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Louise D. McCullough
University of Connecticut School of Medicine and Dentistry

Venugopal Reddy Venna
University of Connecticut School of Medicine and Dentistry

Jun Li
University of Connecticut School of Medicine and Dentistry

Sharon E. Benashski
University of Connecticut School of Medicine and Dentistry

Sami Tarabishy
University of Connecticut School of Medicine and Dentistry

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Venugopal Reddy Venna, PhD[1], **Jun Li, PhD**[1], **Sharon E. Benashski, MS**[1], **Sami Tarabishy, BS**[1], and **Louise D. McCullough, MD/PhD**[1,2,3]

[1]Department of Neuroscience, University of Connecticut Health Center, Farmington, Connecticut 06030, USA

[2]MC-1840, Department of Neurology, 263 Farmington Avenue, Farmington, Connecticut 06030, USA

[3]The Stroke Center at Hartford Hospital, 85 Jefferson Street, Hartford Connecticut 06102, USA

Abstract

Background and Purpose—Ischemic preconditioning (IPC) induces endogenous neuroprotection from a subsequent ischemic injury. IPC involves down-regulation of metabolic pathways. As Adenosine 5′-monophosphate-activated protein kinase (AMPK) is a critical sensor of energy balance and plays a major role in cellular metabolism, its role in IPC was investigated.

Methods—A brief 3 minute middle cerebral artery occlusion (MCAO) was employed to induce IPC in male mice 72 hours prior to 90 minute MCAO. Levels of AMPK and pAMPK, the active form of the kinase, were assessed after IPC. A pharmacological activator or inhibitor of AMPK was utilized to determine the dependence of IPC on AMPK signaling. Additionally, AMPK-α2 null mice were subjected to IPC and subsequent infarct damage was assessed.

Results—IPC induced neuroprotection, enhanced HSP70 and improved behavioral outcomes. These beneficial effects occurred in parallel with a significant inhibition of pAMPK protein expression. Although both pharmacological inhibition of AMPK or IPC led to neuroprotection, IPC offered no additional protective effects when co-administered with an AMPK inhibitor. Moreover, pharmacological activation of AMPK with Metformin abolished the neuroprotective effects of IPC. AMPK-α2 null mice that lack the catalytic isoform of AMPK failed to demonstrate a preconditioning response.

Conclusions—Regulation of AMPK plays an important role in IPC mediated neuroprotection. AMPK may be a potential therapeutic target for the treatment of cerebral ischemia.

Keywords

Preconditioning; MCAO; AMPK; Metformin; Compound C

Corresponding author: Louise D. McCullough, Department of Neuroscience, University of Connecticut Health Center, Farmington, Connecticut 06030, USA., Tel (860) 679-2271, Fax (860) 679-1181, lmccullough@uchc.edu.

Introduction

Adenosine 5′-monophosphate-activated protein kinase (AMPK) is a heterotrimeric protein kinase expressed in most mammalian tissues including brain (Carling et al., 1989; Li and McCullough, 2010). AMPK consists of a catalytic subunit alpha (α) and two regulatory subunits beta (β) and gamma (γ), with differential tissue expression and physiological significance (Hardie, 2003; Weisova et al., 2011). AMPK is activated by phosphorylation of threonine 172 in the catalytic α subunit when energy supplies are low (Hawley et al., 1996; Hardie, 2003) and is increasingly recognized as a major regulator of cellular energy dynamics. At the cellular level, activation of AMPK maintains energy reserves by suppression of ATP consuming anabolic processes with simultaneous activation of ATP generating catabolic pathways, thus initiating a cascade to ensure metabolic adaptation and cell viability (Bungard et al., 2010; Weisova et al., 2011). AMPK is activated in models of reversible middle cerebral artery occlusion (MACO), as shown by an immediate ischemia-induced rise in pAMPK levels that are sustained for 24 hours (McCullough et al., 2005). The aute activation of AMPK is detrimental in focal stroke models, as pharmacological inhibition of AMPK or genetic deletion of the catalytic isoform AMPKα2 is neuroprotective (Li et al., 2007; Li et al., 2010; Li and McCullough, 2010). However, the duration of ischemia and the severity of the insult are emerging as key factors in determining the downstream effect of AMPK activation after injury (Weisova et al., 2011).

Ischemic preconditioning (IPC) leads to adaptive tolerance in mammals, whereby a brief non-injurious stimuli reduces damage from a subsequent severe insult (Stenzel-Poore et al., 2007). Classic or delayed IPC requires several hours or days to fully manifest, and is regulated by gene activation and *de novo* cytoprotective protein synthesis (Stenzel-Poore et al., 2007; Gidday, 2006). Although the neuroprotective effects of IPC have been extensively tested and successfully validated in several species, the induction of IPC is often not practical in clinical situations. Therefore identification of the endogenous mechanisms induced by IPC is critical to development of future pharmacological agents. The beneficial effects of IPC have been attributed to metabolic depression as IPC slows the rate of ATP depletion during subsequent injury (Yenari et al., 2008). Many of the pathways activated by IPC are regulated by AMPK, suggesting that it may be the mediator of ischemic metabolic adaptation (Peralta et al., 2001; Nishino et al., 2004).

In this study we investigated the role of AMPK signaling in IPC. As the beneficial effects of delayed IPC requires several hours to fully manifest and earlier studies have shown that neuroprotection peaks at 72h after IPC (Puisieux et al., 2004), we induced a 90 minute MCAO 72 hours after IPC. The response of AMPK to IPC was evaluated, and manipulation of pAMPK levels with pharmacological and genetic approaches was performed to directly assess effects on infarct size.

Materials and Methods

Ischemic Preconditioning and Middle Cerebral Artery Occlusion Model

All animal work was approved by the Center for Animal Care at University of Connecticut Health Center and was performed in accordance with National Institutes of Health guidelines. Focal transient cerebral ischemia was induced in *C57BL/6* male mice (20-25g) by right MCAO followed by reperfusion as described previously (Li et al., 2010; Deplanque et al., 2011). Wild-type *C57BL/6* mice and AMPK-α2 knockout (KO) and wild-type littermates on a *C57BL/6* genetic background were anesthetized and subjected to a three minute MCAO (IPC) or a sham surgery in which the suture was not advanced into the MCA followed by reperfusion. Seventy two hours later a 90 minute MCAO was induced. Experimental timeline is shown in (Figure 1A). Cerebral blood flow was measured by Laser

Doppler Flowmetry (LDF) (DRT-4, Moor Instruments Ltd, Devon, UK). Wild type mice were purchased from Charles River (Wilmington, Mass). AMPK-α2 KO mice were originally obtained from Dr.Benoit Viollet (Li et al., 2010; Li et al., 2007) and bred at UCHC.

Behavioral Scores

Neurological deficit scores (NDS) were obtained during the intraischemic period and at 24 hours post-stroke. The scoring system was as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to the affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling as described previously (Li et al., 2010). Animals were scored by investigators blinded to treatment group and genotype.

Infarct Analysis

Following 24h of reperfusion animals were euthanized (96h post IPC), brains were removed, cut into five 2-mm coronal sections and stained with 1.5% 2,3,5-triphenyltetrazolium chloride (TTC). The final infarct volumes were analyzed from TTC stained sections and presented as percentage volume (percentage of contralateral structures with correction for edema) and analyzed as described (Li et al., 2010).

Fluoro-Jade and DAPI Staining

Mice were deeply anesthetized with pentobarbital and intracardially perfused with phosphate buffered saline for 1 min followed by 4% paraformaldehyde in PBS for 30 min. Following perfusion, brains were collected and post-fixed in 4% paraformaldehyde for 4h. After post-fixation, brains were cryoprotected in 20% sucrose solution, and subsequently cut into 30 μm coronal sections. Sections were subsequently stained in 0.001% FluoroJade B (Chemicon International, CA) in 0.1% acetic acid for 20 min, washed with water and dipped in 1:5000 concentrated DAPI (Cell signaling, Danvers, MA) for one minute, coversliped with fluoromount (SouthernBiotech, Birmingham, AL). Digital images were collected on a Zeiss (Thornwood, NY) Axiovert 200M fitted with an apotome for analysis was performed as in (Liu et al., 2009).

Drug Treatments

An acute dose of compound C (10mg/kg, Calbiochem, San diego, CA) or metformin (100mg/kg; Sigma, St Louis, MO) or vehicle (saline vehicle), was injected intraperitoneally at the onset of MCAO and injected volumes were 0.2mL/20g body weight.. These doses have been previously shown to either lower (Compound C) or enhance (Metformin) pAMPK levels as measured by Western blot in previous studies (McCullough et al., 2005, Li et al., 2010; Liu et al., 2011).

Western Blot Analysis

Separate cohorts of animals were used for protein analysis. Western blots were performed as described previously (Li et al., 2010). As changes in pAMPK levels are very dynamic, brains were collected by rapid extraction after cervical dislocation and flash frozen. Once homogenized, brain homogenates were aliquoted to avoid freeze thaw cycles and stored at −80°C. Brains were taken four hours after the onset of cerebral ischemia or IPC and 72h after IPC. Western blots are performed as in (Li et al., 2010) using pAMPK (1:500; Cell Signaling, Danvers, MA), AMPK (1:1000; Cell Signaling, Danvers, MA), HSP70 (1:2500; SantaCruz Biotechnology) were assessed. Beta-actin (1:5000; Sigma) was used as a loading control. Blots were incubated overnight in primary antibody at 4°C in Tris-buffered saline containing 4% bovine serum albumin in 0.1% Tween20. Secondary antibodies (goat anti-

rabbit IgG 1:10000 for AMPK, p-AMPK, goat antimouse IgG 1:5000 for HSP70 and Beta-actin; Chemicon) were diluted and incubated for 45 minutes at room temperature, ECL (Pico) detection kit (ThermoScientific) was used for signal detection.

Statistics

Data were expressed as mean±SEM except for NDS, which was presented as median (interquartile range). Statistics were performed with analysis of variance (ANOVA) with Bonferroni post hoc correction when needed for multiple comparisons (for infarct analysis, and p-AMPK densitometry), or by Mann-Whitney U test (NDS) (Li et al., 2010). A p value of p<0.05 was considered to be statistically significant (Li et al., 2010). A separate cohort of control animals were used for each set of experiments. Investigators performing MCAO, behavioral, and infarct size analysis were blinded to treatment conditions.

Results

Preconditioning Led to Enhancement of HSP70 Levels with No Visible Infarct

Occlusion with the IPC stimulus was confirmed by drop in CBF using LDF, all animals showed at least an 80% drop of blood flow at occlusion which was restored to normal at reperfusion. Three minutes of MCAO (IPC stimulus) did not lead to any visible injury on TTC (Figure 1B) or fluoro-Jade B staining (Figure 1C). A significant elevation in HSP70 (Figure 2A), a hallmark of successful IPC (Puisieux et al., 2000; Zhan et al., 2008), was seen (Sham *72h* 1±0.22 vs. IPC *72h* 1.81±0.18, P<0.05, n=6/grp) (Figure 2B).

Preconditioning Induced Neuroprotection and Improved Behavioral Deficits

No differences in LDF were observed between groups (data not shown). IPC 72 hours before MCAO induced significant neuroprotection (Figure 3A). Total hemispheric infarct volume was significantly lower in animals that had undergone previous IPC (Sham+Stroke 45.0±3.4%; n=8 vs. IPC+Stroke 33.1±3.4; n=8; P<0.05) (Figure 3B). Similar findings were seen in cortex (Figure 3B) and striatum (Figure 3B). The protective effect of IPC were also reflected in the NDS (Sham+Stroke 3.0 [0]; n=8 vs. IPC+Stroke 2.0 [1]; n=8; P<0.05; Figure 3C).

Preconditioning Modifies pAMPK Protein Levels

IPC significantly enhanced pAMPK levels 4 hours after IPC (Sham 4h 1.0±0.16; IPC 4h 1.5±0.13, P<0.05, n=6/grp). However, at 72 hours (the time of MCAO), IPC animals had a reduction in pAMPK compared to sham IPC (Sham at 72h 1.1±0.18 versus IPC 72h 0.6±0.13; P<0.05, n=6/grp). As no changes in pAMPK levels were seen with with sham surgery, the data was normalized to the pAMPK levels of pooled shams from all groups (Figure 4). As has been seen previously (McCullough et al., 2005), stroke increased pAMPK levels (Stroke 4h 2.1±0.11, n=6). Importantly, prior IPC significantly inhibited the stroke-induced rise in pAMPK (Stroke+IPC at 4h 1.3±0.11; P<0.05; n=6; Figure 4).

Lack of a Preconditioning in Compound C Treated Mice

To determine if suppression of pAMPK contributes to IPC, mice were treated with the AMPK inhibitor, compound C. Treatment with compound C induced neuroprotection (Total infarct: saline 47.0±3.7 vs. compound C 32.4±2.7; Cortex: saline 59.1±3.7 vs. compound C 36.7±2.9; Striatum: saline 69.7±2.6 vs. compound C 54.6±2.9; n=7 in saline and n=8 in compound C group, p>0.05). When IPC was induced in compound C treated mice, no additional neuroprotection was seen in any brain region (Figure 5). No changes were seen in CBF with drug treatment or with IPC.

Acute Metformin Abolished the Neuroprotective Effects of Preconditioning

To investigate if the effects of IPC can be ameliorated by pharmacologically activating pAMPK we administered the AMPK activator metformin (Zhou et al., 2001) and examined IPC-induced neuroprotection. The protective effects of IPC were abolished by metformin, with loss of the beneficial IPC effects in cortex (IPC+saline 35.7±3.0; IPC+metformin 52.9±1.9; n=6/grp, p>0.05), striatum (IPC+saline 53.0±3.2; IPC+metformin 75.3±1.7; n=6/grp, p>0.05) and the total hemisphere (IPC+saline 35.4±2.2; n=6 IPC+metformin 49.7±2.2; n=6, p>0.05; Figure 6B).

AMPK-α2 KO Mice Do Not Precondition

To more definitively test the hypothesis that suppression of stroke-induced AMPK activation was responsible for the endogenous protection induced by IPC, we examined the effect of IPC in AMPK-α2 KO mice. Consistent with earlier studies (Li et al., 2007; Li et al., 2010) AMPK-α2 KO mice had significantly smaller infarcts after MCAO compared to WT littermates (AMPK-α2 KO 34.8±2.1; n=7 vs. WT 47.1±4.6; n=6, p<0.05). However IPC induced neuroprotection was absent in AMPK-α2 KO mice (total hemisphere; WT littermates+IPC 35.9±1.4; AMPK-α2 KO+sham 34.8±2.1; n=7 vs. AMPK-α2 KO+IPC 32.9±2.8; n=7, p>0.05; Figure 7).

Discussion

The present study demonstrates AMPK signaling is involved in cerebral ischemic preconditioning. A brief ischemic stimulus acutely activated AMPK four hours after IPC; however when pAMPK levels were assessed 72h after IPC a significant reduction in pAMPK levels were seen. Most importantly, IPC led to a significant reduction in stroke-induced pAMPK activation when these mice were subjected to a subsequent stroke 72 hours later. The suppression of pAMPK was seen despite increased HSP70 levels, demonstrating that this is not simply due to an overall down-regulation of protein synthesis. The specificity of this response was then confirmed with pharmacological and genetic studies. When AMPK activity was inhibited by administration of compound C, no additional benefit of IPC beyond that seen with AMPK inhibition alone was seen. This suggests that these effects are mediated by the same signaling pathway. This effect was confirmed in AMPK-α2 KO mice, in which the catalytic isoform known to be responsible for the detrimental effect of AMPK activation in ischemic brain (Li et al., 2007) is absent; these mice failed to precondition. Moreover, acute administration of metformin, an AMPK activator (Zhou et al., 2001), increased infarct size in IPC mice.

Elucidating the endogenous pathways that mediate the beneficial effects of IPC is an area of intense research interest in both the brain and other organs. We chose a brief three minutes IPC to avoid histological tissue damage, as in other models 5 or 10 minutes of ischemia induced microscopic infarcts in 28% of brains (Zhan et al., 2008). Consistent with earlier studies (Puisieux et al., 2004), this brief IPC stimulus did not induce neuronal damage when assessed with TTC (Figure 1B) or fluro-jade (Figure 1C). Fluro-Jade B staining is more sensitive to acute neuronal injury (Schmued and Hopkins, 2000) and reaches a peak similar to that of TTC by 24h (Liu et al., 2009). Since the beneficial effects of IPC have been well documented at 72h post-IPC, as have elevations of HSP70 (Brown, 2007, Zhan et al., 2008) we induced MCAO at this point (Kitagawa et al., 1990; Puisieux et al., 2000; Puisieux et al., 2004). The efficacy of our paradigm was confirmed by observing a reduction in infarct (Figure 3A, 3B), improved NDS (Figure 3C) and increased expression of HSP70 (Figure 2B).

In most rodent models, delayed or classic ischemic tolerance requires several days to develop (Puisieux et al., 2004), suggesting that activation of pro-survival genes is necessary (Brown, 2007; Zhan et al., 2008; Wang et al., 2010). These changes lead to enhanced metabolic function, preservation of ischemic ATP levels, mitochondrial stability and reductions in ischemia induced lactate formation, all of which have been implicated in IPC-induced neuroprotection (Volovsek et al., 1992; Yenari et al., 2008). AMPK activation has been shown to contribute to IPC in peripheral tissues such as heart and liver, however, the role of AMPK in cerebral IPC may differ. In peripheral tissues, activation of AMPK appears to protect tissues from injury, while AMPK activation is detrimental in cerebral ischemia (Li et al., 2010). In the brain, AMPK is highly expressed in neurons and is activated in response to increasing AMP/ATP ratio, an indicator of declining energy reserves (Li and McCullough, 2010). As neurons cannot perform anaerobic glycolysis well (Liu et al., 2009), enhancing AMPK simply increases the drive to produce ATP in a brain that has no available oxygen or glucose, worsening metabolic failure. Thus the profile of protective mechanisms set in action by preconditioning may also differ in the periphery and in brain (Gidday, 2006).

AMPK signaling plays a fundamental role in IPC, but activation status changed over time. AMPK activation occurred at early time points after IPC (4 hours); in contrast pAMPK levels are down-regulated at later time points (72 hours), when MCAO was induced. We propose that this down-regulation of AMPK is beneficial, and contributes to the beneficial effects of delayed IPC. This is consistent with previous work examining the effect of acute or chronic treatment with the AMPK activator metformin (Li et al., 2010). With acute administration, cerebral pAMPK levels rise (Li et al., 2010), and when these animals are subjected to injury they have severe stroke-induced metabolic dysfunction, enhanced lactate production and exacerbation of ischemic injury (Li et al., 2010). In contrast, chronic administration of metformin administered for two weeks prior to stroke leads to both a suppression of both basal and stroke-induced pAMPK, as well as subsequent neuroprotection. This same pattern emerged in this study, with a reduction in pAMPK levels 72h after IPC and neuroprotection after MCAO. Consistent with this work, AMPK activation with pharmacological agents was recently found to reverse the beneficial effects of IPC in other models (Lotz et al., 2011). To ensure that changes in AMPK actually mediated the effects of IPC, mechanistic studies with compound C, metformin, and AMPK-α2 KO mice were performed. The results were consistent with the concept that reductions in AMPK activation represent metabolic tolerance and underlie, in part, the beneficial effects of IPC.

The molecular pathways involved in AMPK-induced metabolic down regulation remain to be elucidated. We have shown that chronic down-regulation of AMPK leads to preserved intra-ischemic ATP levels and reductions in ischemia-induced lactic acidosis (Li et al., 2010). Other groups have found that a brief stimulation of AMPK increases glucose transporter 3 (GLUT3) expression in cultured neurons, leading to ischemic tolerance (Weisova et al., 2011). Interestingly more prolonged activation of AMPK by chronic exposure to the AMPK activator AICAR, or overexpression of AMPK-α1 led to progressive loss of neuronal viability due to increased BH3-only protein Bim promoter activity, a key protein involved in apoptosis and mitochondrial depolarization (Weisova et al., 2011). Other possible downstream mechanisms by which AMPK could mediate IPC include inhibition of the elongation step of translation via activation of elongation factor-2 kinase, reduction in the initiation of protein synthesis via suppression of mTOR (Weisova et al., 2011), or activation of autophagy. Recent studies have shown activation of autophagy is essential for successful preconditioning (Sheng et al., 2010; Vingtdeux et al., 2011) and AMPK has been recently identified as a major mediator of autophagy (Hardie, 2011).

We utilized compound C in these studies, a relatively selective AMPK inhibitor (McCullough et al., 2005), previous studies that have shown significant decreases in brain pAMPK levels after compound C administration (McCullough et al., 2005; Li et al., 2007; Li et al., 2010; Liu et al., 2011). Previous work has demonstrated that compound C induces neuroprotection by inhibiting cerebral AMPK activation (Liu et al., 2011). In this study, mice treated with compound C demonstrated no additional neuroprotective effects with IPC, suggesting that AMPK inhibition is the common neuroprotective pathway. Although a floor effect is possible, this is less likely as mice treated with combination therapy still had substantial infarcts (30%), and we have shown previously that infarct volumes can be reduced to as low as 16% with other neuroprotective agents in earlier studies using this MCAO model (Li et al., 2008). However, it does remain possible that the smaller infarcts are masking an additive effect of AMPK and IPC. Due to this concern, and the possibility of "off-targets" effects of pharmacological treatments, findings were confirmed in mice lacking AMPK-$\alpha 2$, the isoform responsible for the detrimental effects of AMPK activation in stroke (Li et al., 2007).

In summary this work demonstrates that down-regulation of AMPK contributes to the beneficial effects of IPC in cerebral ischemia. The mechanism by which AMPK is induced by IPC, the timing of the response, and the downstream pathways activated by AMPK after IPC remain to be explored. The importance of the early AMPK activation (at 4 hours) after IPC needs further attention. Although immediate preconditioning may eventually have a more important translational role in acute stroke treatment (i.e., graded catheter based reperfusion or immediate remote IPC in the acute patient), the beneficial effects of delayed IPC could have importance in patients at high short-term risk for stroke such as those with transient ischemic attacks and high ABCD2 scores (Giles and Rothwell, 2009). We conclude that mild or short-lived metabolic stress leads to metabolic tolerance by chronically reducing AMPK activation. These studies suggest that finding strategies that lead to chronic down regulation of AMPK activity will protect the ischemic brain.

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Research Highlights

► Brief Ischemic preconditioning can induce brain tolerance and regulate AMPK activity.

► Downregulation of AMPK contributes to the beneficial effects of IPC mediated brain tolarance.

► AMPK activation abolishes the beneficial effects in preconditioned brains.
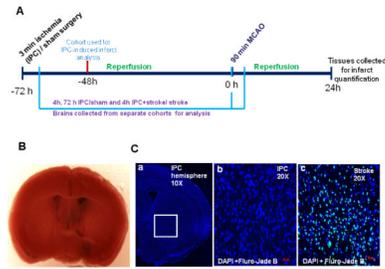
► Lack of preconditioning effects in AMPK-α2 KO animals.

Figure 1.
Timeline and experimental design is illustrated (Fig.1A), Body weights, temperatures are monitored during all the experiments; CBF was monitored during surgery using laser Doppler flowmetry. The brief IPC stimulus did not cause any visible neuronal death, which was confirmed by TTC staining (Fig. 1B), and fluoro-jade, a marker for degenerating neurons (co-labeled with DAPI (Fig.1C)), no fluoro-jade positive cells (green) were present in IPC brains at *10X* magnification (C-a), confirmed at 20X magnification in the core (C-b), in contrast a large amount of fluoro-jade staining was seen after MCAO (C-c).
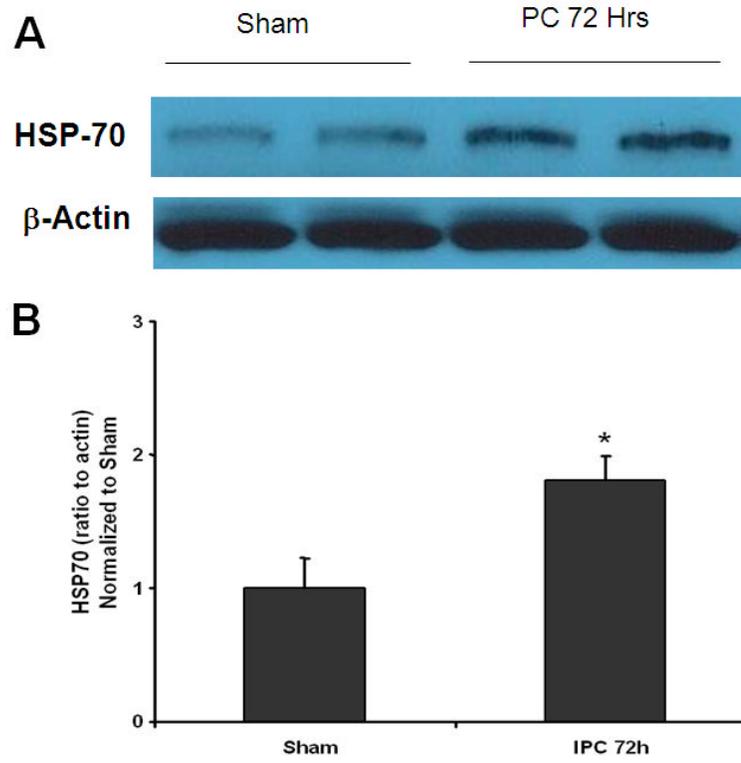
Figure 2.
IPC induced a significant increase in HSP70 levels at 72h n=6, P< 0.05 versus control
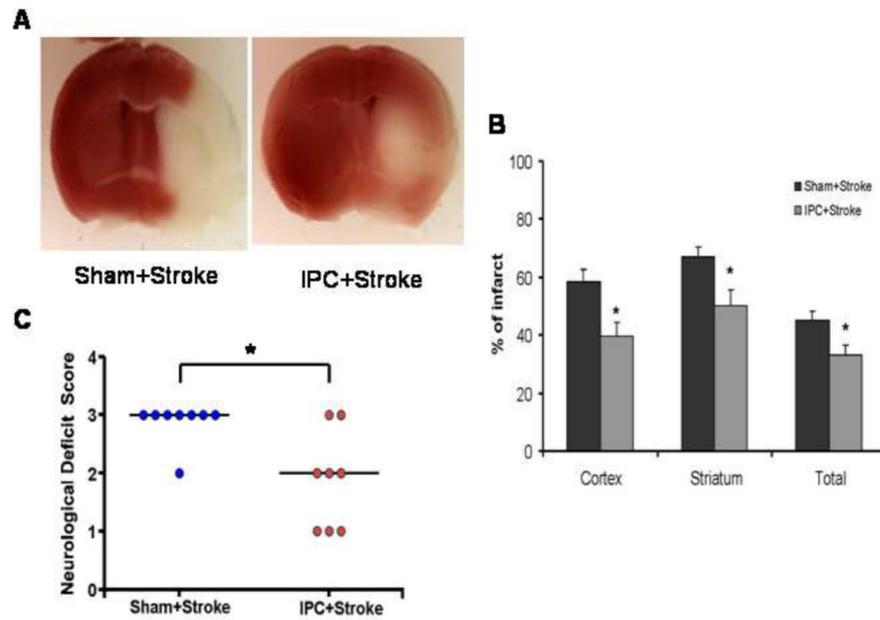(Students t-test); data are expressed as mean ± SEM.

Figure 3.
IPC brains had significantly smaller infarct volumes (A). Neuroprotection was seen in total, cortical and striatal infarcts in IPC+stroke cohorts (B); n=8/grp, *P<0.05 versus control (*Students t-test*); data are expressed as mean ± SEM. IPC improved NDS (C) n=8/grp, P<0.05 versus sham+stroke (Mann–Whitney U test); data are expressed as median (interquartile range).
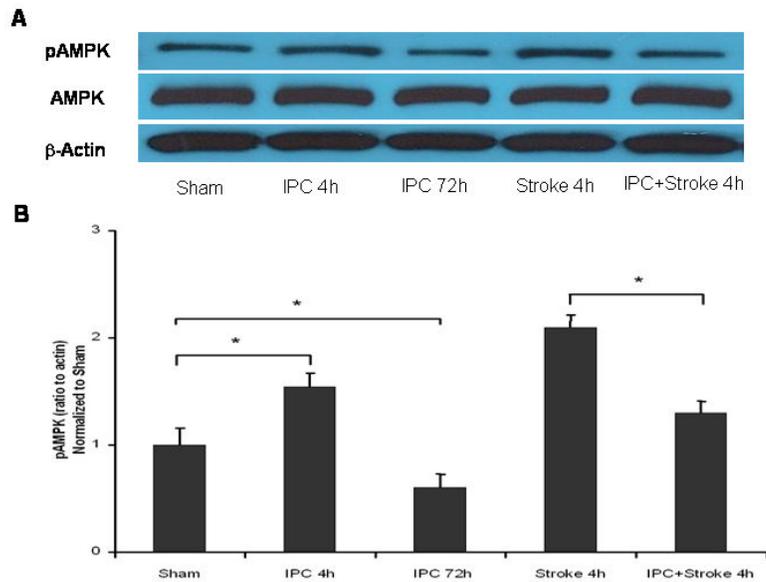
Figure 4.
IPC significantly reduced pAMPK levels at 72h; Brains were collected at 4h and 72h after IPC/sham surgery and 4h after sham+stroke or IPC+stroke. Shams (4h and 72h of each group) were pooled as no differences were seen between groups. Although at the early timepoint (4h) after IPC pAMPK levels were elevated, an inhibitory effect of IPC on AMPK was seen at 72h (one-way ANOVA with Bonferroni post hoc). pAMPK was lower at 4h in IPC+Stroke brains compared to the Sham+Stroke brains; n=6/grp (*one-way ANOVA with Bonferroni correction*). *P<0.05; data are expressed as mean±SEM.
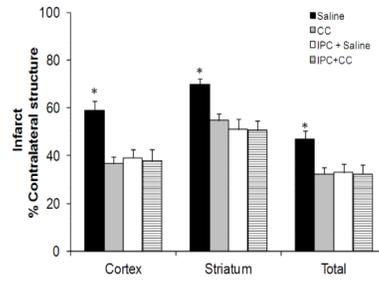
Figure 5.
Acute compound C (CC) injection immediately at stroke onset led to significant neuroprotection after a 90min MCAO and 24h of reperfusion. CC (10mg/kg, IP); n=8 or saline; n=7. IPC 72h prior to stroke induced significant neuroprotection but no additional protective effects were seen in mice treated with IPC+CC. Cortical, striatal, and total hemisphere infarct were calculated (percentage of contralateral hemisphere). Two-way ANOVA identified a significant effect of CC ($P<0.01$), preconditioning ($P<0.02$) and significant interaction of CC and IPC (*$P<0.01$). This significant interaction confirms that CC is neuroprotective, but has no effects in IPC mice. Subsequent t-test confirmed these results (*$P<.05$). Data are expressed as mean±SEM.
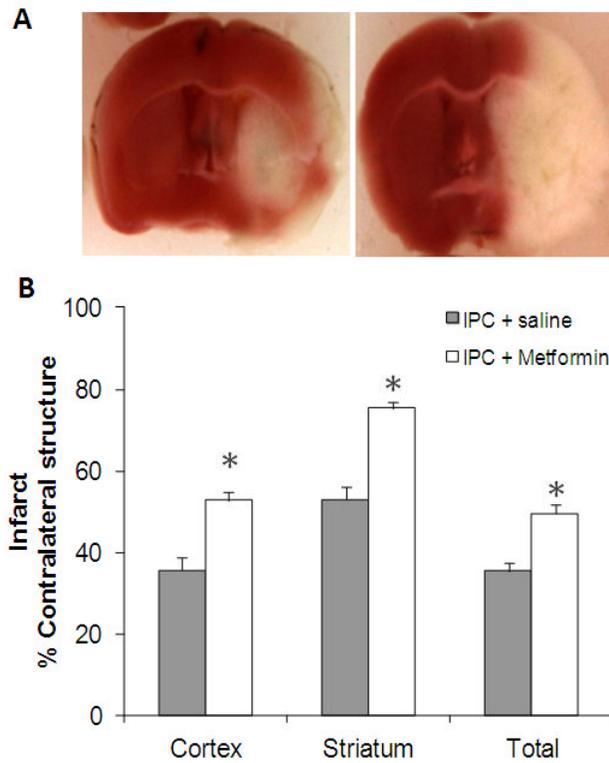
Figure 6.
Acute metformin administration exacerbates IPC induced neuroprotection. Metformin (100mg/kg i.p.) or saline was injected in IPC mice at stroke onset (n=6/group). IPC +metformin treatment increased infarct volume compared to IPC+saline (*P<0.05; Students t-test), suggesting pAMPK activation by metformin could reverse the protective effects of IPC; data are expressed as mean±SEM.
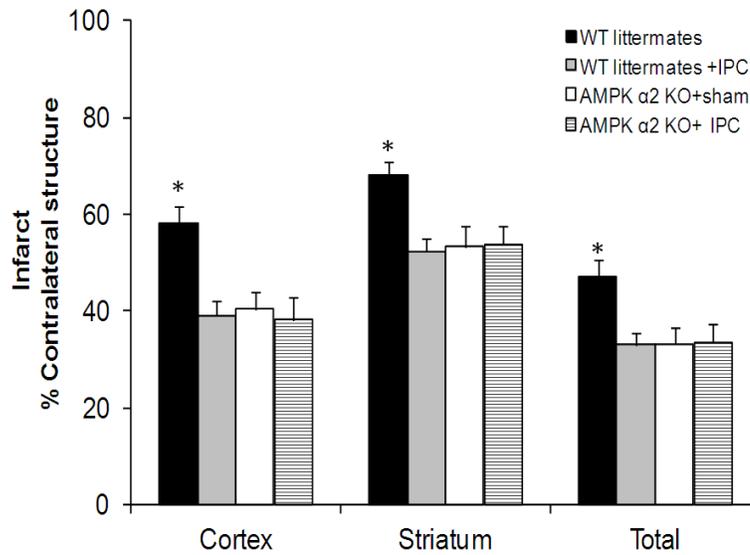
Figure 7.
The effect of IPC was mediated by AMPK. AMPK-α2 KO mice had significantly smaller infarcts compared to WT littermates. IPC had no effect in AMPK-α2 KO mice (n=6 for WT and n=7 /group AMPK-α2 KO with/without IPC). Cortical, striatal, and total hemisphere infarcts were quantified. Two-way ANOVA identified a significant effect of genotype (P<0.02), IPC (P<0.05) and interaction between genotype and IPC (P<0.02). This significant interaction confirms that IPC is protective in WT, but has no effects AMPK-α2 KO mice. Subsequent t-test confirmed these results (*P<.05). Data are expressed as mean±SEM.