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# Akt1 gene deletion and stroke

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**Abstract**

Activation of Akt has been implicated as a major contributor to neuronal survival after an ischemic insult. Numerous neuroprotective agents have been shown to augment Akt activity, suggesting that this protein represents a major mechanism of cellular salvage after injury. Estrogen is known to augment Akt, but the possibility that Akt plays a differential role in the male and female brain has yet to be evaluated. In this study, we employed both pharmacological and genetic approaches to investigate the role of Akt in stroke. Utilizing a focal stroke model we show that deletion of the Akt1 isoform does not affect stroke outcome in either male or female mice. Akt1 deficient mice had equivalent levels of phosphorylated Akt (p-Akt) when compared to their WT controls following stroke suggesting that alternative isoforms can compensate for Akt1 loss. Secondly, estrogen's neuroprotective effect is maintained in Akt1$^{-/-}$ mice and estrogen exposure did not enhance p-Akt levels in WT female mice. Thirdly, we show that inhibiting Akt using the direct pan-Akt inhibitor triciribine has no effect on stroke outcome despite dramatic reductions in p-Akt. Our study demonstrates the limitations of genetic mouse models and suggests that the importance of Akt to ischemic outcome remains unclear.
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*Keywords:* Akt; Triciribine; Estrogen; Stroke

## 1. Introduction

Akt (Protein kinase B) is a subfamily of serine/theonine protein kinase with oncogenic and anti-apoptotic activities [1–3]. Three isoforms of Akt, Akt1, Akt2 and Akt3 have been identified [4]. Akt is activated by extracellular stimuli in a phosphatidylinositol 3-kinase (PI3k)-dependent manner. Activated Akt phosphorylates a variety of downstream proteins, including several associated with cell death pathways such as BAD, caspase-9, Forkhead, CREB and MDM2, leading to diminished apoptotic cell death [1,5].

Akt is activated via phosphorylation. It has been suggested that Akt activation is important to neuronal survival in ischemic brain. Levels of p-Akt (serine-473) transiently increases within hours after ischemia [1,6–9], returning to baseline within 24 h. Increased Akt activation may represent a major mechanism by which a variety of neuroprotective agents protect ischemic brain [8,10,11] as diminished p-Akt levels are associated with cell death [1]. Administration of neuroprotective agents restore Akt activity as measured by elevations in p-Akt levels, however this may simply reflect non-specific effects of tissue salvage.

Akt is activated by PI3-kinase [5]. Consistent with p-Akt's proposed pro-survival role, treatment with the PI3-kinase inhibitors Wortmannin or LY294002, led to significant reductions in p-Akt levels and enhanced cell death *in vitro* [1] and *in vivo* [12]. However, PI3-kinase inhibitors may have additional targets beyond Akt, making these studies difficult to interpret [13].

The strongest evidence that p-Akt plays a direct rather than correlative role in cell death comes from *in vitro* studies that demonstrated a reduction in cell death after transfection with constitutively active Akt and increased cell death after

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transfection with a dominant negative form of Akt. Interestingly, introduction of a dominant negative Akt increased basal, but not NMDA-induced, cell death [1]. Consistent with the hypothesis that Akt activation is involved in ischemic neuroprotection, mice overexpressing neuronal Akt showed significant reductions in infarct volumes compared to wild-type (WT) controls [14]. However, limited data are available on mice lacking Akt, which would be expected to lead to an exacerbation of injury due to the loss of p-Akt mediated neuroprotection. A recent study utilizing Akt1 deficient mice found that deletion protected male mice from stroke damage, and had no effect in female mice [15]. Hormonal effects on Akt have been documented [16] and the contribution of estrogen to this observed gender dichotomy has not yet been investigated.

Estrogen is a female hormone that has potent neurotrophic and neuroprotective roles in immature and adult brains [17]. There is good evidence to suggest that Akt plays a role in estrogen-mediated protection. Estrogen increases Akt phosphorylation *in vivo* and *in vitro* [16] and prevents injury-induced decrease of p-Akt in focal ischemia models [18]. P-Akt levels are reduced in ovariectomized mice, an effect that is reversed by estrogen replacement. However, infarct volumes are strikingly higher in oil-treated animals, and the loss of p-Akt may simply represent a surrogate marker of increased ischemic damage rather than an estrogen-mediated neuroprotective mechanism [19].

The role that Akt plays in the response to cerebral ischemia remains unclear. In addition, the possibility that Akt plays a differential role in the male and female brain, or is related to hormonal exposure has yet to be evaluated. In this study, we employed both pharmacological and genetic approaches to assess 1) the role of Akt1 deficiency in stroke 2) the effect of Akt deficiency in male and female mice 3) the role of estrogen on Akt deficiency and 4) the effect of inhibition of Akt with the direct and specific pan-Akt inhibitor; triciribine [20] on stroke outcome.

## 2. Materials and methods

### 2.1. Akt1 KO mice

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 Lab Animal Care of University of Connecticut Health Center. The Akt1 knockout mice were bred in house from strains previously described [21]. All genetically modified mice were compared to their appropriate WT littermates. The animals used in all studies were age and weight matched (21–25 g, 10–12 weeks of age).

### 2.2. Animal genotyping

Generation of Akt1-targeted mice is as previously described [21] backcrossed to C57BL/6 for at least 10 generations. $Akt1^{+/-}$ (heterozygote) breeder pairs were obtained from Dr Morris J Birnbaum at the University of Pennsylvania. Animals were genotyped by PCR using the following primers in a single reaction: 851, 5′-AGATCTTCTTCCACCTGTCTC-3′; 852, 5′-GCTCCATAAGCACACCTTCAGG-3′; and 853, 5′-GTGGATGTGGAATGTGTGCGAG-3′ [21]. Phenotypically, $Akt1^{-/-}$ mice of both genders are distinguishable from WT mice because of their smaller size after weaning and throughout adulthood [21].

### 2.3. Drug treatment

For estrogen treatment, WT or $Akt1^{-/-}$ female mice were ovariectomized and were implanted subcutaneously with 17β-estradiol (E2) pellets containing 180 μg/ml of E2 in sesame oil (0.062-in. ID/0.125-in. OD) or oil-only control pellets 7 days prior to MCAO as previously described [22,23].

The Akt inhibitor triciribine (2 μl, 3.25 mM, dissolved in 20% DMSO) was injected introcerebroventricularly (icv) to male WT mice at the coordinates (From bregma; −0.9 mm lateral, −0.1 mm posterior, −3.1 mm deep) 1 h prior to the onset of MCAO. Control animals were injected with the equal amount of 20% DMSO.

### 2.4. Focal cerebral ischemia model

Focal transient cerebral ischemia (90 min MCA occlusion) was induced in WT or $Akt1^{-/-}$ mice followed by reperfusion as described previously [23]. At the end of ischemia, the animal was briefly re-anesthetized, and reperfusion was initiated by filament withdrawal. In separate cohorts of $Akt1^{-/-}$ male and $Akt1^{-/-}$ female mice or WT control, as well as the triciribine/vehicle treated animals ($n=4$ p/g), femoral arterial blood pressure and physiological measurements including blood pH, $pO_2$, $pCO_2$, and blood glucose, were obtained. Cortical perfusion using Laser Doppler Flowmetry was evaluated throughout MCAO and early reperfusion as described previously [23].

### 2.5. Behavioral scoring

At 24 h after stroke, animal behavior was scored using the neurological deficits score system 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 as described previously [23].

### 2.6. Histological assessment

At 24 h after stroke, histological assessment was done as follows. Briefly, the animals were killed; their brains were immediately removed, and cut into 5 individual 2-mm slices using a surgical blade. The brain slices were stained with 1.5% 2,3,5-triphenyltetrazolium (TTC) at 37 °C for 30 min

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