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Phosphoinositide-3-Kinase/Akt Survival Signal Pathways Are Implicated in Neuronal Survival After Stroke

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Abstract

In recent years, the phosphoinositide-3-kinase/Akt cell survival signaling pathway has been increasingly researched in the field of stroke. Akt activity is suggested to be upregulated by phosphorylation through the activation of receptor tyrosine kinases by growth factors. Although the upstream signaling components phosphoinositide-dependent protein kinase (PDK)1 and integrin-linked kinase enhance the activity of Akt, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) decreases it. Upon activation, Akt phosphorylates an array of molecules, including glycogen synthase kinase3β (GSK3β), forkhead homolog in rhabdomyosarcoma (FKHR), and Bcl-2-associated death protein, thereby blocking mitochondrial cytochrome c release and caspase activity. Generally, the level of Akt phosphorylation at site Ser 473 (P-Akt) transiently increases after focal ischemia, whereas the levels of phosphorylation of PTEN, PDK1, forkhead transcription factor, and GSK3β decrease. Numerous compounds (such as growth factors, estrogen, free radical scavengers, and other neuroprotectants) reduce ischemic damage, possibly by upregulating P-Akt. However, preconditioning and hypothermia block ischemic damage by inhibiting an increase of P-Akt. Inhibition of the Akt pathway blocks the protective effect of preconditioning and hypothermia, suggesting the Akt pathway contributes to their protective effects and that the P-Akt level does not represent its true kinase activity. Together, attenuation of the Akt pathway dysfunction contributes to neuronal survival after stroke.

Index Entries: Akt; PKB; apoptosis; cerebral ischemia; stroke; preconditioning; neuroprotection; PTEN.

Introduction

The extent of neurological damage following cerebral ischemia is determined by a balance between apoptotic signals and cell survival signals. Although caspase-mediated apoptotic pathways lead to cell death *(1)*, the Akt/PKB survival signaling pathway supports cell survival (ref. *2*; Fig. 1), including its role in blocking neuronal death after stroke *(3,4)*. The number of publications regarding its role in cerebral ischemia has expanded in recent

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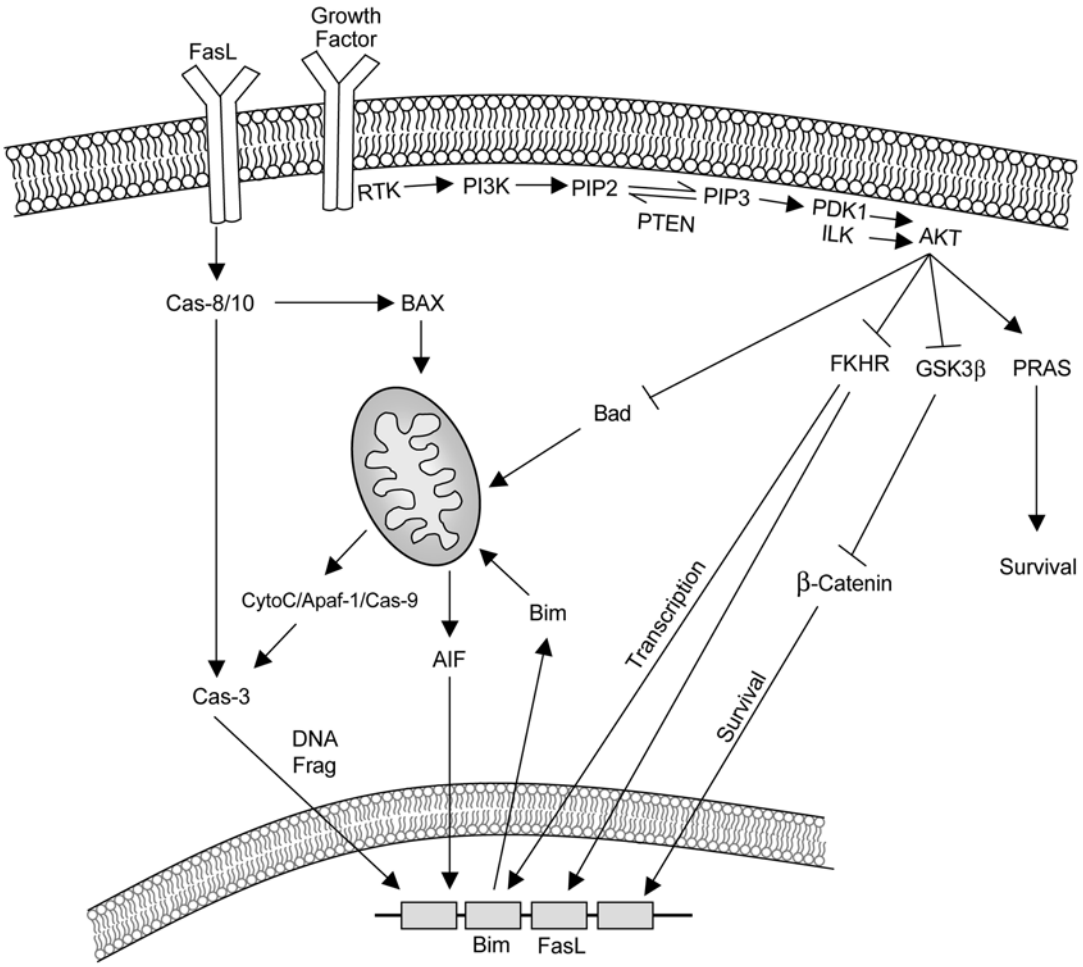

Fig. 1. A diagram of Akt/PKB survival signal pathways. RTK, receptor tyrosine kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP 3, phosphatidylinositol-3,4,5-trisphosphate; PRAS, proline-rich Akt substrate; ILK, integrin-linked kinase; Fas L, Fas ligand; Cyto C, cytochrome *c*; Cas-3, -9, -8/10, caspase-3, -9, -8/10; DNA Frag, DNA fragmentation.

years. These studies have clarified changes in cascades of the Akt pathway after stroke and have explored protective effects of various neuroprotectants on the Akt pathway, including growth factors *(5,6)*, hypothermia *(4)*, preconditioning *(7,8)*, and free radical scavengers *(9,10)*. This article provides a comprehensive review of the roles of the Akt pathway both in cerebral ischemia and in mediating the effects of various protectants. To understand how the Akt pathway is implicated in neuronal survival after stroke, we briefly summarize the cytochrome *c* and caspase-mediated apoptotic pathways and how the Akt pathway is associated with them.

Classic Apoptotic Pathways and Akt Survival Signals

Apoptotic pathways contribute to ischemic damage *(11–16)*. In the cell-intrinsic caspase-

dependent apoptotic pathway (Fig. 1), the pro-apoptotic factor cytochrome *c* is released from injured mitochondria *(17,18)*. Cytochrome *c* forms the apoptosome with apaf-1 and procaspase-9 in the cytosol, which activates caspase-9 and caspase-3 and leads to apoptosis *(18)*. In the extrinsic apoptotic pathway, the binding of extracellular Fas ligands for death receptors can also activate caspase-8 or -10, which then directly activates caspase-3 or causes Bid/Bax activation, inducing cytochrome *c* release *(19)*. Additionally, apoptosis-inducing factor nuclear translocation leads to DNA fragmentation as a part of the caspase-independent apoptotic pathway. The initiation of these cascades by ischemia has been reported *(1,12,13)*. It is unclear how these apoptotic cascades are triggered after stroke. Recent studies have suggested that the occurrence of apoptosis probably results from dysfunction of the Akt survival pathway.

In the first step of the Akt pathway, a receptor tyrosine kinase (RTK) is activated by its cognate growth factor ligand *(2)*. The RTK then activates phosphoinositide-3-kinase (PI3K), which phosphorylates phosphatidylinositol phosphate (PIP)2 to PIP3. The classical theory is that Akt activity is regulated by phosphorylation of Ser 473 (P-Akt) and Thr 308 *(2,20)*. Phosphoinositide-dependent protein kinase (PDK)1 may directly phosphorylate Thr 308 and indirectly phosphorylate Ser 473, probably by interacting with an uncharacterized protein (PDK2). Additionally, some studies have suggested that integrin-linked kinase (ILK) increases the level of P-Akt (Ser 473) *(21,22)*. Conversely, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) dephosphorylates PIP3 to PIP2, thereby inactivating Akt *(2)*. Activated Akt blocks apoptosis by phosphorylating its substrates, such as Bcl-2-associated death protein (Bad), forkhead transcription factor (FKHR), PRAS, and glycogen synthase kinase 3β (GSK3β) *(2,23)*. In the absence of Akt kinase activity, nonphosphorylated Bad translocates into mitochondria and triggers cytochrome c release, caspase-3 activation, and apoptosis *(18)*. Nonphosphorylated FKHR translocates into nuclei and, as a transcription factor, upregulates protein levels of Bcl-2-interacting mediator of cell death (Bim) and Fas ligand *(23,24)*. Similarly to Bad, Bim triggers cytochrome c release, whereas Fas ligand causes extrinsic apoptotic pathway activity *(1)*. Akt also downregulates GSK3β activity by phosphorylating it at Ser 9 *(25)*. Dephosphorylation of GSK3β leads to its activation, which phosphorylates β-catenin, thus marking it for degradation; β-catenin is a transcription factor that plays key roles in cell survival *(26)*.

The Effect of Stroke on the Akt Pathway

This section reviews changes in various molecular signals of the Akt pathway after stroke (summarized in Table 1). First, the effect of cerebral ischemia on different forms of Akt, including P-Akt (Ser 473 or Thr 308) and total Akt are summarized, followed by a summary of the changes in upstream and downstream signals of the Akt pathway. Although hyperphosphorylation of Akt usually indicates enhanced Akt activity, and most studies assume that levels of P-Akt (Ser 473 or Thr 308) after stroke represent its activity, this may not be true under certain conditions *(4)*. Akt activity is regulated by both phosphorylation sites (Ser 473 and Thr 308); P-Akt (Ser 473) is not sufficient to stimulate its activity *(27–29)*. Furthermore, tyrosine phosphorylation is essential for Akt activation *(30)*. Therefore, it is necessary to make a clear boundary between phosphorylation level of Akt and its kinase activity. The levels of various P-Akt isoforms may represent its activity under some, but not all, conditions. Therefore, this article strictly distinguishes between P-Akt and Akt activity.

Changes in P-Akt After Stroke

Akt is normally phosphorylated in nonischemic brains. Most reports demonstrate an increase in P-Akt (Ser 473) after reperfusion in focal, global, and in vitro ischemia models

Table 1
Changes in Molecular Components of the Akt Pathway After Stroke

Model	S	Effects	Reference
Anoxia	M	P-Akt increases 12 h after ischemia	*113*
2-DG/KCN	R	P-Akt increases 2 h after insult	*76*
P-MCAo	R	PI3K and Akt-1 increases at 3–8 h and decreases at 24 h	*39*
1 h MCAo	R	Two waves of P-Akt; no P-Bad (Ser-136) is detected	*49*
1 h MCAo	M	P-Akt increases at 4 h, decreases 24 h.	*31*
1 h MCAo	M	P-Akt decreases in the core but transiently increases at 4 h in the cortex	*50*
90 min MCAo	R	P-Akt reaches a peak at 8 h	*92*
90 min MCAo	R	P-Akt increases at 1-3 h after reperfusion	*114*
90 min MCAo	R	P-PTEN peaks 12 h after reperfusion in the penumbra and in the core, peaks 1 h then decreases by 3 d	*46*
2 h MCAo	R	P-PTEN increases at 24 h	*45*
15 min Global	R	Inhibition of PTEN activats Akt	*18*
60 min MCAo	M	ILK and its interaction with Akt increases after stroke	*9*
P-MCAo	R	P-Akt (Ser-473) transiently increased, P-Akt (Thr-308) does not change; P-PTEN, -FKHR, -PDK1, -GSK3β all decreases	*4*
2 h MCAo	R	P-Akt (Thr308) decreases after stroke	*10*
60 min MCAo	M	P-RARS, and the P-PRAS/Akt interaction decreases after stroke	*53*
60 min MCAo	M	P-PRAS decreases after reperfusion	*52*
30 min Global	R	P-Akt increases after reperfusion	*7*
15 min Global	R	P-Akt peaks at 24 h, decreases at 48 h	*35*
15 min Global	R	Interaction between Bad and Akt decreases after ischemia	*48*
5 min Global	G	P-Akt does not change after reperfusion	*37*
Global ischemia	G/m	FKHR dephosphorylation and translocates into the nucleus, causing Fas and Bim expression	*115*
5 min Global	G	P-FKHR (Ser-256) decreases, its DNA binding increases and upreguates FAS ligand	*47*
15 min Global	R	P-Akt473 increases from 0.5 to 2 h.	*32*
5 min Global	R	P-Akt (Ser-473) and P-GSK3β (Ser-9) increases	*36*
5 min Global	G	P-Akt decreases at 1 h and increases at 6 h; P-Akt expressed in the nuclei	*33*
15 min Global	R	VEGF receptor 1, 2 and P-Akt (Ser-473) increases after ischemia	*40*

Abbreviations: S, species; R, rat; M, mouse; G, gerbil; P-Akt, P-Akt (Ser473); Global, global ischemia; MCAo, middle cerebral ischemia; P-MCAo, permanent MCAo.

(summarized in Table 1). In focal ischemia models, P-Akt (Ser 473) is usually dephosphorylated after ischemia onset but begins to be rephosphorylated after reperfusion, with levels transiently increasing from 1 to 9 h and decreasing at 24 h after reperfusion in the ischemic penumbra. P-Akt (Ser 473) decreases in the ischemic core throughout all the timepoints *(31)*. However, results regarding changes in P-Akt (Ser 473) after transient forebrain ischemia are variable. Although it has been reported that P-Akt (Ser 473) transiently increases during early reperfusion from 0.5 to 6 h *(32,33)*, others have suggested that the phosphorylation is sustained for 24 or even 72 h after reperfusion in the vulnerable regions of ischemic cortex and hippocampus following global ischemia *(34–36)*. Additionally, another

report suggested that P-Akt (Ser 473) did not change after reperfusion following global ischemia in the gerbil *(37)*. Such a discrepancy may be attributed to different degrees of ischemic severity, the ischemic model, and the species used.

Although P-Akt (Thr 308) appears to contribute more directly to Akt activity, few studies have detected its changes after stroke. One study reported its sustained decrease after ischemia onset in a focal ischemic model *(38)*. However, we did not detect its significant decrease until 48 h after stroke *(4)*. More studies are needed to understand the role of P-Akt (Thr 308), and its interactions with P-Akt (Ser 473) to adjust Akt activity after stroke.

Conversely to the rapid changes in P-Akt (Ser 473) after stroke, almost all studies agree that total Akt protein level does not change, suggesting that ischemia/reperfusion modulates neuronal death or survival through phosphorylation of Akt rather than by regulating its protein levels. The only exception was one study that reported nonphosphorylated Akt transiently increased after stroke *(39)*; however, this study employed only immunostaining to evaluate its expression. This is in contrast to most other studies that have used Western blots to control for total Akt levels, which might provide more solid quantitative information.

Regulation of the Molecular Signals Upstream of Akt

To better understand the role of Akt in stroke, it is worthwhile to evaluate changes in other cascades upstream and downstream of Akt. Growth factors, which activate RTKs, are the initial activators of the Akt pathway. However, few studies have linked changes in endogenous growth factors with P-Akt (Ser 473) levels. One study suggested upregulation of vascular endothelial growth factor (VEGF) receptors 1 and 2 after stroke was concomitant with an increase in P-Akt (Ser 473) *(40)*. However, another study suggested that P-Akt (Ser 473) increased as early as 0.5 to 2 h after reperfusion conversely to VEGF levels, which increased only after 6 h in a global ischemia model *(32)*. Because VEGF increases even later than P-Akt (Ser 473), it is unlikely that VEGF is responsible for the Akt phosphorylation. Therefore, the issue regarding whether endogenous growth factors contribute to increase in P-Akt (Ser 473) needs more study.

Growth factors also activate the mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated protein kinase (ERK), P38, and c-Jun N-terminal kinase (JNK). It is generally agreed that activation of P38 and JNK contribute to ischemic damage *(41,42)*; however, the issue of whether ERK is neuroprotective remains extremely controversial *(11,42,43)*. Most recently, Kilic and colleagues *(44)* found that VEGF receptor 2 is expressed in neurons and astrocytes in the ischemic regions. VEGF receptor 2 can upregulate phosphorylation levels of Akt and ERK1/2 and also decreases the activity of MAPK/P38 and JNK1/2. However, VEGF also increases blood–brain barrier permeability by increasing endothelial Akt activity *(44)*. Therefore, VEGF has dual roles in the ischemic brain. Indeed, other studies have indicated that intracerebroventricular delivery of VEGF is neuroprotective, whereas intravenous injection of VEGF is detrimental *(6)*. Nevertheless, the application of growth factors can upregulate P-Akt (Ser 473), which is discussed in another section.

Nonphosphorylated PI3K is reported to transiently increase after stroke, along with the increase in non-phospho Akt *(39)*. However, in that study, protein was quantified only by immunostaining. PTEN is another protein that functions upstream of the Akt. PTEN negatively regulates Akt activity by dephosphorylating PIP3 to PIP2. Phosphorylation of PTEN (P-PTEN) is believed to prevent its interaction with PIP, thus inactivating its activity. Therefore, increases in P-PTEN increase Akt activity and support cell survival *(2)*. We recently reported that P-PTEN immediately decreased during the early time-points following ischemia and returned to normal or slightly elevated levels at 24 h compared with sham animals,

suggesting that decreases in P-PTEN preceded ischemic damage *(4)*. Additionally, P-PTEN was found to be upregulated in endothelial cells 24 h after stroke, which may have regulated the function of blood vessels at later time-points *(4)*. Studies from other groups suggested that P-PTEN significantly increased at 12 or 24 h after focal ischemia *(45,46)*. The neuroprotectant cilostazol reduced infarct size by reducing P-PTEN levels at 24 h and this lead to increased Akt activity *(45)*. However, the underlying mechanisms that caused reduced P-PTEN were not clear in that study *(45)*.

Interestingly, Ning et al. *(18)* found that PTEN physically interacted with the NR1 and NR2 subunits of *N*-methyl-D-aspartate (NMDA) receptors, and downregulation of PTEN blocked NMDA activity. The authors further demonstrated that blocking PTEN expression reduced ischemic damage in vitro and in vivo by blocking NMDA activity and enhancing Akt activity.

PDK1 is another signaling component upstream of Akt that directly phosphorylates Akt at site Thr 308 *(2)*. We found that PDK1 phosphorylation levels decreased after stroke and were accompanied by decreases in P-PTEN, P-FKHR, and P-GSK3. An exception was P-Akt (Ser 473), which transiently increased after stroke *(4)*.

ILK, an upstream signaling component that phosphorylates Akt (Ser 473), transiently increases after focal ischemia *(9)*. Meanwhile, its interaction with Akt also increases. Both the protein level of ILK and its interaction with Akt are inhibited by the PI3K inhibitor LY 294002 *(9)*.

Downstream Molecules of the Akt Pathway: Postischemia

After reviewing upstream signals of the Akt pathway, we review changes in molecules downstream of the Akt pathway after stroke, including FKHR, GSK3β, β-catenin, and Bad. Among these components, dephosphorylation of FKHR is associated with both intrinsic and extrinsic caspase-mediated apoptotic pathways. It has been reported that P-FKHR (Ser 256) decreases at 0, 30, and 60 min after reperfusion following 5 min of forebrain ischemia in gerbil models, and FKHR translocates into the nucleus immediately (at 0 min) after reperfusion *(47)*. This translocation may cause Fas ligand to increase 1 and 2 d later, because the DNA binding activity of FKHR with the forkhead-responsive element within the Fas ligand promoter increases after ischemia. We also observed that P-FKHR decreased from 30 min to 48 h after focal ischemia *(4)*. Additionally, stroke caused dephosphorylation of GSK3β and nuclear translocation of β-catenin *(4)*, which may have contributed to neuronal death, because neuroprotective hypothermia blocked both nuclear β-catenin translocation and neuronal death.

However, conversely to our finding that P-GSK3β decreased after focal ischemia, P-GSK3β increased for 3 d after global ischemia *(36)*. Another downstream component, Bad, was also investigated. Studies showed that the interaction between Bad and Akt decreased after global ischemia *(48)*. In support of this result, another study suggested that phosphorylation of Bad was not detected after stroke, despite the increase of P-Akt (Ser 473) in the same study *(49)*.

The discrepancy between increases in P-Akt (Ser 473) and the absence of concurrent increases in its substrate phosphorylation levels is further evidenced by studies from Chan et al. This group demonstrated that P-Akt (Ser 473) transiently increased after reperfusion *(31,50)*. However, phosphorylation of PRAS, a recently identified substrate of Akt *(51)*, decreased after reperfusion in the same ischemic model *(52,53)*. PRAS phosphorylation created a 14-3-3 protein binding site *(51)*, but its physiological and pathological role was not clear. It was shown that the interaction between PRAS and 14-3-3 protein decreased after stroke *(52,53)*. Interestingly, liposome-mediated PRAS complementary DNA transfection increased P-PRAS and its interaction with 14-3-3 *(53)*, suggesting that increases in 14-3-3 protein/PRAS interactions were associated with neuronal survival. Most importantly,

it significantly reduced infarct size after transient focal ischemia, but little is known about the mechanisms by which PRAS overexpression protected against ischemia damage *(53)*.

Together, all of these studies suggest that the phosphorylation levels of factors downstream of Akt, such as FKHR, GSK3β, Bad, and PRAS, do not increase together with P-Akt (Ser 473); rather, all of them decrease when P-Akt (Ser 473) increases. Therefore, increases in P-Akt (Ser 473) after stroke may not lead to enhancement of Akt activity. However, we can not exclude the possibility that FKHR, GSK3β, and Bad are not substrates of Akt in vivo or that an increase in activity of phosphatases dephosphorylate these substrates.

Overall, it is clear that dysfunction of the Akt pathway occurs after stroke. Several lines of evidence suggest that this may lead to cytochrome *c*/caspase-mediated apoptosis. First, changes in the Akt pathway occur immediately following ischemia onset *(4,31)*. Despite the fact that P-Akt (Ser 473) usually transiently increases after focal ischemia, most substrates of the Akt pathway are not phosphorylated during the early time-points after reperfusion. Second, stroke decreases the interaction between Bad and Akt, which may lead to Bad insertion into the mitochondrial membrane, causing the release of pro-apoptotic proteins from the mitochondria *(48)*. Additionally, there is evidence to suggest that P-FKHR decreases and FKHR translocates into the nuclei, which upregulates expression of Fas ligand, a factor that initiates the extrinsic apoptotic pathway *(47,54)*. Finally, double-immunostaining demonstrates that dephosphorylation of P-PTEN precedes cytochrome *c* release, suggesting that the disorder of the Akt pathway occurs before apoptosis *(4)*. Together, the functional failure of the Akt pathway after stroke may lead to ischemic damage.

Despite the assumption in the aforementioned studies that the increase in P-Akt (Ser 473) is neuroprotective, few have presented convincing evidence to support the statement. To see whether Akt kinase activity is a key survival factor in ischemic tissue after stroke, it is tempting to study whether Akt inhibition causes more damage alone. Unfortunately, there have been no studies showing whether an Akt inhibitor, or PI3K inhibitor (which indirectly reduces Akt activity by blocking PI3K), increases infarct size after stroke. Therefore, a few questions remain. Is the transient increase in P-Akt (Ser 473) a part of an effort by the dying cells to rescue themselves after stroke? Or is such an increase simply a marker or result of cell damage? If P-Akt (Ser 473) is important for neuroprotection, why do neurons in the CA 1 hippocampus die despite sustained increases in P-Akt (Ser 473) after global ischemia *(36)*? Additionally, there is a clear gap between the level of P-Akt (Ser 473) and the phosphorylation levels of its substrates. Despite the fact that P-Akt (Ser 473) increases after stroke, the phosphorylation levels of its substrates all decrease after stroke. Therefore, does P-Akt (Ser 473) alone truly represent its activity? In other words, does an increase in P-Akt (Ser 473) contribute to neuroprotection? To answer these questions, we further analyze results from settings in which neuroprotectants were employed.

Increase in P-Akt (Ser 473) Levels is Associated With Neuroprotective Compounds After Stroke

To understand whether increased levels of P-Akt (Ser 473) contribute to neuroprotection, we review the effect of various compounds that reduce ischemic damage on levels of P-Akt (Ser 473) and their effects on other key survival or death signals after stroke.

Exogenous Growth Factors

As discussed earlier, there are few studies relevant to whether endogenous growth factors are responsible for increases in P-Akt (Ser 473) *(32,40,44)*. Conversely, numerous studies have demonstrated that exogenous application of growth factors protects against stroke (Table 2).

Table 2
The Effects of Growth Factors Treatment on the Akt Pathway

Insult model	S	Treatment	Effect	Reference
NMDA/cortical culture	R	TGF-α	TGF-α enhances P-Akt levels	*55*
Glutamate/hippocampal culture	R	FGF-2	GDNF increases P-Akt level and induces mRNA of Bcl-2	*116*
OGD/HN 33 hippocampal cell line	M	VEGF	PI3K inhibitors blocks protection by VEGF	*34*
5 min Global	G	IGF-1	Prevents decreases in P-Akt immediately after reperfusion	*33*
15 min Global	R	Insulin	Insulin increases P-Akt after reperfusion	*117*
90 min MCAo	M	VEGF	VEGF enhances P-Akt at 24 h after reperfusion	*6*
1 h MCAo	R	TGF-α, MK-801	MK-801, but not TGF-α enhances P-Akt;	*56*
90 min MCAo	R	GDNF	GDNF potentiates P-Akt and blocks caspase-9 and -3	*5*
1 h MCAo	M	NGF, PRAS	Overexpression of PRAS reduces infarct; NGF increases P-PRAS; PI3K inhibitor attenuates P-PRAS	*53*
30 or 90 min MCAo	M	Erythropoietin (EPO) transgenic	EPO enhances P-Akt; inhibition of Akt abolishes protection of EPO	*57*
2.5 h 8% O_2/left CCA occlusion	NR	BDNF	BDNF increases P-Akt; inhibition of PI3K blocks its effect	*60*
1 h Hypoxia (7.7% O_2)	NR	IGF-I	IGF-I increases P-Akt and P-GSK3β and blocks caspase activity	*59*
1 h Hypoxia (7.7% O_2)	NR	Hexarelin	Enhances P-Akt and P-GSK3β	*58*

Abbreviations: NR, neonatal rats; S, species; R, rat; M, mouse; G, gerbil; P-Akt, P-Akt (Ser-473); MCAo, middle cerebral ischemia; OGD, oxygen–glucose deprivation; NMDA, *N*-methyl-D-aspartate; TGF, transforming growth factor; FGF, fibroblast growth factor; GDNF, giant-cell-derived neurotrophic factor; VEGF, vascular endothelial growth factor; IGF, insulia-like growth factor; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; PRAS, proline-rich AKT substrate.

A dozen studies have addressed whether growth factors reduce infarct size by activating the Akt pathway. As shown in Table 2, various growth factors, including fibroblast growth factor, glial cell line-derived neurotrophic factor, VEGF, insulin-like growth factor (IGF)-1, insulin, nerve growth factor, erythropoietin (EPO), brain-derived neurotrophic factor (BDNF), and hexarelin, have been shown to upregulate P-Akt (Ser 473). However, the issue of whether transforming growth factor (TGF)-α enhances P-Akt (Ser 473) level is controversial *(55,56)*. Additionally, a PI3K inhibitor reverses the protective effect of VEGF *(34)*, and EPO *(57)*. Furthermore, IGF and hexarelin not only increase P-Akt (Ser 473) but also enhance P-GSK3β *(58,59)*. Because enhancement of P-Akt (Ser 473) is associated with inhibition of caspases 9 and 3 *(5)*, it suggests that growth factors block apoptosis through upregulation of P-Akt (Ser 473). However, other studies have suggested that Akt inhibition does not block protection caused by TGF-α *(56)* or BDNF application *(60)*, suggesting that P-Akt (Ser 473) enhancement does not induce neuroprotection. From these studies, one may infer that not all growth factors exert protection through Akt.

Free Radical Scavengers

It is well-known that reactive oxygen species (ROS), or free radicals, contribute to ischemic

damage *(61)*. ROS directly oxidize macromolecules such as proteins, lipids, and DNA, thus causing cell necrosis. ROS also induce mitochondrial injury followed by cytochrome *c* release and caspase activity, leading to apoptosis *(62)*. Free radical scavengers have not only served as tools for understanding the cellular and molecular mechanisms of neuronal death and survival after stroke, but efforts have been made to translate their clinical applications from laboratory research *(63–65)*. Superoxide dismutase (SOD) 1 and 2 and glutathione peroxidase (GPX) are endogenously expressed enzymes in the brain that detoxify hydrogen superoxide (H_2O_2) and superoxide (O_2^-), respectively *(61,66 67)*. In recent years, several studies have investigated whether free radical scavengers protect the ischemic brain via the Akt survival pathway. Noshita and colleagues *(50)* first found that P-Akt (Ser 473) decreased in the ischemic core but transiently increased at 4 h in the penumbra in wild-type mice. However, overexpression of SOD1 in transgenic animals enhanced P-Akt (Ser 473) as early as 1 h after stroke and was still demonstrable at 4 and 24 h *(50)*. ILK is a PI3K-dependent serine-threonine kinase whose activity enhances Akt activation *(9)*. The same group demonstrated that similarly to P-Akt (Ser 473), ILK activity transiently increased from 1 to 4 h after reperfusion. Such increases were blocked by the PI3K inhibitor LY294002 *(9)*. However, overexpression of SOD1 also enhances the protein level of ILK. Co-immunoprecipitations indicated that physical interaction between ILK and Akt increased at 2 h after reperfusion. Additionally, the authors demonstrated that SOD1 promoted the interaction between ILK and Akt, which may have contributed to its neuroprotective effect *(9)*. Similarly, overexpression of GPX was also neuroprotective against stroke, and its deficiency exacerbated ischemic damage *(68)*. It was shown that P-Akt transiently increased at 1 and 4 h after reperfusion in a focal ischemia model; however, such increase was not detected in GPX knockout mice *(68)*. These studies suggest that the Akt pathway may contribute to the protective effect of SOD and GPX.

Such protective effects have been supported by studies using other antioxidants. NXY-059 is a free radical trapping agent that is not only effective in ischemic models but is clinically beneficial for human patients suffering from stroke as well *(69)*. Its protection is associated with the Akt pathway, because NXY-059 treatment attenuates the decrease in P-Akt (Thr 308) 24 h after stroke *(10)*. Melatonin, another free radical scavenger, restores phosphorylation of Akt after stroke and blocks caspase-3 activity *(70)*. In conclusion, free radical scavengers reduce ischemic damage, probably by upregulating P-Akt (Ser 473).

Estrogen

The protective effects of estrogen against brain injury have been shown in Alzheimer's disease *(71)*, Parkinson's disease *(72)*, and stroke *(73)*. In the case of stroke, it is well-known that premenopausal women have lower incidence of stroke than postmenopausal women, which is at least partially attributable to the protective activity of estrogen *(74)*. Although beneficial effects from clinical trials of estrogen application have not been reported, neuroprotective effects of estrogen in animal stroke models have been well-established *(73)*. Several protective mechanisms have been reported, including estrogen's effect of blocking apoptosis, inducing neurotrophins, and inhibiting both inflammation and oxidative stress *(75)*.

Several groups have reported that estrogen inhibits ischemic damage by enhancing Akt phosphorylation levels. In a study employing organic slice cultures, ischemia was induced with the metabolic inhibitor 2-DG/KCN *(76)*. P-Akt (Ser 473) levels increased at 0.5, 1, and 2 h, but not at 4 h, postinjury. However, estradiol further increased P-Akt (Ser 473) levels at 1 and 2 h *(76)*, which might contribute to the protective effect of estradiol.

In another study with hippocampal slice cultures, estrogen increased the level of P-Akt (site not shown) at 1 and 24 h after oxygen-glucose

deprivation (OGD) *(77)*. Moreover, the PI3K inhibitor LY 294002 reversed the protective effect of estrogen *(77)*. Such protective mechanisms shown in vitro were then demonstrated using in vivo ischemia models. Estradiol enhances P-Akt (Ser 473) at 24 h following permanent focal ischemia, which corresponds to an increase in physical interactions between P-Bad and 14-3-3 proteins as shown by co-immunoprecipitation experiments *(78)*. Another in vivo study reported that estradiol increased P-Akt (Ser 473), along with other anti-apoptotic proteins, such as cyclic adenosine monophosphate response element binding protein and Bcl-2, whereas it inhibited Bax expression after focal ischemia *(79)*. However, this study also suggested that estradiol protected by reducing P-PTEN, conversely to our report that reduction in P-PTEN had a detrimental effect.

However, the exact mechanisms by which estrogen increases P-Akt (Ser 473) levels after stroke are not clear. Typically, upon stimulation by estrogen, the estrogen receptor enhances gene transcription *(80)*. However, given that total Akt does not increase after stroke, it is unlikely that estradiol treatment upregulates protein levels of Akt through transcription. It has recently been demonstrated that the estrogen receptor physically interacts with PI3K, which increases PI3K activity and leads to hyperphosphorylation of Akt *(80)*. Furthermore, estrogen stimulates the interaction between PI3K and estrogen receptor, which is believed to enhance Akt phosphorylation. Further studies are needed to prove whether estrogen application increases P-Akt through this mechanism after stroke.

Other Neuroprotectants

The Akt pathway is involved in the protective mechanisms of a broad range of compounds (Table 3). In addition to growth factors, preconditioning, hypothermia, free radical scavengers, and estrogen, other neuroprotectants, such as a δ protein kinase C (PKC) inhibitor *(81)* and the immunosuppressant cyclosporine A *(38)*, have been demonstrated to protect the ischemic brain by regulating the Akt pathway.

Adenosine, which is released after stroke because of the consumption of adenosine triphosphate (ATP), reduces ischemic damage *(82)*. Such protection might occur for several reasons. For example, adenosine reduces glutamate release by activating adenosine A1 receptor *(83)*. It also enhances cerebral blood flow after ischemia and inhibits the inflammatory response by stimulating the adenosine A2A receptor *(82)*. The protective effect of adenosine may also be associated with the Akt pathway. In a study employing a hypoxia model, P-Akt (Ser 473) increased following hypoxia *(83)*. However, the adenosine A1 selective agonist cyclohexyladenosine further potentiated this increase, whereas the selective antagonist 8-cyclopentyltheophylline blocked P-Akt (Ser 473) increase, suggesting that adenosine inhibits ischemic damage by regulating Akt activity *(83)*.

The two immunosuppressants, Cyclosporin A (CsA) and FK 506, are well-known for protecting against stroke *(84)*. However, their protective mechanisms are poorly understood. Yoshimoto and colleagues *(38)* demonstrated that both CsA and FK 506 reduced infarct size in a rat transient focal ischemia model. CsA, but not FK 506, prevented a sustained decrease in P-Akt (Thr 308) level after stroke. This study provided two interesting points. First, P-Akt (Thr 308) has no transient increase after reperfusion, as noted for P-Akt (Ser 473), further strengthening our assertion that an increase in P-Akt (Ser 473) alone is not synonymous with an increase in Akt activity *(38)*. Under some conditions, the Akt kinase activity might have decreased because of a reduction in P-Akt (Thr 308). This can occur even if P-Akt (Ser 473) increases at the same time. Second, although enhancement of P-Akt (Thr 308) may be associated with neuroprotection, its decrease does not have to lead to cell death, because FK 506 reduces ischemic damage without enhancing protein levels of P-Akt (Ser 473).

As discussed earlier, the phosphorylation level of Akt is regulated by RTKs. Growth fac-

Table 3
The Effects of Various Compounds on the Akt Pathway

Model	S	Treatment	Results	Reference
8 h OGD, astrocytes	R	Aniracetam	It enhances P-Akt at 8 h	*89*
2 h MCAo	M	Dexamethasone	It causes eNOS activity through PI3K/Akt and reduces stroke	*118*
1 h MCAo	R	Kallikrein gene transfer	It increases NO, P-Akt and Bcl-2; reduces caspase-3, NADPH oxidase activity and superoxides	*119*
2 h MCAo	R	Cilostazol	It decreases P-PTEN and increases P-Akt, P-CREB, Bcl-2 and casein kinase 2 (CK2)	*45*
2 h MCAo	M	STAT1 KO	STAT1 KO enhances P-Akt and decreases procaspase-3 cleavage	*87*
90 min MCAo	R	Electroacupuncture	P-Akt reaches a peak at 8 h, acupuncture enhances P-Akt at 8 and 24 h and blocked caspase 9 cleavage	*92*
Hypoxia/ischemia	R	Adenosine	Adenosine antagonist and agonist blocks or enhances an increase in P-Akt after stroke, respectively (time point not known)	*120*
5 min Global	G	Sodium orthovanadate	It blockes a decrease in P-Akt at 1 h; it increases expression in the cell bodies and dendrites	*33*
2 h MCAo	R	Cyclosporin A and FK 506	Cs A but not FK 506, prevented down regulation of P-Akt (Thr-308)	*38*
Hippocampal culture, 15 h hypoxia	R	Erythropoietin (EPO)	Inhibition of PI3K reverses EPO's protection	*121*
Cortical culture, 2 h OGD	R	Osteopontin (OPN)	OPN increases P-Akt from 1 to 24 h after OGD; blocked by PI3K inhibitors	*88*
15 min MCAo l	M	Rai KO	Rai KO lead to lower P-Akt during the period of the early reperfusion	*122*
2 h MCAo	R	3-AB (PARP inhibitor)	3-AB reduces infarct by blocking inflammation and enhancing P-Akt, P-GSK3β, and reducing caspase-3 activity	*90*
2 h MCAo	R	δV1-1	It enhances P-Akt at 24 h	*81*
2 h MCAo	R	Cilostazol	After stroke, P-PTEN, P-Akt, P-CREB and Bax increased. Cilostazol reduces infarct by blocking P-PTEN and increasing P-Akt	*91*
90 min MCAo	M	t-PA Melatonin	t-PA aggravates injury by decreasing P-Akt without affecting Bcl-XL and caspase 3 activity; Melatonin restores P-Akt, increases P-Akt and reduces cas-3 activity	*93*

Abbreviations: OGD, oxygen–glucose deprivation; S, species; R, rat; M, mouse; MCAo, middle cerebral ischemia; G, gerbil; KO, knockout; eNOS, endothelial nitric oxide syntrase; NO, nitric oxide.

tors enhance protein tyrosine phosphorylation, which can be reversed by protein tyrosine phosphatase (PTP). Therefore, a PTP inhibitor may activate the Akt pathway by enhancing protein tyrosine phosphorylation levels through blocking of PTP activity. Indeed, a couple of reports demonstrated that the potent PTP inhibitor orthovanadate reduced ischemic damage in both global and focal ischemia by regulating the Akt pathway. In the global ischemia study, P-Akt (Ser 473) decreased at 0 h but transiently increased from 2 to 6 h after reperfusion and returned to baseline between 1 and 2 d after 5 min of bilateral CCA occlusion

in the gerbil *(33)*. Orthovanadate blocked the decrease in P-Akt (Ser 473) at 0 h after reperfusion but did not enhance P-Akt (Ser 473) at time-points from 2 h to 2 d. An in vitro Akt kinase assay suggested that Akt activity decreased at 0 h, and this effect was attenuated by orthovanadate. The authors further demonstrated that the PI3K inhibitor wortmannin blocked the protective effect of orthovanadate, suggesting that Akt activity contributed to its protection *(33)*. Work from the same group demonstrated that orthovanadate also reduced infarct size in a focal ischemia model in rats *(85)*. Similarly to the global ischemia model, orthovanadate did not enhance the transient P-Akt (Ser 473) peak at 6 h and did not prevent its decrease at 24 h after reperfusion; however, it did prevent the decrease in P-Akt (Ser 473) at 0 and 2 h after reperfusion. Together, the PTP inhibitor attenuates ischemic damage by enhancing Akt activity at the early time-points but not at later time-points after reperfusion.

The family of signal transducers and activators of transcription (STAT) proteins are phosphorylated in response to cytokines, which play critical roles in cell growth and differentiation *(86)*. Some members of the STAT family are believed to support cell survival, whereas others may contribute to cell death. STAT1 was reported to be phosphorylated and to translocate into nuclei after stroke *(87)*. Because the deficiency in STAT1 in knockout mice reduces infarct size, STAT1 activity is considered detrimental. The authors of the aforementioned study further reported that P-Akt (Ser 473) was upregulated in STAT1 knockout mice at 2 h after reperfusion in a 2-h transient focal ischemic model. It has been suggested that STAT1 functions upstream to suppress the PI3K/Akt pathway, thereby enhancing Bad and caspase activity *(87)*.

Osteopontin (OPN), a secreted cytokine, was reported to increase after stroke and is associated with the activity of β1 integrins, which support cell survival *(88)*. OPN treatment increases P-Akt (Ser 473) after stroke, and inhibition of Akt activity abolishes its protective effect *(88)*.

Aniracetam selectively enhances the sensitivity of the GluR2 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor *(89)*. It has been reported that GluR2 decreases after ischemia, leading to increases in Ca^{2+} permeability, and is potentially responsible for ischemic cell death *(89)*. Aniracetam treatment attenuates cell death after OGD in astrocyte culture, partly by upregulating Akt activity, because P-Akt increases with aniracetam treatment, and inhibition of Akt enhances cell death.

Hyperactivation of poly-(ADP-ribose) polymerase (PARP) is well-known to accelerate ischemic damage by depleting ATP. The PARP inhibitor, 3-aminobenzamide (3-AB) reduces ischemic infarct size after stroke *(90)* and not only inhibits the inflammatory response but also enhances levels of P-Akt and P-GSK3β.

Other neuroprotectants, including δv1-1 (a δPKC inhibitor; ref. *81*) cilostazol *(91)*, and electroacupuncture *(92)*—all of which reduce ischemic damage—also enhance P-Akt (Ser 473) protein level.

Conversely to protective effects of various neuroprotectants, tissue plasminogen activator exacerbates brain injury by decreasing P-Akt (Ser 473) level without affecting Bcl-l and caspase-3 activity *(93)*.

Together, it appears that a decrease in P-Akt (Ser 473) levels correlates with ischemic damage, whereas enhancement of P-Akt (Ser 473) levels is associated with the protective effects of various compounds after stroke. Therefore, the level of P-Akt may reflect its activity under conditions where protective compounds are employed, helping to mediate the protective effects of a broad range of compounds. However, such a correlation does not exist under other conditions, which are reviewed in the following sections.

P-Akt Level May Not Always Represent Akt Kinase Activity

Ischemic damage is definitely associated with lower P-Akt (Ser 473) levels after stroke, at least at later time-points after focal ischemia.

Table 4
Roles of the Akt Pathway in the Protective Effect of Preconditioning

PC	Interval	Ischemic model	S	Results	Reference
Two 2 min + one 3.5 min	1 d	10 min Global	G	PC decreases P-Akt at 10 min after reperfusion following 3.5 min ischemia	*8*
2 min	3 d	10 min Global	G	PC blocks the early peak of P-Akt after lethal ischemia but maintains P-Akt at later time points; Wortmannin blocks preconditioning.	*100*
5 min	2 d	30 min Global	R	PC blocks increases in P-Akt at 30 min after	*7*
3 min	1 d	6 min Global	R	PC blocks dramatic changes in P-Akt after lethal ischemia	*98*
2 min	3 d	10 min Global	G	PC enhances eNOS, probably through Akt	*123*
Two 10-min MCAo	3 d	1 h MCAo	R	PC blocks P-Akt peak at 3 h and inhibits its decrease at 24 h after lethal ischemia	*96*
30 min MCAo	3 d	100 min	R	PI3K inhibition does not block tolerance	*101*
8% O_2 for 3 h	Unknown	Right CCA o/8% O_2 for 2.5 h	NR	PC has no effect on P-Akt, GSK3β	*124*
5% O_2 for 9 h	1 d	Potassium deprivation, glutamate, 3-NP	Cell culture	Inhibition of Akt reverses PC's protection	*102*

PC, preconditioning; MCAo, middle cerebral ischemia; S, species; G, gerbil; R, rat; NR, neonatal rat.

Additionally, numerous studies have demonstrated that neuroprotectants reduce ischemic damage by enhancing P-Akt (Ser 473) levels both at the early and later time-points. However, this is not the case when preconditioning and hypothermia are employed as neuroprotectants.

Controversies Regarding the Role P-Akt in the Protective Effect of Preconditioning

Ischemic preconditioning, a brief nonlethal ischemia performed from hours to several days before the onset of a severe ischemia, reduces subsequent ischemic damage, an effect also referred to as ischemic tolerance *(94,95)*. Preconditioning is considered one of the most robust endogenous forms of neuroprotection. Several studies have recently addressed the role of the PI3K/Akt signaling pathway in the protective effect of preconditioning (Table 3). However, conclusions regarding whether Akt contributes to induction of ischemic tolerance are controversial. In these studies, almost all reports agree that P-Akt (Ser 473) transiently increases after reperfusion and reaches a peak within several hours but decreases at later time-points (Table 1). Surprisingly, preconditioning blocks this early peak *(7,8,96–100)*, despite attenuating the decrease in P-Akt (Ser 473) at the later time-points. There are several interpretations of these results. Some authors have concluded that Akt does not contribute to ischemic tolerance, because the early P-Akt (Ser 473) peak is inhibited by preconditioning *(7,8)*. Conversely, others have concluded that P-Akt (Ser 473) contributes to protection of preconditioning *(98,100)*. In the latter studies, the authors asserted that P-Akt (Ser 473) levels were improved by preconditioning at later time-points, thus P-Akt (Ser 473) enhancement contributes to the protective effect. However, the authors did not interpret the meaning of

the inhibited early peak of P-Akt (Ser 473) after preconditioning *(98,100)*.

Another study showed that the PI3K inhibitor LY294002 did not block the protective effect of preconditioning against focal ischemia *(101)*. Nevertheless, in a global ischemia model, Yano and colleagues *(100)* found that inhibition of the PI3K/Akt pathway by wortmannin blocked the protective effect of preconditioning, despite the finding that preconditioning blocked the early peak of P-Akt (Ser 473). This study has provided more direct evidence that the PI3K/Akt pathway contributes to induction of ischemic tolerance in global ischemia. This conclusion is supported by an in vitro study *(102)*, in which preconditioning is induced by hypoxia of 5% oxygen and the lethal ischemia is generated by potassium deprivation, glutamate, or 3-NP applications.

Despite some conflicting results, the general consensus is that preconditioning blocks the early peak of P-Akt (Ser 473) and attenuates decreases in P-Akt (Ser 473) at later time-points after reperfusion. Whether such inhibition is neuroprotective is not clear. We suggest that the controversy among these studies reflects the lack of an assay for true Akt kinase activity. Among eight articles studying Akt with preconditioning *(7,8,96–100)*, only one used an in vitro Akt kinase assay, suggesting that Akt activity was enhanced by preconditioning *(100)*. Unfortunately, the Akt kinase activity assay was not performed when P-Akt (Ser 473) reached a peak after ischemia, making it unclear whether P-Akt (Ser 473) reflected the level of activity

Hypothermia Inhibits Dysfunction of the Akt Pathway After Stroke

Mild hypothermia is also one of the most effective neuroprotectants against stroke *(103)*. Its protective mechanisms have received extensive study *(104)*. Hypothermia has been reported to inhibit various mediators of ischemic damage, including extracellular glutamate release *(105,106)*, free radical generation *(107)*, cytochrome c release/caspase activity *(108)*, and the inflammatory response *(109)*. However, few reports have studied its effect on neuronal survival signal pathways after stroke. Several groups, including our own, have studied the Akt survival pathway together with apoptotic pathways in a neonatal hypoxia/ischemia model and adult ischemia model. In one study using a neonatal hypoxia/ischemia model, hypothermia blocked cytochrome c release and caspase activity at 24 h after hypoxia/ischemia and simultaneously enhanced P-Akt (Ser 473) *(110)*. Unfortunately, this study did not examine the effects of hypothermia on P-Akt (Ser 473) at earlier time-points. The higher level of P-Akt (Ser 473) at 24 h in the hypothermic group might have been a result of tissue survival, rather than the mechanism responsible for the protective effect of hypothermia. Indeed, another report suggested that hypothermia did not enhance, but actually blocked, the increase in P-Akt (Ser 473) at 1 and 2 h after reperfusion in a similar neonatal hypoxia/ischemia model *(111)*. Because hypothermia also blocked caspase activity and reduced ischemic injury, the authors concluded that the Akt pathway was not important for the protective effect of hypothermia *(111)*. This is a similar phenomenon to the one discussed earlier in the studies of preconditioning, in which preconditioning also blocked the peak of P-Akt (Ser 473) after reperfusion.

However, it may not be appropriate to conclude that Akt activity does not contribute to the protection based on such results. We demonstrated that although hypothermia blocked the dramatic P-Akt (Ser 473) increase in the early reperfusion, it did not mean the Akt activity was also reduced *(4)*. In fact, in vitro Akt kinase assays demonstrated that hypothermia attenuated reduction in Akt activity, suggesting that the level P-Akt (Ser 473) did not represent the true activity of Akt. Furthermore, we found that the PI3K inhibitor LY294002 partially blocked the protective effect of hypothermia, strongly suggesting that Akt activity plays critical roles in the protective effect of hypothermia *(4)*. In our study, the effects of hypothermia on components upstream of Akt,

including PTEN and PDK1, and components downstream of Akt, such as FKHR, GSK3β, and β-catenin, were also studied. Hypothermia generally increases phosphorylation levels of PTEN, PDK1, and FKHR, which is consistent with its ability to improve Akt activity but not P-Akt (Ser 473) levels. However, hypothermia does not attenuate the reduction in P-GSK3β. It appears that activity of GSK3β increases even under hypothermia.

Because other reports have shown that activity of GSK3β leads to ischemic damage *(77,112)*, it appears that our result conflicts with such reports. Therefore, we further studied the effect of hypothermia on β-catenin, a signaling component downstream of GSK3β. Confocal microscopy revealed that β-catenin translocates from the cytosol into the nuclei after stroke, and hypothermia blocks its nuclear translocation in the penumbra but not in the ischemic core *(4)*. Therefore, despite the finding that hypothermia does not block GSK3β dephosphorylation, it inhibits nuclear β-catenin translocation, a downstream effect of GSK3β activity. It appears that hypothermia prevents ischemic damage by regulating at least one downstream target of GSK3β. Therefore, hypothermia reduces ischemic damage by enhancing Akt activity and attenuating a reduction in protein levels of other phosphorylated components of the Akt pathway.

Conclusion

In summary, it is generally agreed that P-Akt (Ser 473) temporarily increases after reperfusion in focal ischemia but may increase steadily after global ischemia. However, few reports have studied changes in P-Akt (Thr 308), which either remains unchanged or decreases after reperfusion. Changes in the phosphorylation levels of other components of the Akt pathway, including PDK1, PTEN, PRAS, GSK3β, and FKHR, are not always consistent with P-Akt (Ser 473). It is well-established that the Akt pathway contributes to the neuroprotective effects of a broad range of compounds. Such neuroprotectants usually attenuate the post-stroke decreases in phosphorylation levels of various effectors of the Akt pathway, and PI3K inhibitors often prevent the protective effects of some of these neuroprotectants. Conversely to individual neuroprotective compounds, mild hypothermia or preconditioning usually blocks the early increase in P-Akt (Ser 473), but Akt kinase activity might be preserved. Therefore, levels of P-Akt (Ser 473) may not accurately reflect the true activity of Akt. In conclusion, the Akt pathway regulates cell death and survival after stroke, making enhancement of Akt activity an attractive target for neuroprotection.

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