Post-Stroke Hypothermia Provides Neuroprotection through Inhibition of AMP-Activated Protein Kinase

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Abstract

Hypothermia is robustly protective in pre-clinical models of both global and focal ischemia, as well as in patients after cardiac arrest. Although the mechanism for hypothermic neuroprotection remains unknown, reducing metabolic drive may play a role. Capitalizing on the beneficial effects of hypothermia while avoiding detrimental effects such as infection will be the key to moving this therapy forward as a treatment for stroke. AMPK is a master energy sensor that monitors levels of key energy metabolites. AMPK is activated via phosphorylation (pAMPK) when cellular energy levels are low, such as that seen during ischemia. AMPK activation appears to be detrimental in experimental stroke, likely via exacerbating ischemia-induced metabolic failure. We tested the hypothesis that hypothermia reduces AMPK activation. First, it was found that hypothermia reduced infarct after middle cerebral artery occlusion. Second, induced hypothermia reduced brain pAMPK in both sham control and stroke mice. Third, hypothermic neuroprotection was ameliorated after administration of compound C, an AMPK inhibitor. Finally, deletion of one of the catalytic isoforms of AMPK completely reversed the effect of hypothermia on stroke outcome after both acute and chronic survival. These effects were mediated by a reduction in AMPK activation rather than a reduction in LKB1, an upstream AMPK kinase. In summary, these studies provide evidence that hypothermia exerts its protective effect in part by inhibiting AMPK activation in experimental focal stroke. This suggests that AMPK represents a potentially important biological target for stroke treatment.

Key words: AMPK; hypothermia; middle cerebral artery occlusion; stroke

Introduction

HYPOTHERMIA IS ROBUSTLY PROTECTIVE in preclinical models of both global and focal ischemia (Zhao et al., 2007). Recent clinical trials have shown that hypothermia is also effective in reducing neuronal damage in patients after cardiac arrest (Gupta et al., 2005; Zhao et al., 2007). However, translation of the therapeutic benefit of hypothermia into patients with focal stroke has been unsuccessful (Lyden et al., 2006). Although induction of hypothermia is feasible, concerns regarding increased infection rates and the complex management of these patients have reduced clinician enthusiasm for this approach (Hemmen et al., 2010). However, the beneficial effects of hypothermia after cardiac arrest and in pre-clinical studies imply that the mechanisms underlying the therapeutic benefit of hypothermia are efficacious (Lampe and Becker, 2010). Despite intensive study, the mechanisms whereby hypothermia produces neuroprotection remain to be elucidated (Erecinska et al., 2003).

AMPK is a master energy sensor that monitors levels of key energy metabolites such as ATP/AMP, and it is activated via phosphorylation when cellular energy levels are low. Once activated, it turns on catabolic processes such as fatty acid oxidation, and inhibits anabolic pathways including cholesterol synthesis, in an attempt to maintain cellular ATP levels (Ronnelt et al., 2009). AMPK activation appears to be detrimental in experimental stroke, as inhibition of this kinase is protective, while its activation exacerbates injury (McCullough et al., 2005). Subsequent studies identified AMPK α 2, one of the two catalytic AMPK isoforms, as the major contributor to AMPK's deleterious effects (Li et al., 2010b, 2007). Acute administration of the AMPK activator metformin enhanced metabolic dysfunction and acidosis after stroke and increased infarct (Li et al., 2010b), and recent studies have shown that prolonged AMPK activation enhances transcriptional activation of the pro-apoptotic Bcl-2 family member *bim* (Weisova et al., 2011). As AMPK is emerging as an important regulator of metabolism, and may influence energy demand and usage during periods of low metabolic demand (i.e.,

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hibernation), we investigated the hypothesis that reductions in AMPK contribute to the neuroprotective effects of induced hypothermia.

Methods

Focal cerebral ischemia model

The present study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals in research, and under protocols approved by the Center for Laboratory Animal Care at the University of Connecticut Health Center. Focal transient cerebral ischemia was induced in male mice (20–25 g) under isoflurane anesthesia by right middle cerebral artery occlusion (90 min of MCAO), followed by reperfusion as described previously (Li et al., 2007). At the end of ischemia, the animal was briefly re-anesthetized, and reperfusion was initiated by filament withdrawal. Wild-type (WT) mice were purchased from Charles River (Wilmington, MA). AMPK α 2-knockout (KO) mice (C57BL6 background) were obtained from Dr. Benoit Viollet in France and re-derived in house (Li et al., 2007), then compared to WT littermates. Previous studies have demonstrated that physiological parameters (i.e., blood pressure, blood glucose, and blood gases) and cerebral blood flow do not differ between KO and WT mice (Li et al., 2007).

Hypothermia treatment (HT)

Immediately following reperfusion, the mice were placed on a heating pad with homothermic control. A blue ice pack was placed on the side of the mouse to cool the animal to target temperature (32°C), which took less than 5 min. Temperature was then maintained for 6 h before passive re-warming; all animals (HT and normothermic [NT]) remained anesthetized during this period. In control animals body temperatures were maintained at 37°C (NT). We measured rectal temperatures in our experiments, as it correlates strongly with brain temperatures (Tsuchiya et al., 2002), and were maintained at 37°C with a feedback control system after 6 h in both HT and NT animals. The animals were divided into subgroups with final numbers as follows: WT for 24 h outcome $n=7$ per group; WT for protein measurement $n=4$ sham and $n=3$ stroke per group; WT for compound C 24 h outcome $n=8$ per group; AMPK α 2 KO for 24 h outcome $n=7$ per group; long-term survival WT $n=7$ per group; long-term survival AMPK α 2 KO $n=7$ per group.

Compound C treatment

Compound C (EMD Chemicals, Gibbstown, NJ) or vehicle (sterile water) was dissolved in water and injected into the mice (IP) at the onset of stroke at 10 mg/kg (volume: 0.2 mL/20 g). Physiological parameters and cerebral blood flow are not affected by compound C treatment, as demonstrated previously, and temperature was maintained in all groups at 37°C (Li and McCullough, 2010; McCullough et al., 2005).

Behavioral measurements

For 24-h survival experiments, neurological deficits (NDS) were scored in the intra-ischemic period and 24 h post-stroke. The scoring system was as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held

by the tail; 2, circling to the affected side; 3, unable to bear weight on the affected side; and 4, no spontaneous locomotor activity or barrel rolling (Li et al., 2007). For 7-day survival experiments, the corner test was carried out as described in Li and associates (2004). The mouse was placed between two pieces of cardboard. The two boards were gradually moved from both sides to encourage the mouse to enter into a corner along the joint between the two boards. When the vibrissae were stimulated the mouse reared forward and upward, and then turned to face the open end. Twenty trials were performed for each mouse and the percentage of right turns was calculated. Only turns involving full rearing along either board were recorded. All experiments were performed by a blinded investigator.

Infarct analysis

After the animals were scored at 24 h after stroke, the brains were removed and cut into five 2-mm slices and stained with 1.5% 2,3,5-triphenyltetrazolium (TTC) solution. The stained slices were fixed with formalin (4%), then digitized, and infarct volumes were analyzed using computer software (SigmaScan Pro, Systat Software Inc., San Jose, CA), and corrected for edema which was defined as the volume difference of the two hemispheres as previously described (Li et al., 2007).

Western blots

Six hours after the onset of stroke, the mice were sacrificed and the brains were removed for protein analysis. To perform Western blots, the brains were homogenized using lysis buffer, and the resulting supernatant was resolved on a 4–15% gradient SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL). The blots were probed for pAMPK (1:500; Cell Signaling Technology, Inc., Beverly, MA) at threonine 172, as described in Li and McCullough (2010), pLKB1 (1:500; Cell Signaling Technology) at threonine 189, and total AMPK (1:1000; Cell Signaling Technology). β -Actin (1:5000; Sigma-Aldrich, St. Louis, MO) was used as a loading control. The blots were incubated overnight in primary antibody at 4°C in TBS containing 4% bovine serum albumin and 0.1% Tween-20. Secondary antibodies (1:5000; goat anti-rabbit IgG for pAMPK, AMPK, and pLKB1, and 1:5000; goat anti-mouse IgG for β -actin; all from Chemicon International, Temecula, CA) were diluted, and the ECL detection kit (Thermo Fisher Scientific) was used for signal detection.

Statistical analysis

Statistical analysis was performed either with one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test for multiple comparisons when appropriate, or Student's *t*-test, except for neurological deficit scores, which was done using the Mann-Whitney *U* test. A *p* value < 0.05 was considered statistically significant. Data were expressed as mean $\pm$ standard error of the mean (SEM), except for the neurological deficit scores, which were presented as median and range (the difference between the third and first quartiles). Investigators performing behavioral and infarct size analysis were blinded to treatment group.

Results

Post-stroke hypothermia provided neuroprotection after stroke

Hypothermic treatment started after reperfusion significantly reduced cortical (hypothermia $32.4 \pm 3.7\%$ versus control $49.6 \pm 3.8\%$; $p < 0.05$), striatal (hypothermia $57.1 \pm 3.7\%$ versus $72.6 \pm 3.4\%$ control; $p < 0.05$), and total (hypothermia $30.0 \pm 1.7\%$ versus control $48.7 \pm 3.2\%$; $p < 0.05$, $n = 7$ per group) infarct volume when compared to controls (Figs. 1 and 2). The neuroprotection was reflected by the improvement of neurological function as measured by NDS (hypothermia 2.0 (0) versus control 3.0 (0.5); $p < 0.05$, $n = 7$ per group; Table 1). There was no mortality and no animals were excluded.

Hypothermia dramatically reduced pAMPK levels

Hypothermia dramatically reduced pAMPK levels (the active form of AMPK) 6 h after the onset of stroke ($p < 0.05$). This effect was also observed in sham-operated mice (Figs. 3 and 4; $p < 0.05$, $n = 4$ in sham and 3 in stroke per group). No effect was seen on total AMPK. We also examined pLKB1, a major upstream kinase of AMPK. Ischemia led to an increase in pLKB1, as described previously (McCullough et al., 2005), but no differences were seen in pLKB1 levels between the HT and NT groups (Fig. 5).

Hypothermia was ineffective when AMPK was inhibited by compound C

To further test the role of AMPK in hypothermic neuroprotection, we initially utilized a pharmacological approach. Compound C (CC) treatment (10 mg/kg) significantly reduced cortical infarct size (CC $27.6 \pm 5.9\%$ versus vehicle $53.6 \pm 5.4\%$; $p < 0.05$), striatal (CC $39.0 \pm 5.2\%$ versus vehicle $67.9 \pm 85.2\%$; $p < 0.05$), and total (CC $30.2 \pm 4.1\%$ versus vehicle $48.7 \pm 4.7\%$; $p < 0.05$, $n = 8$ per group) infarct size (Fig. 6). Compound C treatment reduced neurological deficits [CC 1.5 (1.0) versus vehicle 3.0 (0.25); $p < 0.05$; Table 1].

FIG. 1. Post-stroke hypothermia reduced infarct volume. Mice were subjected to 90 min middle cerebral artery occlusion (MCAO), and hypothermia (32°C) was initiated at reperfusion and lasted for 6 h. In control animals temperatures were maintained at 37°C. 2,3,5-Triphenyltetrazolium (TTC) staining was done to assess infarcts at 24 h after the onset of MCAO. Cortical, striatal, and total hemisphere infarction volumes were calculated (corrected for edema and given as the percentage of the non-ischemic hemisphere; * $p < 0.05$ versus controls by Student's *t*-test).

Control

Hypothermia

FIG. 2. Representative 2,3,5-triphenyltetrazolium (TTC)-stained brain slices. Representative coronal section through the middle cerebral artery territory after TTC staining of a normothermic (control) or hypothermic animal. Infarcted tissue is white.

When hypothermia was combined with CC, there was no additive effect on cortical (hypothermia+CC $32.4 \pm 4.6\%$ versus CC $27.6 \pm 5.9\%$), striatal (hypothermia+CC $35.2 \pm 3.9\%$ versus CC $39.0 \pm 5.2\%$ control), and total infarct size (hypothermia+CC $29.4 \pm 4.7\%$ versus CC $30.2 \pm 4.1\%$, $n = 7$ in the HT+CC group; Fig. 6). Hypothermia did not further reduce neurological deficits in compound C-treated mice

TABLE 1. NEUROLOGICAL DEFICIT SCORES AFTER STROKE

	Pharmacology			
	Wild-type	Knockout	Compound C	Vehicle (water)
Hypothermia	3.0 (0.5) $n = 7$	2.0 (0.5) $n = 7$	2.0 (1.0) $n = 7$	
Normothermia	2.0 (0)* $n = 7$	2.0 (0) $n = 7$	1.5 (1.0)# $n = 8$	3.0 (0.25) $n = 8$

Mice were subjected to middle cerebral artery occlusion (90 min), and hypothermia (32°C) was initiated at reperfusion and maintained for 6 h. Neurological score was assessed 24 h after stroke. Compound C was injected into wild-type mice at the onset of stroke at 10 mg/kg in water (IP). Data are expressed as median (interquartile range).

* $p < 0.05$ versus hypothermia (WT).

$p < 0.05$ versus vehicle control with normothermia (by Mann-Whitney *U* test).

FIG. 3. Representative blot showing reductions in pAMPK levels with hypothermia. Hypothermia (HT) dramatically reduced pAMPK 6h after the onset of stroke. Brains were collected 6h after onset of middle cerebral artery occlusion (approximately 4.5 h into hypothermia). pAMPK and AMPK levels were assessed with Western blots (NT, normothermia; Sh, sham; St, stroke).

(Table 1). In this set of experiments, there was no mortality, and one mouse was excluded in the HT+CC group due to a lack of intra-ischemic neurological deficits during the 90-min MCAO period.

Hypothermia is ineffective at reducing infarct size in AMPK α 2-knockout mice

To investigate if hypothermia induces neuroprotection by inhibiting AMPK, we tested the effect of hypothermia in AMPK α 2-deficient mice. Our data showed that hypothermia was no longer neuroprotective when AMPK α 2 was absent (hypothermia $32.9 \pm 6.2\%$ versus control $36.6 \pm 6.6\%$), striatal (hypothermia $58.5 \pm 7.1\%$ versus $59.3 \pm 8.3\%$ control), and total infarct size ($33.2 \pm 3.7\%$ versus $36.2 \pm 4.3\%$; $n=7$ per group; Fig. 7). Hypothermia did not change neurological deficits in AMPK α 2-KO mice (Table 1). There was no mortality and no animals were excluded in this experiment.

FIG. 4. Densitometry was performed to quantify pAMPK protein levels. The methods for the Western blots were the same as for Figure 3. One-way analysis of variance with Tukey's *post-hoc* test was utilized to compare means. Data were expressed as mean $\pm$ standard error of the mean; * $p < 0.05$ for HT versus NT animals; $n=4$ sham and $n=3$ stroke per group; NT, normothermia; HT, hypothermia).

Hypothermia leads to sustained neuroprotection with 7-day survival

To test if our cooling protocol could provide sustained neuroprotection, we extended the WT animals' survival time to 7 days. Hypothermia reduced cortical (hypothermia $30.2 \pm 5.7\%$ versus control $52.3 \pm 3.0\%$; $p < 0.05$), striatal (hypothermia $46.7 \pm 7.1\%$ versus $71.4 \pm 4.8\%$ control; $p < 0.05$), and total infarct size (hypothermia $34.6 \pm 7.4\%$ versus $55.8 \pm 2.7\%$ control; $p < 0.05$; Fig. 8). The corner test revealed significant functional recovery induced by hypothermia (hypothermia 0.56 ± 0.029 versus 0.77 ± 0.044 control; $p < 0.05$; $n=6$ control and $n=5$ hypothermia per group; Fig. 9). Mortality was 1 and 2 animals out of 9 in the control and HT groups, respectively, and no mouse was excluded.

AMPK α 2 deletion ablated hypothermic neuroprotection with 7 days of survival

To test if AMPK mediates the sustained protection seen in hypothermia, we examined the effect of hypothermia in AMPK α 2-KO mice at 7 days of survival. Hypothermia lost its effect in KO mice, with no reduction seen in infarct size (cortical: hypothermia $31.2 \pm 3.5\%$ versus control $34.9 \pm 3.4\%$; striatal: hypothermia $47.2 \pm 4.8\%$ versus $44.3 \pm 3.3\%$ control; total: hypothermia $36.5 \pm 4.9\%$ versus $33.0 \pm 3.2\%$ control; Fig. 10). This lack of efficacy was also reflected in the corner test scores (hypothermia 0.59 ± 0.029 versus 0.60 ± 0.034 control; $n=6$ control, $n=5$ hypothermia per group; Fig. 11). Mortality was 1 and 2 animals out of 7 in the control and HT groups, respectively, and no mouse was excluded.

Discussion

We have previously shown that suppressing AMPK activation leads to neuroprotection in a reversible focal experimental stroke model (Li and McCullough, 2010; Li et al., 2010a, 2007). In the present study, we have provided the first evidence that hypothermia exerts its protective effect in part by inhibiting AMPK activation. First, we have confirmed the protective effect of post-stroke hypothermia in the focal mouse stroke model. Second, it was found that this level of hypothermia dramatically reduced pAMPK levels in the brain, an effect seen in both sham controls and stroke mice. Third, the decrease in pAMPK is not simply secondary to the reduced damage induced by hypothermia, as hypothermic neuroprotection was ameliorated with the concomitant administration of an AMPK inhibitor. Treatment with both compound C and hypothermia did not lead to additive protective effects, suggesting that hypothermia and AMPK inhibition target the same neuroprotective pathways. Finally, we mechanistically tied the effect of hypothermia to AMPK with non-pharmacological methods by utilizing AMPK α 2-deficient mice with both acute and chronic survival paradigms, which both showed a loss of hypothermia-induced protection.

AMPK is an energy sensor in the periphery and is activated when the energy supply is low. Somewhat surprisingly, AMPK activation was shown to be deleterious in stroke (Li and McCullough, 2010; Li et al. 2010a, 2007). Stroke represents a state of severe energy deficiency, as there is no available substrate for the energy-producing pathways activated by AMPK. Therefore, activating AMPK in the setting of severe

FIG. 5. Representative blot showing no reduction in pLKB1 levels with hypothermia. Hypothermia (HT) did not reduce pLKB1 6 h after the onset of stroke. Brains were collected 6 h after the onset of middle cerebral artery occlusion (approximately 4.5 h into hypothermia). pLKB1 levels were assessed with Western blots ($n=4$ sham and $n=3$ stroke per group; NT, normothermia).

metabolic failure exacerbates injury. The mechanisms through which acute AMPK activation exacerbates stroke injury remain unclear. Studies have suggested that exacerbated lactate accumulation, autophagy, and increased glucose due to unregulated glucose transporters in the reperfusion phase may contribute to stroke damage (Li and McCullough, 2010). Recent work has suggested that the duration of ischemia may be an important factor contributing to the downstream effects of AMPK activation (Weisova et al., 2010), with milder insults providing a preconditioning stimulus, leading to a reduction in damage.

Hypothermia is one of the most potent neuroprotective strategies for ischemic brain injury, and its effect has been replicated in many different studies. It is one of the few therapies that has been translated into a clinically effective therapy for global ischemia (van der Worp et al., 2007). However, moving this protection into a focal stroke model has been a significant challenge. Multiple mechanistic pathways by which post-stroke hypothermia induces neuroprotection have been proposed, including inhibition of apoptotic cell death (via caspase inhibition), necrosis (via calpain inhibition), reductions in inflammation, and en-

hanced maintenance of blood–brain barrier integrity (Zhao et al., 2007).

Hypothermia also appears to have major effects on energy metabolism. Post-ischemic restoration of PCr and ATP levels in the brain occurred sooner and more completely in gerbils and in piglets that underwent hypothermia compared to normothermic controls (Chopp et al., 1989; Kimura et al., 2002). However, the absolute cellular level of ATP is extremely low during ischemia, and is lower than baseline during early reperfusion (Kimura et al., 2002), which is the likely stimulus for the phosphorylation of AMPK. Hypothermia appears to lead to a profound reduction in both baseline and stroke-induced pAMPK, with a subsequent reduction in energy demand, leading to less metabolic stress on ischemic tissue. Studies have shown that post-stroke hypothermia can improve mitochondrial function compared to normothermia (Canevari et al., 1999), and therefore the ischemic brain is in a better position to make ATP when blood flow is restored, as occurs with reperfusion. The fact that pre-stroke hypothermia did not alter ATP levels during ischemia, but restored post-ischemic ATP levels (Chopp et al., 1989; Kimura et al., 2002) supports this notion. It is

FIG. 6. Loss of hypothermic neuroprotection in mice treated with the AMPK inhibitor compound C. Mice were subjected to 90 min of middle cerebral artery occlusion (MCAO), and hypothermia (32°C) was initiated at reperfusion and lasted for 6 h. In the control animals temperatures were maintained at 37°C. 2,3,5-Triphenyltetrazolium staining was done to assess infarcts at 24 h after the onset of MCAO. Cortical, striatal, and total hemisphere infarction volumes were calculated (corrected for edema and shown as the percentage of the non-ischemic hemisphere; $n=8$ NT vehicle, $n=8$ NT compound C, $n=7$ HT compound C). Compound C (Comp C) was injected at the onset of stroke at 10 mg/kg (IP), or sterile water (Vehicle) was injected. Data were expressed as mean $\pm$ standard error of the mean ($\#p < 0.05$ versus Vehicle + NT by analysis of variance with Tukey's *post-hoc* test for multiple comparisons). Compound C was neuroprotective compared to vehicle in the normothermic groups. No additional benefit of hypothermia was seen.

FIG. 7. Loss of hypothermic neuroprotection in AMPK α 2-knockout (KO) mice ($n=7$ per group). The methods were the same as in Figures 1 and 5.

likely that after an ischemic insult, maintaining the functional status of mitochondria is critical to initiate metabolism for any remaining salvageable brain tissue. Reduced lactate formation is seen with post-stroke hypothermia in newborn piglets and gerbils (Amess et al., 1997; Chopp et al., 1989), and in the normal healthy rat brain hypothermia leads to a progressive decrease in lactate production (Nilsson et al., 1975). This is also consistent with our findings, as hypothermia decreases AMPK activity in the brain, and AMPK is a strong stimulator of glycolysis, which produces lactate in the ischemic brain. We have previously demonstrated that ischemic activation of AMPK exacerbates stroke injury by enhancing stroke-induced lactic acidosis (Li et al., 2010b). Therefore it is likely that hypothermia leads to neuroprotection, in part by reducing AMPK activation and subsequent lactate production.

The effect of mild to moderate hypothermia on AMPK has not been previously investigated in the brain. In frog liver, deep hypothermia (2–4°C) increased phosphorylation of AMPK (Bartrons et al., 2004). Temperature preconditioning

(26°C) in isolated rat heart enhanced cardiac ischemia-induced AMPK activation; however, a direct change of pAMPK was not seen with hypothermia (Khaliulin et al., 2007). In hibernating squirrels (5–7°C), pAMPK levels in the brain did not differ from normothermic controls (Horman et al., 2005). The tissue type, animal species, and duration and depth of hypothermia may contribute to these differences. Interestingly, administration of the somewhat non-selective AMPK activator AICAR into hibernating marmots led to a significant increase in food intake and failure to enter torpor (Florant et al., 2010), suggesting that AMPK may play a role in the metabolic downregulation seen in hibernating species (Drew et al., 2007). A dramatic reduction in pAMPK occurred in stroke mice after hypothermia in our experimental setting. Interestingly, hypothermia also inhibited pAMPK in sham (non-stroke) mice. Therefore the beneficial effect of hypothermia in stroke is likely secondary to reductions in pAMPK, and is less likely to be merely correlated with the neuroprotection (smaller infarcts). To confirm this we utilized both pharmacological and genetic methods to reduce AMPK acti-

FIG. 8. Post-stroke hypothermia reduced infarcts with 7 days of survival ($n=6$ control and $n=5$ hypothermia per group). Mice were subjected to 90 min of middle cerebral artery occlusion (MCAO), and hypothermia (32°C) was initiated at reperfusion and lasted for 6 h. In control animals temperatures were maintained at 37°C. 2,3,5-Triphenyltetrazolium staining was done to assess infarcts at 7 days after the onset of MCAO. Cortical, striatal, and total hemisphere infarction volumes were calculated (corrected for edema and shown as the percentage of the non-ischemic hemisphere; * $p<0.05$ versus controls by Student's t -test; NT, normothermia; HT, hypothermia).

FIG. 9. Post-stroke hypothermia improved functional outcome with 7 days of survival ($n=6$ control and $n=5$ hypothermia per group). Mice were subjected to 90 min of middle cerebral artery occlusion (MCAO), and hypothermia (32°C) was initiated at reperfusion and lasted for 6 h. In control animals temperatures were maintained at 37°C . Functional outcome was assessed with the corner test 7 days after the onset of MCAO (* $p<0.05$ versus controls by Student's t -test; NT, normothermia; HT, hypothermia).

vation, and in both cases the neuroprotective effect of hypothermia was lost.

There are several limitations to this study, and the data must be interpreted with these in mind. In this study, brains for Western blot analysis were collected when the animals remained hypothermic (at 6 h), and future studies will be needed to assess pAMPK levels after passive re-warming. As pAMPK levels return to baseline 24 h after MCAO, it is likely that these early events are the most important contributors to outcome (McCullough et al., 2005). The mechanism by which hypothermia reduces AMPK activation is unknown, but is likely to involve a direct effect on reducing energy demand, but we did not directly measure ATP levels in this study, as they have been well characterized previously in other models of hypothermia. Furthermore, we used brief cooling (6 h), while in clinical settings more prolonged hypothermia is often used. Mechanisms and efficacies may differ based on the length of cooling. Brief cooling may act on the mechanisms that predominate at the more acute phase of cell death, such as acute energy metabolism and excitotoxicity, while long-term cooling (e.g., 24 h) may have more marked effects on inflammation and apoptosis. Although our protocol demonstrated sustained neuroprotection at 7 days of survival, the functional

FIG. 10. Post-stroke hypothermia did not reduce infarct in AMPK α 2-knockout mice with 7 days of survival. The methods were the same as in Figure 7 ($n=6$ control and $n=5$ hypothermia per group; NT, normothermia; HT, hypothermia).

FIG. 11. Post-stroke hypothermia did not improve functional outcome in AMPK α 2-knockout mice with 7 days of survival. The methods were the same as in Figure 9 ($n=6$ control and $n=5$ hypothermia per group; NT, normothermia; HT, hypothermia).

role of AMPK in the neuroprotection offered by longer-term cooling in stroke remains to be evaluated. In addition, it is possible that the neuroprotective effect of compound C or AMPK deletion led to a "floor" effect, in which any additional benefit of hypothermia could not be seen. Finally, there are three upstream kinases of AMPK (TAK1, LKB1, and CaMKK; Li and McCullough, 2010), and we only examined pLKB1, which was not reduced by hypothermia after stroke, indicating a direct inhibitory effect of hypothermia on AMPK activation. However, the effect of hypothermia on other major upstream kinases of AMPK remains to be investigated.

AMPK inhibition has a relatively wide therapeutic window and can provide sustained neuroprotection in experimental stroke. Previously we have demonstrated that AMPK inhibition was neuroprotective when treatment was given 2 h after the onset of stroke, and neuroprotection was seen even when outcome was assessed 7 days after cerebral ischemia (Li et al., 2007). In the present study, we provide solid evidence that AMPK is an important mediator of the neuroprotection induced by hypothermia. AMPK inhibitors may be able to provide similar protection by activating the same pathways as hypothermia, while avoiding the deleterious adverse effects such as decreased cardiac output, pulmonary infections, and shivering, seen with cooling (Lampe and Becker, 2010). Our data indicate that AMPK represents a potential therapeutic target for stroke treatment.

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Author Disclosure Statement

No competing financial interests exist.

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