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Adenosine Monophosphate Activated Protein Kinase inhibition is protective in both sexes after experimental stroke

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Abstract

Sex differences in clinical and experimental stroke are now well recognized. Adenosine Monophosphate Activated Protein kinase (AMPK) is an important energy sensor that is activated in times of energy demand. Increasing AMPK is deleterious in experimental cerebral ischemia, at least in males. Interestingly, studies in peripheral tissues have suggested that there are sex differences in the regulation of AMPK in muscle after exercise. PolyADP ribose polymerase (PARP), a key mediator of ischemic cell death, stimulates AMPK activation, yet activation of PARP appears to be selectively detrimental in male brain. As interference with sex specific cell death pathways can determine the efficacy of experimental neuroprotective agents, and AMPK inhibition is a novel neuroprotective target, we examined the effect of AMPK inhibition in male and female mice. In this study, AMPK $\alpha 2$ gene expression (mRNA) and pAMPK protein levels were examined and found to be comparable between both sexes after transient middle cerebral artery occlusion (MCAO). Treatment with the AMPK inhibitor Compound C at stroke onset significantly reduced infarct size and neurological deficits 24 hours after stroke in ovariectomized female mice. Finally, genetic deletion of AMPK $\alpha 2$ in ovariectomized females was neuroprotective as assessed by smaller infarct volumes and improved neurological deficits when compared to wild type littermates. This work demonstrates that AMPK activation is deleterious in experimental stroke, and this effect is independent of sex.

Keywords

Cerebral ischemia; sex difference; AMPK; stroke; animal models

Introduction

Sex differences in clinical and experimental stroke are now well recognized [16]. It is well accepted that variations in the levels of gonadal hormones contribute to ischemic sexual dimorphism. However, the effect of biologic sex on stroke outcome is evident in clinical populations that have minimal differences in hormone levels, such as neonates and the

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elderly [7]. In addition, the response to chemical and hypoxic insults differs *in vitro* based on whether tissue is derived from males or females [2,12]. This has important consequences for the development of effective neuroprotective agents. Females, at least in experimental stroke models, are more sensitive to caspase-mediated cell death than males. Males, in contrast, appear to be exquisitely sensitive to the detrimental effects PolyADP ribose polymerase (PARP) activation [11]. The downstream events triggered by PARP including nuclear translocation of apoptosis inducing factor (AIF) and subsequent caspase-independent cell death play a significant role in male brain as evidenced by the dramatic neuroprotective effects seen after interference with this pathway [17].

AMP-activated protein kinase (AMPK) is a known sensor of peripheral energy balance. AMPK is activated when cellular energy supply is low, as signaled by increasing intracellular AMP and declining ATP levels [15]. In the periphery [12], AMPK acutely regulates cellular metabolism and chronically regulates gene expression, reducing energy storage (fatty acid, lipid and protein biosynthesis) and increasing energy utilization (fatty acid oxidation) [8]. Recently, it has been demonstrated that AMPK activation occurs rapidly in the ischemic brain [8,12]. Somewhat surprisingly, stroke-induced activation of AMPK is deleterious, possibly by enhancing metabolic failure in the ischemic brain. Both pharmacological inhibition and genetic deletion of AMPK are neuroprotective in male mice [8]. Interestingly, there are sex differences in the response of AMPK activation in exercising muscle. Males have a significant increase the activity of AMPK $\alpha 2$, but no increase was seen in women [14]. As activation of PARP, the dominant mediator of stroke-induced cell death in males also stimulates AMPK [4], and we have shown interference with sex specific cell death pathways can determine efficacy of experimental neuroprotective agents [9] we investigated the role of AMPK inhibition, a treatment that is protective in males [10], in female mice after stroke utilizing both genetic and pharmacological techniques [10].

Materials and Methods

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 of University of Connecticut Health Center. Focal transient cerebral ischemia was induced in female mice (20-25 gms) by right middle cerebral artery occlusion (MCAO, 90 minutes duration) followed by reperfusion as described previously [10]. In separate animal cohorts, blood glucose, physiological measurements, femoral arterial blood pressure, and cortical perfusion (Laser Doppler Flowmetry; LDF) were evaluated throughout MCAO and early reperfusion as described previously [10]. Mice used were female wild type mice from Charles River and AMPK $\alpha 2$ KO mice [10] with corresponding littermates. Compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]-pyrimidine, EMD4Biosciences) or vehicle (saline) was injected to the wild type mice at the onset of stroke at dose of 10 mg/kg (i.p, injection volume 0.2ml per 20g animal body weight). Animal body temperatures were controlled at physiological range with set-point heating pads throughout MCAO and reperfusion period.

Ovariectomy was performed to remove gonadal estrogen 10 days prior to MCAO. We utilized ovariectomized animals in this study to control for levels of gonadal hormones and to reduce experimental variability associated with varying estrogen levels during estrous. This is also a more appropriate model for the “at risk” clinical population, as the vast majority of women are postmenopausal at the time of their stroke. Estrogen levels and uterine weights were assessed to confirm loss of estrogenic effects. Blood samples from all female mice were collected at the time of sacrifice, spun at 6,000 r.p.m. for 10 minutes at 4 degrees and assayed by ELISA for estrogen (IBL, Germany). Estrogen levels in four male

wild type mice were also assayed as a control. Uteri of ovariectomized mice were removed at the time of sacrifice and weighed; five intact female uteri were used as a control.

In this focal reperfusion model, MCA occlusion causes measurable neurological deficits. Neurological deficits were scored in the intra-ischemic period and 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 affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling. Any animal without deficit in the intra-ischemic period was excluded from the study.

After the animals were scored at 24 hours after stroke, the brain was removed and cut into 5 2-mm slices and stained with 1.5% 2, 3, 5 triphenyltetrazolium (TTC) solution. The stained slices were fixed with formalin (4%) then digitalized and infarct volumes were analyzed using computer software (Sigma Scan Pro) as previously described [10].

RT-PCR was used to analyze levels of AMPK α 2. Total RNA in the ischemic hemisphere was extracted 6 hours after the onset of 90 minute MCAO with Trizol (Invitrogen) per manufacturer's instructions. RNA purity and concentration was quantified using a nanodrop (Thermo). cDNA was created using the iScript cDNA synthesis kit (Bio Rad), and qRT-PCR was performed using the sSo-Fast Evagreen qRT-PCR kit (Bio-Rad). mRNA was quantified for the following genes: AMPK α 2 and GAPDH as housekeeping genes. Primers were AMPK α 2 forward 5' CGC CAC TCT GCT GAT GCA 3', AMPK α 2 reverse 5' AAA TAG GTA GCT GGG CAA ATC CT 3', GAPDH Forward 5' ACC ACC ATG GAG AAG GC 3', and GAPDH reverse 5' GGC ATG GAC TGT GGT CAT GA 3'.

Protein levels of pAMPK (phosphorylated on threonine¹⁷², the activated form of AMPK) were detected using Western blots as described previously [10]. 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). Blots were probed for p-AMPK (1:500, Cell Signaling Technology). Beta-actin (1:5000; Sigma) and was used as loading controls. Blots were incubated overnight in primary antibody at 4°C in TBS containing 4% bovine serum albumin and 0.1% Tween20. Secondary antibodies (goat anti-rabbit IgG 1:5,000 for p-AMPK, goat anti-mouse IgG 1:5000 for Beta-actin; Chemicon) were diluted and ECL (pico) detection kit (ThermoScientific) was used for signal detection [10].

Statistics were performed either with one-way ANOVA with Tukey post-hoc test (for multiple comparisons, when appropriate) or Student t-test except for neurologic deficit scores, which was done by Mann-Whitney U test. A p value < 0.05 was considered to be statistically significant. Data were expressed as Mean $\pm$ sem except for the neurological deficit scores which were presented as median and range. Investigators performing behavioral and infarct size analysis were blinded to treatment group.

Results

The levels of estrogen in ovariectomized female mice were comparable to male mice control (Table 1a) demonstrating loss of gonadal estrogen. Uterine weights were significantly lower than non ovariectomized controls (Table 1b). There were no significant sex differences in AMPK α 2 mRNA levels either in sham mice or in stroke mice (Figure 1a). Although stroke mice had a trend towards decreasing AMPK α 2 mRNA, this effect was not significant (Figure 1a). Activation of AMPK was seen after stroke, as noted by a robust increase in pAMPK protein in both female ovariectomized and male mice. Densitometry confirmed that

levels of pAMPK were elevated after stroke, but were equivalent between the sexes in both sham and stroke cohorts (Figure 1b and 1c).

Compound C, an AMPK inhibitor known to be protective in male mice after MCAO [10] significantly reduced infarct volumes in female ovariectomized mice (cortex: Comp C 33.9 ± 5.5 vs. vehicle 55.0 ± 4.0 , $p < 0.05$; striatum 39.0 ± 7.8 versus 63.9 ± 5.6 , $p < 0.05$; total comp C 29.4 ± 4.8 vs. 47.1 ± 3.1 , $p < 0.05$). This beneficial effect of compound C was also reflected in the neurological deficit scores (comp C 1.5(1.0) vs. vehicle 3.0(0.5), $p < 0.05$) (Table 2). There was no pre-mature mortality in vehicle treated or Compound C treated groups.

Consistent with the effect of compound C treatment, deletion of one of the subunits of the catalytic isoform of AMPK, AMPK $\alpha 2$, was also neuroprotective as demonstrated by decreased infarct volumes (cortex KO 36.7 ± 4.2 vs. WT 53.6 ± 4.4 , $p < 0.05$; striatum KO 42.8 ± 3.3 vs. WT 60.2 ± 4.7 , $p < 0.05$; total KO 32.9 ± 3.1 vs. WT 49 ± 2.9 , $p < 0.05$) (Figure 3). In addition, AMPK $\alpha 2$ KO mice had reduced neurological deficits (WT 3.0(1.0) vs. KO 2.0(0.5), $p < 0.05$) compared to WT littermates (Table 2). There is no pre-mature mortality observed in WT and KO groups. There were no differences in mean arterial pressure, pO_2 , pCO_2 , or blood glucose between WT mice and AMPK $\alpha 2$ KO mice (Table 3). In addition, LDF was equivalently reduced during ischemia and was restored equally in early reperfusion (Table 3).

Discussion

This study demonstrates several important new findings regarding the role of AMPK in stroke. Previous studies on AMPK in neuroprotection were conducted using male mice or cells derived from mixed sexes [1] [8] [10]. In the present study we found that female mice also benefit from pharmacological inhibition of AMPK or genetic deletion of the $\alpha 2$ catalytic subunit, and confirms that AMPK activation is detrimental in experimental stroke. AMPK inhibition also led to improvement in short-term neurological deficits in ovariectomized mice. In addition, AMPK $\alpha 2$ gene expression and protein levels were comparable between both sexes. Therefore, AMPK inhibition is equally efficacious in male and female animals in protecting the brain from induced stroke. The lack of sex differences suggests that the mechanism of AMPK mediated neuroprotection may involve multiple mechanisms that are independent of sex specific cell death pathways, or more likely, work on more “upstream” pathways mediating ischemic cell death, i.e., stroke-induced metabolic failure.

There has been considerable debate over the role of AMPK in the ischemic brain [8]. Several studies, both in vivo and in vitro have demonstrated that acute AMPK activation is detrimental; however others have suggested that it may play a neuroprotective role. AMPK is an important energy sensor in peripheral tissues and it is activated when the energy level is low. Stroke represents a state of severe energy deficiency, as there is no available substrate for the energy consumptive pathways activated by AMPK. Therefore activating AMPK in the setting of severe metabolic failure exacerbates injury. The mechanisms through which acute AMPK activation exacerbates stroke injury are not yet clear. Studies have suggested exacerbated lactate accumulation, autophagy and increased glucose due to unregulated glucose-transporters in the reperfusion phase may contribute to stroke damage [For review see 8]. As neurons are known to have minimal capacity to perform anaerobic glycolysis, ischemia leads to progressive lactic acidosis and metabolic failure and activating AMPK perturbs metabolism still further. Inhibition of AMPK leads to a reduction in metabolic demand, and under conditions of acute ischemia, protects the brain [8]. The

reduction in AMPK, metabolic drive, and lactate accumulation protects independently of sex specific cell death pathways.

In clinical settings, several reports have shown sex differences in the response to pharmacological agents that are commonly used in stroke patients [5,13]. For example, Tirilazad, a non-glucocorticoid 21-aminosteroid, reduced mortality and enhanced functional recovery after subarachnoid hemorrhage in men, but not in women [5]. In addition, sex differences in the response to acute stroke therapies (i.e., tissue plasminogen activator) are also emerging [3,6]. Most preclinical studies exclusively investigate male animals, in part to avoid the confounding effects of estrogen. The direct effects of estrogen on AMPK remain unknown, as we did not directly examine AMPK levels in ovary-intact or estrogen-supplemented ovariectomized females. We chose to use ovariectomized mice in this study mainly as this may be a more appropriate model of women at highest risk for stroke, the majority of which are postmenopausal. Unlike what is seen in the periphery, where sex differences are seen in AMPK activation in muscle [14], brain mRNA and protein data suggest that this does not occur in the brain. However, differences in tissue types, models, species (human vs. mice) may also contribute to these differences.

In conclusion, this study demonstrates that acute AMPK activation is detrimental in experimental stroke. Inhibition of AMPK is neuroprotective in female mice as shown previously in males [10]. As there is no sex difference in the response to AMPK inhibition after an induced stroke, AMPK may be an important novel target for stroke treatment.

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Figure 1. mRNA and pAMPK levels after stroke are equivalent between the sexes

Brains were collected 6 (for mRNA) or 24 hours (for Western) after MCAO. A. mRNA did not differ between males and females, $n=7$ per group. Data are expressed as Mean $\pm$ SEM. Statistics were performed with one-way ANOVA with Tukey post-hoc test. B and C. pAMPK was induced after stroke in female and male brain and levels between the sexes were not significantly different. pAMPK densitometry was done with comparison to beta-actin controls. Each Western blot represents pooled samples derived from 2 sham and 4 stroke mice, with all experiments repeated in triplicate with three separately derived samples.

Figure 2.

Compound C reduced infarct volume in female ovariectomized mice subjected to 90 minutes MCAO with 24 hour survival. Compound C was injected to mice at the onset of stroke at 10mg/kg. n=8 per group. Cortical, striatal and total hemisphere infarction volumes were calculated (corrected for edema, percentage of non-ischemic hemisphere). * P<0.05 versus control (Student t-test); data are expressed as Mean±SEM.

Figure 3.

AMPK $\alpha 2$ deletion reduced infarct volume in female ovariectomized mice subject to 90 minutes MCAO with 24 hours survival (n=7 per group). Cortical, striatal and total hemisphere infarction volumes were calculated (corrected for edema, percentage of non-ischemic hemisphere). * P<0.05 versus control (Student t-test); data are expressed as Mean $\pm$ SEM.

Table 1
Estrogen levels (a) and uterine weights (b) in female ovariectomized mice

Table 1a.				
Compound C experiment		AMPK α2 KO experiment		
Vehicle	Compound	WT control	KO	Male control
7.3 $\pm$ 0.52	6.6 $\pm$ 0.60	6.1 $\pm$ 0.68	6.7 $\pm$ 0.68	7.3 $\pm$ 0.78
n=8	n=8	n=7	n=7	n=4

Table 1b.				
Compound C experiment		AMPK α2 KO experiment		
Vehicle	Compound C	WT	KO	Intact female control
45.6 $\pm$ 13.7*	39.7 $\pm$ 4.0*	39.4 $\pm$ 5.6*	34.0 $\pm$ 3.8*	75.6 $\pm$ 2.8
n=8	n=8	n=7	n=7	n=5

Estrogen levels in the serum (pg/ml) (table 1a) and uterine weights (mg) (table 1b) measured in Compound C treated groups vs. vehicle control and in AMPK α 2 KO mice vs. WT littermate control. Estrogen levels in the ovariectomized mice were comparable to that of control male mice. Compound C was injected at the onset of stroke at 10mg/kg (i.p.). All serum samples and uterine samples were obtained at 24 hours after stroke at the time of sacrifice. Data are expressed as Mean $\pm$ SEM. Statistics were performed with one-way ANOVA with Tukey post-hoc test.

* p<0.05 vs. intact female controls. All ovariectomized female mice had significantly lower uterine weights than the intact female group.

Table 2
Both compound C treatment and AMPK α 2 deletion reduced neurological deficit scores of female ovariectomized mice after stroke

Vehicle treated	Compound C treated	WT	AMPK α 2 KO
3.0(0.5)	1.5(1.0) *	3.0(1.0)	2.0(0.5) *

Compound C was injected to mice at the onset of stroke at 10mg/kg.

* $P < 0.05$ versus control (Compound C treated versus Vehicle, AMPK α 2 KO versus WT, Mann-Whitney U-test); n= 8 per group in Compound C treatment group, n=7 per group in AMPK α 2 KO/WT groups. Data are expressed as Median (interquartile range).

Table 3

There is no difference in physiological parameters and cerebral blood flow (LDF) between the female AMPK $\alpha 2$ KO mice and WT mice.

		pH	pCO ₂	pO ₂	Glucose	MABP(mm Hg)	LDF % base level	
							Intra-Ischemia	Reperfusion
WT	Pre	7.35±0.03	44.9±1.4	126±6.0	130±14	72.3±5.3	12.4±1.7	82.7±10
	Intra	7.30±0.01	43.5±2.3	114±9.0	138±6.5	78.8±4.5		
KO	Pre	7.38±0.01	43.5±3.0	119±5.5	137±12	81.5±7.2	12.1±1.2	90.0±6.0
	Intra	7.29±0.01	45.5±5.5	116±2.8	129±13	83.5±3.0		

Blood sample were collected from the femoral artery at the onset of MCAO or 60 min from the onset of MCAO. Cerebral blood flow was measured by using Laser Doppler Flow (LDF). Data are expressed as Mean±Sem. n=4 per group. Student's t-test was used to compare the difference of each parameter between the KO mice and WT mice. P<0.05 is considered to be significant statistically.