparisons among time points in the same group and groups at each time point were performed using the Bonferroni correction. The number of damaged axons, which was not normally distributed, was analyzed by the Kruskal-Wallis test, followed by multiple comparisons with the Bonferroni correction. A value of $p<0.05$ was considered to be statistically significant.

Results

General physiological observations

There were no significant differences in baseline body weight and hematocrit among all groups. The rectal temperature in the normothermic groups (groups 1, 2, 2′, 3, and 3′) was maintained at 37°C over the entire experimental period (Fig. 2A and C). The temporalis muscle temperature was approximately 0.5–1.0°C lower than the rectal temperature (Fig. 2B and D). In the hypothermic groups (groups 4 and 4′), the rats were cooled to the target rectal temperature of 33°C in 12.2 ± 0.5 and 12.6 ± 0.5 min, respectively (Fig. 2A and C). The rats were maintained at this level for 60 min, and then they were rewarmed to a normothermic level over a span of 90 min. The rewarming rates in groups 4 and 4′ were 32.8 ± 0.8 and 30.1 ± 1.4 min/°C, respectively. Tables 1 and 2 show the time course measurements of mean arterial blood pressure and blood gas analyses in the Tempol series and the FK506 series, respectively. The mean values of Pao_2 during the hypothermic period (2 h post-injury) in groups 4 and 4′ were significantly higher than those in groups 1, 2, and 3 and those in groups 1, 2′, and 3′, respectively (Table 1 and 2). Other than these physiological changes, all the other physiological variables reported in Table 1 and 2 were within normal physiological limits.

Brain arteriolar reactivity after impact acceleration injury in the Tempol series

The vascular reactivity to ACh at 10^{-7} M and at 10^{-5} M in the Tempol series is shown in Figure 3A and 3B, respectively. There was no significant difference in the vascular reactivity to ACh at 10^{-7} M and at 10^{-5} M at pre-injury among all groups. In group 2, which received delayed treatment with Tempol alone at 30 min post-injury, the vascular reactivity to ACh at 10^{-7} M and at 10^{-5} M revealed partial protection, which was significantly higher than that seen in group 1 at 4 and 6 h post-injury at 10^{-7} M of ACh, and at 4, 5, and 6 h post-injury at 10^{-5} M of ACh (10^{-7} M ACh, 5.7 ± 1.1, 6.2 ± 1.4, and 5.3 ± 0.8% at 4, 5, and 6 h post-injury, respectively; 10^{-5} M ACh, 10.4 ± 1.7, 10.1 ± 1.7, and 11.0 ± 1.1% at 4, 5, and 6 h post-injury). In group 3, which received Tempol alone at 90 min post-injury, there was no vascular protective effect in terms of the ACh vascular reactivity. The vascular reactivity of group 3 was significantly lower than that of groups 2 and 4 (10^{-7} M ACh, 1.0 ± 0.4, 1.2 ± 0.5, and 0.7 ± 0.4% at 4, 5, 6 h post-injury; 10^{-5} M ACh, 1.9 ± 1.1, 2.6 ± 1.0, and 2.0 ± 0.6% at 4, 5, and 6 h post-injury). However, in group 4, which coupled Tempol at 90 min post-injury with the delayed hypothermia, the vascular reactivity to ACh at 10^{-7} M and at 10^{-5} M revealed

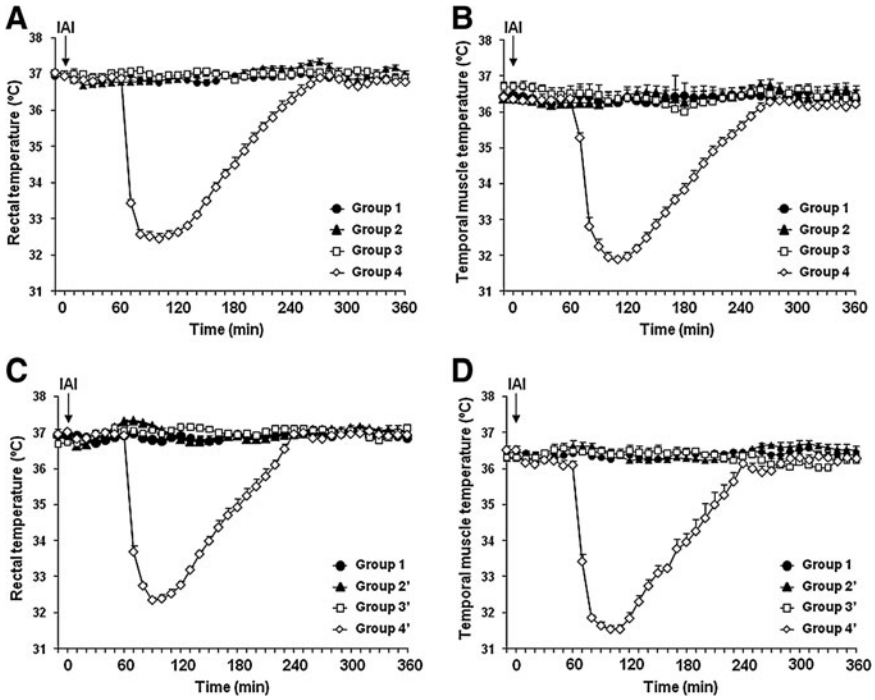

FIG. 2. This graph shows the changes of the mean rectal temperature and mean temporalis muscle temperature throughout the duration of this study in the Tempol series (**A** and **B**, respectively), and FK506 series (**C** and **D**, respectively). The data points represent 5-min intervals. Values are expressed as the mean ± standard error of the mean (IAI, impact acceleration injury).

virtually full protection (10^{-7} M ACh, 7.5 ± 1.0, 6.3 ± 1.5, and 7.6 ± 1.0% at 4, 5, and 6 h post-injury; 10^{-5} M ACh, 16.5 ± 1.0, 15.6 ± 1.0, and 15.4 ± 0.9% at 4, 5, and 6 h post-injury), improving significantly in comparison to those in group 1 (at 4 and 6 h post-injury at 10^{-7} M of ACh, and at 4, 5, and 6 h post-injury at 10^{-5} M of ACh), and group 3 (at 4, 5, and 6 h post-injury at 10^{-7} M and 10^{-5} M of ACh), as well as those in group 2 (at 4 and 6 h post-injury at 10^{-5} M of ACh).

Brain arteriolar reactivity after impact acceleration injury in the FK506 series

The vascular reactivity to ACh at 10^{-7} M and at 10^{-5} M in the FK506 series is shown in Figure 4A and B, respectively. No significant difference in the vascular reactivity to 10^{-7} M and 10^{-5} M ACh was found pre-injury among all groups. In group 1, which included animals subjected to IAI with no treatment,

Table 1. Physiological Parameters in the Tempol Series

		Measurement period						
Variable	*Group*	*Pre-injury*	*1 h*	*2 h*	*3 h*	*4 h*	*5 h*	*6 h*
MAP (mm Hg)	1	109 ± 2	102 ± 4	106 ± 2	108 ± 4	103 ± 4	104 ± 2	102 ± 4
	2	103 ± 4	96 ± 1	98 ± 2	99 ± 3	99 ± 3	96 ± 2	97 ± 2
	3	108 ± 5	106 ± 2	101 ± 3	103 ± 3	107 ± 3	105 ± 5	107 ± 3
	4	107 ± 3	103 ± 1	100 ± 1	101 ± 2	97 ± 3	97 ± 2	101 ± 2
pH	1	7.42 ± 0.01	7.40 ± 0.01	7.40 ± 0.01	7.39 ± 0.01	7.40 ± 0.01	7.38 ± 0.01	7.38 ± 0.01
	2	7.40 ± 0.01	7.41 ± 0.01	7.41 ± 0.01	7.41 ± 0.01	7.41 ± 0.01	7.40 ± 0.02	7.41 ± 0.01
	3	7.41 ± 0.01	7.40 ± 0.01	7.40 ± 0.01	7.38 ± 0.01	7.39 ± 0.01	7.40 ± 0.01	7.40 ± 0.01
	4	7.39 ± 0.01	7.41 ± 0.01	7.39 ± 0.01	7.39 ± 0.01	7.39 ± 0.01	7.41 ± 0.01	7.40 ± 0.01
Pao_2 (mm Hg)	1	90 ± 1	92 ± 3	90 ± 4	91 ± 3	91 ± 1	90 ± 3	90 ± 3
	2	85 ± 4	85 ± 4	85 ± 3	88 ± 7	88 ± 7	90 ± 4	86 ± 5
	3	94 ± 5	90 ± 5	88 ± 4	88 ± 3	87 ± 1	88 ± 2	92 ± 1
	4	83 ± 6	85 ± 4	106 ± 3[a]	78 ± 3	78 ± 3	87 ± 5	86 ± 3
$Paco_2$ (mm Hg)	1	37 ± 1	36 ± 1	36 ± 1	38 ± 1	38 ± 1	38 ± 1	37 ± 1
	2	39 ± 1	36 ± 1	36 ± 1	36 ± 1	36 ± 1	36 ± 1	36 ± 1
	3	37 ± 1	37 ± 1	38 ± 1	38 ± 1	38 ± 1	37 ± 1	36 ± 1
	4	39 ± 1	38 ± 1	38 ± 1	37 ± 1	37 ± 1	36 ± 1	36 ± 1

[a]Significant difference compared with the values of groups 1, 2, and 3 at same measurement point ($p < 0.05$).
Values are expressed as the mean ± standard error of the mean (SEM).
MAP, mean arterial blood pressure; Pao_2, arterial oxygen tension; $Paco_2$, arterial carbon dioxide pressure.

Table 2. Physiological Parameters in the FK506 Series

		Measurement period						
Variable	*Group*	*Pre-injury*	*1 h*	*2 h*	*3 h*	*4 h*	*5 h*	*6 h*
MAP (mm Hg)	1	109 ± 2	102 ± 4	106 ± 2	108 ± 4	103 ± 4	104 ± 2	102 ± 4
	2′	108 ± 3	107 ± 2	105 ± 2	105 ± 2	107 ± 1	105 ± 1	104 ± 2
	3′	101 ± 6	98 ± 5	100 ± 4	105 ± 3	106 ± 4	105 ± 5	96 ± 5
	4′	103 ± 2	101 ± 2	101 ± 3	106 ± 1	99 ± 2	97 ± 3	97 ± 3
pH	1	7.42 ± 0.01	7.40 ± 0.01	7.40 ± 0.01	7.39 ± 0.01	7.40 ± 0.01	7.38 ± 0.01	7.38 ± 0.01
	2′	7.40 ± 0.01	7.39 ± 0.01	7.40 ± 0.01	7.40 ± 0.01	7.40 ± 0.01	7.40 ± 0.01	7.40 ± 0.01
	3′	7.41 ± 0.01	7.40 ± 0.02	7.37 ± 0.02	7.41 ± 0.01	7.39 ± 0.02	7.38 ± 0.02	7.37 ± 0.02
	4′	7.38 ± 0.01	7.40 ± 0.01	7.36 ± 0.01	7.37 ± 0.01	7.38 ± 0.01	7.38 ± 0.01	7.39 ± 0.02
Pao_2 (mm Hg)	1	90 ± 1	92 ± 3	90 ± 4	91 ± 3	91 ± 1	90 ± 3	90 ± 3
	2′	87 ± 2	85 ± 2	83 ± 2	87 ± 4	87 ± 4	92 ± 3	92 ± 2
	3′	92 ± 4	89 ± 7	90 ± 2	91 ± 1	91 ± 1	86 ± 4	89 ± 2
	4′	88 ± 3	85 ± 3	115 ± 9[a]	97 ± 3	93 ± 6	94 ± 2	88 ± 2
$Paco_2$ (mm Hg)	1	37 ± 1	36 ± 1	36 ± 1	38 ± 1	38 ± 1	38 ± 1	37 ± 1
	2′	38 ± 2	37 ± 1	36 ± 1	36 ± 1	36 ± 1	36 ± 1	36 ± 1
	3′	38 ± 1	38 ± 1	36 ± 1	37 ± 1	38 ± 1	38 ± 1	38 ± 1
	4′	39 ± 1	37 ± 1	36 ± 1	38 ± 1	36 ± 1	36 ± 1	38 ± 1

[a]Significant difference compared with the values of groups 1, 2′, and 3′ at the same measurement point ($p < 0.05$).
Values are expressed as the mean ± standard error of the mean (SEM).
MAP, mean arterial blood pressure; Pao_2, arterial oxygen tension; $Paco_2$, arterial carbon dioxide pressure.

the pre-injury vasodilator response to ACh at 10^{-7} M and 10^{-5} M mol/L elicited increases of 7.6% ± 0.7% and 19.2 ± 1.0% in vessel diameter, respectively. The vascular reactivity at 4, 5, and 6 h after injury was significantly decreased compared with the values observed before injury (10^{-7} M ACh, 1.4 ± 0.6, 2.7 ± 0.6, and 0.7 ± 0.4% at 4, 5, and 6 h post-injury; 10^{-5} M ACh, 2.3 ± 0.9, 3.6 ± 0.9, and 0.8 ± 0.4% at 4, 5, and 6 h post-injury). In group 2′, which received delayed treatment of FK506 alone at 30 min post-injury, the vascular reactivity to ACh at 10^{-7} M and at 10^{-5} M revealed almost full protection, and this was significantly higher than that found in group 1 at 4, 5, and 6 h post-injury (10^{-7} M ACh, 5.0 ± 1.0, 6.5 ± 1.0, and 9.3 ± 1.7% at 4, 5, and 6 h post-injury; 10^{-5} M ACh, 14.2 ± 1.3, 14.7 ± 1.4, and 17.2 ± 1.9% at 4, 5, and 6 h post-injury). In group 3′, which received delayed treatment of FK506 alone at 90 min post-injury, the vascular reactivity was significantly higher than that seen in group 1 in ACh at 10^{-7} M at 6 h post-injury and ACh at 10^{-5} M at 4, 5, and 6 h post-injury (10^{-7} M ACh, 3.7 ± 1.0, 5.5 ± 1.0, and 5.3 ± 0.9% at 4, 5, and 6 h post-injury; 10^{-5} M ACh, 11.1 ± 1.6, 12.1 ± 1.4, and 10.7 ± 1.2% at 4, 5, and 6 h post-injury), although this group 3′ protective effect on the vascular reactivity was reduced in comparison to that seen in group 2′. However, in group 4′, which received FK506 at 90 min post-injury with delayed hypothermia, the vascular

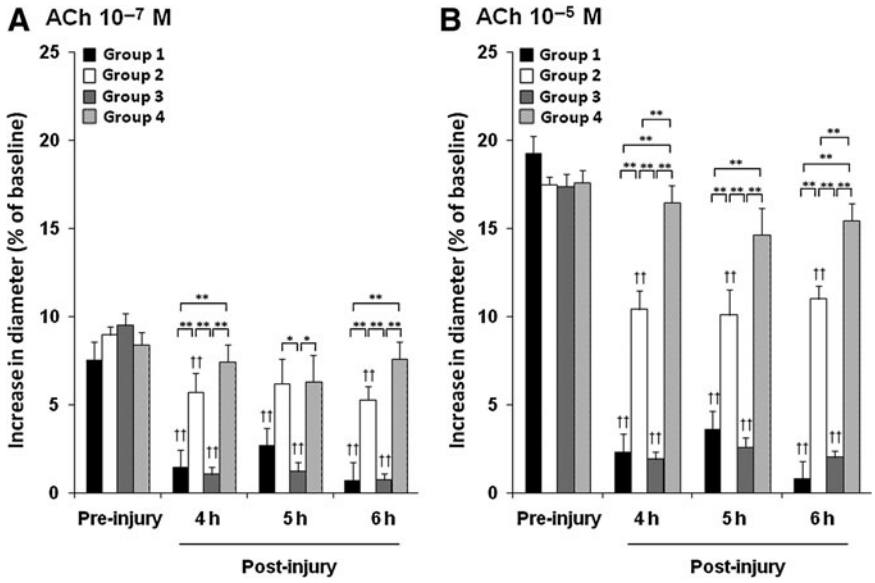

FIG. 3. This bar graph shows the vascular reactivity to acetylcholine (ACh) at 10^{-7} M (**A**) and at 10^{-5} M (**B**) in the Tempol series. Values are expressed as the mean ± standard error of the mean (*$p < 0.05$; **$p < 0.01$; $^{\dagger\dagger}p < 0.01$ compared with the corresponding value at pre-injury in each group).

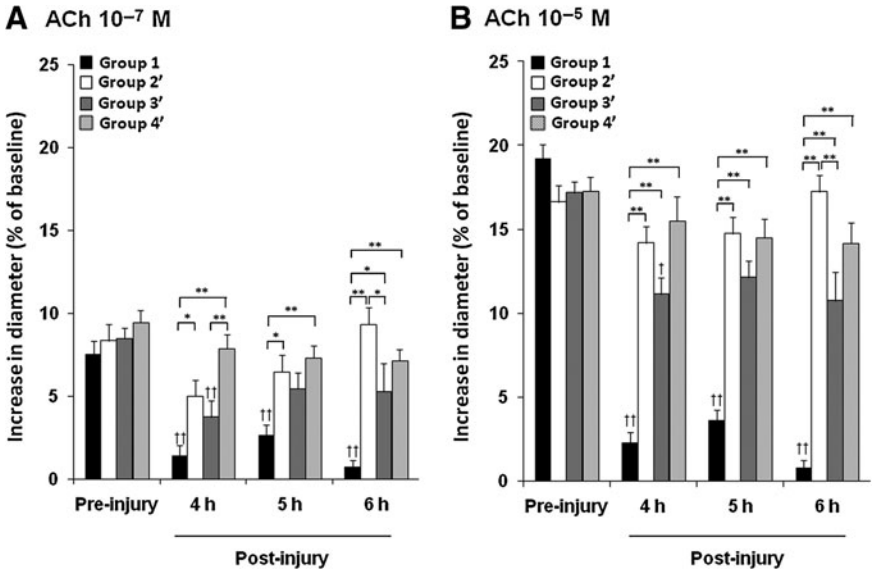

FIG. 4. This bar graph shows the vascular reactivity to acetylcholine (ACh) at 10^{-7} M (**A**) and at 10^{-5} M (**B**) in the FK506 series. Values are expressed as the mean ± standard error of the mean (*$p < 0.05$; **p 0.01; $^{\dagger}p < 0.05$ compared with the corresponding value at pre-injury in each group; $^{\dagger\dagger}p < 0.01$ compared with the corresponding value at pre-injury in each group).

reactivity was improved similarly to those in group 2′, and was significantly higher than those in group 1 at 4, 5, and 6 h post-injury (10^{-7} M ACh, 7.9 ± 0.9, 7.3 ± 0.9, and 7.2 ± 0.6% at 4, 5, and 6 h post-injury; 10^{-5} M ACh, 15.5 ± 1.5, 14.5 ± 1.1, and 14.1 ± 1.2% at 4, 5, and 6 h post-injury).

Axonal injury in the Tempol series

By the quantitative analyses, the numbers of damaged axons in group 1 that had sustained IAI were striking and equaled 285 ± 20 per mm^2 (Fig. 5). The numbers of damaged axons in group 2, which received delayed treatment of Tempol alone at 30 min post-injury, were significantly lower than those in group 1 (29 ± 5 axons/mm^2), with a reduction of approximately 90% (Fig. 5). In groups 3 and 4, which received delayed treatment of Tempol at 90 min post-injury with or without the delayed hypothermia, the numbers of damaged axons were significantly lower than those in group 1 (133 ± 21 and 93 ± 23 axons/mm^2, respectively), but were significantly higher than those in group 2 (Fig. 5). Additionally, there was no statistically significant difference between groups 3 and 4 (Fig. 5).

Axonal injury in the FK506 series

The number of damaged axons in group 2′, which received delayed treatment with FK506 alone at 30 min post-injury, was significantly lower than those in group 1 (61 ± 13 axons/mm^2), with a reduction of approximately 80% (Fig. 6). In groups 3′ and 4′, which received FK506 at 90 min post-injury with or without the delayed hypothermia, the number of damaged axons was significantly lower than those in group 1 (94 ± 13 and 102 ± 16 axons/mm^2, respectively; Fig. 6). However, there was no significant difference between groups 3′ and 4′, nor was a significant difference found between group 2′ or either groups 3′ or 4′.

Discussion

The results of these studies show a relatively complex response achieved through the use of early and delayed post-traumatic Tempol and FK506 administration, either used in isolation or in combination with delayed post-traumatic hypothermia. Following IAI, early, but not delayed, Tempol administration provided significant vascular protection, with the caveat that when the delayed Tempol administration was coupled with delayed hypothermia its vascular protection was restored. In contrast, both the early and delayed administration of FK506 provided significant vascular protection, although this protection was not augmented when coupled to hypothermia. In terms of the burden of axonal damage associated with IAI, the use of early and delayed Tempol administration provided dramatic axonal protection, although

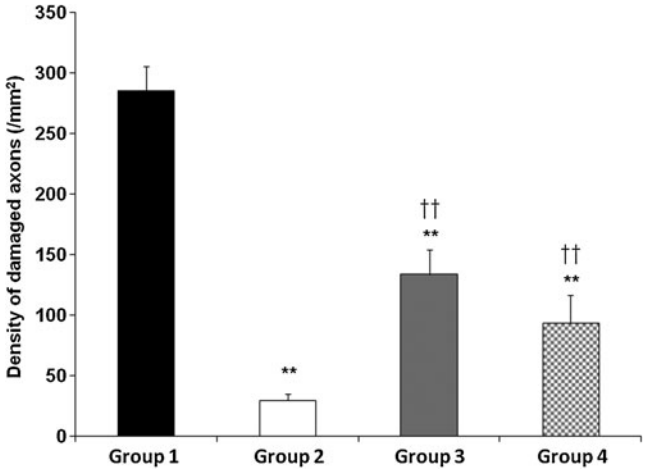

FIG. 5. Bar graph shows a comparison of the mean density of amyloid precursor protein (APP)-immunoreactive damaged axons in the corticospinal tract in the Tempol series. Values represent the means ± standard error of the mean. Statistical differences were analyzed by the Kruskal-Wallis test followed by the Bonferroni test for multiple comparisons (**$p < 0.01$ compared to the value in group 1; $^{\dagger\dagger}p < 0.01$ compared to the value in group 2).

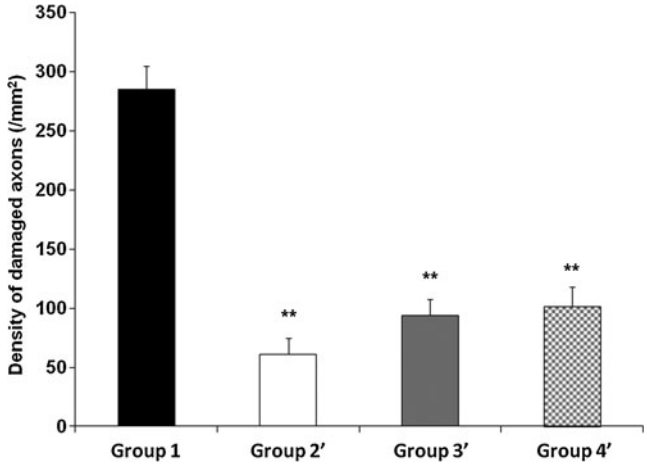

FIG. 6. Bar graph shows a comparison of the mean density of amyloid precursor protein (APP)-immunoreactive damaged axons in the corticospinal tract in the FK506 series. Values represent the means ± standard error of the mean. Statistical differences were analyzed by the Kruskal-Wallis test followed by the Bonferroni test for multiple comparisons (** $p < 0.01$ compared to the value in group 1).

this protection was not enhanced through the use of delayed hypothermia. Similarly, early and delayed FK506 provided significant axonal protection, although once again, this protection was not enhanced by the use of delayed hypothermia. From this perspective, these studies extend our previous reports that support the primary benefits of various reactive oxygen scavengers and the immunophilin ligands. Additionally, these studies support our previous finding that the delayed post-traumatic coupling of hypothermic intervention with the use of oxygen-radical scavengers such as superoxide dismutase provides dramatically enhanced vascular protection (Baranova et al., 2008). Equally important is the fact that the current findings also vary from our previous observations regarding the use of FK506 and its augmentation via hypothermia in rodents subjected to lateral fluid percussion injury. Such differences in the injured brain's biological response may reflect differences in the animal models used (lateral fluid percussion versus impact acceleration injury) and/or structural differences in the brain regions assessed, issues addressed further below.

In terms of the observed microvascular responses to injury, our observation that IAI significantly reduced vascular reactivity to varying concentrations of ACh is consistent with previous studies emerging from our lab (Suehiro et al., 2003; Ueda et al., 2003; Wei et al., 2009). In the present study, the administration of Tempol alone at 30 min post-injury significantly improved and preserved vascular reactivity following IAI, although the administration of Tempol at 90 min post-injury had very limited effects on vascular reactivity. These observations are consistent with the general findings of Hall and colleagues, who have demonstrated that Tempol's overall protective effects diminish rapidly when applied post-injury (Deng-Bryant et al., 2008; Hall et al., 2010). In TBI pathophysiology, it is well known that the production of various reactive oxygen species, including superoxide anion radical, hydroxyl radical, and peroxynitrite, can exacerbate brain damage (Hall et al., 2010; Povlishock, 2000). We have previously reported that superoxide anion radical generation increased in the cerebral microvessels immediately post-TBI and induced vascular damage associated with compromise of vascular function (Kontos and Hess, 1983; Kontos and Povlishock, 1986; Kontos and Wei, 1986). Peroxynitrite, derived from the superoxide anion radical and nitric oxide, has also been reported to cause mitochondrial dysfunction, calcium-mediated calpain overactivation, and neurodegeneration following TBI (Deng-Bryant et al., 2008). Tempol is recognized to scavenge both superoxide anion radical and the peroxynitrite-derived free radicals nitrogen dioxide and carbonate (Carroll et al., 2000; Krishna et al., 1996). Although it is known that free radical production persists several hours after injury, the major portion of this production occurs as an early event that peaks in the first 60 min after injury (Hall et al., 2010), which is consistent with our results of the current study, as well as our previous study, which revealed no vascular protective effects of SOD administration 90 min post-IAI (Baranova et al., 2008).

The combination of delayed hypothermia with Tempol 90 min post-injury was more protective in terms of the pial vascular response to ACh in IAI rats in comparison to the use of Tempol alone at 90 min post-injury. The synergistic effects of the hypothermia combined with Tempol at 90 min post-injury were comparable to the results of our previous studies demonstrating the vascular protective effects of hypothermia combined with SOD or FK506 (Baranova et al., 2008; Oda et al., 2011). It has been previously reported that moderate hypothermia itself can suppresses superoxide anion radical generation and endothelial injury following forebrain ischemia-reperfusion in rats (Koda et al., 2010). Globus and colleagues (1995) have reported that post-traumatic hypothermia suppressed glutamate release and free radical production in lateral fluid percussion injury (LFPI) rats. Additionally, the use of hypothermia could reduce overall brain metabolism while also reducing drug clearance (Tortorici et al., 2007). Accordingly, the combination of hypothermia might enhance the protective effects of Tempol when the administration is delayed.

Tempol administration both 30 and 90 min post-injury also suppressed the axonal damage in the brainstem. The 30 min post-injury administration of Tempol revealed more protective effects than 90 min post-injury Tempol administration, a finding again consistent with the observations of Hall and colleagues. Tempol administration has been reported to reduce brain damage in rats following focal cerebral ischemia and acute subdural hematoma (Kato et al., 2003; Kwon et al., 2003; Rak et al., 2000). Additionally, while not conducting direct axonal counts, Deng-Bryant and colleagues (2008) have also reported comparable axonal protection in mice following traumatic brain injury. Specifically, they demonstrated that Tempol reduced mitochondrial dysfunction, calpain-mediated cytoskeletal degradation, and neurodegeneration, all of which relate to traumatic axonal damage (Büki and Povlishock, 2006; Deng-Bryant et al., 2008; Deng et al., 2007). These data coupled to our current findings indicate not only the utility of Tempol, but also indirectly support the involvement of the superoxide anion and/or peroxynitrite-derived free radicals in the progressive pathology associated with diffuse axonal injury. Of additional importance was our finding that the combination of hypothermia with Tempol revealed a trend for a further suppression of the burden of axonal damage in comparison to Tempol 90 min post-injury

alone. This premise is consistent with the findings of Behringer and associates (2002), who reported that Tempol enhanced hypothermic cerebral preservation during prolonged cardiac arrest in dogs. Thus, the combination of delayed Tempol with hypothermia may be a desirable neuroprotective strategy that obviously merits continued evaluation.

In the current investigation, the administration of FK506 not only at 30 min post-injury, but also at 90 min post-injury, significantly preserved vascular reactivity following IAI. Such a finding is consistent with our previous report that FK506, via its inhibition of the protein phosphatase, calcineurin, can influence endothelial and smooth muscle responsiveness, and therefore explain some of the observed vascular protection (Oda et al., 2011). However, unlike our previous investigations wherein the delayed administration of FK506 achieved significant vascular protection only when combined with delayed hypothermia, the current study showed significant vascular protection when administered at 30 and 90 min post TBI, with little augmentation achieved through the concomitant use of delayed hypothermia. While at first blush such differences appear totally inconsistent, it is instructive to note that in contrast to the current vascular studies conducted following IAI, our previously published works employed assessments of pial vessels directly underlying the injury pulse induced by LFPI (Oda et al., 2011). In our previous studies, we recognized the devastating vascular consequences of LFPI, suggesting that the severity of the injury blunted any potential FK506-mediated protection, with only the addition of hypothermia providing significant vascular protection. In contrast, in the current study employing IAI, it is possible that the less severely injured vessels would benefit maximally from primary FK506 treatment, with the caveat that the obtained vascular protection was so significant as to preclude further improvement with hypothermia. These divergent pial vascular responses seen in two different models of injury—LFPI versus IAI—illustrate that differences in animal modeling and/or injury severity can modify the brain's vascular response to injury, a point that merits continued investigation. Further, these findings suggest that the efficacy of various therapeutic interventions cannot be generalized when discussed in the context of different brain injury models.

Similarly to the above-described divergent vascular responses, we also observed an unanticipated difference in the axonal responses to FK506. In the current study, the use of FK506 alone at 30 and 90 min post-injury provided dramatic axonal protection, which paralleled the axonal protection that we previously reported using the same injury model with a pre-injury drug administration paradigm (Marmarou and Povlishock, 2006; Singleton et al., 2001). In contrast, in rats subjected to LFPI, FK506 alone exerted limited axonal protection in corpus callosum (Oda et al., 2011). Only with the combination of delayed hypothermia and FK506 was significant axonal protection achieved following LFPI (Oda et al., 2011), a finding which stands in stark contrast to our current axonal findings in IAI, for which the combination of delayed hypothermia with FK506 exerted no additional protection. Although the site of axonal injury assessment was different in the current study than that evaluated in our previous report (brainstem versus corpus callosum; Oda et al., 2011), and as such merits further consideration, we believe that the observed differences in the protective effects of FK506 most likely speak to differences in animal modeling and/or model-related injury severity. Theoretically, the less severe injury associated with IAI allows FK506 to exert maximal protection, without the need for hypothermic intervention. Conversely, the more severe LFPI may blunt the initial effectiveness of FK506, with only the subsequent use of hypothermia providing significant benefit. Obviously, these findings constitute important issues that merit continued investigation, with the caveat that additional attention must also be placed on determining those cellular and subcellular mechanisms at work in those axonal responses occurring with the injury and subsequent therapeutic intervention.

Author Disclosure Statement

No competing financial interests exist.

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References

Bales, J.W., Ma, X., Yan, H.Q., Jenkins, L.W., and Dixon, C.E. (2010). Expression of protein phosphatase 2B (calcineurin) subunit A isoforms in rat hippocampus after traumatic brain injury. J. Neurotrauma 27, 109–120.

Baranova, A.I., Wei, E.P., Ueda, Y., Sholley, M.M., Kontos, H.A., and Povlishock, J.T. (2008). Cerebral vascular responsiveness after experimental traumatic brain injury: the beneficial effects of delayed hypothermia combined with superoxide dismutase administration. J. Neurosurg 109, 502–509.

Behringer, W., Safar, P., Kentner, R., Wu, X., Kagan, V.E., Radovsky, A., Clark, R.S., Kochanek, P.M., Subramanian, M., Tyurin, V.A., Tyurina, Y.Y., and Tisherman, S.A. (2002). Antioxidant Tempol enhances hypothermic cerebral preservation during prolonged cardiac arrest in dogs. J. Cereb. Blood Flow Metab. 22, 105–117.

Büki, A., and Povlishock, J.T. (2006). All roads lead to disconnection?—Traumatic axonal injury revisited. Acta Neurochir. 148, 181–194.

Carroll, R.T., Galatsis, P., Borosky, S., Kopec, K.K., Kumar, V., Althaus, J.S., and Hall, E.D. (2000). 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) inhibits peroxynitrite-mediated phenol nitration. Chem. Res. Toxicol. 13, 294–300.

Clifton, G.L., Miller, E.R., Choi, S.C., Levin, H.S., McCauley, S., Smith, K.R. Jr., Muizelaar, J.P., Wagner, F.C. Jr., Marion, D.W., Luerssen, T.G., Chesnut, R.M., and Schwartz, M. (2001). Lack of effect of induction of hypothermia after acute brain injury. N. Engl. J. Med. 344, 556–563.

Deng-Bryant, Y., Singh, I.N., Carrico, K.M., and Hall, E.D. (2008). Neuroprotective effects of Tempol, a catalytic scavenger of peroxynitrite-derived free radicals, in a mouse traumatic brain injury model. J. Cereb Blood Flow Metab. 28, 1114–1126.

Deng, Y., Thompson, B.M., Gao, X., and Hall, E.D. (2007). Temporal relationship of peroxynitrite-induced oxidative damage, calpain-mediated cytoskeletal degradation and neurodegeneration after traumatic brain injury. Exp. Neurol. 205, 154–165.

Ellis, E.F., Wei, E.P., Cockrell, C.S., Choi, S., and Kontos, H.A. (1983). The effect of PGF2 alpha on in vivo cerebral arteriolar diameter in cats and rats. Prostaglandins 26, 917–923.

Gao, G., Oda, Y., Wei, E.P., and Povlishock, J.T. (2010). The adverse pial arteriolar and axonal consequences of traumatic

brain injury complicated by hypoxia and their therapeutic modulation with hypothermia in rat. J. Cereb Blood Flow Metab, 30, 628–637.

Globus, M.Y., Alonso, O., Dietrich, W.D., Busto, R., and Ginsberg, M.D. (1995). Glutamate release and free radical production following brain injury: effects of posttraumatic hypothermia. J. Neurochem. 65, 1704–1711.

Hall, E.D., Vaishnav, R.A., and Mustafa, A.G. (2010). Antioxidant therapies for traumatic brain injury. Neurotherapeutics 7, 51–61.

Jiang, J.Y., Lyeth, B.G., Clifton, G.L., Jenkins, L.W., Hamm, R.J., and Hayes, R.L. (1991). Relationship between body and brain temperature in traumatically brain-injured rodents. J. Neurosurg 74, 492–496.

Kato, N., Yanaka, K., Hyodo, K., Homma, K., Nagase, S., and Nose, T. (2003). Stable nitroxide Tempol ameliorates brain injury by inhibiting lipid peroxidation in a rat model of transient focal cerebral ischemia. Brain Res. 979, 188–193.

Koda, Y., Tsuruta, R., Fujita, M., Miyauchi, T., Kaneda, K., Todani, M., Aoki, T., Shitara, M., Izumi, T., Kasaoka, S., Yuasa, M., and Maekawa, T. (2010). Moderate hypothermia suppresses jugular venous superoxide anion radical, oxidative stress, early inflammation, and endothelial injury in forebrain ischemia/reperfusion rats. Brain Res. 1311, 197–205.

Koizumi, H., and Povlishock, J.T. (1998). Posttraumatic hypothermia in the treatment of axonal damage in an animal model of traumatic axonal injury. J. Neurosurg, 89, 303–309.

Kontos, H.A., and Hess, M.L. (1983). Oxygen radicals and vascular damage. Adv. Exp. Med. Biol. 161, 365–375.

Kontos, H.A., and Povlishock, J.T. (1986). Oxygen radicals in brain injury. Cent. Nerv. Syst. Trauma 3, 257–263.

Kontos, H.A., Wei, E.P., and Marshall, J.J. (1988). In vivo bioassay of endothelium-derived relaxing factor. Am. J. Physiol. 255, 1259–1262.

Kontos, H.A., and Wei, E.P. (1986). Superoxide production in experimental brain injury. J. Neurosurg. 64, 803–807.

Krishna, M.C., Russo, A., Mitchell, J.B., Goldstein, S., Dafni, H., and Samuni, A. (1996). Do nitroxide antioxidants act as scavengers of O_2– or as SOD mimics? J. Biol. Chem. 271, 26026–26031.

Kwon, T.H., Chao, D.L., Malloy, K., Sun, D., Alessandri, B., and Bullock, M.R. (2003). Tempol, a novel stable nitroxide, reduces brain damage and free radical production, after acute subdural hematoma in the rat. J. Neurotrauma 20, 337–345.

Leker, R.R., Teichner, A., Lavie, G., Shohami, E., Lamensdorf, I., and Ovadia, H. (2002). The nitroxide antioxidant Tempol is cerebroprotective against focal cerebral ischemia in spontaneously hypertensive rats. Exp. Neurol. 176, 355–363.

Levasseur, J.E., Wei, E.P., Raper, A.J., Kontos, A.A., and Patterson, J.L. (1975). Detailed description of a cranial window technique for acute and chronic experiments. Stroke 6, 308–317.

Marmarou, A., Foda, M.A., van den Brink, W., Campbell, J., Kita, H., and Demetriadou, K. (1994). A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. J. Neurosurg. 80, 291–300.

Marmarou, C.R., and Povlishock, J.T. (2006). Administration of the immunophilin ligand FK506 differentially attenuates neurofilament compaction and impaired axonal transport in injured axons following diffuse traumatic brain injury. Exp. Neurol. 2, 353–362.

Oda, Y., Gao, G., Wei, E.P., and Povlishock, J.T. (2011). Combinational therapy using hypothermia and the immunophilin ligand FK506 to target altered pial arteriolar reactivity, axonal damage, and blood–brain barrier dysfunction after traumatic brain injury in rat. J. Cereb. Blood Flow Metab. 31, 1143–1154.

Povlishock, J.T. (2000). Pathophysiology of neural injury: therapeutic opportunities and challenges. Clin. Neurosurg. 46, 113–126.

Povlishock, J.T., Marmarou, A., McIntosh, T., Trojanowski, J.Q., and Moroi, J. (1997). Impact acceleration injury in the rat: evidence for focal axolemmal change and related neurofilament sidearm alteration. J. Neuropathol. Exp. Neurol. 56, 347–359.

Rak, R., Chao, D.L., Pluta, R.M., Mitchell, J.B., Oldfield, E.H., and Watson, J.C. (2000). Neuroprotection by the stable nitroxide Tempol during reperfusion in a rat model of transient focal ischemia. J. Neurosurg. 92, 646–651.

Reeves, T.M., Phillips, L.L., Lee, N.N., and Povlishock, J.T. (2007). Preferential neuroprotective effect of tacrolimus (FK506) on unmyelinated axons following traumatic brain injury. Brain Res. 1154, 225–236.

Singleton, R.H., Stone, J.R., Okonkwo, D.O., Pellicane, A.J., and Povlishock, J.T. (2001). The immunophilin ligand FK506 attenuates axonal injury in an impact-acceleration model of traumatic brain injury. J. Neurotrauma 18, 607–614.

Stone, J.R., Singleton, R.H., and Povlishock, J.T. (2000). Antibodies to the C-terminus of the beta-amyloid precursor protein (APP): a site specific marker for the detection of traumatic axonal injury. Brain Res. 871, 288–302.

Stone, J.R., Walker, S.A., and Povlishock, J.T. (1999). The visualization of a new class of traumatically injured axons through the use of a modified method of microwave antigen retrieval. Acta Neuropathol. 97, 335–345.

Suehiro, E., and Povlishock, J.T. (2001). Exacerbation of traumatically induced axonal injury by rapid posthypothermic rewarming and attenuation of axonal change by cyclosporin A. J. Neurosurg. 94, 493–498.

Suehiro, E., Ueda, Y., Wei, E.P., Kontos, H.A., and Povlishock, J.T. (2003). Posttraumatic hypothermia followed by slow rewarming protects the cerebral microcirculation. J. Neurotrauma 20, 381–390.

Tortorici, M.A., Kochanek, P.M., and Poloyac, S.M. (2007). Effects of hypothermia on drug disposition, metabolism, and response: A focus of hypothermia-mediated alterations on the cytochrome P450 enzyme system. Crit. Care Med. 35, 2196–2204.

Ueda, Y., Wei, E.P., Kontos, H.A., Suehiro, E., and Povlishock, J.T. (2003). Effects of delayed, prolonged hypothermia on the pial vascular response after traumatic brain injury in rats. J. Neurosurg. 99, 899–906.

Wei, E.P., Hamm, R.J., Baranova, A.I., and Povlishock, J.T. (2009). The long-term microvascular and behavioral consequences of experimental traumatic brain injury after hypothermic intervention. J. Neurotrauma 26, 527–537.

Wei, E.P., Kukreja, R., and Kontos, H.A. (1992). Effects in cats of inhibition of nitric oxide synthesis on cerebral vasodilation and endothelium-derived relaxing factor from acetylcholine. Stroke 23, 1623–1628.

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