Novel Insights into Vascular Endothelial Growth Factor Receptor 2-Mediated Signaling to the Mammalian Target of Rapamycin/Akt Network in SK-N-SH Neuroblastoma Cells

by

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Abstract

The Role of Vascular Endothelial Growth Factor Receptor 2 in Modulating the Mammalian Target of Rapamycin/Akt Network in SK-N-SH Neuroblastoma Cells

by Jacob Edelstein

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Mammalian target of rapamycin (mTOR) is a central regulator of cell growth and division that exerts many of its effects through regulating protein synthesis. The kinase Akt is a substrate and regulator of mTOR. These proteins are integral to pathological and physiological function in neuronal cells and the Akt/mTOR network is the focus of pharmaceutical interventions. Muscarinic acetylcholine receptors and vascular endothelial growth factor receptor 2 (VEGFR2) can signal protein synthesis but whether they cooperate to mediate mTOR activation has not been demonstrated. Using serum-starved SK-N-SH neuroblastoma cells, we show that the muscarinic receptor agonists carbachol and pilocarpine enhance the activation of the mTOR substrate p70 S6 Kinase (S6K) and its target ribosomal protein S6 (S6) in a VEGFR2-dependent manner. Protein kinase C (PKC) functions in an opposing fashion by positively regulating S6K and S6 phosphorylation and suppressing Akt activation. Treatments with the phosphatase inhibitors sodium orthovanadate and okadaic acid (OA) increase S6, Akt and to a lesser extent S6K phosphorylation, indicating that tyrosine and serine/threonine dephosphorylation also regulates their activity. However, OA elicited a far greater increase in phosphorylation, implicating phosphatase 2A (PP2A) as a critical determinant of their function. Furthermore, PP2A inhibition induces the appearance of novel, high molecular weight, ubiquitinated forms of Akt. The accumulation of phosphorylated Akt induced by PP2A dysfunction causes depletion of total Akt. Rapamycin potentiates Akt phosphorylation and depletion in response to OA through a mechanism regulated by a previously unknown function of VEGFR2. Although hyperactivation of Akt is a common survival mechanism in cancer cells, Akt hyperphosphorylation is associated with induction of a caspase-independent cell death mediated by oxidative stress. Taken together, these results show that the critical role of PP2A in regulating Akt activation also affects Akt ubiquitination, cleavage and removal from the cell. Furthermore, these data

indicate the importance of reactive oxygen species in eliciting cell death and that PP2A promotes survival through a suppression of oxidative stress. Finally, VEGFR2 can stimulate mTOR when stimulated by ligand binding, transactivation or an unknown mechanism induced by rapamycin.

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4EBP1 eIF4E binding protein 1

7-AAD 7-Aminoactinomycin D, DNA dye

Akt protein kinase B/PKB

ANOVA analysis of variance

Bcl-xL B-cell lymphoma-extra large

CREB cyclic AMP-response element-binding protein

EGF epidermal growth factor

EGFR epidermal growth factor receptor

eIF4E eukaryotic initiation factor 4E

ERK1/2 extracellular signal-regulated protein kinases 1 and 2

FBS fetal bovine serum

FRB FKBP12-rapamycin binding domain

G-protein guanine-nucleotide binding protein

GFX GF109203X, PKC inhibitor

GPCR G-protein-coupled receptor

GTP guanosine triphosphate

HMW high molecular weight

IGF-I insulin-like growth factor I

LY LY294002, PI3K inhibitor

mAchR muscarinic acetylcholine receptor

mTOR mammalian target of rapamycin

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

Nac N-acetylcysteine

NGF nerve growth factor

OA okadaic acid

PAGE polyacrylamide gel electrophoresis

PBS phosphate buffered saline

PDGF platelet-derived growth factor

PDGFR platelet-derived growth-factor receptor

PH pleckstrin homology protein domain

PHLPP PH domain and leucine rich repeat protein phosphatase

PI3K phosphoinositide 3-kinase

PIP3 phosphatidylinositol 3, 4, 5 phosphate

PKC protein kinase C

PLC phospholipase C

PP2A protein phosphatase 2A

Rheb Ras homolog enriched in brain

ROS reactive oxygen species

RTK receptor tyrosine kinase

S6 ribosomal protein subunit S6

S6K ribosomal protein subunit S6 kinase

SDS Sodium dodecyl sulfate

SEM standard error of the mean

siRNA small interfering ribonucleic acid

SU SU1498, VEGFR2 inhibitor

TORC target of rapamycin complex

TRAF6 tumor necrosis factor receptor associated factor 6

TSC1 tuberous sclerosis complex protein 1 (hamartin)

TSC2 tuberous sclerosis complex protein 2 (tuberin)

S473 serine 473 (on Akt)

T308 threonine 308 (on Akt)

TTC3 tetratricopeptide repeat protein 3

UPP ubiquitin proteasome pathway

VEGF vascular endothelial growth factor

VEGFR VEGF receptor

ZVAD z-VAD-FMK, pan-caspase inhibitor

Chapter 1

Introduction

1. Introduction

The mammalian target of rapamycin (mTOR) and Akt (also known as protein kinase B) are protein kinases at the hub of a complex, interdependent network that responds to cellular signals such as energy status, growth factors and stress by controlling processes including protein synthesis, growth, proliferation and survival. Akt and mTOR are aberrantly activated in many different cancers and a great deal of research has been focused on the oncogenic properties of these molecules. However, the mTOR/Akt network is relevant in many other settings and has been implicated in neuronal dysfunctions including epilepsy and neurodegenerative diseases.

This work addresses the modulation of the mTOR/Akt pathway in a neuronal model of SK-N-SH neuroblastoma cells. We present a novel example of signaling upstream of mTOR involving crosstalk between a G-protein coupled receptor (GPCR) and a receptor tyrosine kinase (RTK). In this model, protein kinase C (PKC) positively regulates the activation of TOR complex 1 (TORC1) targets while inhibiting TORC2/Akt signaling. We also provide evidence that phosphatase activity plays a central role in modulating mTOR target activation and suppresses an oxidative cell death. Furthermore, phosphatase activity is implicated in maintaining Akt levels, supporting the idea that Akt phosphorylation plays an important role in its degradation. The work presented here contributes to the understanding of this essential signaling network in a neuronal context. Given the clinical significance of the mTOR/Akt network, elucidating the signal transduction pathways that regulate mTOR and Akt may suggest novel strategies for intervention and therapy.

1.1 GPCRs and RTK signaling

Signal transduction in neurons is an essential process in control of cellular functions including communication, growth, proliferation, protein synthesis and response to stress. Transduction occurs primarily through reception of signaling molecules at the cell surface by any of several different classes of membrane-localized receptors. These receptors can be categorized by the class of agonist required for activation as well as the mode of downstream action by which the receptor transduces the extracellular signal. Additionally, signaling by one receptor class can modulate that of another, serving to integrate the effects of external messages. A major focus of the work presented here concerns crosstalk between members of two major platforms for transduction, the GPCRs and the RTKs.

GPCRs make up the largest group of transmembrane receptors in humans and are implicated in mediating numerous cell-signaling events in physiological and pathological conditions (Lappano and Maggiolini 2011). GPCR signaling pathways were the target of intervention in over half of all therapies current in 2002, indicating their importance in aberrant signaling (Pierce et al 2002). All GPCRs have seven transmembrane regions connected by extramembrane loops whose intracellular regions can interact with G proteins. Mammalian guanine-nucleotide binding (G) proteins are classified according to their structure as either heterotrimeric or small and their activity depends on the nature of the bound guanine nucleotide. Guanosine triphosphate (GTP)-bound G proteins are active and can signal to downstream effectors until hydrolysis of the GTP to GDP inactivates the protein. Heterotrimeric G proteins are comprised of three subunits, α, β and γ, which exist in a trimer associated with a GPCR. Agonist binding to the GPCR induces the G_α subunit to exchange GDP for GTP as well as the dissociation of G_α from $G_{\beta\gamma}$, forming two independent signaling entities. The subsequent pathway activation depends on the type of the G-protein subunit. The G_α subunit family is the most diverse and is grouped into four categories, G_s, G_i, G_q and G_{12}. Major effectors of G_α signaling include phospholipase C (PLC), Ca^{2+} flux and protein kinase C (PKC). The diversity of G-protein allows for complex networks to be activated by these receptors.

The muscarinic acetylcholine receptors (mAchRs) are a subgroup of GPCR present in neuronal cells. They are coded for by five genes designated m_1 through m_5 which correlate to the M_1 through M_5 subtypes of mAchRs (Wess et al 2007). The constituent mAchR subtypes vary with cell and tissue type and are found in some combination in the nervous system and many peripheral tissues. Acetylcholine is the physiological agonist for mAchRs and acetylcholine analogs can be used as mAchR agonists. Pilocarpine and carbachol are two such cholinergic drugs and have a number of applications for modulating parasympathetic responses.

Different mAchR subtypes interact with distinct G-protein subunits to signal cellular functions through specific downstream effector proteins. For example, the M_1, M_3 and M_5 receptors couple with $G_{q/11}$ proteins to activate phospholipase C (PLC) while M_2 and M_4 receptors interact with $G_{i/0}$ proteins to inhibit adenylyl cyclase. Subtypes of mAchRs that are coupled to $G_{q/11}$ proteins can protect cells from apoptosis following DNA damage, oxidative stress, and mitochondrial dysfunction (Budd et al 2003, De

Sarno et al 2003). In addition, PLC signaling activates PKC and elevates intracellular calcium (Budd et al 2004). These events can be further enhanced through the activation of different RTKs (Schmidt et al 2000). Moreover, mAchRs can also stimulate mitogenic pathways linked to RTKs, such as epidermal growth factor (EGFR) and platelet-derived growth factor (PDGFR) to mediate cell proliferation, differentiation and survival (Luttrell 2003). This evidence demonstrates that, while mAchRs can signal alone upon agonist binding, they can also integrate multiple inputs of information by acting in concert with pathways stimulated by other receptors including RTKs (Lowes et al 2002).

The receptor tyrosine kinases have a single extracellular region which mediates ligand binding, a transmembrane region, and an intracellular region with intrinsic tyrosine kinase function. Vascular endothelial growth factor (VEGF) is a well-established angiogenic factor that can also confer neuroprotection through its cognate RTK, VEGFR2 (Zachary 2005). In addition to VEGFR2, VEGF can bind to and activate VEGFR1, although the functional significance of this binding is vague. In comparison to VEGFR2, VEGFR1 has a much greater affinity for VEGF but weaker tyrosine kinase activity, suggesting that a major VEGFR1 function is to act as a decoy receptor for VEGF (Hiratsuka et al 1998, Hiratsuka et al 2005). Previous research using our SK-N-SH neuronal model showed that VEGFR2 levels are maximally upregulated 48 h after serum deprivation and VEGFR2 is an important pro-survival factor which can function via paracrine and autocrine signaling (Gomes et al 2007). VEGF binding causes VEGFR2 dimerization, autophosphorylation and kinase activation of the receptor's intracellular region. This attracts docking proteins and allows transduction of the VEGF signal (**Fig. 1.1**). Through these docking proteins, VEGFR2 can activate numerous downstream pathways such as PI3K/Akt, PKC and mitogen-activated protein (MAP) kinases, including MEK/ERK1/2 (Olsson et al 2006).

A number of interactions between GPCRs and RTKs have been elucidated. For example, GPCR stimulation of the EGFR family can occur by mechanisms including proteinase-mediated release of EGFR ligands, Src-mediated phosphorylation of the EGFR, and direct GPCR/EGFR interaction (Rozengurt 2007). VEGFR2 can be transactivated by the bradykinin B2 GPCR to mediate angiogenic processes in endothelial cells (Miura et al 2003, Thuringer et al 2002). In a model of pancreatic cancer, insulin signaling can potentiate GPCR activation and promote tumor growth (Kisfalvi et al 2009). Mammalian TOR was identified as an important point of convergence between GPCR and RTK signaling in these

pancreatic cells and inhibition of mTOR activation hindered tumor growth and crosstalk between GPCR and insulin signaling. Thus, crosstalk occurs between a number of different GPCR and RTK entities and leads to important biological outcomes.

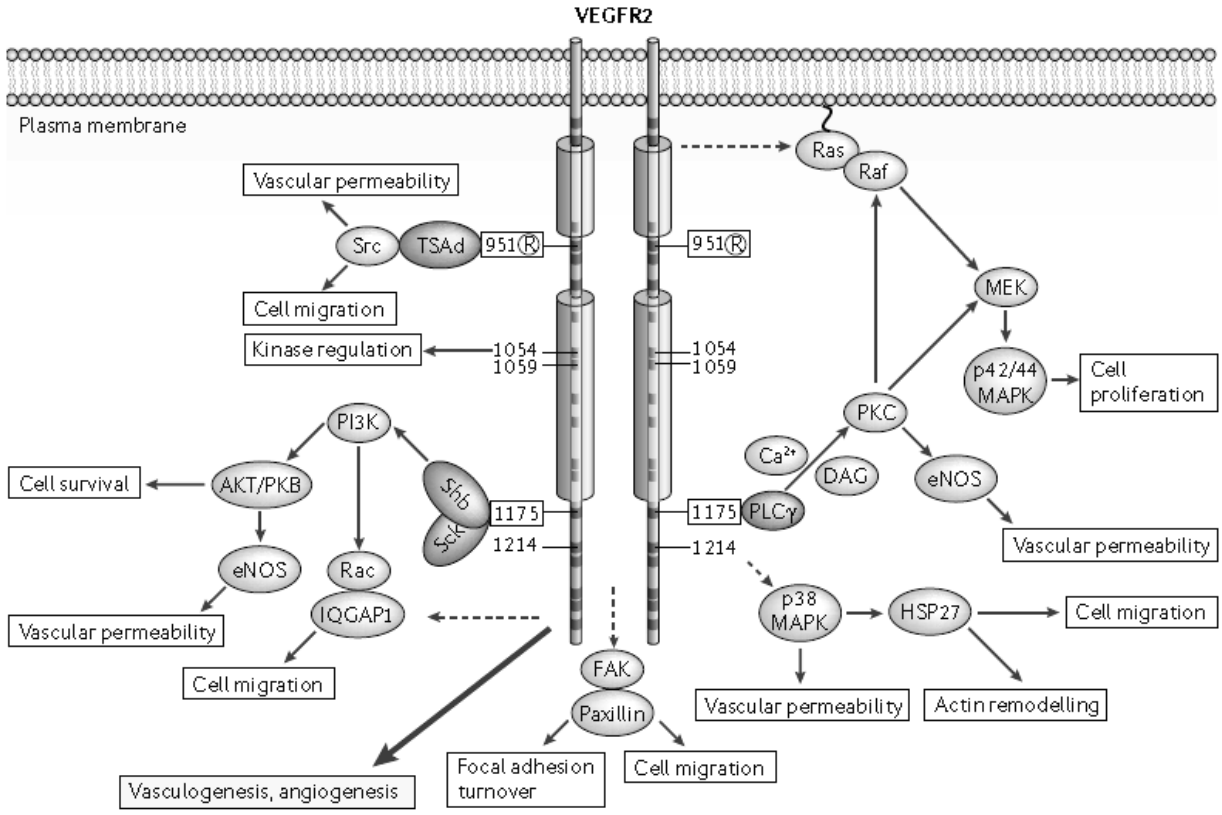


Figure 1.1. Signaling downstream of VEGFR2. The intracellular domain of VEGFR2 is pictured along with signaling cascades associated with specific phosphorylation sites on the receptor. Biological outcomes are indicated in boxes. DAG, diacylglycerol; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; HPC, haematopoietic progenitor cell; HSP27, heat-shock protein-27; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; PLCγ, phospholipase C-; Shb, SH2 and β-cells; TSAd, T-cell-specific adaptor. From Olsson et al 2006.

1.2. The two mTOR complexes

Mammalian TOR is a serine/threonine kinase that senses many inputs to cellular homeostasis such as hypoxia, stress and nutrient status (Sandsmark et al 2007, Sarbassov et al 2005a). In response, mTOR regulates translation, metabolism, cell growth and proliferation and plays a major role in neuronal development, synaptic plasticity, survival and long-term memory formation (Kelleher et al 2004, Kumar et al 2005, Richter and Klann 2009, Tang et al 2002). Under pathological conditions, aberrant mTOR activity is associated with seizure induction in neurological disorders such as tuberous sclerosis, cortical dysplasia and temporal lobe epilepsy (Crino 2009, Zeng et al 2009). In mammals, the mTOR protein functions in two different multimeric complexes, TORC1 and C2.

The two TOR complexes differ in their constituent proteins, their upstream effectors and their susceptibility to inhibition by the bacterial product rapamycin. TORC1 activity targets regulators of protein synthesis such as the eIF4E-binding protein 1 (4EBP1) and S6 kinase (S6K). In its hypophosphorylated form 4EBP1 sequesters the eukaryotic initiation factor 4E, blocking a rate-limiting step in translation. TORC1 promotes translation by phosphorylating 4EBP1 and inducing the release of eIF4E (Mamane et al 2006). S6K is also linked to the ability of mTOR to affect cell growth and proliferation. Activated TORC1 phosphorylates S6K which phosphorylates a number of substrates involved in translation including the ribosomal subunit S6, however it is not clear how this affects protein synthesis (Foster and Fingar 2010)

The main endogenous regulator of mTORC1 is a complex of the hamartin (TSC1) and tuberin (TSC2) proteins (Tee et al 2002). Together, TSC1 and 2 act as a GTPase-activating protein for the G protein Ras homologue enriched in brain (Rheb), which in its GTP-loaded form is an essential activator of TORC1 (Long et al 2005, Tee et al 2003). Much less is known about the regulation of TORC2 although it can act as a serine 473 (S473) kinase of Akt, often downstream of growth factor receptors (Hresko and Mueckler 2005, Sarbassov et al 2005b).

In neuronal models, mTOR pathway activation can be controlled by GPCRs and RTKs. Agonists for mAchRs have been shown to stimulate activation of S6K in 1321 astrocytoma cells (Tang et al 2003), SH-SY5Y (Deguil et al 2008) and PC12 cells (Chan et al 2009) and S6 ribosomal protein via PKC in serum-deprived SK-N-SH neuroblastoma cells (Slack and Blusztajn 2008). Furthermore, VEGF was

shown to elevate intracellular calcium and the activation of mTOR, S6K and Akt in hippocampal neurons (Kim et al 2008a).

1.3. Regulation of Akt Signaling and Stability

Akt is a central regulator of major cellular functions including cellular proliferation, cell cycle progression, metabolism and apoptosis. Akt activation is a complex process centering on the production of the phospholipid phosphatidylinositol 3, 4, 5 phosphate (PIP3) mediated by PI3K. The pleckstrin homology (PH) domain of Akt interacts with PIP3 causing membrane recruitment and a change out of inactive conformation that allows PDK1 to phosphorylate Akt at threonine 308 (Calleja et al 2007). Akt is not fully active unless it is also phosphorylated at the serine 473 site in its C-terminal hydrophobic motif. Once Akt is at the membrane TORC2 acts as the S473 kinase (Hresko and Mueckler 2005, Sarbassov et al 2005b) although in some circumstances other kinases or autophosphorylation may also play this role (Bozulic and Hemmings 2009, Franke 2008). While targeting T308 appears to be the key step in Akt activation, it is not clear if an established order of T308 and S473 phosphorylation exists (Bhaskar and Hay 2007, Huang and Manning 2009, Polak and Hall 2006).

Activated Akt is under tight control by two phosphatases, protein phosphatase 2A (PP2A) and PH domain and leucine rich repeat protein phosphatase (PHLPP). These target T308 and S473 respectively although some research indicates that PP2A can also act on S473 (Liao and Hung 2004). PP2A and PHLPP are in distinct subfamilies and, while PP2A is inhibited by the poriferan toxin okadaic acid (OA), PHLPP is not (Gao et al 2005).

Several lines of research indicate that the activation state of Akt is an important determinant of its stability. Some research shows that unphosphorylated Akt is subjected to increased ubiquitination and degradation (Facchinetti et al 2008), but more recent experiments suggest that it is active Akt that is preferentially targeted by the ubiquitin proteasome pathway (UPP). For example, myristoylated Akt was polyubiquitinated to a much greater degree than inactive Akt by the E3 ubiquitin ligase TTC3 (Suizu et al 2009). S473 phosphorylation has also been shown to induce Akt polyubiquitination and proteasomal degradation (Wu et al 2011).

In addition to the proteasome, Akt can be digested by caspases (Rokudai et al 2000). Hydroxynonenal, a product of oxidative stress, induces a caspase-dependent Akt dephosphorylation by

PP2A later leading to Akt degradation (Liu et al 2003). In addition, caspase activation has been identified as a necessary event for the ubiquitination and UPP-mediated degradation of Akt in 3T3-L1 adipocytes (Medina et al 2005). These studies show that the phosphorylation state of Akt is an important determinant of its stability and both caspases and the UPP can degrade Akt. However, whether Akt activation results in elevated Akt degradation appears to vary with cell type and proteolytic pathway.

1.4. Akt/mTOR Interactions

Akt and mTOR signaling are connected in a complex loop of interactions that places the two kinases both upstream and downstream of each other. As described above, mTORC2 is the main S473 kinase directly upstream of Akt. Additionally, Akt positively regulates mTORC1 by an inactivating phosphorylation of TSC2, a negative regulator of Rheb. However, mTORC1 signaling also engages mechanisms that reduce its activity. S6K, a canonical target of mTORC1, can dampen upstream signaling to Akt by inhibiting receptor signaling pathways such as that of the insulin receptor and PDGFR thus reducing the activity of PI3K (Haruta et al 2000, Zhang et al 2007). This signaling allows mTORC1 to modulate its own activation through negative feedback (**Fig. 1.2**).

Isolation of rapamycin led to the discovery of TOR and has been the basis for much research concerning TOR function. The mTOR/S6K/Akt feedback loop has significant consequences for rapamycin-based inhibition of mTOR. Rapamycin functions by complexing with FK506-binding protein 12 (FKBP12) and binding to the allosteric FKBP12-rapamycin binding domain of TOR (Kim et al 2002). This association weakens the raptor/mTOR interaction and reduces or ablates the phosphorylation of mTORC1 targets (Kim et al 2002, Oshiro et al 2004). However, rapamycin does not inhibit all mTOR kinase activity. The compound interferes with mTORC2 only in some cell lines under chronic exposure and leaves TOR-mediated Akt activation intact in other instances (Sarbassov et al 2006). Furthermore, mTORC1 possesses rapamycin-resistant functions. Rapamycin-mediated inhibition of mTORC1 does not categorically inhibit 4EBP1 phosphorylation and, presumably for this reason, has limited effects on protein synthesis (Choo and Blenis 2009, Feldman et al 2009, Thoreen et al 2009). This effect and feedback on PI3K/Akt appear to be of clinical importance and may underlie the modest results obtained from rapamycin-based cancer treatments (Shor et al 2009). To address these issues, new approaches to inhibiting mTOR have been developed.

A new mode of non-allosteric, ATP-competitive inhibition of mTOR has been formulated in recent years (Guertin and Sabatini 2009). These drugs target the TOR kinase domain and thus inhibit both mTOR complexes. Use of this inhibition strategy was shown to have greater efficacy than rapamycin in some cancer models and revealed the importance of 4EPB1 in driving protein synthesis, survival and tumorigenesis (Hsieh et al 2010). This approach also prevents the activation of Akt by mTORC1 inhibition by simultaneously compromising TORC2's Akt kinase activity (Feldman et al 2009).

1.5. Pilocarpine Model of Epilepsy

Pilocarpine is used in animal models to replicate temporal lobe epilepsy and this effect appears to be mediated by specific mAchR subtypes (Bymaster et al 2003). Pilocarpine induces status epilepticus which causes acute neuronal damage. This is followed by a latent period of days to weeks, then the onset of spontaneous seizures leading to a chronic epileptic state (Leite et al 2002). During the events preceding the chronic state it appears that dysfunction of components of cholinergic reception is a key contributor to the development of disease (Friedman et al 2007). In addition, cell death and the formation of new neuronal circuitry generate significant changes in the brain (Cavazos and Cross 2006, Sutula 2002). Interestingly, hyperactive mTOR signaling is associated with both acquired and inherited epilepsies and rapamycin has been shown to be an effective treatment in several epilepsy models (Ehninger et al 2008, Huang et al 2010, Wong 2010).

Oxidative stress is known to follow acute seizure activity and reduction of cellular antioxidant activity occurs during the latent period (Waldbaum et al 2010, Waldbaum and Patel 2010). An excessively pro-oxidant environment can cause cell death in a number of ways including induction of apoptotic pathways and inactivating mitochondrial enzymes (Patten et al 2010, Starkov 2008). Neurons are highly susceptible to oxidative stress due to energy depletion resulting from mitochondrial dysfunction (Du et al 2003, Zeng et al 2007). However, the relative contribution of oxidative stress to neuronal cell death in models of epilepsy is unclear.

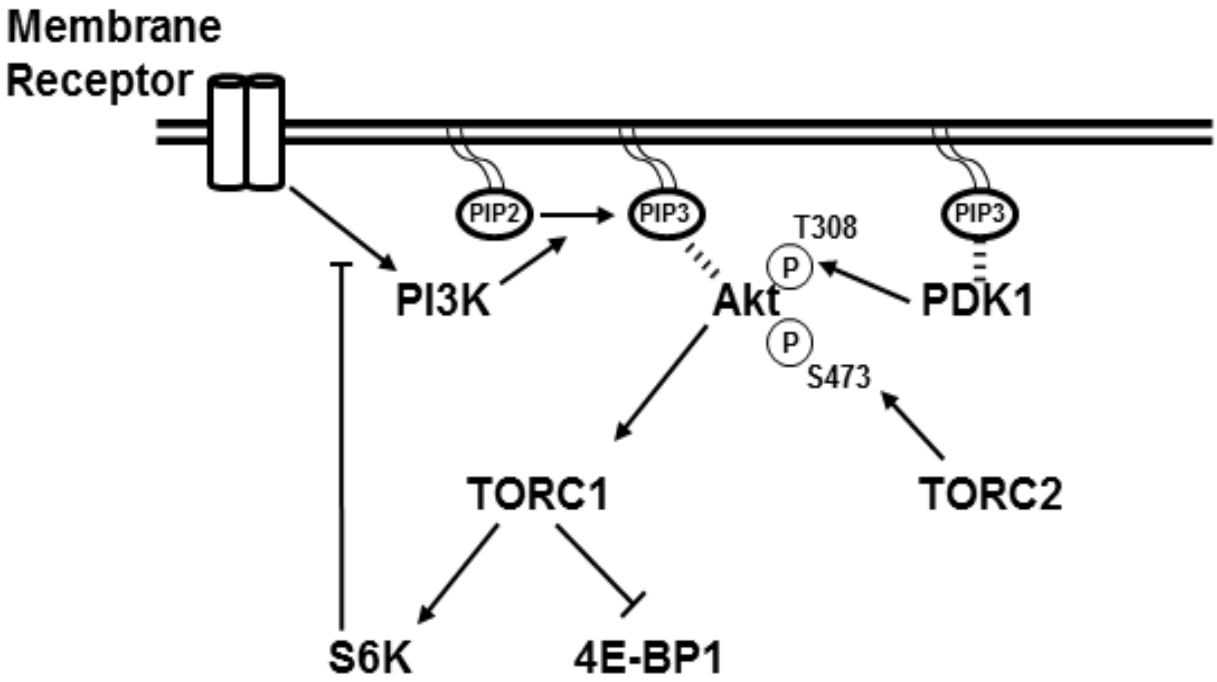


Figure 1.2. Akt is upstream and downstream of mTOR signaling. Receptor activation signals to PI3K through adaptor proteins and generates phosphatidylinositol 3, 4, 5 triphosphate (PIP3) from PIP2. PIP3 recruits Akt to the plasma membrane by interacting with the Akt pleckstrin homology domain and facilitating the assumption of an "open" Akt conformation allowing PDK1 (which is also recruited to the membrane by PIP3) to phosphorylate Akt at T308. TORC2, also an Akt kinase, will phosphorylate Akt at S473. Activated Akt can indirectly stimulate TORC1 in part by inhibiting the TSC1/2 complex. TORC1 phosphorylates and activates S6K and inhibits 4E-BP1. Active S6K can inhibit Akt activation by negatively regulating upstream signaling to PI3K. Dashed lines represent intermolecular interactions (adapted from Huang and Manning 2009).

1.6. *Rationale for Addressing RTK/mAchR Crosstalk*

The interplay between RTKs and GPCRs has been shown to have significant effects in several models of cancer. As described in 1.1, GPCR signaling can potentiate insulin pathway response and drive tumor growth in pancreatic cancer cells (Rozengurt 2007). Furthermore, an interaction between EGFR and neurotensin receptor-mediated signaling induces mitogenesis in prostate cancer cells (Amorino et al 2007). Interactions between RTKs and GPCRs have also been implicated in other disorders in addition to cancer. For example, renal and cardiovascular pathologies associated with metabolic syndromes such as obesity and diabetes have been linked to crosstalk between insulin and angiotensin II pathways (Kisfalvi et al 2007). Together these studies indicate the prevalence of these RTK/GPCR interactions and their biological relevance.

Some shared overlap between pathways is necessary for crosstalk to occur. In the case of VEGFR2 and mAchR signaling, evidence exists showing that agonists for either receptor can activate the PI3K/Akt/mTOR network as well as PKC (Slack and Blusztajn, 2008, Kim et al 2008). A growing body of research demonstrates that VEGF has neuroprotective properties and mTOR activation is implicated as a pro-survival factor in a number of different cancers (Guertin and Sabatini 2007, Ruiz de Almodovar et al 2009). The SK-N-SH neuronal model is widely used in assessment of neurotoxicity and neuroprotection and these cells express all five mAchR subtypes (Slack and Blusztajn 2008). These properties first led us to investigate mAchR agonists in SK-N-SH cells for possible use as an *in vitro* model of epilepsy and to propose to investigate the effects of mAchR agonists on cell viability. Our results demonstrating the common targets between VEGFR2 and mAchRs as well as the general importance of GPCR/RTK interactions led us to hypothesize that VEGFR2/mAchR crosstalk regulates mTOR/Akt and this signaling is neuroprotective in serum-starved SK-N-SH cells. This hypothesis forms the basis for the work described in chapter 3.

1.7. *Rationale for Addressing the Effects of PP2A on Akt Phosphorylation*

The work in chapter 4 started with our results from chapter 3 showing that the efficacy of VEGF-mediated Akt stimulation is much greater in the presence of the phosphatase inhibitor OA. Furthermore, Akt phosphorylation was increased to a much greater extent by inhibition of PP2A than by tyrosine phosphatase inhibition. Akt is a crucial effector of mTOR function (Guertin and Sabatini 2007) as well as

neuronal survival, growth and neurodegeneration (Chow et al 2009, Hoeffer and Klann 2010). The central role of Akt in pathological and physiological signaling has generated a considerable interest in the regulation of Akt phosphorylation. These considerations led to further investigation into the relationship between phosphatase activity and Akt activation.

Chapter 2

Materials and Methods

2.1 Materials

Recombinant human VEGF 165 was obtained from PeproTech (Rocky Hill, NJ). SU1498, LY294002, GF-109203X, rapamycin and okadaic acid were obtained from LC Labs (Woburn, MA). Pilocarpine was from MP Biomedicals (Solon, OH), carbachol was from Calbiochem (La Jolla, CA), N-acetylcysteine in thiol form (Nac) and sodium orthovanadate (Na_3VO_4) were from Sigma (St. Louis, MO). ZVAD was procured from Axxora (San Diego, CA) while PP242 was from ChemDea (Ridgewood, NJ).

2.2 Cell Culture and Inhibitor Treatment

Human neuroblastoma SK-N-SH cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained at 37℃ in MEM supplemented with 5% fetal bovine serum, non-essential amino acids, sodium pyruvate, L-glutamine and vitamins (Invitrogen; Carlsbad, CA).

For each experiment in Chapter 3, cells were grown to 80% confluence followed by treatments under serum starvation for a period of 48 h as described in Gomes et al. (2007). For inhibitor studies, serum starved cells were pretreated for 1 h with okadaic acid (OA) or Na_3VO_4, 48 h with rapamycin, or for 2 h with SU1498, LY294002 and GF-109203X (GFX). Unless indicated otherwise, these treatments were followed by 15-min incubations with VEGF or the agonists for mAchRs, carbachol and pilocarpine. In the cell viability experiments, all agonists and inhibitors were incubated for 24 h.

For inhibitor studies in Chapter 4, cells were grown and serum-starved as above then treated for 2 h with either PP242, SU1498, Nac or ZVAD followed by a 1-h OA treatment where indicated. Rapamycin treatments were for 48 h.

2.3 siRNA

Cells were transfected with 20 μM of KDR/Flk-1/VEGFR2 SMARTpool siRNA duplexes or non-specific (scrambled) siRNA duplexes (Dharmacon RNA Technologies; Lafayette, CO) according to the manufacturer's directions. Cells were then incubated without serum for 48 h before lysis.

2.4 Protein extraction and immunoblotting

Cells were harvested in lysis buffer and quantified for protein content using the protocol described previously (Rockwell et al 2004). Equal amounts of protein from each total cell lysate were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were blocked

then incubated overnight at 4℃ with primary antibody. All primary and secondary antibodies were from Cell Signaling (Danvers, MA) except for the antibodies against actin (Sigma), ubiquitin (Dako; Houston, TX) and phosphorylated and total VEGFR2 (Santa Cruz Biotechnology; Santa Cruz, CA). Immunoblots were visualized with the SuperSignal West Pico chemiluminescent Substrate (Pierce; Rockford, IL) and quantified where indicated using ImageJ (NIH, http://rsb.info.nih.gov/ij/). Phosphorylated proteins were normalized to their respective total protein levels and expressed as the fold difference of the appropriate control.

2.5 Protein Immunoprecipitation

VEGFR2 was immunoprecipitated from equal concentrations (500 µg) of total cell lysates using an anti VEGFR2 antibody (Millipore; Billerica, MA). Antibody antigen complexes were selectively removed by binding to protein A magnetic beads (Millipore) according to the manufacturer's directions and then incubated overnight at 4℃ in a final volume of 200 µl. Precipitates were eluted in 2x SDS loading buffer and analyzed by immunoblotting. Akt was immunoprecipitated using antibody specific to either p-Akt T308 or Akt (Cell Signaling) from 400 µg of cell lysates as described above.

2.6 Cell viability

Cells were plated in 96-well plates at a concentration of 5 X 10^4 cells/well and treated as indicated under serum deprivation for 48 h at 37℃. Cell viability was determined using a colorimetric MTS assay (Promega Corp; Madison, WI) and quantified according to manufacturer's instruction. Survival measurements are expressed as the percent of the untreated or vehicle control.

2.7 Caspase 3/7 activity

Caspase activity was measured in cells plated in a 96-well format using the fluorescence cell-based Apo-ONE Homogeneous Caspase 3/7 Assay (Promega) according to manufacturer's directions. Caspase activity was quantified using the Molecular Dynamics Typhoon 9410 Imaging System with ImageQuant software (Amersham; Piscataway, NJ). Data were normalized as fluorescent units/µg protein.

2.8 Nuclear and Cytoplasmic Fractionation

Cells were serum-starved for 48 h and treated over a one-h time period with 400-nM okadaic acid then collected with dissociation buffer (0.3 mM EDTA and 5% glycerol in Hank's Balanced Salt Solution) and fractionated with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce; Rockford, IL) according to manufacturer's instructions.

2.9 Immunofluorescence

Cells were plated in 8-well glass chamber slides and serum-starved for 48 h with or without rapamycin then treated with OA for 1 h as indicated. Cells were then fixed in 3.7% formaldehyde and permeabilized with 0.1% saponin for 20 min and incubated overnight with antibody against p-Akt T308 (1:900, Cell Signaling) and detected with fluorescein-conjugated secondary antibody (Invitrogen). Nuclei were detected with 7-aminoactinomycin D (7-AAD, Invitrogen). Images were captured at 40X using a Leica TCS SP2 Laser Scanning Spectral Confocal Microscope.

2.10 Statistical analyses

Data are expressed as the mean ± SEM of cell treatments that were replicated at least three times. Statistical significance was assessed with GraphPad Prism version 5.00 using a one-way (Fig. 3.7) or two way (Fig. 3.6) analysis of variance (ANOVA) with Bonferroni's posttest; $P < 0.05$ was considered significant (GraphPad Software; San Diego, CA).

Chapter 3

Crosstalk between VEGFR2 and muscarinic receptors regulates the mTOR pathway in SK-N-SH cells

The work in Chapter 3 has been published (Edelstein et al 2011).

3.1. Muscarinic and VEGF receptors signal to mTOR

In these studies we investigated whether the VEGF/VEGFR2 pathway would couple with mAchRs in SK-N-SH cells to activate the PI3K/Akt/mTOR pathway as a response to serum deprivation. Since mAchRs signal to the TORC1 target S6 in SK-N-SH cells (Slack and Blusztajn 2008), serum-starved cells were treated with the mAchR agonist carbachol at 1mM (Slack and Blusztajn, 2008) and examined over a 30-min time frame for the phosphorylation of S6K at the TORC1 site T389 and Akt activation at T308 (**Fig. 3.1A**). Carbachol elicited opposing effects on S6K and Akt in that an increase in S6K phosphorylation, which peaked at 15 min, was accompanied by a concomitant decrease in Akt activation (lane 8). Experiments were then performed to evaluate the phosphorylation of mTORC1 targets and Akt in serum-starved cells incubated with VEGF for 15 min (**Fig. 3.1B**). VEGF also increased the phosphorylation of S6K and S6 at the S6K activation sites S235/236 while mediating a slight increase in Akt phosphorylation at positions T308 and S473. The overlap of downstream targets between mAchRs and VEGFR2 in SK-N-SH cells led us to hypothesize that crosstalk between these receptors signals to the mTOR pathway.

3.2. G-protein-coupled mAchRs stimulate VEGFR2 activation upstream of mTOR

To address whether mAchRs cooperate with VEGFR2 to signal S6K and S6 activation, serum-starved SK-N-SH cells were treated for 10 or 15 minutes with VEGF or carbachol in the presence and absence of 10-μM SU1498, a pharmacological inhibitor of VEGFR2 (**Fig. 3.2A**). SU1498 at this concentration has been shown to inhibit VEGF-mediated activity in both neuronal and endothelial cells (Jin et al 2002, Shen et al 1999). Treatments with SU1498 (**Fig. 3.2A**, lanes 4, 5, 8 and 9) or VEGFR2 siRNA (**Fig. 3.2B**) attenuated the phosphorylation of S6K and S6 induced by VEGF or carbachol, suggesting that mAchRs crosstalk with VEGFR2 in serum deprived SK-N-SH cells to activate mTOR. Immunoprecipitation of VEGFR2 from VEGF and carbachol treated cells indicated that both agonists stimulated an increase in the autocrine signaling of VEGFR2 phosphorylation by VEGF (**Fig. 3.2C** lanes 2 and 3) (Gomes et al 2007). These results suggest that mAchRs transactivate VEGFR2 to mediate mTOR activation in serum deprived SK-N-SH cells.

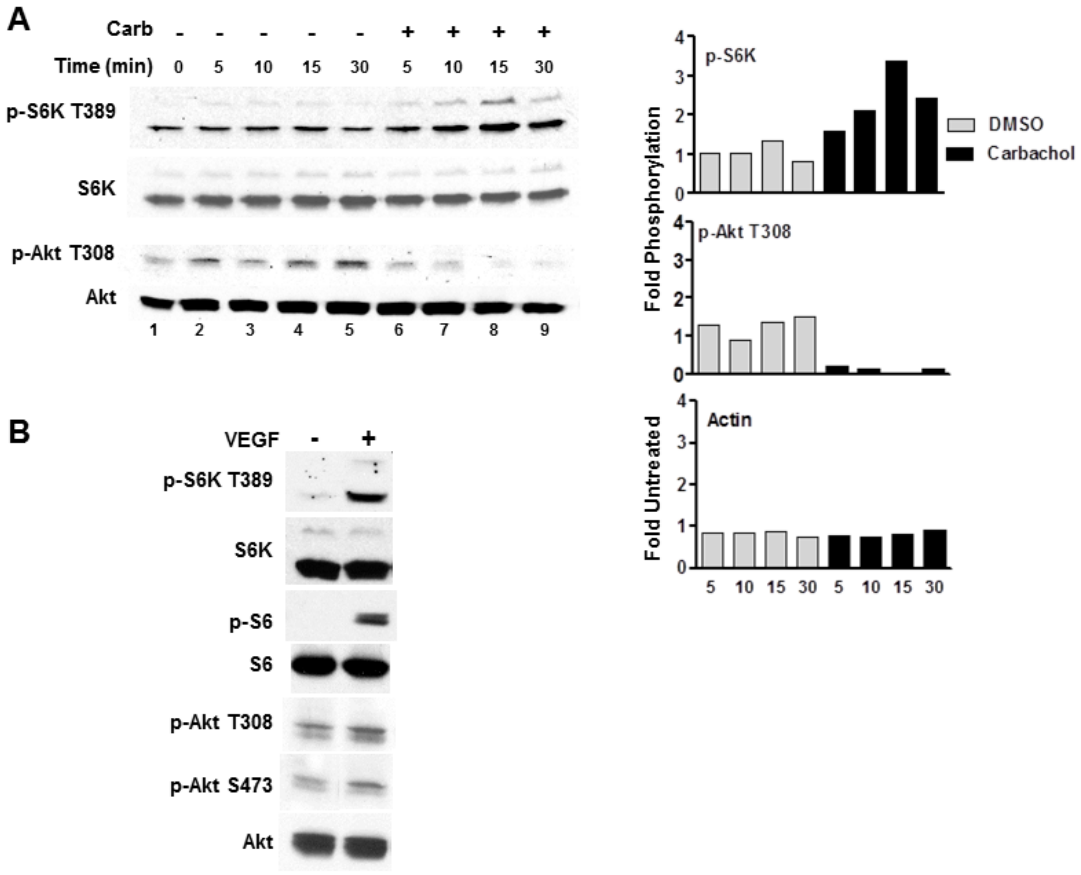


Figure 3.1. Carbachol and VEGF induce activation of mTORC1 targets with different effects on Akt phosphorylation in serum deprived SK-N-SH cells. (A) SK-N-SH cells were serum-deprived for 48 h as described in Chapter 2. Cells were treated without and with 1-mM carbachol during the final 30 minutes of serum deprivation as indicated. Cell lysates were analyzed by immunoblotting as described in Chapter 2. Blots were probed with antibodies that specifically recognize the phosphorylation of S6K on Thr389 and Akt on Thr308. (B) Cells were treated with 10 ng/ml VEGF for 15 minutes and analyzed for phosphorylation of S6K on Thr389, S6 on Ser235/236 and Akt on Thr308 and Ser473. Blots in (A) and (B) were stripped and reprobed with antigen-specific antibodies to detect total protein levels for use as loading controls. Data for each time frame were quantified as the fold difference of normalized phosphorylation relative to the untreated control for the blot shown. Results are typical of the phosphorylation levels observed in three independent experiments.

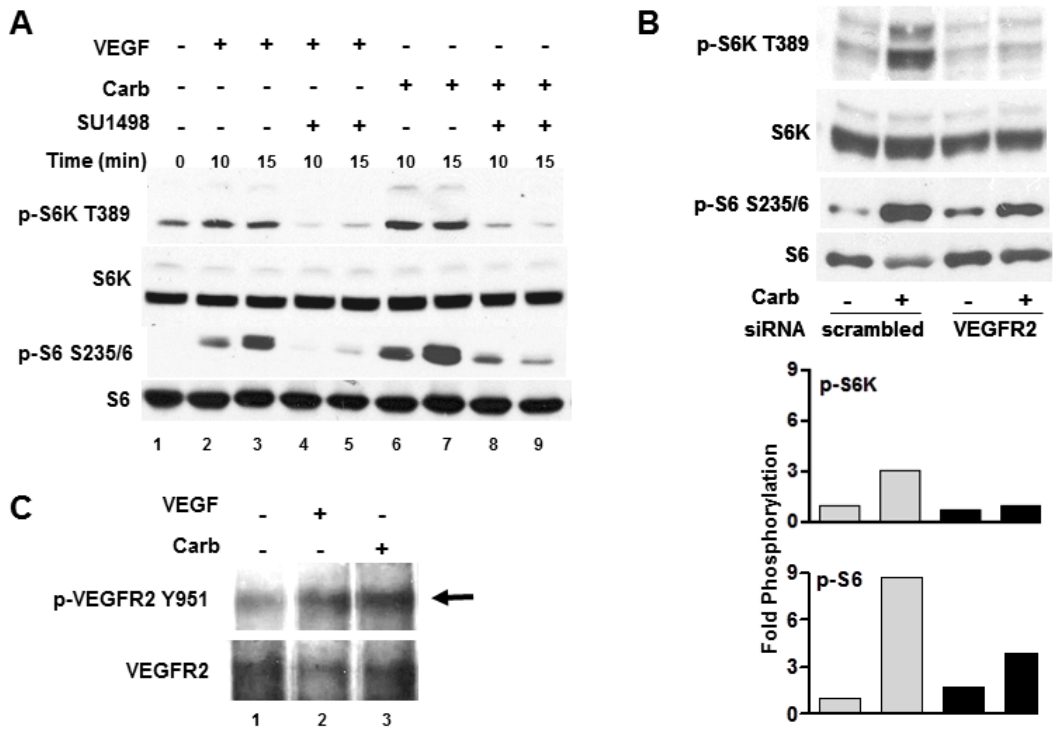


Figure 3.2. VEGFR2 signals VEGF and carbachol mediated S6K and S6 phosphorylation. (A) Serum deprived cells were pretreated for 2 h with 10-μM SU1498 followed by stimulation with either 10-ng/ml VEGF or 1-mM carbachol as indicated. Immunoblotting was carried out as described in Fig. 1 for detection of total and phosphorylated S6K and S6. Control cells were treated with vehicle (DMSO). (B) Cells were transfected with either scrambled (non-targeting) or VEGFR2 siRNA and then serum deprived for 48 h. During the final 15 min of serum deprivation, cells were treated with 1-mM carbachol for 15 min. Immunoblotting and the quantification of phosphorylation were carried out as described for Fig. 1. (C) VEGFR2 was immunoprecipitated from lysates of serum-starved cells treated with either 10-ng/ml VEGF or 1-mM carbachol for 15 min. Control cells were treated with vehicle (DMSO). Immunoprecipitates were analyzed by immunoblotting and probed with antigen-specific antibodies that detect VEGFR2 phosphorylation on Tyr951 and total protein and performed by Tianfeng Hao.

3.3. PKC exerts opposing effects on the signaling of mTORC1 and Akt by VEGF and mAchR agonists

Agonists that stimulate G_q-protein coupled receptors typically activate PKC as a downstream effector of signaling events (Gliki et al 2002, Keely et al 2000). In addition, prior evidence shows that PKC modulates the carbachol mediated phosphorylation of S6 and Akt in different cell lines (Berna et al 2009, Slack and Blusztajn 2008, Ueda et al 2004). Therefore, we addressed a role for PKC in agonist-mediated signaling to mTORC1 targets and Akt by pre-incubating serum-starved cells for 2 h with GFX, at a concentration (Toullec et al 1991) that inhibits conventional PKC isoforms (α, β and γ), followed by a 15-min stimulation with VEGF or carbachol alone or in combination (**Fig. 3.3A**). Treatments with SU1498 were included for comparison. Western blot analyses showed that carbachol induced S6K and S6 phosphorylation to levels that were 2- and 3-fold greater than that induced by VEGF (compare lanes 4 and 7). In combination, both agonists further augmented S6 activation by VEGF and carbachol to levels that were 5-fold and 2-fold greater, respectively (compare lanes 4 and 7 with 10). However, Akt phosphorylation on S473 was increased only slightly by VEGF and suppressed by carbachol (lanes 4 and 7). PKC inhibition mimicked SU1498 in preventing S6K and S6 phosphorylation in all cell treatments while increasing Akt phosphorylation in untreated, VEGF- and carbachol-treated cells (lanes 3, 6 and 9). Additionally, these experiments showed that SU1498 (lane 2) and GFX (lane 3) also blocked an endogenous phosphorylation of S6K and S6. These results were also confirmed using the PKC inhibitors G□6976 and rottlerin (data not shown). Together, these data suggest that PKC modulates the activation of TORC1 targets and Akt in an opposing fashion in serum starved SK-N-SH cells.

To assess whether these results are specific for carbachol, the phosphorylation levels of S6K, S6 and Akt were evaluated in serum-starved cells treated with pilocarpine, another agonist for mAchRs. Like carbachol, pilocarpine stimulated S6K and S6 in a PKC and VEGFR2 dependent fashion (**Fig. 3.3B**, compare lane 2 with lanes 3 and 4) while PKC inhibition augmented Akt phosphorylation (**Fig. 3.3C**, lane 3). Together, these data suggest that mAchR stimulation can signal mTOR activation through VEGFR2 and that PKC is an important downstream mediator of this event in serum-deprived SK-N-SH cells.

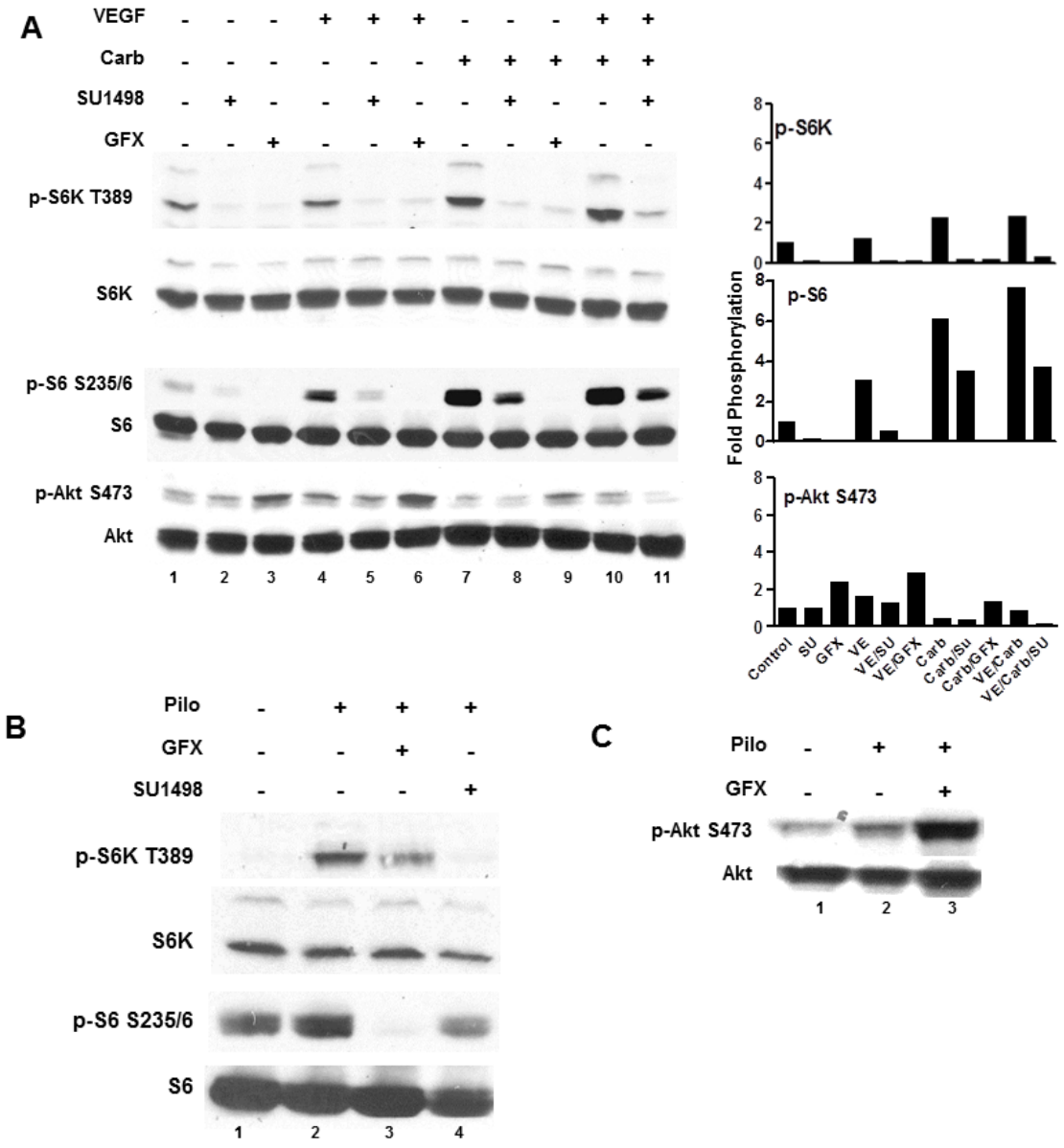


Figure 3.3. PKC positively regulates S6K and S6 phosphorylation and negatively regulates Akt activation. (A) Serum deprived cells were treated for 2 h with 10-μM SU1498 or 1-μM GFX followed by 15 min incubations with either 10-ng/ml VEGF (VE) or 1-mM carbachol (Carb) alone or in combination as indicated. Immunoblotting and data quantification for total and phosphorylated S6K, S6 and Akt were performed as described for Fig. 3.1. (B, C) Cells were treated with inhibitors as described in (A) followed by a 15-minute exposure to 1-mM pilocarpine (Pilo). Immunoblots were probed for total and phosphorylated S6K and S6 (B) and Akt (C). Control cells were treated with vehicle (DMSO) for each experiment.

3.4. S6 and Akt are downstream from PI3K

Since PI3K is a well-established mediator of TORC1 activation, we examined the relationship among PI3K, mTOR and PKC in the endogenous signaling to S6 and Akt. To this end, serum-starved cells were treated with inhibitors of PKC (GFX), PI3K (LY294002) and mTOR (rapamycin) either alone or as combined treatments (**Fig. 3.4**). Whereas S6 activation was inhibited by all three inhibitor treatments, Akt phosphorylation was increased at T308 and S473 by GFX (lane 2) and only at T308 following a prolonged (48 h) treatment with rapamycin alone or with GFX (lanes 4 and 6). However, PI3K inhibition attenuated the GFX-mediated increase in Akt phosphorylation (lanes 5 and 7), placing PI3K as an upstream activator of S6 and Akt phosphorylation. The rapamycin induced loss of the Akt S473 phosphorylation is likely due to an attenuation ofTORC2 assembly (Sarbassov et al 2006).

3.5. Phosphatase activity modulates S6 and Akt activation

In addition to kinase activity, dephosphorylation plays an essential role in the signaling cascades mediated by cell surface receptors. In particular, serine/threonine as well as tyrosine phosphatases can affect receptor signaling upstream of mTOR and directly dephosphorylate the pathway members S6K, S6 and Akt. To address these mechanisms, SK-N-SH cells were treated with either the serine/threonine phosphatase inhibitor OA at a concentration that is selective for PP2A activity (Millward et al 1999) or Na_3V0_4, a general inhibitor of tyrosine phosphatases at a concentration that blocks activity in a PC12 neuronal system (Wu et al 2002). Whereas both inhibitors increased S6K, S6 and Akt phosphorylation, OA alone elicited a 2-fold or greater increase in phosphorylation than Na_3V0_4 (**Fig. 3.**5A, compare lanes 2, 5 and 8 with 3, 6 and 9). Moreover, OA-induced S6 phosphorylation was enhanced by VEGF and carbachol by 5- and 8-fold, respectively, while Akt activation increased by 7- and 12-fold (compare lanes 2 with 5 and 8). A similar pattern of increased activation was induced by Na_3V0_4 but to a lesser extent (compare lanes 3 with 6 and 9). In contrast to these data, S6K activation was enhanced no greater than 3-fold.

Given these findings, we reexamined the dependency of S6K, S6 and Akt activation on mTOR and PI3K when PP2A was inhibited in the absence and presence of rapamycin or LY294002 (**Fig. 3.5B**). Consistent with PI3K as an upstream regulator, LY294002 abrogated the activation of S6K and Akt in OA-

treated cells (lane 3). Rapamycin only blocked S6K phosphorylation in the presence of OA (lane 2) supporting the notion that TORC1 is a critical regulator of S6K function (Hay and Sonenberg 2004). In contrast with the data in **Fig. 3.4**, Akt phosphorylation on S473 in OA-treated cells was not eliminated by rapamycin while S6 activation at S235/236 was both PI3K and mTOR independent. These data suggest that there is an alternative means of phosphorylating S6 when mTOR is inhibited by rapamycin.

3.6. Treatments with pilocarpine induce cell death in a time- and dose-dependent manner

Since VEGF and mAchRs mediate neuronal survival (De Sarno et al 2003, Ruiz de Almodovar et al 2009), we examined the effects of increasing concentrations of carbachol or pilocarpine (0.01 to 1.0 mM) on the viability of serum-starved SK-N-SH cells over a 24-h period of exposure as measured by an MTS assay (**Fig. 3.6A**). Interestingly, pilocarpine, unlike carbachol, induced a dose- and time-dependent loss in viability at 20 and 24 h that was accompanied by sustained activation levels of S6K and S6 phosphorylation (**Fig. 3.6B**, compare lanes 2 and 3). To evaluate the contribution of PKC at 24 h, serum-starved cells were also treated with either agonist in the presence of GFX (**Fig. 3.6B**, lanes 4 and 5) and analyzed for the phosphorylation levels of S6K, S6, Akt at S473 and the PKCα/βII isoforms at T638/641 together with the total levels of PKCα. Consistent with the data in Fig. 3.3, PKC inhibition elicited a decrease in the phosphorylation levels of S6K and S6 with a parallel increase in Akt (lanes 5 and 6). These GFX-mediated events were accompanied by reduced levels of phosphorylated PKCα/βII and total PKCα, suggesting that PKC activation influences the phosphorylation levels of S6K, S6 and Akt.

3.7. Pilocarpine-induced cell death is blocked by antioxidant treatment independent of TORC1 activation

Pilocarpine is used to induce seizures in a rat model of epilepsy and recent evidence shows that this event is associated with a hippocampal neuronal cell death involving oxidative stress and caspase activation (Liu et al 2010). To assess whether pilocarpine mediates a similar cell death mechanism in our neuronal model, we assessed viability, caspase activation and mTOR activation in cells treated for 24 h with 1-mM pilocarpine without and with the antioxidant Nac alone or in combination with SU1498 (**Fig. 3.7**). Consistent with *in vivo* studies, the loss in viability induced by pilocarpine was accompanied by caspase-3/7 activation and these events were prevented by treatments with Nac independent of SU1498 (**Fig. 3.7A**). However, the phosphorylation of S6K and S6 persisted in pilocarpine-treated cells in the

presence of Nac (**Fig. 3.7B**, lane 2) but was attenuated by the inclusion of SU1498 (lane 3). These findings suggest that TORC1 activation by VEGFR2 was dispensable for survival in pilocarpine-treated cells. Taken together, these results suggest that the duration and magnitude of stimulation by mAchRs can affect cell fate independent of mTOR signaling in serum-deprived SK-N-SH cells.

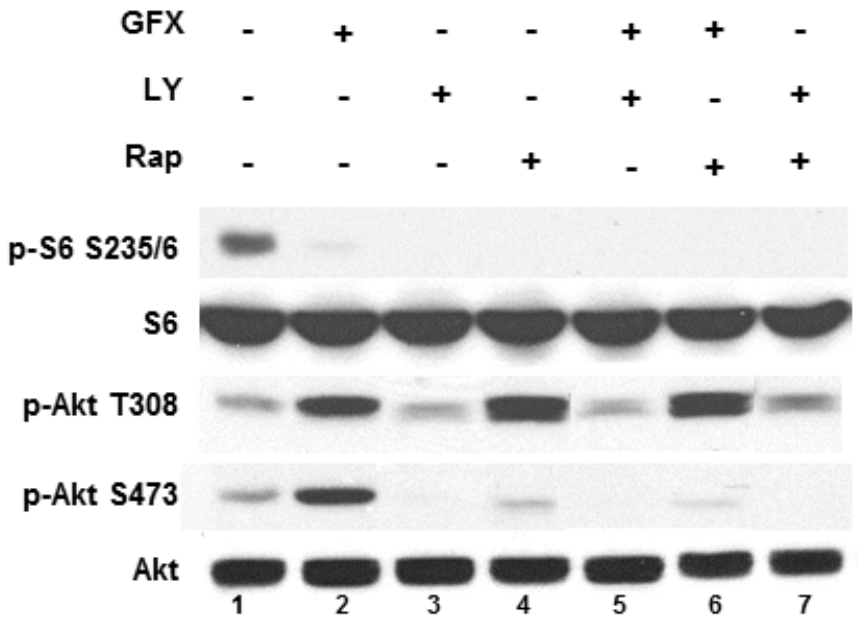


Figure 3.4. PI3K is upstream from TORC1 and Akt. Serum starved cells were treated with either 1-μM GFX or 20-μM LY294002 (LY) for 2 h or 1-μM rapamycin (Rap) for 48 h either alone or in combination. Lysates were then analyzed by immunoblotting for detection of total and phosphorylated S6 and Akt as described for Fig. 3.1. Control cells were treated with vehicle (DMSO).

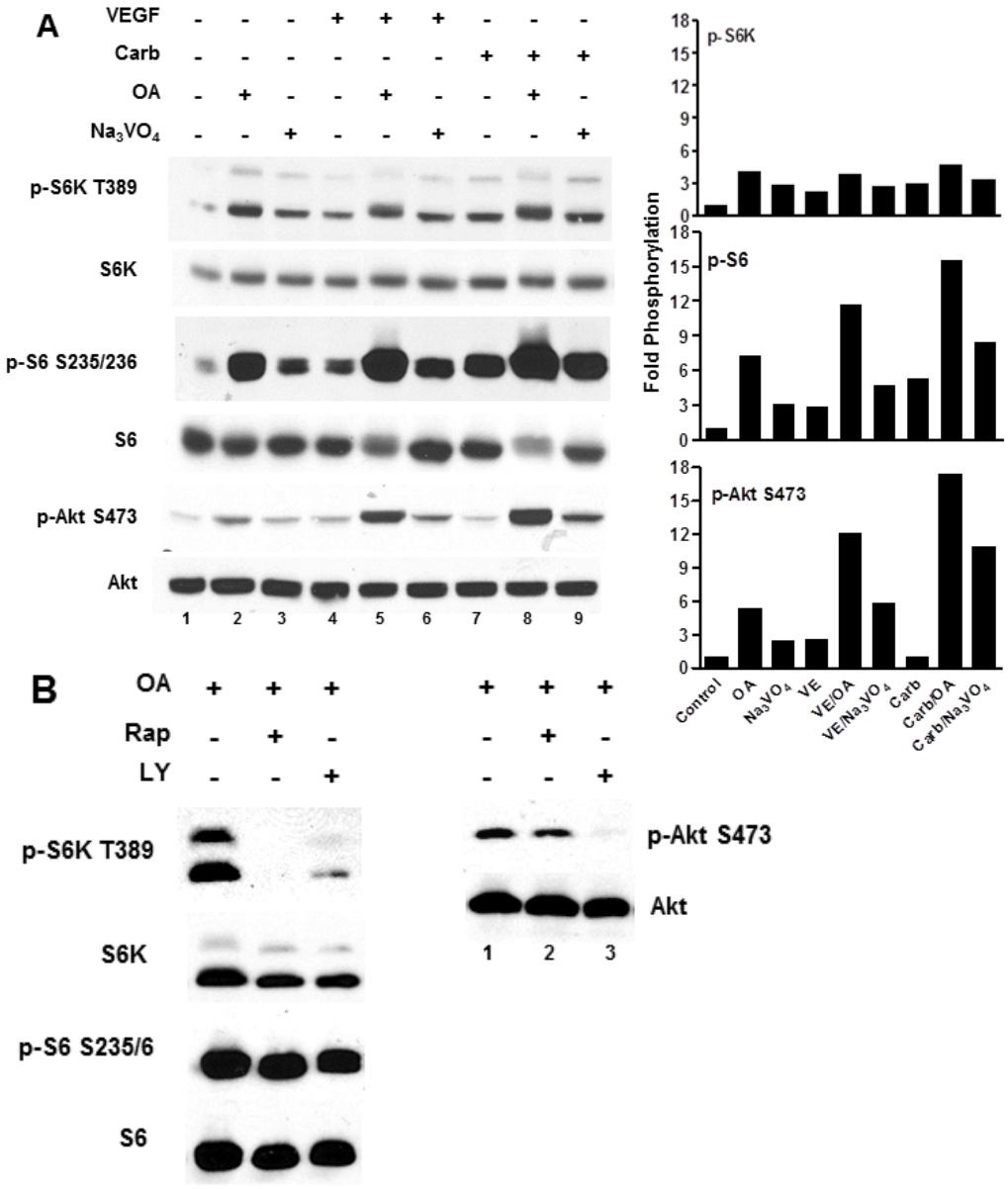
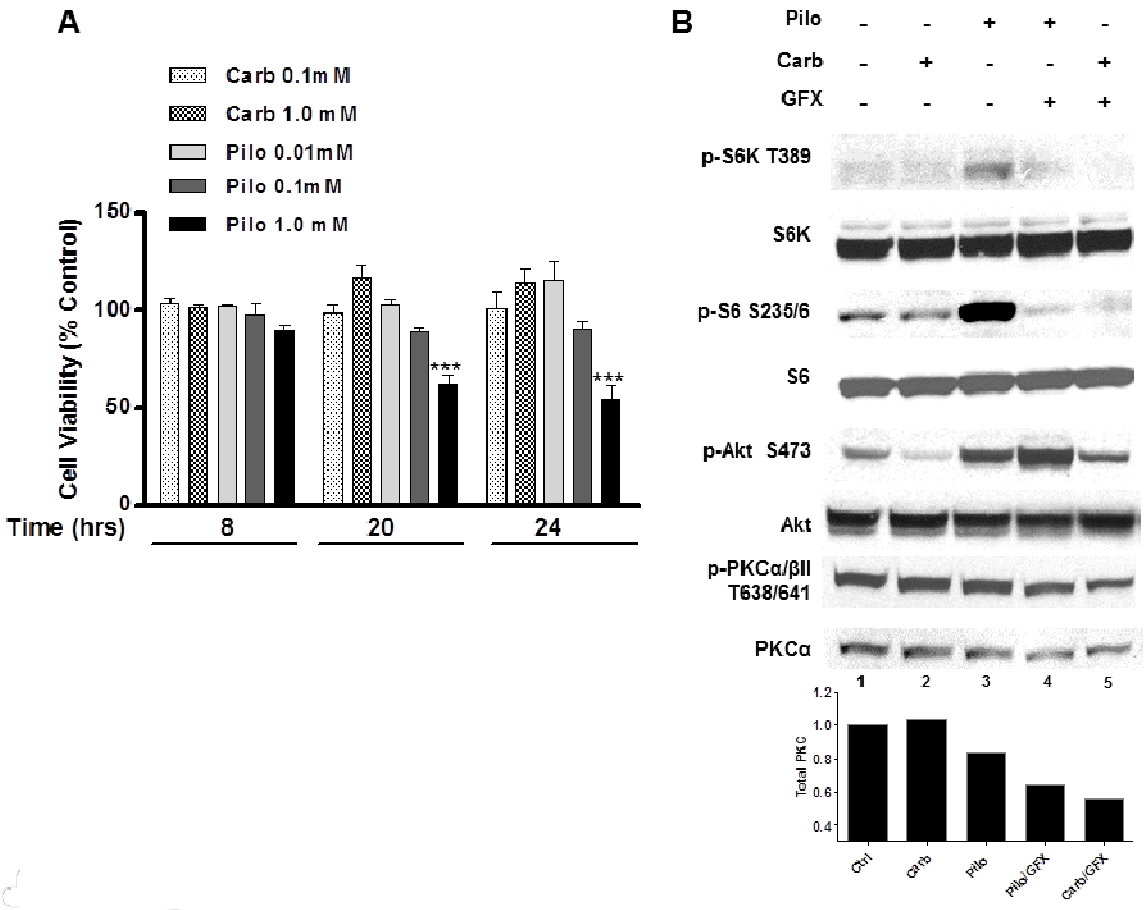


Figure 3.5. Okadaic acid has a greater effect than Na_3VO_4 on phosphorylation levels of S6K, S6 and Akt (A) Serum-starved cells were treated with either 400-nM OA or 50-μM Na_3VO_4 for 1 h followed by a 15-minute exposure to 10-ng/ml VEGF or 1-mM carbachol as indicated. Cell lysates were analyzed by immunoblotting for detection of total and phosphorylated S6K, S6 and Akt followed by data quantification as described for Fig. 3.1. Control cells were treated with vehicle (DMSO). (B) Cells were treated with 400-nM OA in combination with 1-μM rapamycin (Rap) or 20-μM LY294002 (LY) as indicated and analyzed by immunoblotting for phosphorylated S6K, S6 and Akt as in (A).



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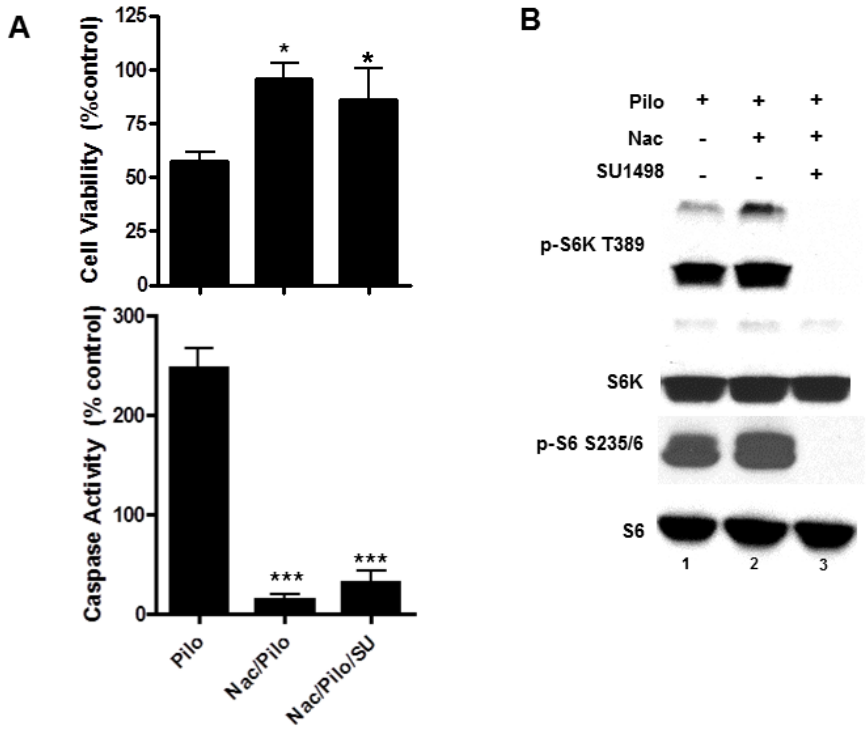


Figure 3.7. Pilocarpine-induced cell death is blocked by antioxidant treatment independent of TORC1 activation. Serum-deprived cells were treated for 24 hours with 1-mM pilocarpine alone or with 5-mM Nac or Nac in combination with 10-μM SU1498. (A) Cell viability and caspase 3/7 activation was measured as described in Chapter 2. Results represent the percent cell viability or caspase activity relative to the vehicle treated control ±S.E.M from at least three independent experiments. The asterisk indicates a significant difference between pilocarpine treatments with Nac versus pilocarpine alone (*P<0.05; ***P<0.001). (B) Immunoblots from cells treated as in (A) were analyzed for detection of total and phosphorylated S6K and S6 as described for Fig. 3.1.

3.8. Discussion

In this study, we employed serum-deprived SK-N-SH cells as a neuronal model to investigate whether mAchRs and the VEGF/VEGFR2 pathway crosstalk to activate mTOR. We based these studies on previous work showing that VEGF and carbachol, an agonist for mAchRs, signal activation of mTOR targets in different neuronal models (Kim et al 2008a, Slack and Blusztajn 2008). We reveal a previously unknown link between mAchRs and VEGFR2 in which carbachol and pilocarpine enhance the activation of the TORC1 targets S6K and S6 in a VEGFR2-dependent manner. This interpretation is based on our findings that 1) carbachol treatment increases VEGFR2 phosphorylation (**Fig. 3.2**), 2) the combined action of VEGF and carbachol augments S6 phosphorylation to levels greater than either agonist alone (**Fig. 3.3**) and 3) inhibition of VEGFR2 blocks S6K and S6 activation not only by VEGF, but also by carbachol and pilocarpine (**Figs. 3.2** and **3.3**).

Crosstalk between GPCRs and RTKs serves as a mechanism whereby signaling components are shared to presumably potentiate cellular responses. Our findings suggest that mAchRs signal the activation of TORC1 through a transactivation of VEGFR2 to enhance the activation of downstream targets. Activated GPCRs have been shown to couple with RTKs for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and insulin-like growth factor (IGF) to signal cell proliferation, differentiation, and other cellular functions (Luttrell 2003, Waters et al 2004). For example, upregulation of NGF by mAchRs was shown to enhance acetylcholine release from synaptosomes in the brain (Knipper et al 1994). In non-neuronal cells, mAchRs were shown to interact with PDGFR or transactivate EGFR to enhance ERK1/2 activation (Alderton et al 2001, Keely et al 1998, Lin et al 2008). In our paradigm of serum deprivation in SK-N-SH cells, carbachol- and pilocarpine-mediated activation of mAchRs may augment TORC1 signaling through VEGFR2 to enhance the cellular response to VEGF.

The similarity in the regulatory pattern of S6K and S6 activation by VEGFR2 and PKC raises the possibility that, in our studies, PKC is a downstream effector of the coupled muscarinic receptor/VEGFR2-mediated signaling to mTOR. The observation that PKC inhibition exerts inverse effects on the phosphorylation patterns of TORC1 substrates and Akt in cells treated with VEGF, carbachol or pilocarpine (**Figs. 3.3** and **3.6**) as well as untreated cells (**Fig. 3.4**) suggests that PKC targets these

pathways independent of agonist treatment. These opposing effects of PKC on TORC1 and Akt activation are supported by evidence that EGFR signals TORC1 through PKC independently of Akt activation in different cancer cell lines (Fan et al 2009). In addition, the suppression of Akt phosphorylation by PKC is consistent with reports that PKCα negatively regulates VEGFR2-stimulated phosphorylation of Akt at S473 and GPCR-mediated transactivation of EGFR in response to carbachol in non-neuronal cells (Rask-Madsen and King 2008, Santiskulvong and Rozengurt 2007). However, signaling between PKC and Akt is complex as PKC can also positively regulate Akt activation induced by carbachol in astrocytoma and PC12 cells (Tang et al 2002, Wu and Wong 2006). Also, our results cannot preclude the possibility that carbachol negatively regulates Akt phosphorylation through PKC-independent mechanisms (Berna et al 2009, Ueda et al 2004).

Nevertheless, one explanation for these opposing effects is that VEGF and muscarinic receptor agonists independently signal PKC through their respective receptors. Both mAchRs and VEGFR2 can signal through PLC and intracellular Ca^{2+} mobilization to activate PKC as a critical effector of downstream signaling events (Hubbard and Hepler 2006, Xiong et al 2009). It is also possible that the agonist mediated stimulation of mAchRs and VEGFR2 leads to the activation of several isoforms of PKC that simultaneously modulate mTOR and Akt function in an opposing fashion. For example, PKC isoforms were shown to confer distinct regulatory effects on Akt phosphorylation in keratinocytes (Li et al 2006). Thus, a given PKC isoform would positively regulate TORC1-mediated S6K phosphorylation and the activation of its downstream target S6 while another isoform would suppress the phosphorylation of Akt on T308 via PI3K and its phosphorylation on S473 by TORC2. Interestingly, in pilocarpine-treated cells incubated with GFX, the increase in Akt S473 phosphorylation and the loss of S6K and S6 activation are coincident with decreased levels of total PKCα and T638/664 phosphorylation of the PKCα/βII isoforms (**Fig. 3.6**). This reduction in activated and total PKC may result from its dephosphorylation and subsequent degradation by the ubiquitin/proteasome pathway (Lee et al 1996). Consequently, the negative and positive effects of PKC on TORC1 and Akt activation may result from a decrease in the functional levels of PKC. Taken together, our studies reveal a novel pathway in SK-N-SH cells in which mAchRs cooperate with VEGFR2 to mediate contrasting effects on mTOR and Akt activation through a PI3K pathway that is further modulated by PKC.

Our studies also revealed that the extent of S6K, S6 and Akt phosphorylation by VEGF and carbachol was enhanced by blocking tyrosine and to a greater extent serine/threonine dephosphorylation (**Fig. 3.5**). Consequently, these findings suggest that in serum-starved SK-N-SH cells, phosphorylation by RTKs and dephosphorylation by PP2A coordinate as mechanisms to control TORC1 and Akt function induced by muscarinic/VEGFR receptor signaling. Interestingly, carbachol was 3-fold more effective than VEGF in augmenting the phosphorylation levels of S6 and Akt when combined with tyrosine or serine/threonine phosphatase inhibition. However, the far greater increase (12- to 15-fold) in S6 and Akt activation by VEGF and carbachol in OA-treated cells implicates dephosphorylation by PP2A as a critical regulatory mechanism in the response to serum starvation. This line of reasoning is supported by evidence that PP2A can interact with Akt and mediate dephosphorylation of S473 (Ivaska et al 2002, Resjo et al 2002). Furthermore, a regulatory link between PKC and PP2A was provided by evidence that PKC activates PP2A and mediates the association of Akt with the PP2A catalytic subunit for subsequent dephosphorylation (Li et al 2006). In agreement with our findings, this study also showed that PKC inhibition induced an increase in Akt activation at S473 that was attributed to a blockade of PKC-mediated dephosphorylation of Akt. Whether PKC coordinates with PP2A to regulate Akt in serum starved SK-N-SH cells is currently under investigation. Although PP2A can directly interact with S6K (Peterson et al 1999), S6K was not as responsive as S6 or Akt to the stimulatory effects of phosphatase inhibition on phosphorylation.

Our inhibitor studies also provide insight regarding the capacity of upstream kinases to phosphorylate endogenous S6K, S6 and Akt when dephosphorylation by PP2A is blocked (**Figs. 3.4** and **3.5B**). These results place PI3K as an upstream regulator of S6K and Akt phosphorylation while mTOR alone modulates the activation of S6K. In this context, Akt activation is mTOR-independent which is consistent with Akt residing upstream from TORC1 (Hay and Sonenberg 2004). Interestingly, the observation that S6 phosphorylation at the S6K target site S235/236 is independent of PI3K and mTOR with OA-treatment is supported by reports that kinases such as p90RSK can serve as compensatory regulators of S6 phosphorylation (Pende et al 2004, Ruvinsky and Meyuhas 2006). Given these findings, an alternative explanation for the divergent phosphorylation patterns of TORC1 targets and Akt in GFX- and rapamycin-treated cells is that this response results from a negative feedback loop in which activated

S6K inhibits the PI3K-mediated Akt phosphorylation at T308 (Efeyan and Sabatini 2010, Hay and Sonenberg 2004). Accordingly, the blockade of S6K activation by PKC and mTOR inhibition, as observed in our studies, would inactivate this feedback inhibitory loop, leading to enhanced PI3K activity and an increase in phosphorylated Akt.

Since VEGF and mAchRs elicit neuroprotection under stress conditions (de Freitas et al 2010, Gomes et al 2007, Zhou et al 2008), it seemed reasonable to assume that mAchRs would couple with VEGFR2 to protect against apoptotic cell death induced by serum starvation in SK-N-SH cells (Gomes and Rockwell 2008). Unexpectedly, pilocarpine, but not carbachol, induced a concentration and time dependent loss in viability that was manifested by caspase activation and oxidative stress. Interestingly, this mode of cell death is also associated with the neuronal cell loss mediated by pilocarpine-induced seizures in a rat model of epilepsy (de Freitas et al 2010, Liu et al 2010). Similarly, the sustained activation of S6K and S6 induced by pilocarpine in SK-N-SH cells is consistent with the evidence that aberrant mTOR expression contributes to pilocarpine-induced seizures (Zeng et al 2009). However, neither the presence of S6K and S6 activation nor its suppression by VEGFR2 inhibition affected the survival mediated by the antioxidant in our model, suggesting that the pilocarpine stimulation of mTOR activation through VEGFR2 is dispensable for survival. This finding suggests that pilocarpine mediates cell death and mTORC1 activation through different mAchRs.

Muscarinic receptors vary in their stimulatory effects depending on the cell type, the receptor subtype activated as well as the agonist and extent of stimulation (Rozengurt 2007). For example, pilocarpine was shown to promote neuroprotection against glutamate-induced apoptosis in retinal neurons through the M_1 mAchR (Zhou et al 2008). Furthermore, divergent pilocarpine and carbachol signaling to ERK1/2 has been documented in salivary cells, with receptor subtype M_3 and EGFR downstream of pilocarpine while both M_1/M_3 subtypes and PKC acted downstream of carbachol (Lin et al 2008). In general, carbachol appears to affect a broader spectrum of mAchR subtypes than pilocarpine, thus the mAchR profile of target cells is an important determinant of their response to mAchR agonists (Grant and El-Fakahany 2005). In support of this idea, differences in carbachol versus pilocarpine in inducing seizure-like activity in brain slices have been attributed to the agonists targeting different

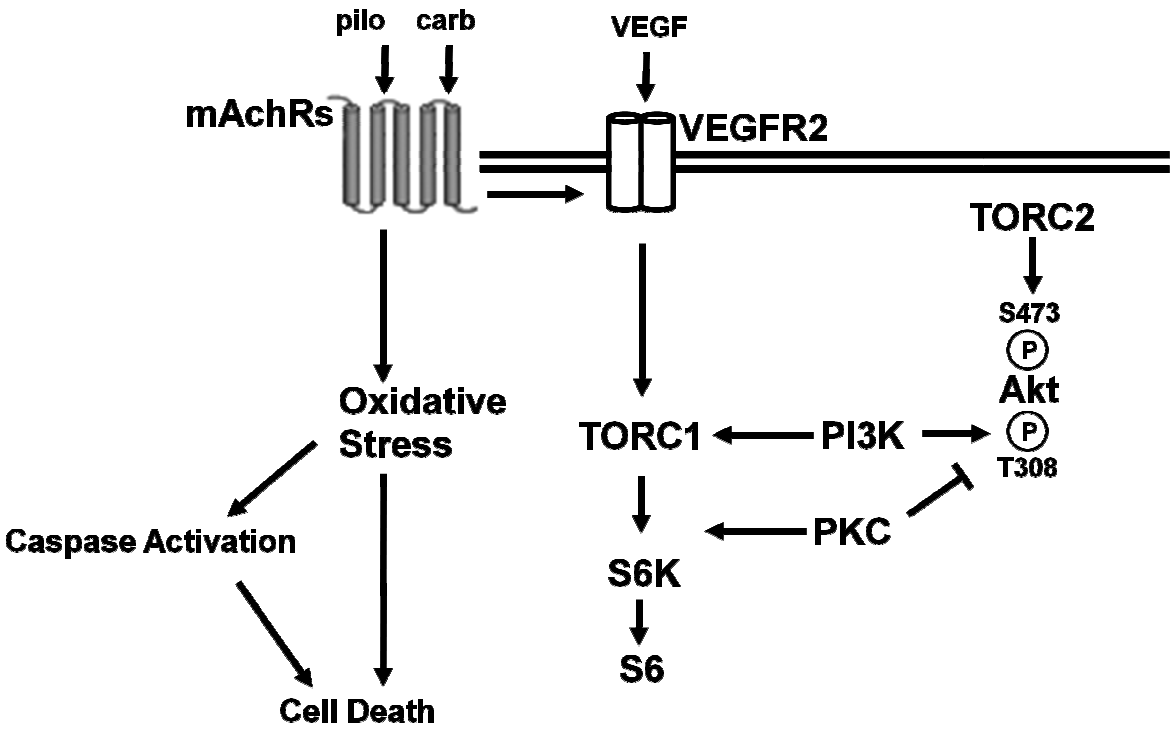
neuronal cell types (Marchi et al 2007). Our results suggest that both agonists stimulate VEGFR2 to signal mTOR activation but use different mechanisms to regulate survival.

The ability of Nac to prevent pilocarpine-induced cell death (**Fig 3.7A**) suggests that oxidative stress is mediating this effect. Consistent with this interpretation, redox state has been linked to neuronal survival in tissue and cellular models (Kim et al 2011, Kovacs et al 2002). Furthermore, pilocarpine induces oxidative species, lipid peroxidation and cell death in rat models of status epilepticus (Kovacs et al 2002, Liu et al 2010). These reports, in conjunction with our data, lead us to conclude that the pro-survival effects of Nac are due to its antioxidant capabilities. However, in addition to its antioxidant activity, Nac can also alter glutamate receptors after metabolic conversion to cysteine which can have profound effects on neuronal homeostasis (Lafleur et al 2006, Moran et al 2005). We cannot rule out the possibility that similar effects may also contribute to neuroprotection by Nac in our model.

This theory of the mechanism of pilocarpine-induced cell death may be extrapolated to models of status epilepticus. The extent to which the alteration of neuronal circuitry contributes to neuronal loss in these models is an open question. Our result that pilocarpine can induce cell death associated with oxidative stress in a cell line clearly indicates that the pro-oxidant effects of mAchR stimulation contribute directly to neuronal loss in the absence of aberrant neuronal growth.

3.9 Conclusions

In summary, we present evidence for a novel signaling mechanism in serum-starved SK-N-SH neuroblastoma cells in which mAchRs crosstalk with the VEGF/VEGFR2 pathway to regulate the activation of the PI3K/Akt/mTOR pathway. This model hypothesizes that muscarinic receptor agonists share a common mechanism to stimulate protein synthesis in response to serum starvation (**Fig. 3.8)**. In this context, activated mAchRs enhance the phosphorylation of S6K and S6 activation by VEGF through a signaling pathway that is PI3K-dependent and further modulated by PKC and PP2A. PKC positively regulates TORC1 targets S6K and S6 and negatively modulates the TORC2 substrate Akt while the magnitude and duration of this response is determined by dephosphorylation via PP2A. However, our findings also revealed that the length and extent of agonist mediated stimulation confer divergent effects on survival.



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Chapter 4

Phosphatase Inhibition Depletes Akt Levels and Induces a Caspase-Independent Cell Death in SK-N-SH Neuroblastoma Cells

4.1 Okadaic acid induces hyperphosphorylated Akt that exists in normal and high molecular weight forms

The data reported in the previous chapter suggest that PP2A is a critical regulator of mTOR signaling in our neuronal model. Treatment of serum-starved cells with okadaic acid (OA) for 1 h at a concentration known to inhibit PP2A resulted in hyperphosphorylation of Akt at serine 473 (S473) as well as S6K and S6 (**Fig 3.5A**, lane 2), suggesting that PP2A is a critical regulator of mTOR/Akt signaling under stress. Notably, this inhibition induced Akt phosphorylation at S473 to a much greater extent than a general inhibitor of tyrosine phosphatases (**Fig. 3.5A**, compare lanes 2 and 3). Akt is an important participant in mTOR-mediated regulation of protein synthesis (Ma and Blenis 2009) and Akt mediates survival in a number of neuronal and cancer models (Dudek et al 1997, Vivanco and Sawyers 2002, Zhao et al 2006). Because phosphorylation at threonine 308 (T308) is a key event in Akt activation (Alessi et al 1996) and PP2A preferentially dephosphorylates Akt T308, the studies from Chapter 3 were expanded to examine the effects of 400-nM OA on Akt phosphorylation at T308 over a 60-min time frame.

Western blot analyses of total lysates from OA-treated cells revealed that Akt phosphorylation increased over time and high molecular weight (HMW) forms were detected starting at 45 minutes that ranged between 72 and 130 kDa (**Fig. 4.1A**, lanes 4 and 5). Notably, the HMW forms were not dissociated by the denaturing and reducing conditions of SDS-PAGE, suggesting that they existed as covalently modified complexes of Akt. Reprobing these blots with antibody against total Akt did not detect the HMW forms, possibly because the levels of OA-induced phosphorylated Akt represent a small fraction of the total Akt present in whole-cell lysates. The blots for total Akt also showed that Akt levels decreased with the appearance of the HMW phosphorylated forms (lanes 4 and 5).

To further examine the relationship between the phosphorylated HMW forms and OA, the level of Akt phosphorylation at T308 and S473 was assayed after 1 h of exposure to OA at concentrations of 100, 200 and 400 nM (**Fig. 4.1B**). OA induced a concentration-dependent increase in the monomeric and HMW forms of phosphorylated Akt (lanes 3 and 4). Consistent with **Fig. 4.1A**, the level of total Akt decreased at an OA concentration of 400 nM. Additionally, the HMW forms could be detected by a long exposure of blots probed with antibody against Akt phosphorylated at S473 (**Fig. 4.1B**, lanes 3 and 4). The phosphorylated HMW forms were detected to a greater extent at T308 than S473 (**Fig. 4.1B**,

compare lane 4 top and middle blots). This may reflect a greater sensitivity of Akt phosphorylation at T308 to PP2A inhibition since PP2A preferentially dephosphorylates Akt at this site while Akt dephosphorylation at S473 is performed by either PP2A or an OA-resistant phosphatase PHLPP (Gao et al 2005). Nevertheless, the monomeric (60-kDa) Akt S473 was highly responsive to OA treatment suggesting that PP2A is a critical regulator of S473 phosphorylation in our model. It is important to note that the blots presented in this chapter were probed with a phospho-Akt S473 antibody generated by a new method (XMT technology, Cell Signaling, Danvers MA) which detects phosphorylation at S473 with a far greater sensitivity than that used for the data in Chapter 3.

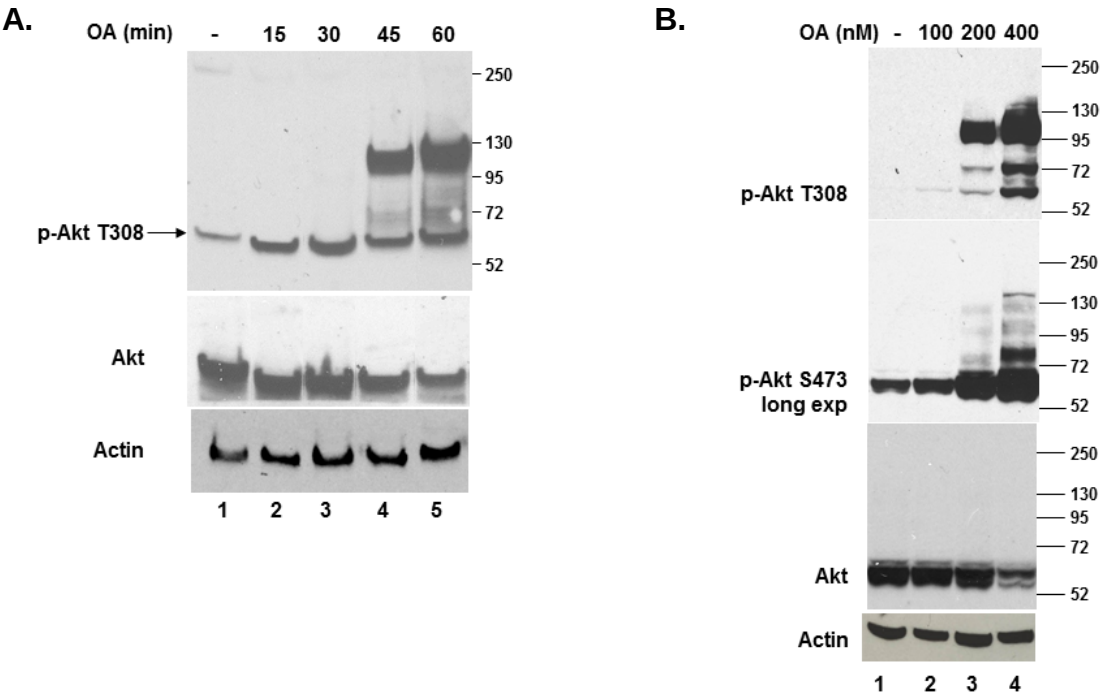
4.2 Phosphorylated high molecular weight forms can be immunoprecipitated by antibodies against Akt and their accumulation is PI3K-dependent

To address the specificity of these HMW forms, total Akt was immunoprecipitated from lysates of OA-treated cells using an antibody against the Akt C-terminus. Blots were then probed with antibodies against either p-Akt T308 (**Fig. 4.2A**) or S473 (**Fig. 4.2B**) or total Akt (**Fig. 4.2C**). In each case, monomeric Akt was detected along with HMW forms of approximately 125 and 150 kDa. In contrast with probing total lysates (**Fig 4.1)**, antibody against total Akt did detect HMW forms in a long exposure (**Fig. 4.2C**). The immunoprecipitation of Akt may concentrate the Akt to the point that detection of the HMW forms is within the limit of sensitivity of our antibody.

Since Akt is a known substrate for the ubiquitin/proteasome pathway (Suizu et al 2009) and the HMW forms resist denaturation by SDS-PAGE, we hypothesized that these forms of phosphorylated Akt are also ubiquitinated. In agreement with this, reprobing the blot of immunoprecipitate brought down by antibody against total Akt showed that the HMW forms of Akt were conjugated to ubiquitin (**Fig. 4.2D**).

It is well established that the lipid kinase PI3K is an essential upstream regulator of Akt activation by the kinase PDK1 (Huang and Manning 2009). To determine whether this pathway regulated the HMW forms of phosphorylated Akt, an antibody against phospho-Akt T308 was used to immunoprecipitate Akt from lysates of OA-treated cells pre-incubated without and with the PI3K inhibitor LY294002 (LY). Western blot analyses revealed that the monomeric and HMW forms of Akt phosphorylation at T308 were immunoprecipitated from cells treated with OA but not from cells co-treated with OA and LY (**Fig. 4.2E**), suggesting that PI3K activity is necessary for Akt hyperphosphorylation under phosphatase inhibition.

Taken together, Figures 4.1 and 4.2 suggest that, in serum-starved SK-N-SH cells, OA induces a PI3K-dependent hyperphosphorylation of Akt that shifts to higher molecular weight forms due to covalent binding of ubiquitin. These HMW forms cannot be detected in the absence of OA.



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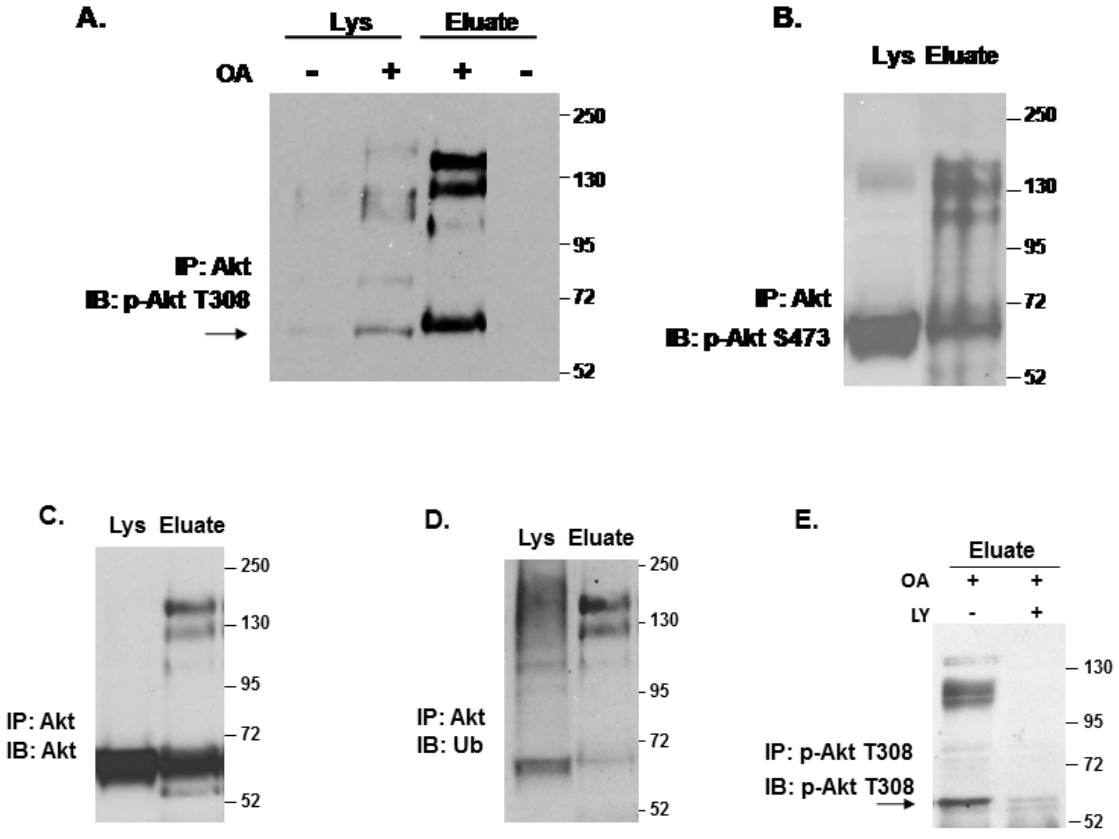

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4.3 Phosphatase inhibition induces accumulation of Akt in the nucleus and cytoplasm

Recent evidence suggests that Akt can be ubiquitinated in the cytoplasmic and nuclear compartments of the cell by different ubiquitin E3 ligases that target activated Akt for proteasomal degradation. (Suizu et al 2009, Wu et al 2011). To investigate the location and timing of the formation of HMW Akt, nuclear and cytoplasmic fraction of cells exposed to OA over a 60-min time frame were assayed for phosphorylated Akt. OA-induced Akt phosphorylation in both the cytoplasmic and nuclear fractions increased concurrently after 45 minutes with greater levels in the cytoplasm (**Fig 4.3**, compare lanes 4 and 8). The OA-induced depletion of Akt observed in **Fig 4.1** was also apparent in the nucleus and the cytoplasm after 60 minutes (lanes 4 and 8). The concomitant Akt depletion and appearance of the HMW forms in the nucleus and the cytoplasm suggest these processes are not limited to a single compartment.

4.4 Rapamycin enhances Akt phosphorylation and depletion in OA-treated cells

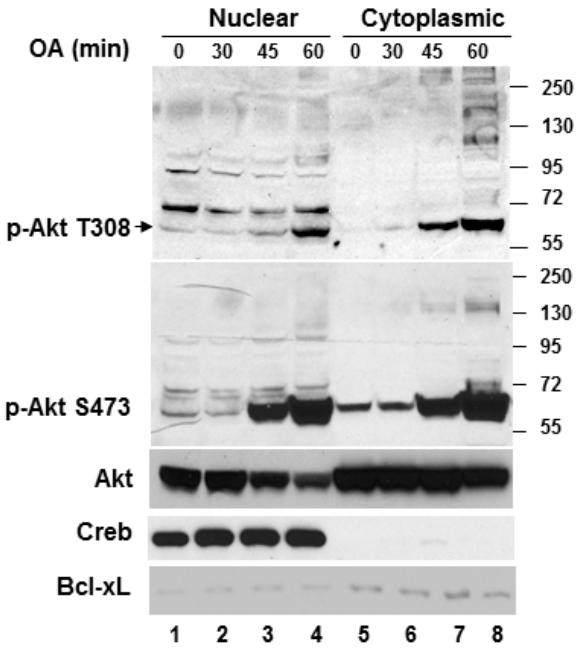
Our findings in Chapter 3 showed that a prolonged (48-h) treatment with rapamycin inhibited the TORC1 targets S6K and S6, and possibly TORC2, while increasing Akt phosphorylation at T308 (**Fig. 3.4**, lane 4). Given that mTOR can regulate Akt phosphorylation through a negative feedback loop mediated by S6K (Manning 2004), we attributed the increased Akt activation to an interruption of this loop. To assess the effects of PP2A inhibition on the Akt phosphorylation potentiated by rapamycin, serum-starved cells were incubated with OA for 45 min in the presence and absence of long-term rapamycin treatment. The distribution of Akt T308 in these cells was examined with immunofluorescent imaging. Consistent with our Western blot data (**Fig 4.1**), OA increased the detection of Akt T308 (**Fig 4.4A**, compare panels 1 and 3). Notably, phosphorylated Akt T308 assumed a punctate distribution in the cells under OA treatment (white arrows, **Fig. 4.4A** panel 3 inset). The combination of rapamycin with OA strongly intensified the Akt T308 signal (**Fig. 4.4A**, compare panels 3 and 4). Furthermore, the increased overlay of green and red in the nucleus indicates that the level of phosphorylated Akt T308 is increased in cells treated with OA and rapamycin (**Fig 4.4A** panel 4).

To further test the effect of mTOR inhibition on Akt activation, Western blots of lysates from cells treated for 1 h with OA in the presence or absence of long-term rapamycin were probed for phosphorylated Akt. In accordance with the immunofluorescence data, OA strongly augmented the

rapamycin-induced increase in Akt phosphorylation and this was accompanied by elevated levels of HMW phosphorylated forms (**Fig. 4.4B** compare lanes 2 and 3). The rapamycin-induced potentiation of Akt T308 phosphorylation is consistent with the model that rapamycin blocks negative feedback from mTORC1 to Akt. In addition to potentiating Akt phosphorylation, the addition of rapamycin increased the decline in total Akt detected in OA-treated cells (compare lanes 2 and 3). The correlation between higher levels of Akt phosphorylation and decreased levels of total Akt suggests that Akt hyperphosphorylation causes the loss of total Akt.

Akt can be degraded by the ubiquitin proteasome pathway (UPP) and by caspase activity. A long exposure of the blots of the phosphorylated Akt forms showed lower molecular weight bands of approximately 40 and 44 kDa corresponding with fragments generated by caspase cleavage (Rokudai et al 2000). Since Akt is a known target of caspases and the greatest decline in Akt was observed in cells incubated with OA and rapamycin, the effect of caspase inhibition on Akt levels was investigated in this treatment. Specifically, serum-starved cells were exposed to 48-h rapamycin and treated with OA for 1 h in the presence or absence of a 2-h pretreatment with the caspase inhibitor ZVAD. ZVAD treatment slightly increased total Akt levels indicating that caspases contribute to but are not the only factor in the Akt loss induced by OA (**Fig. 4.4C**). Similarly, ZVAD had little effect in altering levels of total Akt in cells treated with OA alone (data not shown).

The limited effect of ZVAD on the OA-induced decrease in Akt suggested that the UPP might be an important contributor to Akt degradation when PP2A is inhibited. This decline in Akt is accompanied by increased levels of ubiquitinated, phosphorylated HMW forms, further implicating the UPP in this process (**Figs. 4.1 and 4.2**). To investigate the role of the UPP in decreasing Akt levels, cells were treated as in **4.4C** with ZVAD replaced by 100-nM epoxomicin, an exposure shown to reduce proteasome activity by 85% in the SK-N-SH line (Groll et al 2008). While epoxomicin did increase the general levels of ubiquitinated conjugates, this had very little effect on the levels of total or phosphorylated Akt (**Fig. 4.4D**). These data suggest that an additional mechanism to the UPP and caspase cleavage contributes to Akt depletion induced by phosphatase inhibition.



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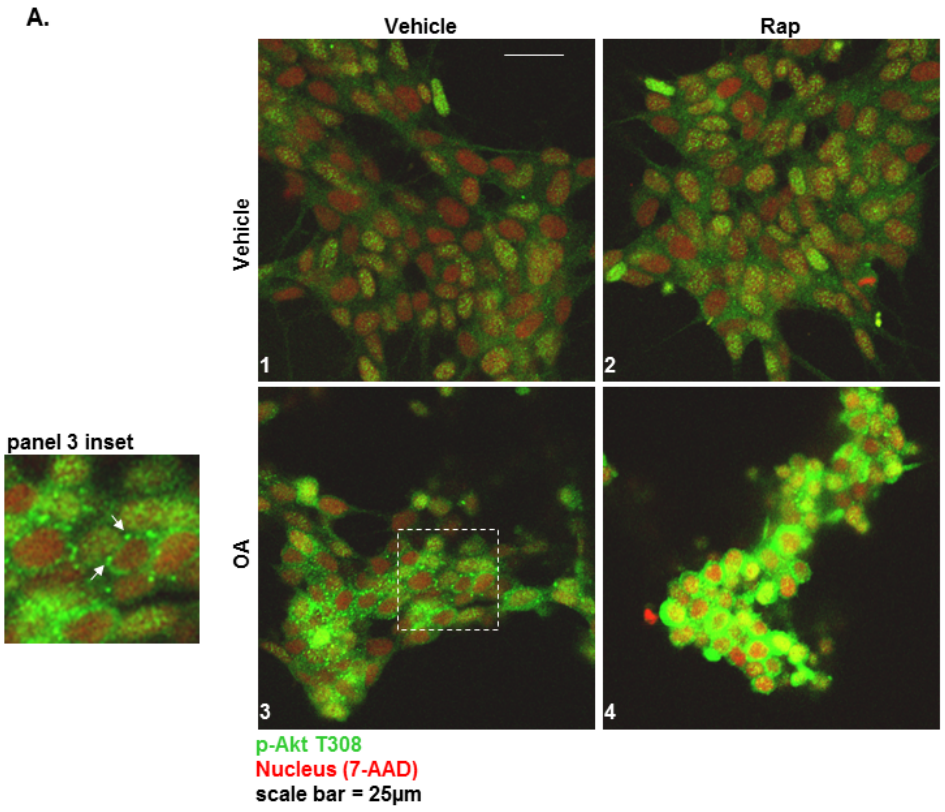

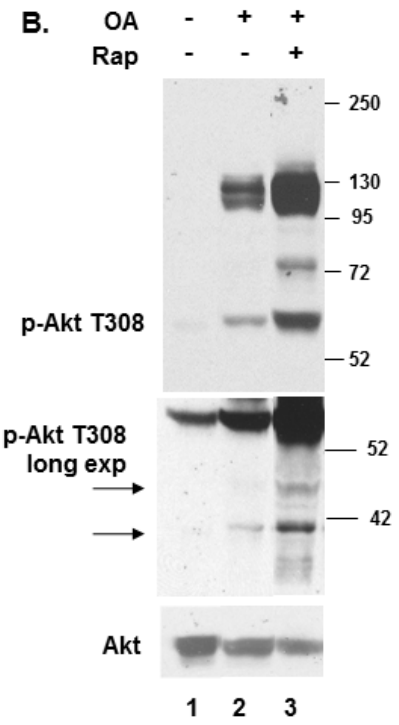

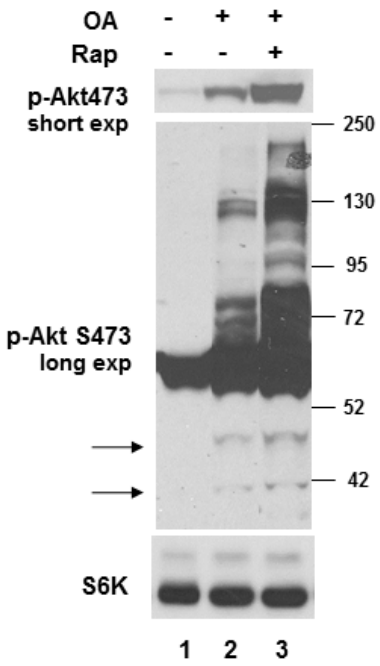

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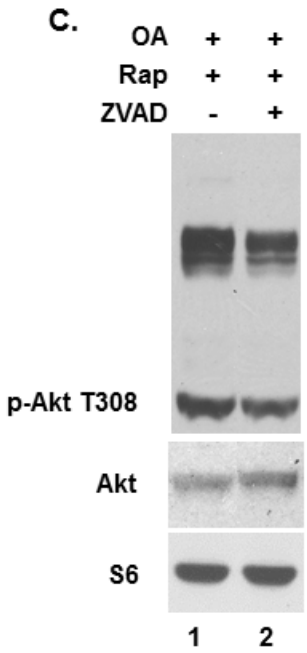

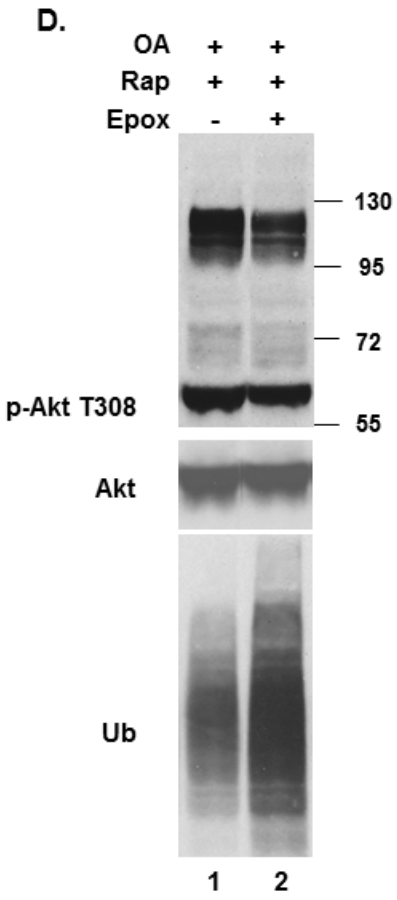

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4.5 Akt hyperphosphorylation induced by rapamycin is mediated through VEGF/VEGFR2 signaling

Previous work in our lab showed that serum starvation induced an upregulation in VEGF and VEGFR2 in SK-N-SH cells which concomitantly served as a pro-survival signaling pathway (Gomes et al 2007). Furthermore, the data in chapter 3 demonstrate that VEGF/VEGFR2 signaling regulates mTOR activation in these cells and activates the TORC1 substrate S6K through autocrine and paracrine mechanisms. These data led us to hypothesize that VEGFR2 plays a role in modulating OA-mediated hyperphosphorylation. Because S6K is an important intermediary in the feedback inhibition of Akt by mTOR, the dependence of S6K phosphorylation on VEGFR2 activity was examined in OA-treated cells. Western blots of lysates from serum-starved, OA-treated cells in the presence or absence of a 2-hr pretreatment with the VEGFR2 inhibitor SU1498 showed that VEGFR2 inhibition prevents S6K phosphorylation at the TORC1 phosphorylation site T389 (**Fig. 4.5A**, lane 3). OA-treated cells pre-incubated with rapamycin for 48 h were included as a negative control (lane 2).

These data suggest that VEGFR2 may play a regulatory role in the potentiation of Akt phosphorylation by rapamycin. To investigate this, OA-treated cells in the presence or absence of long-term rapamycin or 2-hr SU1498 pretreatments were incubated with VEGF for 15 min before harvesting. Lysates were immunoblotted and probed for phosphorylated Akt as well as ERK1/2, a known target of VEGFR2 (Gomes et al 2007). A blockade of VEGFR2 reduced ERK1/2 phosphorylation in cells treated with VEGF and OA, demonstrating that activation of targets downstream of VEGFR2 was inhibited (**Fig. 4.5B**, compare lanes 2 and 3). In contrast, SU1498 had only a negligible effect on Akt phosphorylation in these cells. However, SU1498 was very effective in preventing the increase of HMW phosphorylated Akt levels induced by rapamycin (**Fig 4.5B** compare lanes 2 and 4). Cells under VEGFR2 inhibition also showed a reduced depletion in total levels of Akt compared to cells treated with OA and rapamycin. In conjunction with preventing the decrease in Akt, the appearance of 40 and 44kDa Akt fragments in cells treated with OA and rapamycin was greatly reduced in the presence of VEGFR2 inhibition (**Fig 4.5B** compare lanes 2 and 4). Taken together these data suggest that a blockade of PP2A activity in combination with rapamycin elevates Akt phosphorylation through VEGFR2 signaling and results in depletion of total Akt.

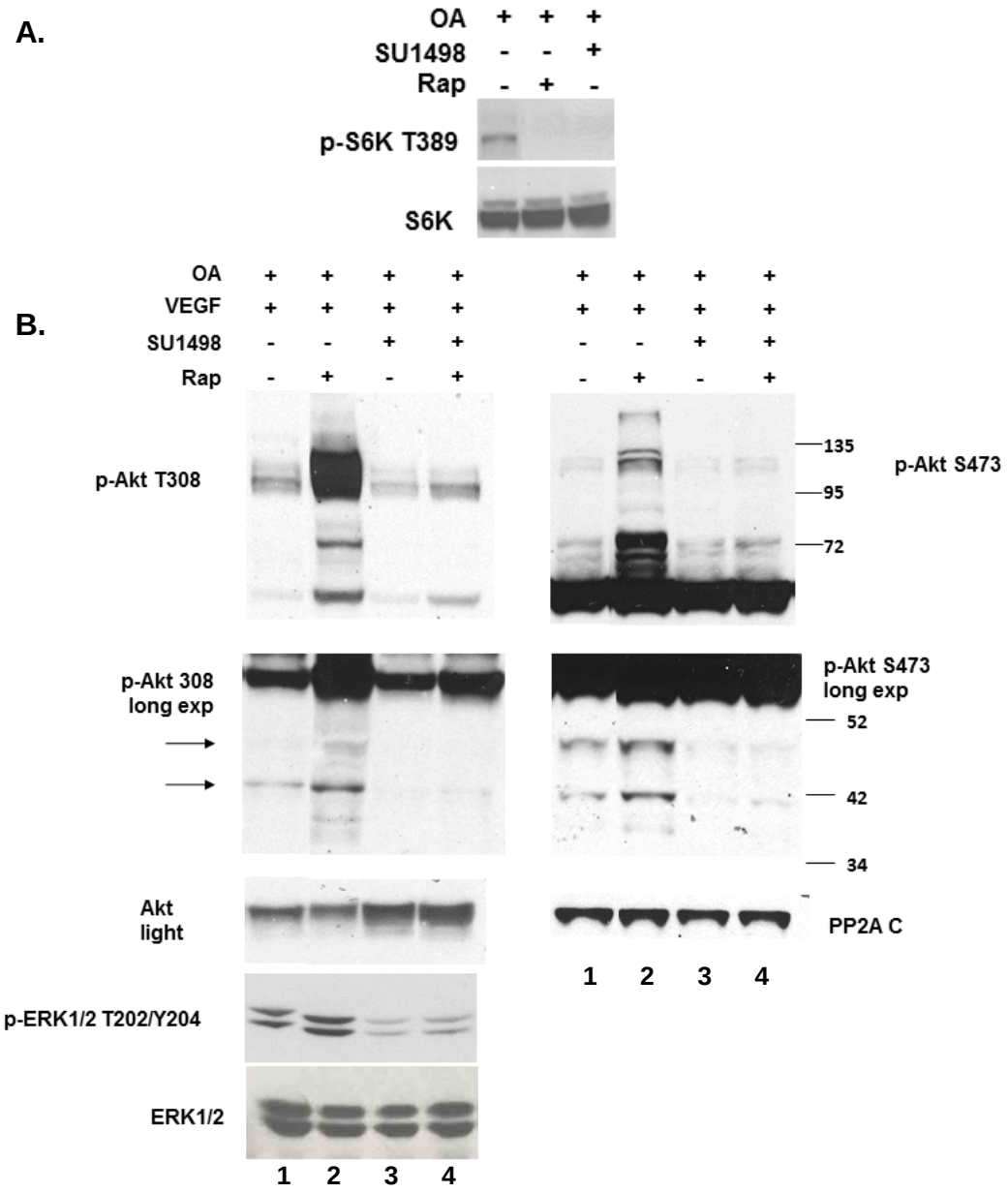


Figure 4.5. Akt hyperphosphorylation induced by rapamycin is mediated through VEGF/VEGFR2 signaling. (A) Serum-starved SK-N-SH cells were treated with or without 1-μM rapamycin (Rap) as in 4.4B or with SU1498 for 2 h. At the end of this period cells were treated with 400-nM OA for 1 h. and lysates were subjected to immunoblotting. (B) Cells were treated with rapamycin or SU1498 alone or in combination, then incubated with 400-nM OA for 1 h before harvesting. Lysates were subjected to immunoblotting as in **Fig. 4.1** and blots were probed for phosphorylated and total Akt as indicated. Arrows indicate putative caspase-cleaved Akt fragments.

4.6 Differing modes of TOR inhibition elicit distinct effects on TOR signaling and ubiquitinated proteins in OA-treated cells

To address whether the rapamycin-induced potentiation of Akt hyperphosphorylation was a general response to mTOR inhibition, OA-treated cells were pre-incubated with the TOR kinase site inhibitor PP242 (Feldman et al 2009). This strategy was employed since PP242 inhibits both mTORC1 and mTORC2. In contrast, rapamycin acts on mTOR signaling through an allosteric mechanism via FKBP12 that primarily targets the function of TORC1. To this end, the effects of PP242 were tested on the phosphorylation of the primary TORC1 targets S6K and the translational inhibitor 4EBP1. While rapamycin only inhibited S6K phosphorylation, PP242 blocked the phosphorylation of S6K and 4EBP1 in OA-treated SK-N-SH cells (**Fig. 4.6A**).

Furthermore, PP242 was effective in reducing Akt phosphorylation at both T308 and S473 and greatly reduced the accumulation of HMW T308-phosphorylated Akt (**Fig. 4.6B**). Taken together, these results suggest that PP242 is an effective inhibitor of both TORC1 and TORC2 activity in our model. This is consistent with evidence in a mouse model showing that rapamycin and PP242 have opposite effects on Akt phosphorylation (Feldman 2009). In addition, TOR kinase inhibition in the presence of OA does not further decrease the detection of Akt, further supporting the idea that Akt hyperphosphorylation causes the reduction in Akt levels observed in cells treated with OA and rapamycin (**Fig 4.6B**, compare lanes 3 and 4).

Subsequent studies addressed the contribution of individual mTOR complexes on rapamycin-mediated potentiation of Akt phosphorylation with siRNA against components specific to TORC1 (raptor) or TORC2 (rictor) but we were unable to sufficiently reduce the levels of these proteins to affect their downstream targets to allow a reliable measurement of the role of each complex in Akt phosphorylation (data not shown).

The control of protein synthesis by mTOR is a tightly regulated process that can be modulated by damaging stimuli (Ma and Blenis 2009). Recent findings show that rapamycin increases protein ubiquitination as a stress response in myocardial cells and this event is accompanied by an elevation in Akt phosphorylation (Harston et al 2011). These findings raised the possibility that the increased Akt phosphorylation by rapamycin in OA-treated cells may be accompanied by increased levels of ubiquitin

conjugates. To investigate this, the levels of ubiquitinated proteins were examined in lysates from OA-treated cells incubated in the presence or absence of either rapamycin or PP242. Interestingly, phosphatase inhibition induced a two-fold increase in the level of ubiquitinated proteins relative to the control (**Fig 4.6C** compare lanes 1 and 2) that was further increased to four-fold levels by rapamycin (**Fig. 4.6C**, lane 3). In contrast, mTOR inhibition with PP242 generated total ubiquitin protein levels that matched those of OA treatments alone (**Fig 4.6C**, lane 4). This accumulation of ubiquitinated proteins may result from the partial inhibition of mTOR by rapamycin which does not block protein synthesis (Feldman et al 2009).

4.7 OA induces a loss of viability in serum-starved cells that is associated with caspase activation and blocked by an antioxidant

PP2A inhibition can cause cell death associated with reactive oxygen species (ROS) overproduction and caspase activation in neuronal cells (Yi et al 2009). Therefore the effects of OA on viability and caspase activity were examined in serum-starved SK-N-SH cells pretreated with or without the antioxidant Nac. To investigate the effects of distinct mTOR inhibitory mechanisms on viability, these experiments also included Nac pretreatments co-incubated with PP242 or rapamycin. The data from these studies show that OA induced a significant loss of cell viability that correlated with significant increases in caspase-3/7 activation (**Fig. 4.7A, B** compare columns 1 and 5). Co-treatments of rapamycin and OA induced a significant additional loss in cell viability relative to OA alone or OA with PP242 (**Fig. 4.7A**, compare columns 5, 6 and 7). Pretreatments with the antioxidant Nac attenuated both the loss in viability and caspase activation induced by OA, suggesting that PP2A inhibition mediates cell death via oxidative stress (**Fig. 4.7A, B** compare lanes 5-7 vs. 8-10). Together, these results demonstrate that PP2A is a critical regulator of survival in serum-starved SK-N-SH cells and a blockade of mTOR function by rapamycin further sensitizes these cells to a greater loss in viability.

4.8 Cell death induced by okadaic acid is independent of caspase activity and accumulation of phosphorylated Akt

The observation that caspase activation accompanies the cell death induced by OA raised the possibility that this event is mediated by apoptosis. To address this idea, viability was measured in cells pretreated with the pan-caspase inhibitor ZVAD alone or in combination with rapamycin or PP242 prior to

1-hr incubation with OA. Unlike Nac, ZVAD failed to rescue serum-starved cells from the toxicity elicited by OA despite a significant reduction in caspase-3/7 activity (**Fig. 4.8A and B,** compare column 1 vs. 4, 5 and 6). Notably, the greater loss in viability in cells co-treated with rapamycin and OA (**Fig. 4.7A**) persisted in the presence of ZVAD (**Fig. 4.8A**, compare columns 5, 6 and 7). Thus, cell death was able to proceed when caspase activity was blocked despite the significantly elevated caspase activity induced by OA.

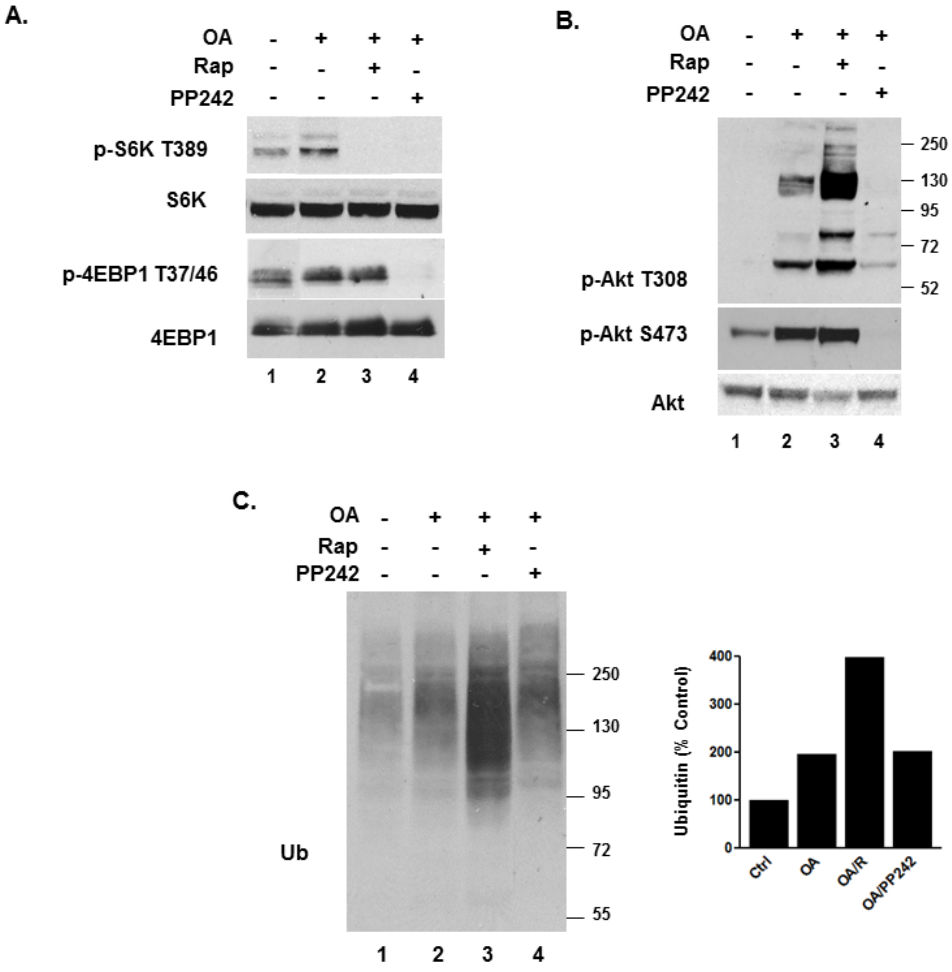
The findings that HMW phosphorylated forms of Akt accompany the cell death induced by OA (**Fig. 4.1** and **Fig 4.7A**) and that these events are potentiated by rapamycin (**Fig. 4.4** and **4.7A**) suggested that in addition to improving survival, pretreatments with Nac would modulate Akt activation levels. Therefore, cells exposed to rapamycin were treated with Nac before 1-hr incubation with OA (as in **Fig 4.7**) then analyzed by Western blotting for Akt phosphorylation at T308. Western blots showed that Nac treatments potentiated the detection of the monomeric 60-kDa and HMW phosphorylated forms of Akt (**Fig. 4.8C**, compare lanes 1 and 2). Taken together, these results suggest that the accumulation of phosphorylated HMW and 60-kDa forms of Akt together with caspase activation are dispensable for OA-induced cell death.

4.9 Discussion

The data presented in this chapter show that PP2A inhibition by OA enhances Akt phosphorylation at T308 and S473 and this Akt exists in monomeric and HMW forms. These forms are ubiquitinated and present in the nucleus and cytoplasm of serum-starved SK-N-SH cells. In addition, the accumulation of phosphorylated Akt induced by PP2A dysfunction causes a depletion of total Akt. Rapamycin potentiates both Akt phosphorylation and depletion in response to OA through a mechanism regulated by VEGFR2. Rapamycin is widely reported to upregulate Akt phosphorylation by disrupting a negative feedback between mTOR and PI3K. The results presented here identify a novel role for VEGFR2 as an activator of Akt in rapamycin-treated cells. Akt is an important pro-survival factor and hyperactivation of Akt is a common survival mechanism in cancer cells. However, we show that Akt hyperphosphorylation is associated with induction of a caspase-independent cell death mediated by oxidative stress. Taken together, these results show that the critical role of phosphatases such as PP2A in regulating Akt activation also affects Akt ubiquitination, cleavage and removal from the cell.

Furthermore, these data indicate that PP2A promotes survival through a suppression of oxidative stress. It should be noted that most reports addressing Akt degradation by the ubiquitin/proteasome system have employed transfected constructs that overexpress either ubiquitin or Akt to demonstrate the accumulation of Akt as ubiquitinated HMW forms (Facchinetti et al 2008, Lee et al 2008, Suizu et al 2009). The data in this report demonstrate that PP2A inhibition is sufficient to induce the accumulation of ubiquitinated HMW phosphorylated Akt without introducing exogenous protein.

Our findings suggest that Akt reaches a critical threshold of activation that triggers its ubiquitination in both cellular compartments. Recent reports have shown that Akt is a substrate for degradation by the UPP in both neuronal and non-neuronal cells (Facchinetti et al 2008, Lee et al 2008, Suizu et al 2009, Yan et al 2006). The E3 ubiquitin ligases TTC3 and BRCA1 preferentially target activated Akt (Suizu et al 2009, Xiang et al 2008). In agreement with these findings, phosphorylation at S473 was recently shown to direct Akt to the proteasome (Wu et al 2011). Furthermore, the signaling of Akt phosphorylation by the growth factors PDGF and IGF-1 was shown to induce a time-dependent downregulation in total Akt that was attributed to degradation by the ubiquitin/proteasome pathway (Adachi et al 2003). These results suggest that there is a causal link between Akt activation by phosphorylation and its subsequent ubiquitination. We propose that blocking PP2A prevents the dephosphorylation of Akt at T308 and S473 (Liu et al 2003), thus increasing the cellular pool of phosphorylated Akt and causing its ubiquitination and shift to HMW forms. Consistent with this notion, inhibiting Akt phosphorylation by blocking PI3K or mTORC2 signaling prevents OA from inducing HMW forms of phosphorylated Akt and the depletion of total Akt (**Fig. 4.2E** and **4.6B**). Thus our paradigm of PP2A inhibition mimics the effects of persistent growth factor signaling of Akt activation (Adachi et al 2003, Wu et al 2011) and provides a connection between phosphatase activity and the targeting of phosphorylated Akt for ubiquitination.



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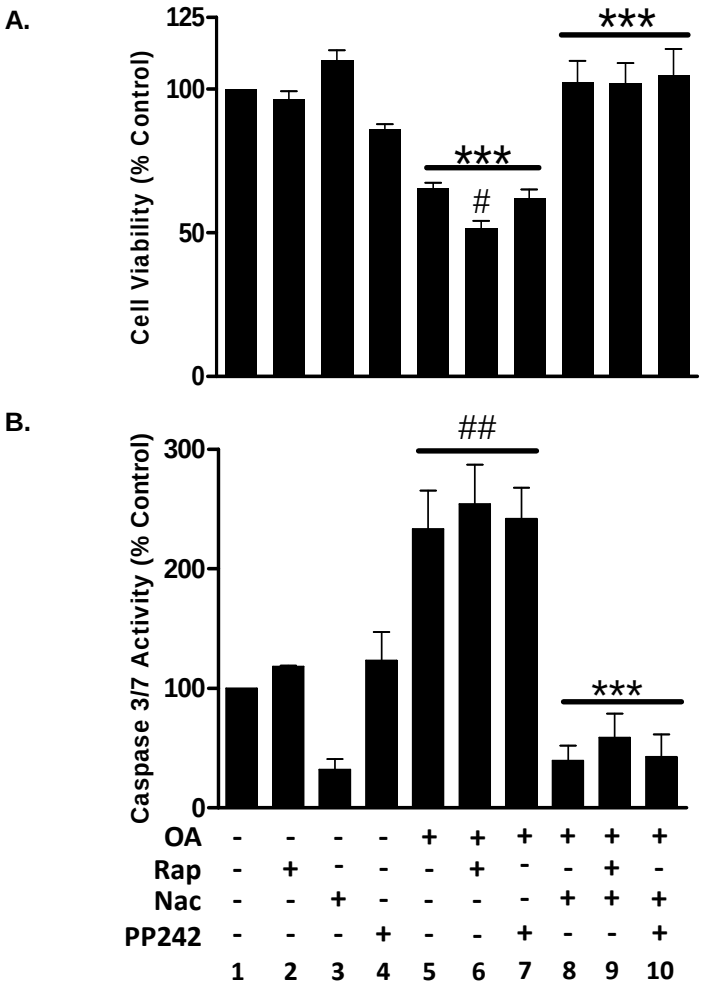
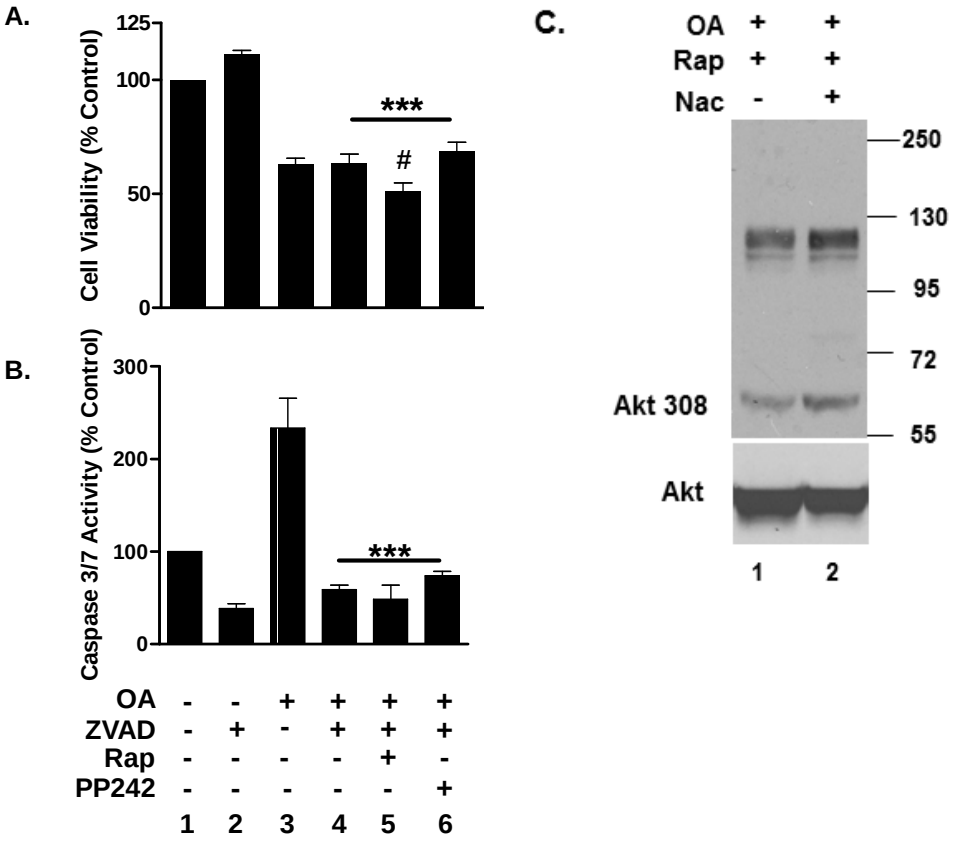


Figure 4.7. OA induces a loss of viability in serum-starved cells that is associated with caspase activation and blocked by an antioxidant. Cells were pre-treated with 1-µM rapamycin for 48 h or for 2 h with Nac (5 mM) or PP242 (2.5 µM) before incubation with 400-nM OA or vehicle. (A) Cells were then assayed for viability as described in Chapter 2 and survival was expressed as the percent viability relative to the vehicle-treated control (100%) ±S.E.M from at least three independent experiments. *** (P<0.001) Indicates a significant difference between OA, OA/Rap, OA/PP242 versus control and between OA/Nac, OA/Rap/Nac and OA/PP242/Nac versus the same treatments without Nac. # (P<0.05) indicates significant difference between OA/Rap and OA. (B) Cells were treated as in (A) and caspase 3/7 activity was assayed as described in Chapter 2. Results represent the percent caspase activity relative to control ±SEM from a minimum of 3 independent experiments. ## indicates significant difference between OA, OA/Rap or OA/PP242 versus the corresponding treatment without OA (P<0.01).



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The model described above predicts that other means of modulating Akt phosphorylation should affect levels of Akt in the cell. In support of this idea, a blockade of mTOR activity in VEGF-treated endothelial cells leads to caspase activation and proteasome-mediated degradation of Akt (Riesterer et al 2004). Rapamycin has a well-established effect of enhancing Akt activation via inhibition of a negative feedback loop between mTOR and Akt (Huang and Manning 2009). In our model, rapamycin caused an increase in the phosphorylation and accumulation of activated Akt in OA/VEGF co-treated cells and this correlated with reduced levels of total Akt. Consequently, the increase in Akt depletion with rapamycin may be a physiological response to the excessive levels of phosphorylated Akt that accumulate from a failure to undergo dephosphorylation.

Our investigation into the rapamycin-induced increase in HMW Akt revealed a novel role for VEGFR2 in mediating negative feedback from TORC1/S6K to Akt. While blocking VEGFR2 in the presence of OA only slightly reduced HMW Akt T308 forms, the increase in the levels of HMW Akt by rapamycin was completely prevented by VEGFR2 inhibition (**Fig. 4.5B**). The observation that VEGFR2 inhibition also reduced ERK1/2 phosphorylation is consistent with the previous demonstration that VEGF regulates ERK1/2 activation in serum-starved SK-N-SH cells through VEGFR2 (Gomes et al, 2007). The effect of VEGFR2 on Akt is likely to involve signaling through a negative feedback loop that functions via S6K suppressing PI3K and Akt activity (reviewed in Efeyan and Sabatini 2009). This feedback loop was originally determined to affect the PI3K/Akt axis via inhibition of IRS-1, an important mediator of insulin signaling to PI3K (Haruta et al 2000). A role for VEGFR2 in this feedback mechanism is supported by evidence that VEGF signaling in endothelial cells can cause VEGFR2 to co-localize with IRS-1 and stimulate PI3K (Feliers et al 2005). On the other hand, there is evidence that this loop can occur independently of IRS-1. Activated mTOR signaling can inhibit the ability of other receptor tyrosine kinases such as PDGFR (Zhang et al 2007) to activate PI3K, so it is conceivable that the role of VEGFR2 in the feedback loop does not involve IRS-1. Although we did not identify the direct target of S6K that inhibits VEGFR2, our data support the idea that blocking S6K activation with rapamycin induces a molecular switch in which VEGFR2 becomes a critical regulator of Akt.

OA effected an increase in ubiquitinated Akt as well as the formation of 40 and 44 kDa fragments. These Akt fragments (**Fig. 4.5B**) are consistent with the demonstration that caspase-3 cleaved Akt into

fragments of similar sizes *in vitro* and *in vivo* and can be induced by oxidative stress (Liao and Hung 2010). Thus it follows that both ubiquitination and caspase-mediated degradation contribute to the observed decline in Akt in cells subject to OA. However, it is not clear that these mechanisms account for the depletion of Akt in our model. For example, Akt degradation may result from a caspase-dependent ubiquitination but our findings that HMW forms of phosphorylated Akt persist in the presence of ZVAD or Nac which attenuate caspase-3/7 activation (**Fig. 4.8C**) rules against this possibility. Additionally, in cells treated with OA and rapamycin proteasome or caspase inhibition had minimal effects on levels of total or phosphorylated Akt (**Fig. 4.4C** and **D**). These results indicate that there may be an additional mechanism responsible for the disappearance of Akt under PP2A inhibition. Our findings that rapamycin in OA/VEGF treated cells leads to the accumulation of activated Akt as HMW and cleaved forms with reduced levels of total protein is consistent with the demonstration that blocking mTOR activity in VEGF treated endothelial cells leads depletes Akt levels (Riesterer et al 2004). Therefore, although the mechanism is unknown, mTOR activation through the VEGF/VEGFR2 pathway appears to regulate Akt stability in addition to its activation levels.

The deleterious effects of PP2A inhibition in our neuronal model underscore the importance of dephosphorylation in regulating Akt activation as well as the consequent effects of oxidative stress on survival (Yi et al 2009, Yoon et al 2002). This notion is reinforced by the demonstration that antioxidant treatment but not caspase inhibition can promote survival in OA-treated cells, suggesting that ROS mediates cell death in our model.

Notably, our viability data are in contrast to the notion that a rapamycin induced increase in the phosphorylation of Akt promotes survival under pathological conditions (Bove et al 2011, O'Reilly et al 2006). Instead, the hyperphosphorylation of Akt induced by OA in serum deprived SK-N-SH cells results in caspase-3/7 activation and a cell death that is induced by oxidative stress and potentiated by rapamycin independent of caspase inhibition (**Fig. 4.7** and **4.8**). In support of this finding, hyperphosphorylated Akt induced by rapamycin can sensitize cancer cells to oxidative stress-induced apoptosis by decreasing antioxidant protein levels and promoting ROS overproduction through increased oxidative phosphorylation (Nogueira et al 2008). Consequently, rapamycin may sensitize SK-N-SH cells to a greater loss in viability under pro-oxidant conditions. The TORC1/TORC2 inhibitor PP242 (Feldman

et al 2009) blocked the Akt phosphorylation induced by OA but not the cell death. These findings suggest that that Akt hyperphosphorylation is possibly mTORC2 dependent but dispensable for survival.

Autophagic cell death may explain the OA-induced loss of viability in our paradigm as it has been linked to oxidative stress and can proceed independently of caspase activation (Chen et al 2008, Xu et al 2006). In this regard, rapamycin alone or a combination of caspase 3 and mTOR inhibition can induce autophagy and potentiate cell death in cancer cells (Codogno and Meijer 2005, Kim et al 2008b). Furthermore, cell death by autophagy has been linked to oxidative stress and proceeds independent of caspase activation. During autophagy protein aggregates are sequestered into membrane-bound autophagosomes (Yao 2010). Our observation that phosphorylated Akt accumulates in a punctate pattern in the cytoplasm and nucleus of OA-treated cells (**Fig. 4.4A**) suggests that, due to a lack of dephosphorylation, activated Akt also accumulates into aggregates (Basso et al 2002). In support of this concept, OA was shown to induce autophagosomes in rat neurons (Yoon et al 2008). Consequently, our findings underscore a cellular requirement for phosphatase function to prevent oxidative insults. Consistent with this interpretation of our data, PP2A activity protects against apoptosis induced by oxidative stress (Antony et al 2010) and reduces levels of HMW phosphorylated α-synuclein and associated motor function deficits in a mouse model of Parkinson's disease (Lee et al 2011).

Cell death associated with neurodegenerative diseases has long been correlated with the induction of ubiquitination and subsequent accumulation of ubiquitin–protein conjugates (Huang and Figueiredo-Pereira 2010). Interestingly, OA also increased total levels of ubiquitinated proteins and this effect was further augmented by pretreatments with rapamycin but not PP242 (**Fig. 4.6D**). In support of these findings, rapamycin alone induces increased ubiquitin conjugate levels in stressed cardiomyocytes (Harston et al 2011). The enhancement in ubiquitin protein conjugates by rapamycin in OA-treated cells may result from several possible Akt-dependent mechanisms. Numerous Akt targets are ubiquitinated in response to phosphorylation including TSC2 and FOXO3a (Inoki et al 2002, Lin et al 2002, Plas and Thompson 2003). Additionally, Akt can activate several E3 ligases for protein ubiquitination (Zhou et al 2001). Alternatively, the failure of rapamycin to block 4E-BP1 phosphorylation would further elevate the pool of ubiquitinated proteins through increased translation. Under the combined effect of OA and rapamycin, active translation in the cell may produce newly synthesized proteins which are preferentially

targeted to the UPP (Medicherla and Goldberg 2008). The oxidative modification of these and other proteins may overwhelm cellular stress responses and potentiate the cell death caused by PP2A inhibition (Haynes et al 2004). Thus active protein synthesis in the presence of oxidative stress may account for the potentiation of cell death by rapamycin in OA-treated cells.

4.10 Conclusions

This work presents critical roles for PP2A in mediating Akt ubiquitination and suppressing cell death associated with pro-oxidant conditions in a neuronal cell model. We show that phosphorylated, ubiquitinated HMW Akt is induced by PP2A inhibition, a finding consistent with recent work showing preferential ubiquitination of activated Akt. We also present a novel role for VEGFR2 in potentiating Akt hyperphosphorylation by rapamycin. Finally, despite the presence of high levels of activated Akt, the cell death caused by a blockade of PP2A proceeds through a caspase-independent mechanism.

Chapter 5

Conclusions and Future Directions

5.1. Conclusions

The work presented in this thesis contributes to elucidating the regulation of mTOR and Akt in neuronal cells. We demonstrated that VEGF/VEGFR2 mediates mTOR activity when stimulated by 1) ligand binding, 2) transactivation by mAchRs and 3) interruption of the S6K/PI3K feedback loop. Downstream of mAchR/VEGFR2 crosstalk, PKC-family signaling had opposing effects on targets of the two different mTOR complexes. In addition, we demonstrated the critical role that PP2A activity plays in modulating signaling from VEGF and VEGFR2 to targets of the mTOR pathway. Finally, we identified ROS as essential mediators of cell death associated with aberrant signaling in our model. A diagram depicting these signaling interactions is presented in **Fig. 5.1**.

Interrupting VEGF signaling is a well-established strategy for anti-angiogenic treatments and the mTOR/Akt network is a focus of intense study for developing therapeutic interventions. The S6K/PI3K/Akt signaling loop has clinical ramifications in certain cancers and metabolic syndromes and the existence of this loop has been reported in neuronal cells (Meikle et al 2008, Zoncu et al 2011). By furthering the understanding of the connection between these signaling platforms in neuronal cells our work may assist in the development and rational application of these established and emerging therapies.

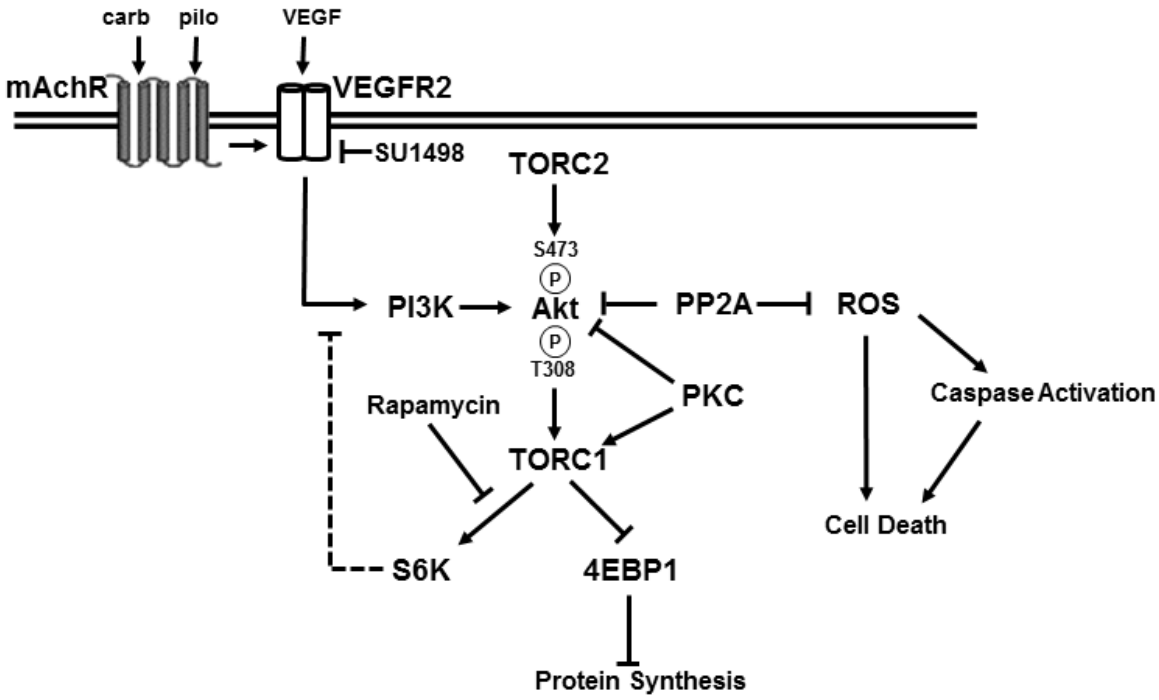
Our work also identified that the cellular concentration of Akt phosphorylated at T308 and/or S473 is a prime determinant of Akt levels. PP2A inhibition greatly increased the phosphorylation of Akt at the activation loop and hydrophobic motif, two sites essential for activation of the kinase. Hyperphosphorylation induced the depletion of Akt, a result that is congruent with a recent report that enhancing S473 phosphorylation caused a loss in overall Akt (Wu et al 2011). The work of Wu et al. is also consistent with our demonstration that inhibiting upstream signaling to Akt prevents its depletion in the presence of phosphatase inhibition. The loss of Akt induced by PP2A inhibition may represent a negative feedback regulation that is intended to reduce the amount of active Akt in the cell when aberrant levels are detected. Our work makes the novel demonstration that this response can be triggered not only by upstream stimulation of Akt but also by preventing phosphatases from acting on Akt. Thus, we have expanded the list of regulators of cellular Akt levels to include PP2A. The targeted degradation of Akt protein and the modulation of phosphatase activity show promise as strategies for treating cancer and neurodegeneration (Lee et al 2011, Perrotti and Neviani 2008, Vintonyak et al 2009). Our work

elucidating the cellular mechanisms regulating Akt levels provides support for further pursuit of this avenue of research.

A notable similarity between our two studies was the efficacy of antioxidant treatment in preventing the cell death induced by OA and pilocarpine. These results highlight the essential role of redox levels in maintaining neuronal cell viability. In this regard, previous work links both drug treatments to the generation of free radicals (Santos et al 2011, Waldbaum et al 2010, Yi et al 2009). The most common means of disrupting the oxidative balance of the cell is through perturbation of mitochondrial function. OA and pilocarpine have been shown to induce mitochondrial dysfunction, providing a general link between the effects of these agonists (Waldbaum et al 2010, Yoon et al 2006). Mitochondrial ROS can be generated by disruption of the electron transport chain and associated catabolic reactions and by lowering its capacity to act as a sink for free radicals (Starkov 2008). These mitochondrial functions are highly complex and involve many enzymes. Thus, while both OA and pilocarpine can impair the mitochondria, the specific mechanism by which these molecules affect the mitochondria in our model is unlikely to involve the same targets.

5.2 Future Directions

Akt is a central regulator of numerous cellular functions and maintaining physiological levels of this protein is essential to proper cell function. The ubiquitin/proteasome and caspase pathways are the prime means by which Akt is degraded. Our work with Akt and PP2A inhibition showed an accumulation of high molecular weight forms of phosphorylated, ubiquitinated Akt concomitant with a depletion of the normal molecular weight form, suggesting that we were perturbing normal metabolic control of this protein. There are some puzzling observations associated with this data. Neither the levels of total Akt nor the appearance of HMW forms were increased by inhibiting the proteasome. Furthermore, caspase inhibition had only a limited effect on Akt levels suggesting that some other mode of proteolysis was operating in this scenario. The Akt might be driven into the insoluble fraction of the cells or an autophagic response may be induced by phosphatase inhibition. The strategy of enhancing Akt degradation to control aberrant Akt signaling has been employed with success in a mouse model (Jo et al 2011). Pursuing these lines of experimentation could identify an additional mode of Akt degradation in the cell and further contribute to our understanding of Akt metabolism.



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Chapter 6

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6. Literature Cited

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