Pharmacological Inhibition of AMP-activated Protein Kinase Provides Neuroprotection in Stroke*

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The restoration of energy balance during ischemia is critical to cellular survival; however, relatively little is known concerning the regulation of neuronal metabolic pathways in response to central nervous system ischemia. AMP-activated protein kinase (AMPK), a master sensor of energy balance in peripheral tissues, is phosphorylated and activated when energy balance is low. We investigated whether AMPK might also modulate neuronal energy homeostasis during ischemia. We utilized two model systems of ischemia, middle cerebral artery occlusion in vivo and oxygen-glucose deprivation in vitro, to delineate changes in AMPK activity incurred from a metabolic stress. AMPK is highly expressed in cortical and hippocampal neurons under both normal and ischemic conditions. AMPK activity, as assessed by phosphorylation status, is increased following both middle cerebral artery occlusion and oxygen-glucose deprivation. Pharmacological inhibition of AMPK by either C75, a known modulator of neuronal ATP levels, or compound C reduced stroke damage. In contrast, activation of AMPK by 5-aminoimidazole-4-carboxamido ribonucleoside exacerbated damage. Mice deficient in neuronal nitric-oxide synthase demonstrated a decrease in both stroke damage and AMPK activation compared with wild type, suggesting a possible interaction between NO and AMPK activation in stroke. These data demonstrate a role for AMPK in the response of neurons during metabolic stress and suggest that in ischemia the activation of AMPK is deleterious. The ability to manipulate pharmacologically neuronal energy balance during ischemia represents an innovative approach to neuroprotection.

Despite significant advances in our understanding of neuronal responses to ischemia, interventions for stroke remain elusive. A reduction in oxygen and glucose in cells causes a disruption of protein synthesis, depletion of intracellular energy stores, destabilization of the cell membrane, opening of voltage-gated Ca2+ channels, and activation of the N-methyl-D-aspartic acid receptor. These conditions lead to excitotoxic and oxidative damage (1). Stimulation of nitric-oxide synthase (NOS) by increasing Ca2+ levels causes accumulations of nitric oxide (NO), superoxide, peroxynitrite (ONOO), and free radicals, which further damage the cell membrane and may lead to DNA damage (1, 2). In the attempt to repair damage and return neurons to homeostasis, numerous energy-consuming processes are activated (3, 4). Overactivation of these pathways during ischemia can lead to complete energy failure and cell death (5, 6). The mechanisms by which neurons attempt to restore energy balance are largely unknown.

In peripheral tissues, AMP-activated protein kinase (AMPK), a member of a metabolite-sensing protein kinase family (7, 8), is activated by energy deficiency to coordinate a switch from anabolic to catabolic pathways to produce a positive energy balance. AMPK is composed of a catalytic α subunit (α1 or α2) and two regulatory subunits (β and γ) (9) and is activated via phosphorylation by an upstream kinase (10, 11) without which there is no detectable activity (12). AMPK monitors AMP and ATP levels. A rise in AMP levels or an increase in the AMP/ATP ratio signals declining energy stores, activating AMPK. This activation can acutely regulate cellular metabolism and chronically regulate gene expression to restore ATP levels (13–16). AMPK also enhances catabolic processes such as fatty acid oxidation and restricts anabolic processes such as fatty acid, cholesterol, and protein biosynthesis to restore ATP levels (7, 8).

Initial work on AMPK demonstrated its importance in the physiological response to energy demand, for example in exercising muscle (17, 18). Over the past several years, the importance of AMPK in the regulation of cellular energy dynamics has greatly expanded (8). Although responsive to rising AMP levels, AMPK is also sensitive to changes in cellular pH, redox...
status, and reactive nitrogen species and can be activated independently of changes in AMP (8, 19–22). In non-neuronal cells, AMPK is activated by a variety of signals not directly related to metabolism, including cellular stressors, such as hypoxia (23, 24), glucose deprivation (7, 25, 26), oxidative stress (19, 20, 23, 27), nitric oxide (28, 29), and the potent oxidant peroxynitrite (23), a key player in induction of ischemic damage (4, 30). In turn, AMPK activates numerous transcription factors and proteins, such as hypoxia-inducible factor 1 (HIF-1) (24) and endothelial NOS (31, 32), which play critical roles in the ischemic response. This suggests that AMPK, in addition to being a key regulator of physiological energy dynamics, may affect neuronal survival after ischemic stress.

AMPK is abundantly expressed in cultured cortical and hippocampal neurons (33, 34) as well as in cortical, hippocampal, and striatal neurons in vivo (34–36). The catalytic subunit of AMPK (α) has two major isoforms α1 and α2, both of which are present in neurons (35). The α1 isoform is primarily cytoplasmic, whereas α2 is predominantly nuclear and plays a role in transcriptional regulation (35, 37, 38). It has been demonstrated recently that AMPK is highly expressed in neurons in the hypothalamus, where it appears to play a role in the regulation of food intake (36, 39, 40). AMPK phosphorylation is increased in the hypothalamus after starvation, indicating that hypothalamic AMPK is responsive to feeding status. We investigated the role of AMPK in neuronal energy sensing using reagents that alter neuronal energy balance to assess whether neuronal AMPK is responsive to energy status. C75, a synthetic fatty-acid synthase (FAS) inhibitor (41, 42) and carnitine palmitoyltransferase-1 (CPT-1) stimulator, increases ATP levels in a number of cell types, including neurons (33, 43). Treatment of mice with C75 results in dephosphorylation and inactivation of AMPK and induces profound anorexia (36). Therefore, manipulation of neuronal energy dynamics may be possible with compounds that alter FAS or CPT-1 activity and represent a novel tool with which to modulate neuronal energy balance.

Sensing ATP levels may be important in the neuronal response to ischemia, but the consequences of persistent activation of AMPK in neurons that have compromised energy supplies are unknown. At present, it is unclear whether neuronal AMPK is activated by ischemia, and whether its activation would be protective or damaging. In the present study, we utilize an in vivo MCAO and an in vitro OGD model of ischemia to delineate changes in AMPK activity incurred as a metabolic stress. We demonstrate that AMPK is elevated and activated after ischemic insults and that this activation may be mediated by NO. Moreover, a reduction in stroke volume occurs after ischemic insults and that this activation may be mediated by NO.

**Experimental Procedures**

**Experimental Animals**—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 Animal Care and Use Committee of The Johns Hopkins University.

**Drug Treatments**—Mice were injected intraperitoneally with the indicated compounds and doses at the onset of MCAO. C75, compound C (FASgen, Inc.), and AICAR (Toronto Research Chemicals Inc.) were dissolved in RPMI 1640, such that the desired dose or vehicle could be administered in a fixed volume of 200 μl.

**Ischemia Model**—Cerebral ischemia protocols approved by the Animal Care and Use Committee of The Johns Hopkins University.

**Histopathology**—Infarction volume was analyzed by CNC techniques in vivo (2–4 mm slices). Infarction volume was determined by video microscopy and image analysis (Inquiry 3, Loats Associates), as described previously (44, 45).

**Western Blot Analysis**—Mice and rat brain samples were obtained by rapid removal of the brain from the skull, resec-
tion of the cerebellum, followed by immediate dissection of the right (R; ischemic) and left (L; non-ischemic) hemispheres. Samples were flash-frozen in liquid nitrogen. Samples from rats were further sub-
dissected into core and penumbral regions for both hemispheres. Sam-
ple were stored at −80 °C until use. Samples were prepared as re-
ported previously (36). Briefly, each sample was homogenized in 200 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM dithio-
reitol, 0.5 mM, 0.1 mM benzamidine, 50 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor). SDS detergent was added to a final concen-
tration of 0.2%, and lysates were boiled for 5 min. After the supernatant was harvested, and protein concentration was determined using an BCA kit (Bio Rad). 40 μg of protein was loaded per lane on a 4–15% gradient SDS-polyacrylamide gel and transferred to a polyvinylidene difluo-
ride membrane. The blots were successively blocked, stripped, and re-
probed for antigen detection. Phosphorylation of AMPKα was determined using an anti-phospho-AMPKα (α1 and α2 Thr172) antibody (1:1000, Cell Signaling). ACC (1:500) and phosphorylated ACC (1:1000) were obtained from Upstate Biotechnology, Inc., Lake Placid, NY. Anti-AMPKα antibody (α1 and α2, 1:1000; Cell Signaling), hsp-70 (1:1000; Santa Cruz Biotechnology), and β-actin (1:5000, Sigma) were used as loading controls. Membrane filters were incubated overnight in primary antibody at 4 °C in TBS buffer containing 5% bovine serum albumin and 0.1% Triton X-100. The secondary antibody (goat anti-rabbit IgG, Chemicon) was diluted to 1:5,000, and ECL (pico) detection kit (Amersham Biosciences) was used for signal detection.

**Immunohistochemistry**—Floating brain sections were prepared as described previsously (36). Free-floating sections (35 μm) were blocked in phos-
phate-buffered saline containing 5% goat serum, 1 mg/ml bovine serum albumin, 0.05% Triton X-100, and 1 mM NaF for 1 h at room tempera-
ture followed by incubation with anti-phospho-AMPKα antibody (1: 100), glia fibrillary acidic protein (GFAP) (1:1000, DAKO; for astrocy-
tes), NeuN (Chemicon 1:100; a marker for mature neurons), or von Willebrand factor (Santa Cruz Biotechnology, 1:400 for endothelial cells) in phosphate-buffered saline containing 1% goat serum, 1 mg/ml bovine serum albumin, 0.05% Triton X-100, and 1 mM NaF overnight at 4 °C. The secondary antibody (1:200; Vector Laboratories, Burlingame, CA) was incubated in phosphate-buffered saline containing 1% goat serum for 1 h 30 min (Texas Red, fluorescein isothiocyanate). The signal was visualized with immunofluorescence confocal microscopy using Zeiss image acquisition software (Zeiss LSM 510).
10 HEPES, and 10 glucose) and returned to the incubator to equilibrate for 30 min. Slice cultures were rinsed twice with warm deoxygenated glucose-free BSS, transferred to an air-tight chamber, flushed with anoxia (5% CO2, 85% N2, and 10% H2), and maintained at 37°C for 60 min. Controls were rinsed with BSS and transferred to a normal aerated incubator for 1 h. The period of anoxia was terminated by returning the cultures to fresh medium (50% minimum Eagle’s medium, 25% Hanks’ balanced salt solution, 25% heat-inactivated horse serum, 6.5 mg/ml d-glucose, 5 units/ml penicillin G, and 5 μg/ml streptomycin sulfate), and samples were prepared for Western analysis at 2, 4, 6, and 24 h (as above). Control and OGD-treated slices were included in each experiment with triplicate wells or 15 slices per condition for each experimental group.

Statistical Analysis—All data are expressed as mean ± S.E. Physiological variables and histology were analyzed by one-way analysis of variance with a post-hoc Newman-Keuls to correct for multiple comparisons. Post-ischemic neurological scores were analyzed by the Mann-Whitney U test.

RESULTS

AMPK Is Expressed in Neurons Post-ischemia—AMPK expression has been demonstrated previously in neurons (33–36) both in vivo and in vitro. To determine the localization of AMPK in ischemic brain, immunohistochemistry for AMPK and pAMPK was performed by using brain sections from mice subjected to 2 h of MCAO followed by 2 h of reperfusion (Fig. 1, A–C) and pAMPK (Fig. 1, D–F) localized to cortical neurons in the middle cerebral artery distribution, as shown by double labeling immunofluorescence using the mature neuronal marker NeuN. Higher magnification of cortical neurons immunostained for NeuN and pAMPK (Fig. 1, G–I) shows nuclear and cytoplasmic immunostaining for the α isoform (35), the predominant form expressed in neurons (35). Whereas AMPK may be only minimally expressed in non-stimulated astrocytes in intact brain (35), previous studies have found AMPK immunoreactivity in cultured astrocytes (49, 50). To clarify the localization of AMPK and pAMPK in vivo, we examined both normal non-ischemic tissue as well as brains harvested both 4 and 24 h after infarction (at 22 h of reperfusion) to determine whether AMPK is expressed in astrocytes after stroke. There was only minimal co-localization of GFAP with AMPK or pAMPK 4 h after MCAO (Fig. 1, J–L and M–O, respectively). Similar to 4-h tissue, no co-localization of AMPK or pAMPK with GFAP was seen in cortex 24 h after stroke (Fig. 1, P–R). In addition, no co-labeling of either AMPK or pAMPK with the endothelial marker von Willebrand factor was seen in intact or ischemic brain (data not shown). To determine whether this pattern of pAMPK immunolocalization was retained regionally, images of pAMPK and NeuN double labeling were obtained at 24 h after infarction (22 h of reperfusion) (Fig. 2). Similar levels of staining were seen in both the cortex (Fig. 2, A–C) and the hippocampus (Fig. 2, D–F), with prominent pAMPK co-labeling with NeuN in both regions. No signal was seen in control sections without addition of primary antibody (data not shown). These results suggest that AMPK is expressed primarily in neurons in both the ischemic and non-ischemic brain, where it may function in a cell-autonomous manner to influence neuronal energy balance.

AMPK Is Increased after MCAO—Male wild type (WT) C57Bl6 mice were subjected to 2-h right MCAO or sham surgery as described previously (44) and sacrificed at a variety of reperfusion times. There were no significant differences in mean arterial pressure, pO2, pCO2, glucose, or hemoglobin measurements between stroke and sham animals (Table I). As can be seen in Fig. 3, pAMPK (as detected by a Pan-α subunit antibody to Thr172 phosphorylation site (11)) was elevated in ischemic brain (stroke) as early as 90 min following ischemia compared with animals that had undergone sham surgery (sham) (n = 6 per time point for ischemic animals; 4 per time point for sham-operated controls). pAMPK levels were elevated in the right (R) ischemic hemisphere and in the left (L), non-ischemic hemisphere and remained elevated for 24 h (22 h of reperfusion). In contrast, pAMPK levels in sham brains remained stable over the 24-h period. Total AMPK levels showed minimal changes. Appropriate elevations of hsp-70 were seen in the ischemic hemisphere, although these were most notable at later time points (i.e. 6, 12, and 24 h). The global increase in pAMPK levels suggests that AMPK is activated secondary to metabolic derangements and compensatory responses not only in the ischemic area but also in the contralateral non-ischemic side.
**Table I**

| Parameter | Vehicle | Cpd C | Vehicle | C75 | Vehicle | AICAR |
|-----------|---------|-------|---------|-----|---------|-------|
| pH        | 7.37 ± 0.05 | 7.36 ± 0.07 | 7.30 ± 0.10 | 7.34 ± 0.08 | 7.37 ± 0.05 | 7.33 ± 0.11 |
| pCO₂      | 45.2 ± 2.8  | 421 ± 3.1  | 47.2 ± 4.7  | 47 ± 7.1    | 42 ± 3.6    | 39.7 ± 4.9  |
| pO₂       | 140 ± 9.8   | 1446 ± 14.3 | 144.7 ± 11.6 | 135 ± 14.2  | 147 ± 15.9  | 128 ± 15.2  |
| MAP       | 80.7 ± 4.8  | 82.2 ± 5.9  | 81.5 ± 3.9  | 79.1 ± 6.5  | 84 ± 7.1 at 15 | 69 ± 12.4 at 15 |
| Hg        | 12.6 ± 1.9  | 12.7 ± 1.3  | 12.8 ± 1.8  | 12.9 ± 0.5  | 13.8 ± 2.3  | 13.4 ± 0.9  |
| HCO₃      | 23.5 ± 1.1  | 23.1 ± 0.8  | 22.6 ± 2.4  | 22.8 ± 1.2  | 23.6 ± 2.6  | 22.9 ± 2.3  |
| LDF (%) BL | 9.8 ± 1     | 10.6 ± 0.9  | 10 ± 1.3    | 10.6 ± 0.88 | 11.3 ± 8 RP  | 11.1 ± 1.2 RP |

**FIG. 3. Time course of elevation of pAMPK after stroke.** Western blot analysis was performed on lysates derived from whole hemisphere homogenates from either stroke animals (S) (R = right/ischemic hemisphere and L = left/non-ischemic hemisphere) or non-stroke sham animals (NS) at various times after reperfusion. Antibodies detecting either phosphorylated or unphosphorylated AMPKα (top two panels), hsp-70 (third panel), and β-actin, which serves as a loading control (bottom panel), were used.

**AMPK Is Activated after OGD in Vitro**—To confirm our in vivo findings, we investigated whether AMPK was activated in vitro in hippocampal slice cultures after OGD, an in vitro model of stroke. We found a robust increase in pAMPK levels 2 h after OGD compared with normoxic normoglycemic control cultures (Fig. 4A). This increase in pAMPK persisted at 6 h, but normalized by 24 h, as was seen in our in vivo studies. A major downstream target of AMPK activation is acetyl-CoA carboxylase (ACC), which is the pace-setting enzyme in the de novo synthesis of fatty acids. Increasing AMPK enhances phosphorylation and inactivation of ACC limiting the anabolic process of fatty acid synthesis in times of energy deficiency. Evaluation of slice samples 4 h following OGD demonstrated an increase in the level of phosphorylated (inactive) ACC (Fig. 4B), supporting the idea that the observed increases in pAMPK are physiologically relevant. Total AMPK and ACC levels changed minimally at all time points examined in both OGD and control slices.

**AMPK Elevation Is Seen in the Ischemic Penumbra**—Assuming that metabolic derangements in stroke may be more severe in the necrotic core of an infarct, whereas pro-survival pathways may be activated in the penumbra in an attempt to salvage marginally functioning brain (51), we dissected out core (CC) and penumbral (PC) tissues from both the ischemic right hemisphere (IH, ischemic hemisphere) and non-ischemic left hemisphere (NIH, non-ischemic hemisphere) for Western blot analysis from rat brains (n = 6/group) 6 h after stroke (45) (Fig. 5). Rats were used for this experiment, as dissection of core and penumbra is more rapidly and reliably done using the larger rat brain; this also allowed us to confirm our findings in a different species. The areas of ischemia and of dissection were visualized using TTC staining at 24 h (Fig. 5A). pAMPK levels were dramatically elevated in the cortical penumbra of the ischemic hemisphere and in the contralateral non-ischemic hemisphere (PC IH and CC NIH, respectively), compared with pAMPK levels in either hemisphere in the sham brain (PC and CC) (Fig. 5B). pAMPK levels in the ischemic core (CC in the ischemic hemisphere) were lower than in the other areas of brain but were elevated compared with surgical shams (sham CC and PC).

It is possible that the lower level of AMPK in the core represents failure of protein synthesis and energy-dependent phosphorylation in the ischemic core of the infarct. However, expected increases in heat-shock protein 70 (52) were seen. Alternatively, AMPK activation could represent a compensatory protective pathway in the penumbra. pAMPK could be up-regulated in neurons that are damaged, but remain viable, as a strategy to increase available energy supply. The rise in contralateral AMPK suggests that neurons far removed from the area of the insult may also be receiving a signal to increase energy availability. Therefore, increases in pAMPK levels could represent an adaptive, endogenous neuroprotective pathway. To address this issue, we proceeded to pharmacologically manipulate AMPK levels to determine the effect of changing pAMPK levels to stroke outcome.

**Compound C Reduces pAMPK Levels and Is Neuroprotective**—To investigate the functional effects of AMPK activation, we examined the effect of compound C, a selective pharmaco-
logical AMPK inhibitor (24, 53), in mice subjected to MCAO and reperfusion. The effect on stroke outcome in animals treated with compound C (20 mg/kg delivered intraperitoneally; n = 14) or vehicle (n = 8) at stroke onset (2-h MCAO with 22 h reperfusion) was determined (Fig. 6). Compound C significantly reduced total infarct volume (as measured by % non-ischemic hemisphere, corrected for edema using TTC), as well as striatal (CP, 64 ± 5.7 versus 21 ± 3.9; p < 0.001) and cortical (CTX, 63 ± 3.4 versus 19.4 ± 3.3; p < 0.001) infarct volumes compared with vehicle treated animals (Fig. 6A). A reduction in severity of behavioral deficits at 24 h reflected these differences (vehicle: 3.3 ± 0.3 versus compound C, 1.9 ± 0.5, p < 0.01). There was no difference in relative cerebral blood flow (CBF) as measured by laser Doppler, either during the intra-ischemic period (vehicle: 9.8 ± 0.54 versus compound C; 10.6 ± 0.9; % of base line; p = not significant), or during the 30 min after reperfusion, suggesting that differences in blood flow could not account for the differences in ischemic damage. No differences in physiological measurements were seen between treatment groups (Table 1) demonstrating the equivalent contribution of these factors to stroke outcome.

Additional groups of vehicle- or compound C-treated mice subjected to either 2-h MCAO (n = 6/group) or sham surgery (n = 4/group) were sacrificed 4 (Fig. 6B) or 24 h (Fig. 6C) after infarction for determination of AMPK and pAMPK levels. Lysates from either the ischemic (right) or non-ischemic (left) hemispheres were analyzed by Western blot analysis in both whole hemisphere homogenates (R = right, ischemic hemisphere; L = left, non-ischemic hemisphere) were obtained 4 (B) and 24 h (C) following stroke or sham surgery. Western blot analysis was performed using antibodies that detect the following: phosphorylated and unphosphorylated AMPK, hsp-70, and β-actin, serve as loading controls.

**FIG. 5.** pAMPK is elevated in the ischemic and non-ischemic hemispheres. A, TTC staining of a rat brain stained 24 h after stroke demarcates the typical vascular distribution of right middle cerebral artery stroke. Pale areas in the right hemisphere indicate non-perfused/nonviable tissue. Circles show the areas sub-dissected out at 6 h for Western analysis. PC, penumbral cortex; PS, penumbral striatum; CC, core cortex; and CS, core striatum. B, Western blot analysis was performed on pooled cortical lysates taken 6 h after stroke using antibodies that detect phosphorylated or unphosphorylated AMPK, hsp-70, and β-actin, serve as loading controls.

**FIG. 6.** Compound C treatment causes a significant decrease in regional and total stroke volume. Mice were treated intraperitoneally with 20 mg/kg/body weight of compound C (n = 14) or vehicle at the onset of 2 h MCAO (Stroke) or sham surgery (Sham). A, 24 h following stroke, total, striatal (caudate-putamen; CP), and cortical (CTX) infarct volumes were measured by using TTC staining and are reported as the % of the non-ischemic hemisphere, corrected for edema. Lysates from whole hemisphere homogenates (R = right, ischemic hemisphere; L = left, non-ischemic hemisphere) were obtained 4 (B) and 24 h (C) following stroke or sham surgery. Western blot analysis was performed using antibodies that detect the following: phosphorylated and unphosphorylated AMPK, hsp-70; β-actin. ***, p < 0.001. Error bars represent S.E.
Inhibition of AMPK in Stroke Is Neuroprotective

FIG. 7. Administration of the FAS inhibitor C75 leads to significant neuroprotection. Mice were treated intraperitoneally with 20 mg/kg/body weight of C75 or RPMI vehicle at the onset of 2-h MCAO (Stroke) or sham surgery (Sham). A, 24 h following stroke (22 h of reperfusion), total, striatal (caudate-putamen; CP), and cortical (CTX) infarct volumes were measured by using TTC staining and are reported as the % of the non-ischemic hemisphere, corrected for edema. Lysates from whole hemisphere homogenates (R = right, ischemic hemisphere; L = left, non-ischemic hemisphere) were obtained 4 (B) and 24 h (C) following stroke (2 or 22 h of reperfusion, respectively) or sham surgery. Western blot analysis was performed using antibodies that detect the following: phosphorylated and unphosphorylated AMPKα, β-actin, hsp-70; β-actin. ***, p < 0.001. Error bars represent S.E.

...total and regional infarction volume seen in the C75-treated group (p < 0.001). Improvements in behavioral scores were seen (vehicle 3.2 ± 0.05 versus C75 2.0 ± 0.03, p < 0.01). As for compound C, no differences in LDF or physiological variables were seen (n = 4). Additional groups of vehicle- or C75-treated mice subjected to either 2-h MCAO and reperfusion (n = 6/group) or sham surgery (n = 4/group) were sacrificed 4 (Fig. 7B) or 24 h (Fig. 7C) following stroke for analysis of AMPK and pAMPK levels. The anticipated increase in pAMPK at 2 h of reperfusion was seen in both and C75-treated and vehicle-treated mice (Fig. 7B, lanes 1 and 2 versus lanes 5 and 6, respectively), although the increase in pAMPK levels was blunted by C75 treatment. As with compound C, the reduction in the increase in pAMPK levels was more pronounced in the ischemic right hemisphere compared with the non-ischemic left hemisphere. Compared with vehicle-treated mice, C75 reduced pAMPK levels in both sham and stroke mice prominently 24 h following stroke (Fig. 7C, lanes 1–4 versus lanes 5–8, respec-

...tively), indicating a more lasting central effect of C75 (compared with compound C) in reducing pAMPK levels.

AMPK Activation with the AMPK Activator AICAR Exacerbates Stroke—The widely used AMPK activator, AICAR (54), was administered at stroke onset (500 mg/kg intraperitoneal; n = 11/group). AICAR is taken up into cells and phosphorylated to form 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, which mimics the effects of AMP on AMPK activation (54). There was a significant exacerbation of total and cortical (CTX) stroke volumes in AICAR-treated animals (Fig. 8A). Physiological monitoring demonstrated a significant drop in mean arterial pressure (MAP) in AICAR-treated animals that self-corrected by 15 min after injection (MAP vehicle 84 versus AICAR 69 mm Hg, p < 0.05). There were no differences in intra-ischemic CBF, but a reduction in CBF was seen in AICAR-treated mice compared with controls at 30 min after reperfusion (98 versus 82.7% of base line, p < 0.05). This suggests that part of the detrimental effects of AICAR may be secondary to decreased perfusion pressure, in addition to its effects on AMPK. Western blot analysis showed elevations of pAMPK in AICAR-treated versus vehicle-treated sham animals with little additional elevation of pAMPK levels in stroke mice (2 h of reperfusion) (Fig. 8B, lanes 5–8 versus lanes 1–4, respectively). These effects were not seen at 24 h (data not shown).

Loss of nNOS Prevents Stroke-induced Elevations in pAMPK—As ONOO is a significant contributor to stroke-induced neuronal damage, and AMPK is activated by ONOO as well as hypoxia in vitro (23, 24), we evaluated the role of nNOS...
in AMPK activation after stroke (Fig. 9). nNOS-deficient (nNOS<--/--) or WT male mice were subjected to 2 h of Stroke or Sham surgery and 2 or 22 h of reperfusion, respectively. Lysates from whole hemisphere homogenates (R = right, ischemic hemisphere; L = left, non-ischemic hemisphere) were obtained 4 (A) and 24 h (B) following infarction. Western blot analysis was performed using antibodies that detect the following: pAMPK or unphosphorylated (AMPK) AMPK; ACC; hsp-70; and β-actin.

FIG. 9. AMPK and pAMPK levels in WT and nNOS<--/--> mice. nNOS-deficient (nNOS<--/--> or WT male mice were subjected to 2 h of Stroke or Sham surgery and 2 or 22 h of reperfusion, respectively. Lysates from whole hemisphere homogenates (R = right, ischemic hemisphere; L = left, non-ischemic hemisphere) were obtained 4 (A) and 24 h (B) following infarction. Western blot analysis was performed using antibodies that detect the following: pAMPK or unphosphorylated (AMPK) AMPK; ACC; hsp-70; and β-actin.

FIG. 10. AMPK and pAMPK levels in WT and PARP-1-deficient mice. PARP-1-deficient (parp--/-) or WT male mice were subjected to 2 h Stroke or Sham surgery as described for previous experiments. Lysates from whole hemisphere homogenates (only ischemic hemisphere shown) were prepared 24 h following surgery. Western blot analysis was performed using antibodies that detect the following: pAMPK and unphosphorylated (AMPK) AMPK; and β-actin.

2 h after OGD in vitro. Increased levels of AMPK were seen throughout the brain, in both penumbral areas as well as in areas far removed from the ischemic insult. Initially, this increase was thought to represent an adaptive response to neuronal stress, as a mechanism whereby the brain could increase ATP production in times of increased energy demand. However, our pharmacological studies clearly demonstrate that inhibition of AMPK activation under conditions of neuronal ischemia reduced stroke damage. Administration of the AMPK inhibitor compound C (53) or the FAS inhibitor C75 (which is known to increase neuronal energy levels and decrease AMPK phosphorylation and activation both in vitro and in vivo (36, 41)) provided significant neuroprotection in our model. In contrast, administration of the AMPK activator AICAR exacerbated stroke damage. Finally, nNOS-deficient mice had both smaller infarcts and lower pAMPK levels after stroke, whereas mice deficient in PARP-1 had similar pAMPK levels to WT despite smaller infarcts. This suggests that the nNOS and ONOO pathways are important activators of AMPK in vivo under conditions of neuronal stress. These results show that activation of neuronal AMPK is detrimental in an in vivo model of ischemia and reperfusion injury and that prevention of stroke-induced phosphorylation of AMPK is neuroprotective.

AMPK is known to play a critical role as an energy sensor in peripheral tissues (7, 8), and more recent evidence indicates a similar role centrally (36, 38). The finding that hypothalamic AMPK is responsive to peripheral energy status and is activated when peripheral signals indicate that energy supply is low (36, 39, 40) suggested a possible role of AMPK in the neuronal response to ischemia. The observation that AMPK is also activated in vitro by oxidative stress signals in addition to physiological stresses (starvation) implied that AMPK may play a more far-reaching role in cellular homeostasis and survival. In vitro, AMPK is responsive to numerous ischemic and oxidative stress signals, such as ONOO (23), hydrogen peroxide (27), NO (29), and hypoxia (19, 20, 24, 50), all of which lead to an increase in pAMPK levels and enhancement of catabolic, ATP-generating pathways. In addition, AMPK regulates other molecules, such as Akt and hypoxia-inducible factor (HIF-1), that are known to activate cell survival pathways after ischemic insults (24, 55). Although classically thought to be most responsive to AMP levels, hypoxic regulation of AMPK may be independent of AMP levels (53) and could represent a secondary pathway of AMPK activation that is triggered by oxidative rather than metabolic stress.

One well characterized cytotoxin formed in ischemic cells is ONOO, a potent oxidant by-product formed when superoxide and NO are produced at near equimolar amounts (56). There is
AICAR was utilized as an adenosine activator (70). In addition, AICAR reduced delayed cell death in the astrocytic apoptosis (68), suggesting that the effects of AICAR are ameliorated by adenoviral overexpression of superoxide dismutase or administration of NOS inhibitors, suggesting that the ischemic heart as cardiac injury induced by H$_2$O$_2$ can be partially ameliorated by treatment with compound C (71).

Although activation of AMPK may be beneficial in some peripheral tissues in response to hypoxia (19), the situation in the central nervous system may be quite different. For example, in hypoxic endothelial cells, eNOS is activated by direct phosphorylation by AMPK (28, 32) to presumably alter vascular tone to enhance blood flow. However, in stroke, this presumably protective effect of AMPK may be of limited benefit, as CBF is severely reduced during the acute occlusion (by the filament), making eNOS-induced vasodilatation a futile energy-consumptive process. In addition, under hypoxic conditions, enhanced NO production (via eNOS) can lead to increased levels of ONOO propagating free radical injury (72).

The consequences of AMPK activation to cell survival after ischemic insults in vivo were unknown previously. The majority of work has been done in vitro, often with transformed cell lines. AMPK activation with AICAR enhanced survival under conditions of reduced energy availability (glucose deprivation and glutamate excitotoxicity) in cultured hippocampal neurons (34). However, numerous investigators have now documented a pro-apoptotic effect of AICAR on numerous cell lines, including hepatocytes (63), neuroblastoma cells (64, 65), and mouse MIN cells (66) through stimulation of c-Jun N-terminal kinase, c-Myc, and caspase 3. In contrast, AICAR is protective in ischemic cardiomyocytes (67) and prevents ceramide-induced astrocytic apoptosis (68), suggesting that the effects of AICAR are dependent on both the cell type and model system used. AICAR is taken up into the cell and accumulates in the cytoplasm as the monophosphorylated nucleotide, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside, which mimics the effects of AMP and activates AMPK without altering cellular ATP levels (54). However, AICAR is nonspecific and has numerous other effects in the cell, including induction of adenosine, a known vasodilator via competition for nucleoside transport (69). AICAR reduced delayed cell death in the hippocampus after global ischemia in gerbils, but in this case, AICAR was utilized as an adenosine activator (70).

Our data demonstrate that activation of AMPK occurs in stroke and that this response, when augmented by AICAR, exacerbates tissue damage. A limitation to the use of AICAR in vivo studies was demonstrated by our physiological data, which revealed a transient, yet significant drop in MAP in AICAR-treated mice. The reduction in MAP may have increased infarction independently of the effects on AMPK by a decrease in perfusion of peri-infarct areas. Given the brevity of the drop, the maintenance of CBF (via LDF), and the dramatic neuroprotection seen with pharmacological inhibition of AMPK, this is unlikely to be the primary explanation. In addition, recent data suggest that AMPK activation may be detrimental to the ischemic heart as cardiac injury induced by H$_2$O$_2$ can be partially ameliorated by treatment with compound C (71).
suggesting that brain water content can vary in areas remote from the original injury (78, 79). Communication with non-ischemic brain can occur through transcallosal or intrahemispheric “diachisis” (80), leading to impairments in CBF, electrophysiological activity, and metabolism (81). It is likely that the signal for AMPK activation is transmitted globally throughout the brain as is suggested by the robust contratralateral rise in pAMPK levels.

Clearly, physiological responses are different in ischemic tissue compared with tissue that continues to receive relatively “normal” blood flow. Therefore, it is not at all unusual for reagents to affect the physiology predominantly in the ischemic area. However, in this case, we feel that there is good evidence in our data that the pharmacological treatments used did indeed have effects throughout the brain. As can be seen in Figs. 6–8, each of the pharmacological manipulations led to changes in pAMPK that can be seen on Western blot analysis. For example, compound C treatment appears to reduce pAMPK at 2 h of administration in both the ischemic and non-ischemic side compared with vehicle-treated mice. The effect does appear to be more prominent in the contratralateral (non-ischemic) hemisphere but is clearly present in the ischemic hemisphere as well. As can be seen in Fig. 7, C75 also appeared to have an effect on pAMPK levels in both the ischemic and non-ischemic hemispheres. These effects are delayed compared with compound C (seen at 24 h rather than 4 h after stroke). There also appears to be an effect of C75 at 24 h in the sham mice in that vehicle-treated animals have small elevations in pAMPK levels at 24 h compared with C75-treated sham mice. This may be because of the effects on reduced food intake, as sham animals undergo the same surgical procedures and anesthesia as do the stroke mice, although the suture is not advanced into the internal carotid. These animals also have a reduction in food intake in the first post-surgical day. We have shown previously that a 24-h period of food deprivation will indeed lead to pAMPK activation (36), and this demonstrates our ability to modulate both physiological and pathological elevations in AMPK. Finally, administration of the AMPK activator AICAR led to an increase in pAMPK levels in sham mice but did not clearly increase the stroke-induced rise in pAMPK. It is possible that the increase in pAMPK induced by stroke “masks” possible effects of AICAR as both conditions would be expected to increase pAMPK. The additional AICAR effect appears minimal, but it is important to recognize the limitations of Western blots when searching for small changes in protein expression. In addition, in these studies, we examine a homogenized sample of the ischemic and non-ischemic hemispheres, rather than dissecting out specific areas. Therefore, some “dilution” of signal could be expected, as some areas may have dramatic increases in pAMPK, whereas others have decreases in pAMPK due to either failure of protein synthesis or lack of stimuli to turn on pAMPK. Certainly changes in flow dynamics, drug delivery, and blood-brain barrier breakdown could lead to differential effects of pharmacological agents throughout the brain. However, we feel that there is strong evidence of “downstream effects” of our pharmacological manipulations as shown by pAMPK levels.

We hypothesize that activation of AMPK increases stress in already compromised neurons. Interfering with cellular energy production may augment the ischemic threshold, allowing cells to survive in energy-deficient but quiescent state until energy production can be restored. As in all neuroprotective studies, concern exists that when damage is evaluated at short time points after stroke (i.e., 2 h of reperfusion) that the neuroprotection may be transient. This is clearly relevant with AMPK, as in some models AMPK activation has clear anti-apoptotic effects. As apoptotic death is delayed and energy-dependent (82), the possibility exists that these compounds could simply delay neuronal death and enhance later apoptosis. Investigations are currently underway to address these issues. We postulate that reducing AMPK activation encourages “neuronal hibernation” at early points after the onset of ischemia, reducing neuronal energy failure and prolonging neuronal viability until blood and nutrient supply can be restored. Improving energy dynamics in ischemic neurons allows for the possibility of prolonging the “therapeutic window” in clinical stroke. The suggestion that neuronal metabolic pathways may represent an important and novel target for neuroprotection is demonstrated by our findings.

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46. fascinating, interesting, and important research in the field of neuroprotection following stroke.
Pharmacological Inhibition of AMP-activated Protein Kinase Provides Neuroprotection in Stroke
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