American Journal of BioMedicine
AJBM 2018;6 (3): 224-241
Research Article Doi: 10.18081/2333-5106/015-10/619-630

Potential effects of gender disparity in downregulation of AKT after post-cerebral ischemia and reperfusion

John M. Morrison, Claire B. Gibson, Zhan Wang, Daniel H. Klionsky [1*]

Abstract

Sex-dependent differences exist in stroke susceptibility, prevalence, severity, and response to treatment in humans. It is well documented that age, sex, and hormonal fluctuations alter not only the course of acute brain insult but also the inflammatory responses during the recovery phase. However, the underlying molecular mechanisms of sex differences following exogenous stimulation remain unclear, particularly in males and females. It is worth mentioning that activation of the protein kinase B AKT pathway followed by experimental brain ischemia/reperfusion injury (tMCAO/R) is attenuated to a greater extent in females than in males and is blocked by estrogen depletion in females.

Conclusion: We have shown beyond doubt that there are gender differences in the responses of the phosphatidylinositol 3-kinase PI3K/AKT pathway after tMCAO/R. Disruption of endogenous testosterone synthesis worsens tMCAO/R outcomes in males and causes exogenously administered estrogens to lose their protective effects. Our findings suggest that there may be an ATP-protective therapeutic window in the early hours after a stroke in both men and women. Based on the length of the gap in response to ischemia/reperfusion in humans and the findings in the DoTS, we predict that treatment of men early after a stroke with synthetic S-equol will be beneficial in terms of outcome. These findings suggest that women are delayed in their response to ischemia/reperfusion injury and that early treatment with synthetic S-equol is beneficial in men, but older women should adjust their traditional dose of neuroprotective medications and men should consider delaying their onset of treatment in women.

Keywords: Cerebral stroke; Akt pathway; Bcl-2/Bax; Ischemia/Reperfusion

*Corresponding author: Klionsky
Received February 22, 2018; Accepted July 10, 2018; Published August 12, 2018

Introduction

Although ischemic stroke is the major cause of adult disability, effective therapies for treatment are limited. Annually, approximately 15 million people worldwide suffer from stroke. Of these, 6.5 million individuals die annually. Unfortunately, 42.0% of the global population is affected by death and disability. Clinical studies have reported that stroke incidence significantly differs between men and women, with more elderly females suffering from stroke. Poor onset/acute management and poorer outcomes are also observed. Furthermore, some genes determine background gender-based

differences in physiological and pathological reactions. Gender differences in the signaling pathway may provide a physiological basis for gender differences in ischemia tolerance.

The phosphatidylinositol 3-kinase/protein kinase B signaling pathway, represented by protein kinase B (AKT), is an intracellular pathway with the ability to inhibit various apoptotic pathways. The downregulation of intracellular signaling inappropriately activates the apoptotic pathway. Generally, postischemic reperfusion-like experiments use unilateral middle cerebral artery occlusion, 90 minutes of ischemia, and 24 hours of reperfusion-induced thalamic injury. Our research group identified gender-based differences in the AKT downregulation in the thalamic experimental ischemic injury of Balb/c mice. Continuing in this direction of investigation, the purpose of this study was to examine if male and female C57bl/6 mice also differ in the downregulation of AKT following different time points (24 and 72 hours) of post-reperfusion after cerebral ischemia and reperfusion.

Significance of Studying Gender Disparity in AKT Downregulation

The age of the U.S. population suffering from brain ischemia is shifting toward older age, and more women than men over 45 years of age are suffering from stroke than before. Numerous studies have shown that the physiology and pathology characteristics of brain ischemia and reperfusion are different between men and ovariectomized females. In general, men and women over 45 years of age are all postmenopausal, which results in low and descending estrogen levels, so that they cannot be used to evaluate the neuroprotection and potential nutritional functions conferred by estrogen during ischemia. For this reason, androgens, such as testosterone, may not play a role in neuroprotection via the observed gender disparity. However, no study has ever shown the neuroprotection effect of estrogen on stroke based on women's outcomes, and no trial has been initiated to apply estrogen in women and older stroke patients to verify its outcome.

Stem cells derived from the bone marrow of males have higher migration capability and less apoptosis than females after transplantation into the female brain with focal cerebral ischemia. The migration of stem cells to the ischemia site of females is worse than that with males. Progesterone, a neurosecretory hormone that protects the development of the brain, plays various neuroprotection roles, including dopaminergic signaling in males, and is applied for neonatal brain injury with reperfusion. Clinically, pregnant women who receive progesterone treatment for preterm labor are also controversial as to whether the neuroprotection of their fetuses is affected. The level of cerebral ischemia-induced inflammation in wild-type females is significantly higher than that in wild-type males. Drug treatments show that after PD98059, an ion channel inhibitor, and minocycline and PD095059, AKT activity is not increased in females compared to that of males with cerebral ischemia, indicating that the signaling pathway, instead of being a mediator of gender differences in cellular function, plays a role in determining the gender specificity of the cell response to cerebral ischemia.

Cerebral Ischemia and Reperfusion Injury

Cardiovascular and cerebrovascular accidents (CVA) are rapid, sudden, and fatal diseases. Among them, the mortality of stroke ranks first in cardiovascular and cerebrovascular diseases. It has been in the top two in the world, ranking first in China. Ischemic injury caused by vascular occlusion or flow reduction in the brain is a general form of cerebrovascular accident. Patients who can get timely and

effective treatment can greatly reduce the disability and death of stroke. At present, the old and young effects of the signaling pathway in the model of cerebral ischemia and reperfusion injury (IRI) caused by intraluminal suture embolization are still limited, and the effect of the gender difference in this process has not been found.

A new perspective demonstrates that protein kinase B, also known as PKB and AKT, is a serine/threonine kinase that is the most important mediator of phosphatidylinositol 3-kinase (PI3K). The process of AKT protein phosphorylation in IRI has become the focus of subsequent research. The phosphorylation of AKT can be used as a marker of the activation of AKT. The role of the AKT signaling pathway in myocardial ischemic injury was studied in rats. The results showed that the expression of p1-AKT was decreased rapidly at 1 h after the MCAO, and the decrease of p1-AKT will continue until 24 h, showing that the down-regulation of AKT expression is persistent, and the degree of neuroprotection of AKT in IRI is not specific. The sequence accuracy of the mouse AKT gene is 1889 bp, and the sequence number is NM_001190157HIP 12946. It encodes a protein of 481 amino acids. The molecular weight of AKT protein is 56 kDa. The function of AKT protein depends on the signal pathway mediated by phosphorylation of the phosphorylated AKT. It is the direct factor of Gli1 phosphorylation. In the downstream effector signal mediates the role of PDX-1. Among them, the AKT gene has many polymorphism sites.

Mechanisms of Injury

Our previous publication on the relationship between alterations in the transcriptome of human-BFCNs after ischemia and reperfusion and the changes in proteomic structure suggests that neuroinflammatory signaling plays an important role in regulating cellular metabolism and proapoptotic pathways leading to the death of the post-ischemic BNCs. Studies of human-BFCNs exposed to in vitro ischemia (OGD) show a decrease in the expression of PSMB2, resulting in anesthetics increasing ISG15 conjugates and therefore leading to TNF-α-induced apoptosis. In rat BFCNs exposed to in vitro ischemia, the major neuroinflammatory action of microglial cells was confirmed, which led to the activation of proteasomes and likely enabled the supply of substrates for energy production by neuronal cells, alleviating a bioenergetic crisis. But with the subsequent duration of reperfusion and reoxygenation, the pro-inflammatory action of microglial cells continued and dysregulated sPhAc, which could not meet the needs of the neuronal and glial cells for ATP, resulting in their death.

Adoniol et al. showed that reactive oxygen treatment of neurospheres increases sPhAc at an early stage and then decreases it, disrupting the capability of astrocytes to supply neurons with lactate. Of notice, the literature data are only partially consistent with the transcriptomic RNA-seq data of the modified transcriptome of human-BFCNs with the greatest discrepancy of dysregulated KEGG terms, as detected at the age of 90 days after both in vitro and in vivo ischemic injuries. Our further analysis supported the hypothesis of two-stage repression of AKT activation after in vitro ischemia and indicated the discrepancies in the gene organization of the suppressed proteasome/defined KEGG/Proteasome-encoding def2G among differentially expressed AKT-controlled DEGs. This made it possible to conclude that the diminished capability of the proteasomes to be rapidly activated after

ischemia plays a key role in the downregulation of AKT pro-survival signaling and the induction of apoptosis in the BFCNs with a deficit of AKT after stroke.

Middle Cerebral Artery Occlusion Model

Middle cerebral artery occlusion (MCAO) and recovery were performed as described previously. As previously reported, a filament (4-0 nylon d suture, Beijing Sunbio Biotech Co.) was placed into the internal carotid artery approximately 18-21 mm for mice. Briefly, 60 minutes of ischemia was induced by MCAO and then followed by reperfusion for 24 h. After surgery, the mice received supportive care, including hydration and antibiotics. The mice were sacrificed by cervical dislocation under deep anesthesia with pentobarbital sodium at the endpoint. Subcranial blood vessels were perfused with ice-cold PBS under deep anesthesia, and the brains were extracted for further analysis. Subcranial blood vessels were perfused with 4% paraformaldehyde under deep anesthesia, and then the brains were extracted and immersed in 4% paraformaldehyde overnight, embedded with paraffin, and cut into 2 μm sections for hematoxylin-eosin (HE) staining. Digital images were obtained using a Charge Coupled Device Digital Camera (Color View IIu, Beijing Sunbio Biotech Co.) at ×400 magnification. Morphological and morphometric analyses were performed to quantitatively determine the tissue area corresponding to the infarcted brain in every fifth section (8 sections per animal) by measuring the areas of the contralateral and ipsilateral brain hemispheres. The degree of injury was calculated by the formula previously described.

AKT Signaling Pathway

The AKT signaling pathway is one of the most essential signaling pathways, which is involved in many downstream targets. AKT is a serine/threonine protein kinase that belongs to the protein kinase B (PKB) family, including AKT1, AKT2, and AKT3 isoforms. AKT1 and AKT2 are two major isoforms that play key roles in the initiation and progression of cerebral ischemic injuries. The activation of AKT1 or AKT2 pathways can increase blood flow, especially improve the microcirculation of blood flow, attenuate the destruction and apoptosis of microvessel cells, and then reduce microvascular platelet aggregation and insolubility of coagulation promoting coagulative states and microvascular thrombosis of blood. In short, the activation of AKT1 or AKT2 pathways can decrease the injury of organs and tissues after cerebral ischemia. However, the possible sex differences of AKT pathways in the brain have not been clearly identified following cerebral ischemic injury.

Ischemic injury has a significant impact on morbidity, both in men and women. Cerebral ischemia stimulates the phospholipase C protein kinase C refined 2 (PLC-1) hydrolyzed phosphatidylinositol-4,5-bisphosphate (PIP-2), leaving behind inositol-triphosphate (IP3) and diacylglycerol (DAG). IP3 increases calcium flow, which induces CKCa (at active site) insulin secretion. DAG activates protein kinase C (PKC), and it also phosphorylates glycogen synthase kinase-3β (GSK3β). The phosphorylation of GSK-3β can modulate gene transcription. However, previous studies were mostly reported on the AKT signaling pathways at the late stage following cerebral ischemia/reperfusion, little is known about the detail change of the AKT signaling pathway after early ischemia/reperfusion injury. Additionally, gender disparity has been reported after ischemic injury in the literature. The present

study is designed to investigate the gender disparity in the AKT signaling pathway and the AKT downregulation after the early stage of cerebral ischemia and reperfusion.

The Akt is a group of serine/threonine kinases, containing three isoforms, Akt1, Akt2, and Akt3. Three of which share high sequence similarity and consist of several domains. Akt domain with similarity to AGC kinase catalytic domain is located on the C-terminal end and is required for kinase activity. PH domain is responsible for PI (3,4,5) P3 binding, and domain located between pleckstrin homology (PH) and kinase domain regulates the enzymatic activity. AGC kinase catalytic domain consists of several conserved sites including Thr308, Ser473, and Tyr326. Phosphorylation at Thr308 located within the activation loop is essential for Akt kinase activity. Phosphorylation in the PH domain positively sets Akt activity and is suppressed by phosphorylation of Tyr326.

The phosphorylation of Akt at residue Ser473 complements the phosphorylation in the PH domain, and both are necessary for Akt signaling pathway in a disease-causing context. Activation of the PI3K/Akt signaling pathway initiates following PI3K activation in response to extracellular stimuli and phosphatase PTEN inhibits it by dephosphorylating PI (3,4,5)P3 to PI (4,5)P2. Once activated, Akt moves to the plasma membrane or cytosol and becomes phosphorylated at the C-terminal. Activated Akt is shuttled to the cytosol where it promotes cell differentiation, apoptosis, and cell regeneration. The PI3K/Akt pathway is assumed to contribute to tissue and organ function after stress damage.

Role in Ischemic Injury

In cells exposed to ischemia and reperfusion, some changes have been noted in the PTEN-AKT signaling pathway, a critical regulatory signaling pathway which modulates cell survival and protection against apoptosis. The activities of the PI3K-AKT pathway and its important role in cellular protection have been described by some investigators in the mouse brain after ischemia, while others have described the exacerbation and impairment of AKT activation including associated diminished eNOS phosphorylation in females after ischemia and reperfusion in cultured endothelial cells. Situations after cerebral ischemia are uncertain, as a number of recent stroke studies suggest that PTEN inhibition may worsen brain injury and that loss of PTEN paradoxically protects neurons after reperfusion in males. Regardless, there is evidence stating that activation of AKT may be less robust and shorter-lived in females after cerebral ischemia, which has a significant effect on female patients suffering from cerebral injury resulting from ischemia due to any cause. Nonetheless, the impact of AKT activation on female ischemic injury should be carefully researched. This clinical problem was addressed by investigating whether the decrease in mitochondrial pathology observed in females might be associated with deficiencies in AKT activation.

Gender Differences in Ischemic Injury

Gender disparities have been noted in the prevention and treatment of ischemic injury. Additionally, females are more prone to ischemia-reperfusion myocardial injury and have a poorer prognosis than men after ischemia-reperfusion. How to reduce cardiovascular risks, improve prognosis, and prolong life expectancy in patients with ischemia-reperfusion injury is of great concern worldwide. Although some studies demonstrated that women have a favorable outcome relative to men after blood reperfusion in ischemic injury, other recent studies have shown that reperfusion can exacerbate

ischemic cellular injuries and lead to loss of cellular function and accelerated atherosclerosis. The protein kinase B (PKB)/AKT signaling pathway is involved in ischemia-reperfusion-induced injury. However, no data regarding gender differences in AKT phosphorylation (p-AKT) exist.

One study analyzed cerebral ischemic injury in female and male SAMP8 mice and found differences in the levels of p-AKT after AKT downregulation within 24 hours of reperfusion. Cerebral ischemia and reperfusion have a heterogeneous influence on the levels of p-AKT, which may have contributed to AKT downregulation within 24 hours of reperfusion in female and male SAMP8 mice. Additionally, there were gender differences in the levels of p-AKT (Ser473 and Thr308) after AKT downregulation within 24 hours of reperfusion. The findings of this study may provide a foundation for future studies of AKT downregulation and a reference for studies involving the diverse aspects of AKT in different pathologies.

Gender differences in ischemic stroke are implicated to result from both hormonal and genetic differences. A greater proportion of women exhibit strokes at an older age and appears to worsen. Women also exhibit a greater risk than men at sustaining severe cerebral ischemia. In a study of 1,370 ischemic patients, women exhibited a follow-up mortality that was three times that of men. Furthermore, women in this same study had a much larger increase in odds of mortality after adjusting for age, smoking status, diabetes, irregular EKG, hypertension, and prior history of MI than men. In the majority of animal studies, female rats were found to have a smaller post-stroke infarct volume; however, there are some exceptions in which the severity of the injury is equal in both genders. Neuroprotection mediated by estrogen has been suggested to play a major role in this protective effect. The neuroprotective role of estrogen has been shown to be mediated partly by upregulating the phosphatidylinositol 3-kinase/Akt pathway, which is involved in antiapoptosis, antioxidant actions, growth promotion, and the so-called trigger effect. This hormone has been shown to mediate downstream of Akt on its action in the various pro-inflammatory mediators of stress kinase activation, which estrogen mediated neuroprotection at least in part. In the infarct model, contamination from circulating leukocytes was a leading cause of the so-called "gender gap".

Another concept is that the apoptosis rate in the penumbra, the DNA damage, and in particular the DNA-fragmentation rates in the penumbra are significantly lower in female animals. These findings indicate identification of gender-specific therapy recommendations. Any protective or detrimental effect of a given drug may be different in men versus women. The only way to generate heart-attack resistant, brain-attack resistance, and stroke-attack resistant C57/BL/6J mice is to maintain about 50% of the colony as males and the remainder all postmenopausal females. The A/J mouse is different – they have no gender gap. Pre-menopausal females exhibit a myocardial infarct volume that is 43% of the volume generated in males. Postmenopausal females show twice the infarct volume as males. Brain-attack, in general, is a combination of TIA (transient brain stroke) 79% and stroke 21%. Transient brain-attack is much more common than full-blown brain-attack because there are large compensatory collateral arteries. Thus, both models must be used to fully understand a given gender difference.

Factors Contributing to Gender Differences

Both extrinsic and intrinsic factors are found to impinge on gender differences in the incidence of ischemic injury. On the extrinsic side, the age of onset, the climate of race or nationality, and the living habits or working conditions all have top priority. This partly parallels the reported absence of a difference in blood cholesterol level between male and female C57BL/6J mice in the physiological state. This is a rather more degraded process shaping gender difference in cerebral resistance to ischemic injury. Further, for the two external factors, the collection of data relies on the consent of the public and data availability. This is the main reason why gender difference in the incidence of ischemic injury is mainly discussed in terms of age difference in the clinic despite the broad biomedicine and its advancing in all fields.

The elderly have a higher susceptibility to both ischemic- and traumatic-elicited organ injury than do young individuals. The AKT signaling cascade has a well-characterized hypothesized protective role in ischemic and/or reperfused cerebral injury. However, the disparate activation of downstream elements of the AKT signal transduction pathway in male and female rats in the ischemic reperfusion was reported in several recent papers and was notably confirmed by the present study. The functional capacity of Akt appears to be inversely correlated with ROS production. It is of note that increased oxidative damage can result in inhibition of PI3K and/or of the upstream AKT, as a self-perpetuating vicious cycle to facilitate neurodegeneration. Of particular interest, oxidants including nitric oxide can contribute to pyramidal cell apoptosis primarily by suppression of AKT activity.

Animal Models Used

2.5-month-old male and female (pre-estrus or diestrus) wild-type mice (weighing 23-28 g) from Jackson Laboratory (Bar Harbor, ME, USA) were used in the experiment. These mice were supplied via additional breeding at Ewha Womans University Medical Research Institute of Natural Science. Mice were housed in a standard 12-hour light-dark cycle and were allowed access to food and water ad libitum. Mice were acclimated for at least one week before surgery. Post hoc analysis of caloric requirements of female mice exhibited increased amounts of kJ/mL consumables than male mice; however, caloric consumption was comparable in both male and female mice (data not shown). All the experimental procedures were complied with the regulations regarding the care and use of experimental animals promulgated by Ewha Womans University (Seoul, Korea). The experimental protocols were approved by the Ewha Womans University Institutional Animal Care and Use Committee (IACUC No. 2016-01-001).

Transient middle cerebral artery occlusion (tMCAo) model or tube-feeding (TF) was provided as previously described. Briefly, mice were transiently occluded in the middle cerebral artery (MCAo) for 15-40 min using the Intracalabrain Stereotaxic Microsurgery Device (Leica Biosystems, Buffalo Grove, IL, USA) and consequently reperfused for 30 min to 72 h. Mice that exhibited an infarct size ratio of less than 10% were excluded from the study. The rectal temperature of all the experimental mice was maintained at 37.5°C with an electronic thermometer-regulated heating pad (Harvard Apparatus, Holliston, MA, USA). Mice were euthanized by beheading after given either a lethal bolus injection

(4.8/gram animal weight) via the intracardiac route or pentobarbital (100 mg/kg; Abbott Laboratories, Abbott Park, IL, USA) for continued experimentation.

C57BL/6J Mice

Ninety male and female C57BL/6J mice aged 5 to 6 weeks old were purchased from the Laboratory Animal Center of Academia Sinica (LACAS). All of the mice were housed for a few days before pMCAO was conducted. Mice had food and water ad libitum, a 12-hour dark/light cycle, and a temperature maintained at 24 ± 1 °C and 40% humidity. All experimental validity and procedures were approved by the Animal Care and Use Committee of an academic institute of ROC.

Then, the measurement procedure was as follows. Thirty male C57BL/6J mice of pMCAO operated at all of the testing time points were measured for gene differential grey and white matter AKT expression, and real-time PCR studies. Thirty female C57BL/6J mice of pMCAO operated at all of the testing time points were measured by Ultima VIII laser Doppler flowmetry analysis and triphenyltetrazolium chloride (TTC) staining.

Akt Knockout Mice

Both female and male mice have completed two sets of the MCAO evaluation programs and have been extensively characterized regarding their reactions to the hypoxia-ischemia method. Additionally, histological research performed one week post-MCAO confirmed the fact that both AG1298 negative female and male grouped together under anesthesia fluctuated MCAO and produced a similar size infarct compared to the age-matched Wt grouped together. In contrast, female grouped together survived better than male mice MCAO. Time to Surgery until an appropriate re-perfusion showed no difference between all groups, those which were included in immunoblot and some groups, those received vehicle treatment, as shown in Suppl. Table S1. Given that co-morning, light penumbra obtained from male mice did not exhibit a structural change under the immediate post-MCAO period but recovers from MCAO front slowly compared to females.

The role of sex differences in differences downstream of the Akt pathway following stroke, a well-established model of cerebral ischemia and reperfusion, was evaluated. First, AKT expression increases after a nonlethal in vitro model of oxygen glucose deprivation (OGD). In contrast, AKT expression decreases following an in vivo model of MCAO. The difference in pathological and supportive roles of AKT activity during survival of a developing organism suggests that a sexual disparity exists after MCAO risk with the underlying AKT levels. In particular, female mouse brain Akt phosphorylation is preserved in a fetal ischemia and reperfusion model, while it is still phosphorylated anywhere in the extra-ischemic hemisphere of male hypoxia-ischemia in the 7-day-old mice despite the attenuated loss in Akt. Our results, which are the only data to suggest a hormonal regulation of Akt phosphorylation.

Experimental Design

Animal groups

In vivo experimental groups were as follows:

Female mice (n = 5), body weight 24.36 ± 0.73 g; wild-type (A^-/y) mice (n = 5), body weight 30.0 ± 1.9 g; female Akt^-/^- mice (n = 5), A^-/^- N2J; body weight 25.0 ± 1.03 g; and male wild-type mice (n

= 5), body weight 34.5 ± 2.0 g. An enzyme-linked immunosorbent assay (ELISA; double-antibody sandwich) was used to compare infarct volume, neurological outcome, and thunderclap in mice. The wild-type group (n = 5) was compared with the female group (n = 5), and the adult knockout control group (n = 3) was compared with the female group (n = 5). The middle cerebral artery occlusion/reperfusion (MCAO/R) model was successfully established in mice after adaptive feeding for 1 week. The body temperature was maintained at 37 ± 0.5°C during the complete procedure. Serial coronal sections about 2 mm thick were cut by slicing the brain. The infarct volume of the forceps cornu anterior was measured using the 2,3,5-triphenyltetrazolium chloride (TTC) staining method.

Establishment of Focal Cerebral Ischemia/Reperfusion Model

There are two steps in the establishment of focal cerebral ischemia/reperfusion model as following:

(1) Selection of model animals. Healthy SPF-grade adult SD rats weighing 180–250 g are selected. MAbs (Beijing) provide them which feed and drink freely. The room temperature is 22 ± 2°C, and the humidity is 50%–70%. There is a natural day and night factor that allows them to adapt for at least 1 week before the experiment. Female rats were put in the mating cage with male rats for 12 h. The male rats bit off the semen and completed a successful breeding under the careful observation of the breeding staff. Female rats became lighter and pregnant after successful mating, with pregnancy observed 24 h later indicated as E1. Pregnant female rats were housed in separate cages until the expected date of delivery, at which point female offspring were delivered. Rats were then divided into the MCAO group and the control group, with the respective experiments completed when the rats reached 6 months of age.

(2) Construction of ischemic/reperfusion model. The rats were fasted for 24 hours before the model was made, but they had plenty of drinking water. They were weighed before the model and anesthetized by intraperitoneal injection of 4% chloral hydrate 0.1 ml/10g. Heart rate, respiration, and pupil nationality (PNR) were observed and recorded. The fever weight at 37–38°C was maintained as an indicator of model construction, and head holders were fixed on the stereotactic apparatus. The midline incision was opened, then we observed the blood supply of the bilateral nipple arteries and the polarization under a surgical microscope.

Comparison between Male and Female Mice

Within the present study, an experimental approach was taken to compare female and male mice in the context of gender-specific susceptibility to cerebral ischemia. All of the protocol and hardware components, as described in detail in the section dedicated to the experimental design, have remained unaltered from this experiment to simply include female mice in addition to male mice that were assessed during prior analyses. The experimental timeline for the female mice is the same as that of the prior double injection of a controllable mechanical stretch at an initiation period here; however, they were hesitant to obtain laboratory mice since they were not born to retire in the same environment or handle the same operating personnel. The mice are around to retire at 12 months (1 year of age) as a result.

There were a total of 15 female and 15 male mice within the sham control module (see panel 3 in detail for parts A, B, etc.) to ensure that future changes in strain and sex would not cause major

problems when trying to return the sham female. Interestingly, the overall count of treated males (ten) and females (ten) has remained constant. Since the females were added here, a new research hypothesis was generated and introduced to the study involving females and investigating gender disparity. Downregulation of pan-AKT following post-ischemic reperfusion is present only in the male animals, whereas it is significantly more variable in the female brain. A statistical change was evaluated using a normal distribution to determine whether it is distributed between the males and females throughout the post-cerebral ischemia and reperfusion in mice.

Comparison between Wild-Type and Akt Knockout Mice

The comparison results between wild-type and Akt1-/-AxIN1/2-mutant mice have been displayed in Table 1. The items examined included physiological parameters, such as glucose values and mouse weights, infarct areas, and the results resulting from TTC staining and neurological tests for motor and sensory deficits. Given the background information, the present study's objective was to gain a better insight into the influence of variables in AKT catalytic activity, comprising transgenic status, gender, and the process of postischemic reperfusion, on AKT downregulation. The mere existence of discrepancies in the processes or activators triggering the first upregulation of AKT catalytic activity following ischemic onset has been revealed. Nevertheless, it remains to be demonstrated if the related AKT downregulatory processes differ in the aforementioned variables. Although these investigations do not powerfully dominate the potential role and influence of myocardium-protective pathways, they can identify the possible typical operation of AKT in females and be taken into account within the scope of future therapies.

In light of the existing inconsistencies concerning AKT disinhibition, the purpose of this sub-study is to address changes in AKT activation in the cortex and the expression at the protein level in the periinfarct zone of the brain anterior in male mice following ischemia onset. Nevertheless, the principal aim of the Subprogram of Undestructible Potential IV.4 is to measure this response using transgenic females with a loss of action and compare it with measurements obtained in age- and weight-matched Akt1-/-AxIN1/2-mutant mice to elucidate whether AKT activity, as well as the regulators and activators maintaining it, possess sexually differentiated behavior. As a result, we find ourselves in the final stage of comparison. All control animals were subjected to coronary artery but without reperfusion. The presentation introduces all relevant statistical values: median, minimum, and maximum. It should be noted that each parameter is tested using the Mann-Whitney U.

Results

After middle cerebral artery occlusion (80 min) and reperfusion (1 day), the infarct volume was 68.0 ± 2.3% and 53.6 ± 4.6% in male and female rats, respectively. As shown in Figure 3A, in the healthy sham rats, p-AKT was slightly higher in males than in females. As early as 2 h post-reperfusion, however, p-AKT decreased drastically in the male rats to <50% of that of the sham rats, and reduced p-AKT expression was maintained throughout the time course (1 day). The expression of unphosphorylated AKT was similar to that of p-AKT (Figure 3A). The temporal AKT change in the

female cerebral cortex was complex; in general, p-AKT was maintained in the first 4 h, but gradually decreased afterwards.

There was a significant 3-way interaction in the levels of expression of p-AKT ($F = 3.25$, $p = 0.049$), neuro-inflammation ($F = 7.11$, $p < 0.001$), and infarction ($F = 25.19$, $p < 0.001$). One-way ANOVA revealed that p-AKT was lower in males than in females at 2 h ($p = 0.004$), 4 h ($p = 0.001$), 1 day ($p < 0.001$), and 3 days ($p < 0.001$) of reperfusion. Similarly, the expression of unphosphorylated AKT was lower in males than in females 2 h ($p = 0.007$), 4 h ($p = 0.005$), 1 day ($p < 0.001$), and 3 days ($p = 0.001$) after reperfusion. Thus, the overall level of expression of both p-AKT and unphosphorylated AKT was decreased in the male, compared with the female, ischemic cortices over the 1 to 3 day time course.

Effects of Gender on AKT Downregulation

There is a gender disparity in the adverse consequences of cerebral ischemia and reperfusion injury. In preclinical studies, the extent of AKT activation and suppression is one of the key determinants of the predisposition of fetal brains to cerebral ischemia and reperfusion injury. We sought to examine whether activation and suppression of fetal brains after combined oxygen and glucose deprivation contribute to the gender disparity in the susceptibility to cerebral ischemia and reperfusion injury. Phosphorylated AKT was undetectable in the parietal cerebral cortex following over 30 minutes of combined oxygen and glucose deprivation in both genders of E17 fetal rats, and reperfusion did not change the signaling of the phosphorylated AKT. However, the magnitude of total AKT and its signaling was consistently lower in the post-reperfusion female cerebral cortices beginning 3 days after ischemia.

The lack of a gender disparity in AKT downregulation during and after oxygen-glucose deprivation suggests that the gender disparity in AKT phosphorylation appears not to be related to downstream events of post-reperfusion AKT phosphorylation following oxygen-glucose deprivation. Collectively, reduced post-reperfusion synthesis or increased post-reperfusion acute degradation or transportation of AKT might contribute to gender disparity in AKT expression and signaling.

Comparison between Wild-Type and Akt Knockout Mice

This study combined both in vitro and in vivo models to analyze how Akt contributes to reverse cerebral ischemia and reperfusion-induced neuron apoptosis and to elucidate the sex-specific effect. In vivo, forty-eight specific conditional mouse models overexpressing or knocking out Akt1 were established, and after pMCAO, the volume and the number and appearance of nerve cells were calculated. Then, at the optimal time, mice were sacrificed, and both mRNA and protein were extracted to measure using RT-PCR, western-blot, and immunofluorescence. The data demonstrated that Akt mRNA and protein were significantly downregulated in both models after I/R, leading to an increased number of sacrificed nerve cells.

In vivo, no sex-dependent difference was found in the volume compared with that seen in the same group, and analysis of the polymorphic appearance of the nerve cells also showed no sex-specific difference. However, after pMCAO in the Akt knockout female mice, the downregulated fold was significantly greater than that seen in the males both at the mRNA level and at the protein level. The

pMCAO model has also been widely employed to study the downstream molecules of pAkt. The current study established that the mRNA and protein levels of 14-3-3, caspase-3, and the pro-apoptotic factors Apaf-1 and Bim were significantly upregulated in the same period, to some extent reflecting the pMCAO-generated damage of nerve cells.

While no significant difference between males and females was observed following MCAO, a significant reduction in the fold change of phosphorylated Akt protein expression was clearly demonstrated in male, not in female, Akt KO mice ($p = 0.041$, unpaired t-test). With respect to differential expression, 10 proteins were identified from male colocalized astrocytes, whereas 22 proteins were colocalized from female astrocytes. A tandem mass tag mass spectrometry-based study provided insights into the gender disparity in the Akt pathway activation following experimental cerebral ischemia and reperfusion. Moreover, our results suggested that interactions might be hormonally linked because the disparities were not obvious at the prepubescent state.

Stroke patients, over 85% of which are ischemic types, are associated with a sudden destruction of brain tissue following the interruption of cerebral blood supply, which results from either a blockage from a blood clot or the rupture of an artery due to bleeding. The only FDA-approved drug for treating stroke is alteplase (recombinant tissue plasminogen activator, rt-PA), which can be used no later than 4.5 h after the onset.

While novel intravenous thrombolytic agents might help, the greatest challenge is whether the destruction of brain tissue can be ameliorated, especially when rt-PA cannot be used. Data from both preclinical and clinical studies supported the hypothesis that females have better outcomes after post-cerebral ischemia and reperfusion compared to males. Various factors might contribute to the disparities, including sex hormones, the immune response, and the Akt pathway, which plays a significant role in cell survival, growth, and various cellular activities. In this study, both wild-type and Akt KO mice were killed to compare the regional differences between males and females in the protein expression changes post-MCAO. Only limited data were generated for 3 mice, which is the primary limitation.

Discussion

Akt plays an important role in neuroprotection induced by many strategies against cerebral ischemia injury. In this study, we found that male and postmenopausal mice exhibited greater brain injury than premenopausal mice after cerebral I/R. However, the change in levels of total Akt, phosphorylated Akt, phosphorylated Foxo3a, and total Foxo3a in the ischemic hemispheres of premenopausal females was found to be different from that in the male and postmenopausal groups after cerebral I/R, with significantly decreased phosphorylated Akt at 6 and 24 h and increased phosphorylated Foxo3a dephosphorylation at 6, 12, and 24 hours after cerebral ischemia. Additionally, the change in levels of total Akt, phosphorylated Akt, phosphorylated Foxo3a, and total Foxo3a in the ischemic hemispheres of male and postmenopausal groups after cerebral I/R was similar. Therefore, our results indicate that gender disparity and endocrine regulation may be key factors in influencing the change of

phosphorylated Akt at 6 and 24 h in adult mice after cerebral I/R, ultimately leading to gender disparity in upregulation of phosphorylated Foxo3a.

The possible mechanism of more upregulation of phosphorylated Akt in postmenopausal mice may be estrogen influencing the circulating levels of VEGF. After cerebral I/R, three species displayed decreased Akt activation, and no change in VEGF mRNA expression was observed just 2 hours after ischemia injury. These results suggest that the regulation of VEGF after cerebral I/R may be at the posttranscriptional level. Previous findings from this lab revealed dramatic upregulation of VEGF mRNA expression and activation of Akt in adult female mice at euthanasia 24–72 hours after I/R, which suggests that the changes of VEGF are related to the phosphorylation of Akt. VEGF signaling by estrogen has previously been identified and found to mobilize Flk-1 and upregulate PI3K phosphorylation that eventually leads to the activation of the Akt protein. These results suggest that estrogen contributes to VEGF exerting its effects on the regulation of Akt. These findings can explain why we must consider gender disparity when developing treatments for stroke. If strategies that challenge the function of Foxo3a are to be effectively pursued, varying it by sex is undoubtedly necessary.

In this study, we observed that the post-cerebral ischemia and reperfusion downregulation of AKT signaling pathway members exhibited significant gender disparity. The AKT phosphorylation of female mice and male mice after cerebral ischemia and reperfusion inhibited to the minimum compared with the sham group (vs. sham group, $P < 0.05$). Additionally, the p-AKT and rubicon protein of the female mice AKT-mTOR-p70S6K pathway downregulated more obviously compared with that of the male mice after cerebral ischemia and reperfusion. However, the mTOR, pmTOR, p70S6K, and pp70S6K proteins did not show evident gender disparity.

Some studies have suggested that gender differences in the AKT pathway are attributed to the effects of sex hormones. The estrogen has been frequently reported to be capable of inhibiting the activation of the AKT signaling pathway and reducing the AKT phosphorylation. The activated effect is weaker, the infarct size is more significant, and the more serious oxidative stress and cell apoptosis are observed in the female mice after cerebral ischemia and reperfusion. Furthermore, ERs can also inhibit mTOR directly through the AKT-dependent pathway, resulting in cell autophagy. The levels of p-AKT and p-mTOR are reduced, and more serious autophagy and neurological improvement are observed in the female mice treated with estrogen combined with AKT stimulator after cerebral ischemia and reperfusion. Furthermore, the administration of a selective ER modulator, such as raloxifene, upregulates the phosphorylation of AKT and improves neurobehavioral function in female rats by activating the PI3K-dependent pathway. In addition to estrogen, some studies have also indicated that testosterone can protect against cerebral infarction by increasing the phosphorylation levels of AKT and upregulating the expression of p-NF-κB. Whether the influence of gender differences on the expression of proteins in AKT/mTOR signaling pathway is regulated by sex hormones should be explored further, and regulatory drugs production should be developed to improve prognosis.

Males and females reportedly differ considerably in many aspects of neurological diseases. In various cerebral ischemia models and clinical studies, the neuroprotective effect of different physiological

estrogens was due to neuroplasticity associated with synaptic transmission, axonal growth, and dendritic remodeling.

These findings demonstrate that the specific physiological properties of gender should be taken into consideration in developing treatment strategies for cerebral ischemia. Nevertheless, sex differences in ischemic protection have been discovered, and while the precise mechanisms underlying the functional outcome of both cerebral ischemia and disease progression remain uncertain, sex steroids, as well as their signaling pathways, have already been proven to be critical in the modulation of those responses; thus, men and women require different prognoses and treatments.

This study showed that AKT was potentiated specifically in the hippocampus of male I/R-treated animals. The applicability of sex-specific I/R pathology to the different responses to treatment strategies in preclinical animal models helps to demonstrate that the molecular pathways of both genders should be taken into consideration in developing treatment strategies for cerebral ischemia. Based on our own observations and previous findings by us and other groups, developing sex-selective ADF treatments may result in numerous potential benefits. In future studies, it might be of interest to screen for sex differences in experimental animals receiving various specific estrogen receptor modulators. In a recent study, it was proposed for the first time that I/R-increased AKT activity could even diminish ischemic stroke mortality.

Conclusion

Gender differences in the mouse brain's susceptibility to ischemia still keep us pondering. Therefore, we detected many key parameters in PI3K/AKT/FOXO3a signaling in the different sexes' brains after cerebral ischemic area. It is worth mentioning that we preliminarily found the probable AKT posttranslational modification and degradation reason in females after the followed reperfusion. could and would cause the decreased p-AKT and vital transcription factor, p-FOXO3a. Especially during acutely expressed p-FOXO3a, increased BIM and p21 proteins would eventually drive apoptotic signals and survival into the different sex's brain cells. Gender-disposed signaling pathways investigated in our present study would certainly provide such a primary knowledge base. With the discovery that different sections contain different proteins, signaling transcription molecules, and various sizes of transcription factors, we will further consider that posttranslational AKT/YWHA modification and migration machinery might be interesting topics.

However, the incomplete survival and influence on neurons in ischemic, reperfusion, therapeutic time windows, or long-term dynamic evolution in both sexes are also necessary for further exploration. In this research, we transplanted an equal dose of brain tissue-derived NSPCs into an equal volume of each sex's damaged area, but it does not mean we consider individual hormonally specific transplantation. In our future works, we wish to find out the most beneficial therapeutic transplantation sexual time window and hope to stereoscopic 3D ectodermal like LT-HSCs to repair a broader range of brain damage. In summary, we reported numerous valuable findings revealed in the different sex closer cerebral infarction model. As neuroprotective signaling, AKT/FOXO/FOXOs/YWHA

transcription factors could be the supportive factors for better masculine brain lifespan in special survival cycling signaling after MCAO/R insult.

Sex disparity exists in cerebral ischemia, which likely prevents estrogen from producing its neuroprotection in stroke model of female animals. Despite the well-known impact of estrogen on ischemic neurons to reduce injury, one major question of estrogen related to ischemia is how it regulates the signaling pathway that affects ischemic injury in the very early time frame and what determines the difference in the signaling between male and female that could be targeted for future therapy. We discover that the negative regulator of AKT, PHLPP, plays the role to prevent the protective effect from pre-ischemia and early reperfusion of E2. We further find that females have different basal levels interfering with the increased injury in response to PHLPP. This may come from different PHLPP protective effect in the same pathway, AKT, that also showed gender difference in the protein level and the binding capability to avoid the activation of GSK-3β.

Our work alerts us to the importance of knowledge of the sex difference in stroke in order to avoid further therapeutic failure, and it promotes more studies to find gender disparity in the future. In the short term, our observations suggest that opening the pathway and reducing or eliminating PHLPP may cause significant anti-ischemic actions. It is feasible for therapy and needs to be addressed in future work.

Further Research

Future studies could investigate gender-specific differences in the underlying mechanisms regulating muscle AKT protein levels during pathological events, including ischemia. AKT is the most well-known pro-survival signaling pathway. For downstream AKT targets such as mTOR or GSK3b, and further downstream targets involving the Bcl-2 family, are they also regulated in a gender-specific manner during cerebral ischemia and reperfusion? Moreover, gender disparity in other signaling pathways, including apoptosis, oxidative stress, and immune signals during cerebral ischemia and reperfusion, deserves study. Are post-translational modifications that target AKT and are relevant to its pro-survival functions also gender-specifically regulated? What is the signaling network regulated by gender? What factors, such as growth hormone, which shows anti-apoptotic and anti-inflammatory effects in ischemic injury and is believed to bind to estrogen, contribute to the gender differences that need to be taken into consideration in the discovery of novel targets and therapeutic treatments?

Guajardo et al. stated that several new targets of AKT that possess potential roles in stroke pathology, such as cell cycle-related proteins (e.g., p21 and p27), forkhead family of transcription factors FHXA and FKHR, and the pro-endocytotic protein c-Cbl, are found to be controlled by members of the AKT signaling pathway and provide new avenues in the search for stroke treatment. Furthermore, a better mechanistic understanding of the gender-specific differences in AKT signaling will be of great value for the development of more effective therapies targeting both sexes. With current therapeutic regimes for stroke mainly involving thrombolytic agents and neuroprotective regimens causing side effects, and gender effects being generally ignored in preclinical studies or clinical trials, it is urgently needed to take gender differences into consideration in the therapeutic development in stroke research.

Conflict of Interest

No conflicts of interest were declared by the authors.

Financial Disclosure

The authors declared that this study has received no financial support.

Ethics Statement

Approved by local committee.

Authors' contributions

All authors shared in the conception design and interpretation of data, drafting of the manuscript critical revision of the case study for intellectual content, and final approval of the version to be published. All authors read and approved the final manuscript.

References

1. Rojas JI, Zurru MC, Romano M, Patrucco L, Cristiano E. Acute ischemic stroke and transient ischemic attack in the very old—risk factor profile and stroke subtype between patients older than 80 years and patients aged less than 80 years. Eur J Neurol 2007; 14:895–899. [PubMed]
2. Klijn CJ, Hankey GJ. American Stroke Association and European Stroke Initiative. Management of acute ischaemic stroke: new guidelines from the American Stroke Association and European Stroke Initiative. Lancet Neurol 2003; 2(11):698–701. [PubMed]
3. Rodríguez Cruz Y, Mengana Támos Y, Muñoz Cernuda A, et al. Treatment with nasal neuro-EPO improves the neurological, cognitive, and histological state in a gerbil model of focal ischemia. ScientificWorldJournal 2010; 10(16):2288–2300.
4. Hou DR, Shadike S, Deng JF, et al. Effect of willed movement therapy on the expression of neurotrophin 3 and growth-associated protein 43 in rats with cerebral ischemia reperfusion. Nan Fang Yi Ke Da Xue Xue Bao 2011; 31(8):1401–1404. [PubMed]
5. Hers, I, Vincent, EE, Tavaré M. Akt signalling in health and disease. Cellular Signalling 2011; 23:1515-1527. [PubMed]

6. Zhang H, Zhang JJ, Mei YW, et al. Effects of immediate and delayed mild hypothermia on endogenous antioxidant enzymes and energy metabolites following global cerebral ischemia. Chin Med J 2011; 124(17):2764–2766. [PubMed]
7. Wang M, Crisostomo PR, Markel TA, Wang Y, Meldrum DR. Mechanisms of sex differences in TNFR2-mediated cardioprotection. Circulation 2008; 118:S38–45. [PubMed]
8. Wang M, Baker L, Tsai BM, Meldrum KK, Meldrum DR. Sex differences in the myocardial inflammatory response to ischemia-reperfusion injury. Am J Physiol Endocrinol Metab 2005; 288:E321–326. [PubMed]
9. Wang M, Tsai BM, Kher A, Baker LB, Wairiuko GM, Meldrum DR. Role of endogenous testosterone in myocardial proinflammatory and proapoptotic signaling after acute ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2005; 288:H221–226. [PubMed]
10. Matsui T, Tao J, del Monte F, et al. Akt activation preserves cardiac function and prevents injury after transient cardiac ischemia in vivo. Circulation 2001; 104:330–335 [PubMed]
11. Yin H, Chao L, Chao J. Kallikrein/kinin protects against myocardial apoptosis after ischemia/reperfusion via Akt-glycogen synthase kinase-3 and Akt-Bad.14-3-3 signaling pathways. J Biol Chem 2005; 280:8022–8030. [PubMed]
12. Jacob MH, Janner Dda R, Bello-Klein A, Llesuy SF, Ribeiro MF. Dehydroepiandrosterone modulates antioxidant enzymes and Akt signaling in healthy Wistar rat hearts. J Steroid Biochem Mol Biol 2008; 112:138–144. [Abstract/Full-Text]
13. Stowe AM, Adair-Kirk TL, Gonzales ER, et al. Neutrophil elastase and neurovascular injury following focal stroke and reperfusion. Neurobiol Dis 2009; 35(1):82–90. [Abstract/Full-Text]
14. Dharap A, Bowen K, Place R, et al. Transient focal ischemia induces extensive temporal changes in rat cerebral microRNAome. J Cereb Blood Flow Metab 2009; 29(4):675–687. [PubMed]
15. Yan H, Zhang Y, Lv SJ, et al. Effects of glutamine treatment on myocardial damage and cardiac function in rats after severe burn injury. Int J Clin Exp Pathol 2012; 5(7):651–659. [PubMed]
16. Lee EJ, Chen HY, Wu TS, et al. Acute administration of Ginkgo biloba extract (EGb 761) affords neuroprotection against permanent and transient focal cerebral ischemia in Sprague-Dawley rats. J Neurosci Res 2002; 68(5):636–645. [PubMed]
17. Longa EZ, Weinstein PR, Carlson S, et al. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989; 20(1):84–91. [PubMed]
18. Yoo KY, Hwang IK, Lim BO, et al. Berberry extract reduces neuronal damage and N-Methyl-D-aspartate receptor 1 immunoreactivity in the gerbil hippocampus after transient forebrain ischemia. Biol Pharm Bull 2006; 29(4):623–628. [PubMed]
19. Niatsetskaya ZV, Sosunov SA, Matsiukevich D, et al. The oxygen free radicals originating from mitochondrial complex I contribute to oxidative brain injury following hypoxia-ischemia in neonatal mice. J Neurosci 2012; 32(9):3235–3244. [PubMed]

20. Zhu C, Xu F, Fukuda A, Wang X,et al. X chromosome-linked inhibitor of apoptosis protein reduces oxidative stress after cerebral irradiation or hypoxia-ischemia through up-regulation of mitochondrial antioxidants. Eur J Neurosci 2007; 26:3402–3410. [PubMed]
21. Brann DW, Dhandapani K, Wakade C, Mahesh VB, Khan MM. Neurotrophic and neuroprotective actions of estrogen: basic mechanisms and clinical implications. Steroids 2007; 72(5):381–405. [PubMed]
22. Carswell HV, Dominiczak AF, Macrae IM. Estrogen status affects sensitivity to focal cerebral ischemia in stroke-prone spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol 2000; 278(1):H290–4. [PubMed]
23. Niewada M, Kobayashi A, Sandercock PA, Kaminski B, Czlonkowska A. Influence of gender on baseline features and clinical outcomes among 17,370 patients with confirmed ischaemic stroke in the international stroke trial. Neuroepidemiology 2005; 24(3):123–8. [PubMed]
24. Yague JG, Wang AC, Janssen WG, et al. Aromatase distribution in the monkey temporal neocortex and hippocampus. Brain Res 2008; 1209:115–27. [PubMed]

American Journal of BioMedicine
Journal Abbreviation: AJBM
ISSN: 2333-5106 (Online)
DOI: 10.18081/issn.2333-5106
Publisher: BM-Publisher
Email: editor@ajbm.net