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Effects of Astragaloside IV on GSK-3β and S6K1 Phosphorylation in C2C12 Muscle Cells

Kyle James Tuohy

Abstract

Saponins are a broad class of functionally diverse phytochemicals that have been shown to exhibit antihyperglycemic, antihyperlipidemic, anti-inflammatory, and anti-cancer effects. Through *in vitro* and *in vivo* experimentation, Astragaloside IV (AsIV) has been shown to regulate glycogen synthesis through protein kinase B (AKT)-mediated phosphorylation of glycogen synthase kinase 3-beta (GSK-3β). Along with control of glucose homeostasis, AKT activation is also an important regulator of protein synthesis via the downstream phosphorylation of mammalian target of rapamycin (mTOR) and ribosomal protein S6 kinase beta-1 (S6K1). Because AsIV can activate this pathway to stimulate glucose metabolism, it is possible that it can also promote protein synthesis as well since these two regulatory processes utilize the same pathway. In order to determine the effect of AsIV on protein synthetic pathways, C2C12 murine muscle cells were treated with AsIV and the phosphorylation levels of key enzymes were assessed.

We found that AsIV induced phosphorylation of key protein synthetic targets in a time-dependent manner, with the greatest increases in phosphorylation after two hours of AsIV treatment. However, this effect was not seen when cells were serum-starved prior to AsIV treatment. Lastly, AsIV was able to increase GSK-3β phosphorylation in cells treated with dexamethasone first. These findings help to further understand the molecular changes that occur in muscle cells treated with AsIV.

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Effects of Astragaloside IV on GSK-3β And S6K1 Phosphorylation in C2C12 Muscle Cells

By

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Introduction

Atrophy of skeletal muscle is a serious consequence of many conditions, such as COPD, HIV/AIDS, chronic kidney and heart diseases, cancer, and diabetes (Schiaffino and Mammucari 2011). There is an association between Cachexia, the involuntary body wasting caused by chronic disease states, and poorer outcomes from these chronic diseases (Evans et al. 2008). As such, it poses a major public health issue (Farkas et al. 2013). While the specific mortality rates vary with each different disease, from 10-15% in COPD, to 60-80% in cancer patients (Farkas et al. 2013), cachexia occurs in 30% of patients who die from these diseases overall (von Haehling and Anker 2010). Within the industrialized nations of North American, Europe, and Japan, the prevalence of cachexia is estimated to be around 1% of the populations, or nine million people (von Haehling and Anker 2010).

Maintaining healthy muscle mass is a balance between control of synthesis and degradation. While there is currently no single, established pathway by which body wasting occurs, the regulation of protein synthesis may play an important role. There appears to be an "anabolic-catabolic dysbalance" seen with cachexia, as a result of dysfunction to multiple body systems (von Haehling and Anker 2010). In patients with cancer or COPD cachexia, a reduction in muscle protein synthesis was an important factor (Morrison et al. 1988), while increased muscle breakdown was also present in cardiac cachexia (von Haehling and Anker 2010).

There are currently no medical interventions to treat muscle atrophy, only strategies to help reverse it by either blocking the activation of pathways that lead to protein degradation and atrophy, or stimulate the pathways that lead to protein synthesis

and muscle hypertrophy (Glass 2003). However, this is easier said than done due to the necessity of both growth and degradation for proper muscle homeostasis. Therefore, it is important to understand the molecular mechanisms behind these signaling pathways (Sitnick 2006) and to identify ways to reverse muscle wasting via these pathways.

Regulation of protein synthesis via Akt

The regulation of muscle mass requires a constant equilibrium between protein synthesis and breakdown, and studies have shown that the Akt/mammaliam target of rapamycin (mTOR) signaling pathway is the mechanism controlling this balance (Glass 2003). The pathway is stimulated by extracellular insulin, insulin-like growth factor 1 (IGF1), or other structural analogs that are capable of binding to and activating receptors on the cell membrane. The IGF1-Akt1 and insulin-Akt2 pathways are interconnected and share many of the same components. In addition, IGF1 is capable of binding to the insulin receptor and insulin is capable of binding to the IGF1 receptor (Schiaffino and Mammucari 2011). However, different ligands can lead to slightly different downstream effects as IGF1 is more involved with muscle growth and insulin is more active in glucose metabolism (Schiaffino and Mammucari 2011).

IGF binding to the IGF1 receptor (IGFR) leads to downstream activation of Akt, which leads to increased protein synthesis, decreased protein degradation, and increased glucose metabolism and transport (Figure 1). When IGF1 binds to its receptor, it leads to autophosphorylation of the receptor via activation of its intrinsic tyrosine kinase activity. This creates a docking site for insulin receptor substrate (IRS), which is then phosphorylated by the IGF1 receptor as well. Phosphorylated IRS then activates phosphatidylinositol-3-kinase (PI3K), which converts phosphoinositide-4,5-biphosphate

(PIP2) into phosphoinositide-3,4,5-triphosphate (PIP3). PIP3 acts a docking site for phosphoinositide-dependant kinase 1 (PDK-1) and Akt, allowing for PDK-1 to activate Akt by phosphorylating it at threonine 308. These actions all occur at the inner surface of the cell membrane.

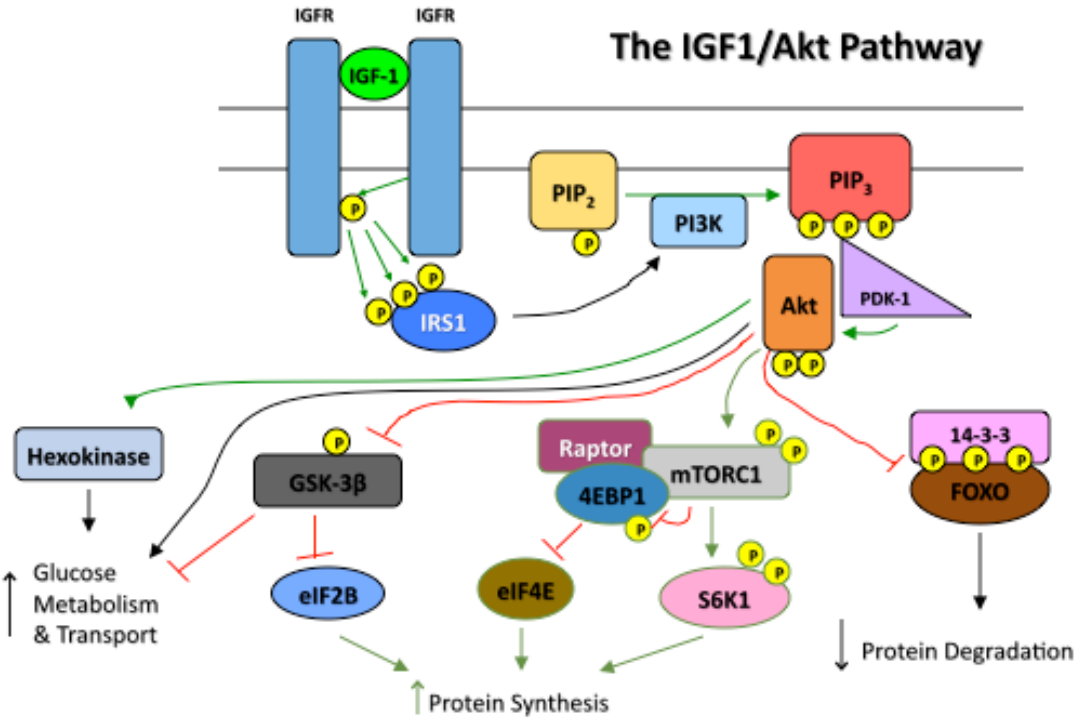


Figure 1: Overview of the IGF1/Akt pathway. This pathway controls protein synthesis, degradation and glucose metabolism and transport via activation of downstream target proteins. Arrows designate one protein activating another, red lines with a perpendicular red line at the end designates one protein inhibiting another. Modified from Sartorelli V and Fulco M. Sci. STKE 244:1-7 (2004).

Akt then increases the rate of protein synthesis via two related mechanisms. First, Akt indirectly activates mTOR signaling, which includes two complexes: mTORC1 and mTORC2. mTORC2 is important for Akt activation via positive feedback that

phosphorylates Akt at serine 473, which is necessary for optimal activation of Akt. mTORC1 is bound to Raptor and phosphorylates ribosomal protein S6 kinase beta-1 (S6K1), which promotes protein translation efficiency. mTORC1 also increases protein synthesis via phosphorylation and subsequent inhibition of eukaryotic translation initiation factor 4E-binding proteins (4EBP1). Inactivation of these binding proteins blocks the inhibition of translation initiation factor 4E, allowing for the assembly of the ribosomal complex and translation initiation.

Second, Akt promotes protein synthesis via phosphorylation and inhibition of glycogen synthase kinase 3 beta (GSK-3β). This protein inhibits another translation factor: eukaryotic translation initiation factor 2B, which is important for the formation of the initial tertiary complex of mRNA and the ribosomal subunits, allowing for the binding of the $tRNA_i^{Met}$ to the ribosome. Therefore, inhibition of GSK-3β by mTORC1 also leads to increased protein translation.

Akt also regulates protein metabolism by decreasing the rate of protein degradation. This is achieved via phosphorylation and inhibition of the forkhead box O (FoxO) family of transcription factors. Specifically, FoxO3 acts on both the proteasomal and autophagic-lysosomal pathways to induce protein degradation (Schiaffino and Mammucari 2011). First, FoxO3 regulates the transcription of ubiquitin ligases muscle atrophy F-box (MAFbx) and muscle ring finger 1 (MuRF1), which cause ubiquitylation of myosin and other muscle proteins, marking them for degradation by the proteasome (Lecker et al. 2006). Second, FoxO3 also regulates the transcription of proteins needed to activate the autophagy-lysosome pathway. Normal autophagic activity is necessary for removing dysfunctional organelles and preserving the integrity of muscle fibers. The net

effect of Fox03 activation is an increase in muscle atrophy, which is inhibited by IGF1-mediated Akt activation (Sandri 2011).

Significance for control of muscle growth and loss

Genetic knockout studies have provided insight into the importance of the IGF/Akt pathway on muscle metabolism. Mice lacking the IGF1 gene, *igf1,* had severe growth retardation with most mice dying shortly after birth (Lui et al. 1993). In addition, mice possessing a mutated, inactive IGF receptor controlled by a muscle-specific promoter had delayed growth up to three weeks of age (Fernandez et al. 2002) and impairment of muscle regeneration in adulthood (Heron-Milhavet et al. 2010). Deletion of the *akt1* gene in mice led to growth retardation (Chen et al. 2001; Cho et al. 2001a), while *akt2* deletion caused deficits in glucose metabolism without affecting growth (Cho et al. 2001b). When both genes were knocked out, there was significant growth retardation and atrophy of skeletal muscle (Peng et al. 2003). Additionally, *in vivo* studies have confirmed that this pathway is suppressed in mouse models of muscle atrophy (Sitnick 2006; 2009)

In comparison, activation or over-expression of this pathway can lead to muscle growth. First, Coleman et al. (1995) used the avian skeletal α-actin gene to create a myogenic expression vector to direct the expression of IGF1 cDNA in skeletal muscle. This resulted in localized over-expression of IGF1 in skeletal muscle and resulted in striated muscle hypertrophy. Second, Lai et al. (2004) transfected mice embryos with an Akt cDNA vector controlled by the human skeletal actin promoter, creating transgenic mice with constitutively active Akt expressed in their skeletal muscle. Again, this

transgenic model led to increased hypertrophy of muscle fibers, as well as increased activation of the downstream protein target S6K1.

These studies highlight the importance of these proteins in maintaining muscle mass, and provide evidence that increased activation of the IGF1/Akt pathway can promote protein synthesis and muscle hypertrophy. Akt is not only involved in protein synthesis, however, it is also the pathway controlling glucose metabolism and transport.

Regulation of glucose metabolism and transport via Akt

The PI3K/Akt pathway is a highly conserved cell-signaling pathway that facilitates glucose uptake and metabolism. It is initiated primarily by insulin and is especially important for insulin-dependent cells like muscle, but may also be activated by other extracellular growth factors like IGF1 (Ward and Thompson 2012; Schiaffino and Mammucari 2011). The initial steps in this pathway are similar to those that affect protein synthesis (see Figure 1). Extracellular growth factors bind to their cell membrane receptors and lead to activation of PI3K. This enzyme then phosphorylates phosphatidylinositol lipids, which eventually leads to phosphorylation and activation of Akt.

Akt increases glucose uptake by increasing the expression of glucose transporters (GLUT) at the cell surface. Class I facilitative glucose transporters are divided into four isoforms: GLUT1 – GLUT4. The insulin-responsive GLUT4 is the predominant type found at the cell surface of skeletal muscle (Navale and Paranjape 2016). In non-stumulated cells, GLUT4 is found mostly on the interior of the cell within cytoplasmic vesicles. Akt phosphorylates AS160 (Akt substrate of 160 kDa), which inhibits it and

causes increased exocytosis and deceased endocytosis of GLUT4 (Swiderska et al. 2018). The net effect is increased GLUT4 transporters at the cell surface.

Akt also activates enzymes necessary for glycolysis and glycogenesis. First, Akt increases hexokinase activity by promoting its association with mitochondria (Gottlob et al. 2001). Hexokinase catalyzes the first reaction of glycolysis, converting glucose to glucose 6-phosphate. Second, Akt phosphorylates and inhibits GSK-3β just like it does in the protein synthesis pathway. In the synthesis of glycogen, GSK-3β phosphorylates and inhibits glycogen synthase (GS). GS is a key enzyme in glycogen formation; it catalyzes the incorporation of glucose into the glycogen chain via a $\alpha(1\rightarrow4)$ glycosidic bond. When active, GSK-3β inhibits glycogenesis by inhibiting GS. However, Akt inhibits GSK-3β, so the net effect of Akt activation is an overall increase in glycogen synthesis.

It is clear that both glucose metabolism and protein synthesis share the same upstream pathway of PI3K/Akt phosphorylation. If a compound or chemical is capable of stimulating this pathway to improve glucose metabolism and transport, it is theoretically possible for such a compound to stimulate this pathway to improve protein synthesis. If so, this mechanism may represent a therapeutic target for treating muscle wasting caused by various medical conditions and aging. Therefore, it is important to identify substances that may in this manner. A group of compounds that may potentially elicit this effect are called saponins.

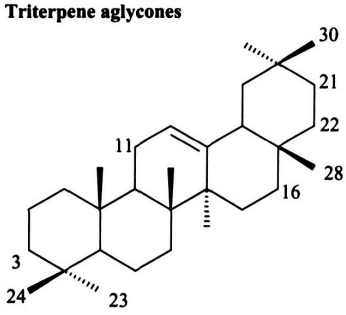
Saponins overview

Saponins are a class of naturally occurring phytochemicals that are widely distributed among various plant species. They are glycosides containing one or more sugar chains attached at varying locations to an aglycone backbone. They can be broadly

classified as either triterpenoid (Figure 2A) or steroidal (Figure 2B) based on the structure of their aglycone backbone, called a sapogenin Both backbone types are derived from the same 30-carbon linear precursor (Moses et al. 2014), but subsequent processing creates one of the two backbone subtypes. Steroidal sapogenins (27C) may possess as 6-ring spirostane or 5-ring furostane skeletons. The triterpenoid sapogenins contain four or five ring backbones and are more structurally diverse (Podolak et al. 2010).

Saponins can be further classified as mono, di-, or tridesmosidic based on the number of sugar chains they possess (Güçlü-Ustündağ and Mazza 2007). The combination of the lipid-soluble backbone and water-soluble sugar residues makes saponins generally amphipatic with emulsifying and foaming properties (Wang et al. 2005). However, the complexity and structural diversity of these compounds leads to a number of other biological effects that vary from one saponin to the next, some of which are not completely understood (Moses et al. 2014).

A: **Triterpene aglycones**



Aglycone	-OH	=O	-COOH
Glycyrrhetinic acid	3β	11	30
Gypsogenin	3β	23	28
Oleanolic acid	3β		28
Quillaic acid	3β, 16α	23	28
Soyasapogenol A	3β, 21β, 22β, 24		
Soyasapogenol B	3β, 22β, 24		
Soyasapogenol E	3β, 24	22	

B: **Steroid aglycone**
Diosgenin

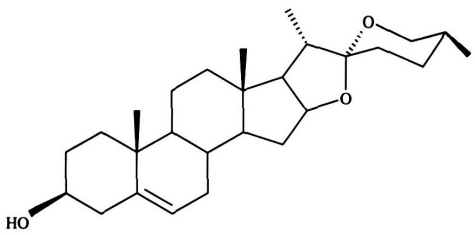


Figure 2: Structural difference between steroidal and triterpenoid saponins. Structure of the triterpene aglycone backbone (A), containing 30 carbons, compared to the steroid aglycone (B) containing 27 carbons. In either case, the backbone is called a sapogenin. The addition of one or more sugar chains makes the molecule a saponin. Modified from Güçlü-Ustündağ and Mazza 2007.

Several studies have attempted to correlate biological activity with the structural variations between different saponins (Francis et al. 2002). Collectively, saponins are commonly researched for their cytotoxic, lipid-lowering, anti-inflammatory, and immune system effects. Most importantly for our study, however, is the effects that saponin have been shown to exert in glucose metabolism and transport. Matsuda et al. (1999) found various oleanolic acid oligoglycosides to have hypoglycemic effects that acted by increasing the release of insulin. These compounds also suppressed the transport of glucose from the stomach to the small intestine, and glucose transfer across the brush border of the small intestine.

Other studies have helped to delineate the molecular mechanisms by which saponins regulate glucose metabolism and transport. Bhavsar et al. (2009b) treated diabetic and obese mice with saponins derived from the *Helicteres isora* plant and found a reduction in serum glucose and cholesterol levels. They also found changes in the gene expression of adipsin, Glut4, and PPARγ, genes regulating glucose and lipid transport and metabolism. In addition, Bhavsar et al. (2009a) identified changes in protein phosphorylation in C2C12 muscle cells as a result of treatment with saponins. They found that saponins activated the PI3k/Akt pathway to inhibit GSK-3β and stimulate glycogen synthesis.

Astragaloside IV

Given the evidence that saponins can activate the Akt pathway to improve glucose metabolism and transport, it strongly suggests that these glycosides can also activate this pathway to stimulate protein synthesis. While saponins have varying effects due to their structural variety, we will specifically be using Astragaloside IV (AsIV). AsIV is the main active component of the *Astragalus membranaceus* plant. This plant has been used in traditional Chinese medicine for many years for the treatment of cardiovascular disease and metabolic disorders (Ren et al. 2013), though it wasn't until recently that AsIV was isolated from the plant in the lab and shown to be the compound providing these beneficial effects.

Studies have shown that AsIV exerts cardioprotective effects via inhibition of GSK-3β and activation of the Akt pathway. First, AsIV was shown to reduce cell death after ischemia/reperfusion injury to cardiac muscle cells via GSK-3β inhibition by preventing mitochondrial permeability transition pore opening (He et al. 2012). AsIV also decreased infarct size, mitochondrial swelling, and GSK-3β activity in an *in vivo* model of rat myocardial ischemia/reperfusion injury (He et al. 2014). Lastly, AsIV increased angiogenesis and cell migration in human epithelial cells subjected to hypoxia (Zheng et al. 2011). This effect was mediated by PI3K/Akt pathway activation.

AsIV also provides its effects on glucose metabolism and transport via activation of Akt. Du et al. (2018) used high-fat diet feeding in mice to induce adipose dysfunction, which led to increased endogenous hepatic glucose production and lipolysis. Subsequent AsIV treatment limited hepatic lipid accumulation and excess hepatic glucose production via Akt activation. In addition, Zhu et al. (2016) examined the effect of AsIV in a cellular model of insulin resistance. In C2C12 cells exposed to palmitate for 16 hours, AsIV

facilitated glucose uptake by increasing mRNA and protein levels of the GLUT4 glucose transporter, in turn reversing the effects created by palmitate. Again, this effect was mediated by phosphorylation and activation of the IRS/Akt pathway.

These studies consistently show that AsIV can activate Akt and inhibit GSK-3β via phosphorylation of the Akt pathway to provide downstream cardioprotective and glucose regulatory effects. We therefore hypothesize that AsIV activation of the IGF/Akt pathway in C2C12 muscle cells can also lead to downstream stimulation of protein synthesis.

Materials and Methods

Cell Culture

C2C12 cells (CRL-1772, American Type Culture Collection (ATCC), Rockville, MD), a murine drived skeletal muscle cell line, were grown in proliferation media (PM): Dulbecco's Modified Eagle Medium (DMEM) (ATCC, Rockville, MD) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin (P/S) (10,000 U/ml, Thermo Fisher Scientific, Waltham, MA). As C2C12 cells exited the proliferatory phase upon confluency, cells were passaged at 70-80% confluence in order to expand cell populations. To perform experimental treatments, the cells were allowed to reach 100% confluency, after which the culture media was switched from PM to differentiation media: DMEM supplemented with 2% horse serum (Thermo Fisher Scientific, Waltham, MA) and 1% P/S to induce differentiation into contractile myotubes (Figure 3). Cells were then allowed to differentiate for four days prior to treatment. All cell cultures were incubated at 37° C and 5% CO_2 at all times.

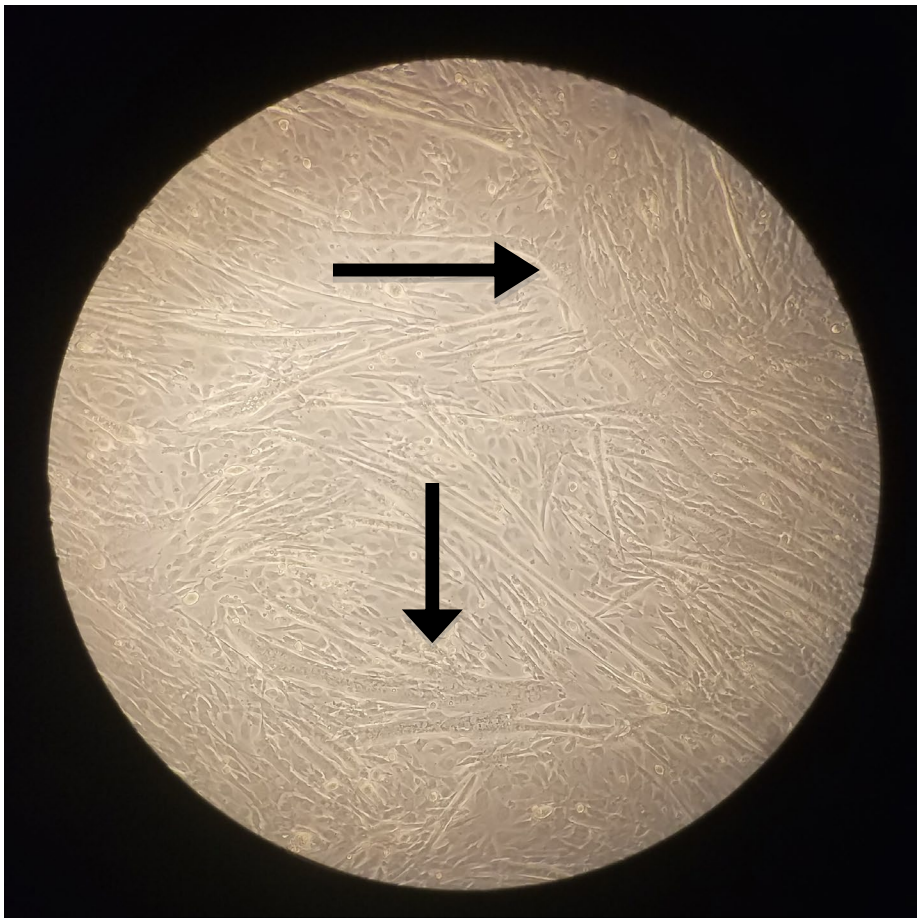


Figure 3: Representative image of cultured cells after differentiation. After reaching 100% confluence, cells were induced to differentiate for 4 days in DMEM supplemented with horse serum. This resulted in differentiation into contractile myotubes visible under light microscopy (see arrows).

Reagents

AsIV and Dexamethasone (Dexa) were obtained from Cayman Chemical Company (Ann Arbor, MI) in powdered form. AsIV was dissolved in dimethyl sulfoxide (DMSO) to create stock concentrations of 12.5 mM. Dexa was dissolved in DMSO and maintained in stocks of 10 mM and 100 mM. Long R3 IGF-1 (IGF1) (Millipore Sigma, St. Louis, MO) is a recombinant analog of the human IGF-1 and was diluted in H_20 at 1 mg/ml. All stock chemicals were stored at 4°C.

Antibodies

Phosphorylation of AKT was determined with rabbit polyclonal anti-AKT (BD Biosciences, San Jose, CA) and mouse phospho-AKT (Ser473) (Cell Signaling Technologies, Danvers, MA). Phosphorylation of GSK-3β was determined using rabbit polyclonal anti-GSK-3β (BD Biosciences, San Jose, CA) and phospho-GSK-3β (Ser9) antibodies (Cell Signaling Technologies, Danvers, MA). Phosphorylation of S6K1 was determined using rabbit polyclonal anti-S6K1 (BD Biosciences, San Jose, CA) and phospho-S6K1 (Thr389) antibodies (Cell Signaling Technologies, Danvers, MA). IRDye® 800 CW Infrared Dye goat anti-mouse and IRDye® 680 CW Infrared Dye goat anti-rabbit secondary antibodies (LI-COR Biosciences, Lincoln, NE) were used for fluorescent quantification.

Cell Treatment

Time-Course

In order to determine the ideal treatment conditions, a time-course experiment was performed. Based on previous research, each plate was exposed to a vehicular control (DMSO) or 12.5 μM of AsIV.). The 12.5 μM concentration of AsIV was chosen based on previous studies that have shown AKT activation in C2C12 myotubes using similar concentrations (Du et al. 2018; Zhu et al. 2016). Cells were treated for zero hours (control), 1, 2, 4, and 6 hours before harvesting the cells. Each time point was run in duplicate. The highest level of phosphorylation was found after two hours (Figure 4), so two hours was chosen as the optimal incubation time.

Treatment

From the result of the time course, two hours was chosen as the optimal incubation time for all treatments in the study. For treatment groups, cells were grown to confluency in PM and switched to DM to induce myotube formation (Day 0). At Day 4, cells were treated with fresh DM with 0.2% DMSO (VC), 12.5 μM AsIV, 100 ng/ml IGF1 alone or in combination with 10 μM Dexa (Dexa alone, Dexa + AsIV or Dexa + IGF1). Groups treated with Dexa were treated 48 hours prior to harvest starting on Day 2 to adequately inhibit Akt phosphorylation and protein synthesis prior to AsIV or IGF1 treatment for two hours (Latres et al. 2005).

Exposure to DMSO was normalized at 0.2% for all treatment groups. Each series of experiments was repeated twice using different passages of C2C12 myotubes (p6 and p7). Groups treated with IGF1 were also incubated for 2 hours to maintain consistency with the AsIV treatment time. The same concentration we used, 100 ng/ml, is capable of phosphorylating and activating mTOR in less than two hours (Miyazaki et al. 2010).

Protein Isolation and Immunoblot Analysis

After two hours of treatment, cells were lysed in a modified RIPA buffer containing 50 mM Tris-HCl, pH 7.4, 150mM NaCL, 1 mM EDTA, 25 mM NaF, 1 mM PMSF, 5 mM $NaVO_4$, and 0.1% SDS. Protease Inhibitor Complex, Phosphatase Inhibitor Complex 2 and Phosphatase Inhibitor Complex 3 (Millipore/Sigma) were also added at 10 μl/ml of buffer. Cell slurries were then centrifuged for 20 minutes at 20,000 *x g* at 4ºC and the supernatants containing protein were removed to a fresh tube and stored at -80ºC until use. Protein concentrations were quantified using Bradford Reagent (Thermo Fisher Scientific, Waltham, MA) according to manufacturer directions using a microplate reader (BioTek Instruments, Winooski, VT).

Westerns

For immunoblot analysis, 20-30 µg of protein from the soluble fraction was denatured for ten minutes at 70° C in SDS loading buffer. The protein was then loaded onto a 4-15% NuPAGE BIS-TRIS protein gel (Invitrogen, Carlsbad, CA) and electrophoresed for 60 minutes at 200 V. After separation, proteins were transferred to an Immobilion-Fl PVDF membrane in a Transblot turbo for 30 minutes at 25V and 1 mA. Membranes were air-dried before continuing the blotting process.

Membranes were re-wet with methanol, rinsed twice in DDH_2O and stained using the ReVERT Total Protein Stain (LI-COR Biosciences, Lincoln, NE) according to manufacture protocol. Following the staining procedure, membranes were imaged using the 700 channel on an Odyssey CLx Infrared Imaging System (LI-COR Biosciences) and total lane protein was quantified to use for normalization of western blots according to manufacturer recommendations.

Following protein staining, membranes were rinsed and incubated in Odyssey Blocking Buffer (OBB)(LI-COR), for 1 hour. Blots were then incubated at 4^oC overnight in primary antibody solution (OBB with 0.01% Tween) with primary antibody. Following primary incubation, membranes were washed 4 x 5 minutes in 1X TBS with 0.01% tween (TBST). After washing, membranes were incubated with secondary antibody at 1:30,000 in OBB with 0.01% tween and 0.01% SDS for 1 hour. Membranes were washed 4 x 5 minutes in TBST, and then rinsed in 1X TBS to remove residual tween before air-drying. All membrane incubations were performed rocking at room temperature unless noted. Membranes were quantified on the Odyssey CLx and

normalized to the corresponding normalization factor, represented as relative signal units using Image Studio Software (LI-COR).

Statistical Analysis

All data are expressed as means ± SD. Statistical significance for treatment groups was determined by using a two-way analysis of variance for multiple comparisons followed by a Tukey's post hoc test. A P value of <0.05 was considered significant.

Results

AsIV optimally increases phosphorylation of Akt (S473), GSK-3β, and S6K1 after two hours of treatment

We first performed time course experiments to determine the optimal length of AsIV treatment for activation of the protein synthetic pathway. We found that Akt, GSK-3β, and S6K1 had the highest levels of phosphorylation after 2 hours of treatment (Figure 4). Compared to controls, there were 1.4, 1.6, and 4.4-fold increases in phosphorylation at the 2-hour time point for Akt (S473), GSK-3β, S6K1, respectively. These results suggest that two hours is the optimal length of time for AsIV treatment for the C2C12 differentiated myotubes we are working with. Therefore, 2 hours of incubation time was used for all subsequent experiments.

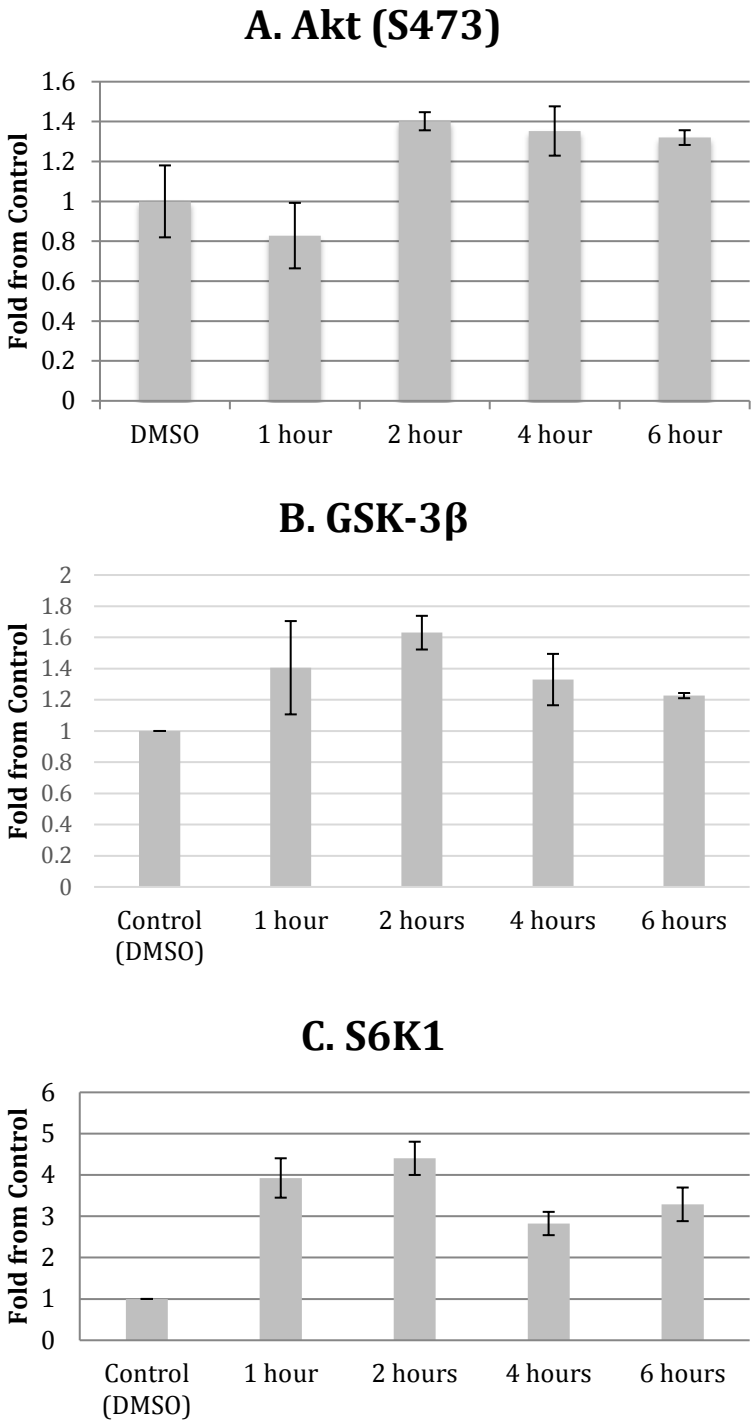


Figure 4: Time course treatment of C2C12 cells with AsIV (n=2; DMSO control: n=2). All three target proteins showed the highest levels of phosphorylation after 2 hours of treatment. A. There was a 1.4 fold increase in Akt (S473) activation at 2 hours. B. There was a 1.6 fold increase in GSK-3β phosphorylation, indicating inhibition of GSK-3β. C. Lastly, there was a 4.4 fold increase in phosphorylation of S6K1.

AsIV does not affect target protein levels in serum-starved conditions

In order to further evaluate the effects of AsIV, cultures were placed in plain DMEM, starved of horse serum for 2 hours, then treated based on the group they belonged to as previously described (Miyazaki 2010). In theory, this should drop the level of baseline phosphorylation and we should therefore see a more pronounced stimulatory effect in our AsIV and IGF1 treatment groups. IGF1 showed significantly higher activation of Akt ($P<0.001$), and S6K1 ($P=0.010$) compared to controls. There was greater phosphorylation of GSK-3β as well, but this was not statistically significant. Treatment with AsIV, however, resulted in a non-significant *decrease* in phosphorylation of all target proteins, compared to controls (Figure 5).

Cultures were then treated with Dexa for 48 hours to induce atrophy and inhibit the protein synthetic pathway. Cells were then incubated with either AsIV or IGF1 in an attempt to recover phosphorylation levels. AsIV was able to very modestly increase phosphorylation levels of both GSK-3β and S6K1, but did not increase Akt phosphorylation, compared to Dexa only cultures. IGF, on the other hand, was able to significantly stimulate phosphorylation of Akt ($P<0.001$) and S6K1 ($P<0.001$) even after Dexa treatment, as well as a non-significant increase in GSK-3β. These cultures recovered their phosphorylation levels and showed Akt pathway activation similar to the results found in cells only treated with IGF1.

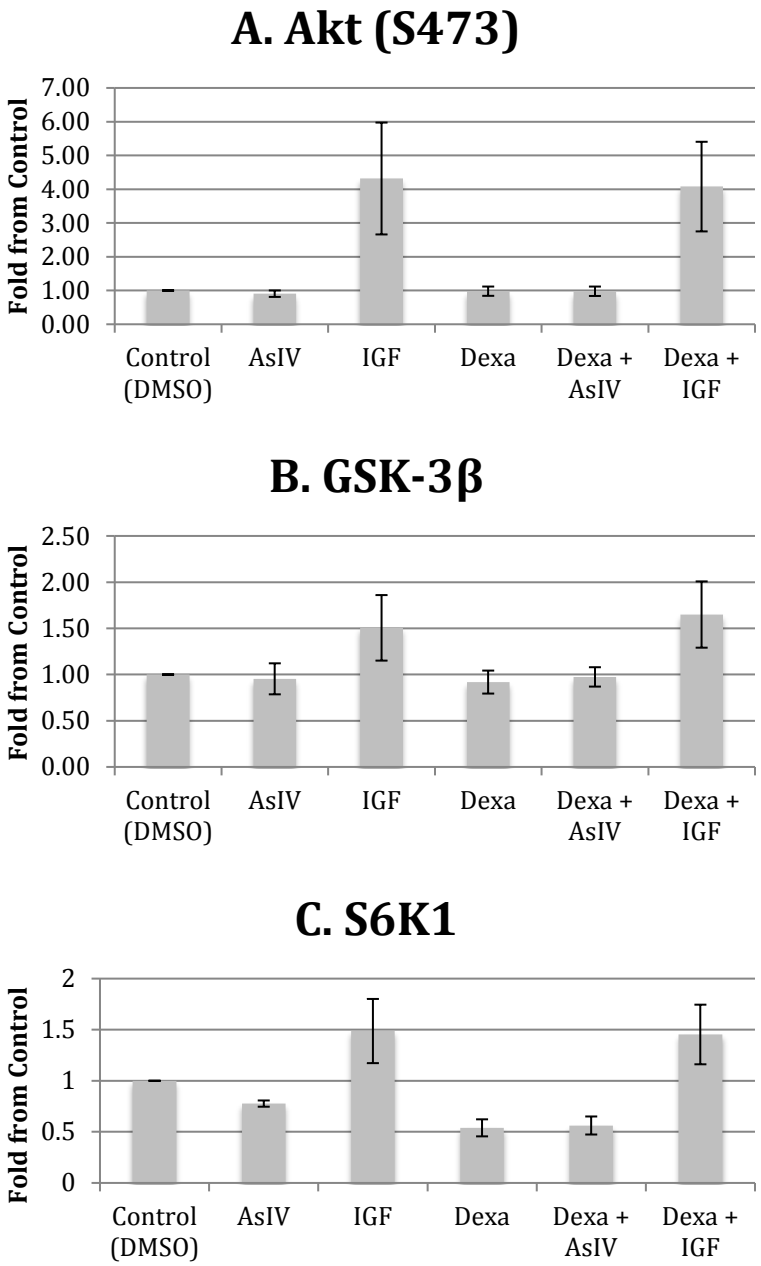


Figure 5: Cells treated in serum-starved conditions (n=3). There was a decrease in phosphorylation of all target proteins when subjected to AsIV, compared to controls. In comparison, IGF was still able to induce phosphorylation in these conditions. However, after cells were treated with Dexa, AsIV was able to modestly increase phosphorylation levels of both GSK-3β (B) and S6K1 (C), but did not noticeably increase Akt (A) phosphorylation. IGF was able to recover activation after Dexa treatment in all targets, to levels similar to cells not initially treated with Dexa.

AsIV may affect target protein phosphorylation levels after initial treatment with dexamethasone

In light of the results we obtained with serum-starved trials, we repeated the experiments using cultures maintained in DMEM and 2% horse serum at all times prior to harvest (Figure 6). As a result, we found very modest increases in Akt and GSK-3β phosphorylation compared to controls, which were not statistically significant. However, this effect was not seen in S6K1, which had no change in phosphorylation levels when compared to controls. IGF1 was again able to consistently increase phosphorylation, especially in Akt (P=0.039) and S6K1 (P=0.038), with only modest increases in GSK-3β.

Cells were then incubated with Dexa for 48 hours prior to AsIV/IGF treatment. There was no difference in Akt phosphorylation after AsIV treatment, but considerable increases after IGF that approached significance (P=0.067). Phosphorylation of GSK-3β was also greatly increased in the Dexa + AsIV group (P=0.021) compared to cells only treated with Dexa. This was the only group in which AsIV was able to stimulate phosphorylation in a target protein to levels nearly as high as IGF. Lastly, phosphorylation of S6K1 was mildly increased in the Dexa + AsIV group, but significantly increased in the Dexa + IGF group (P<0.001). In no AsIV treatment group was phosphorylation levels higher than those seen with IGF.

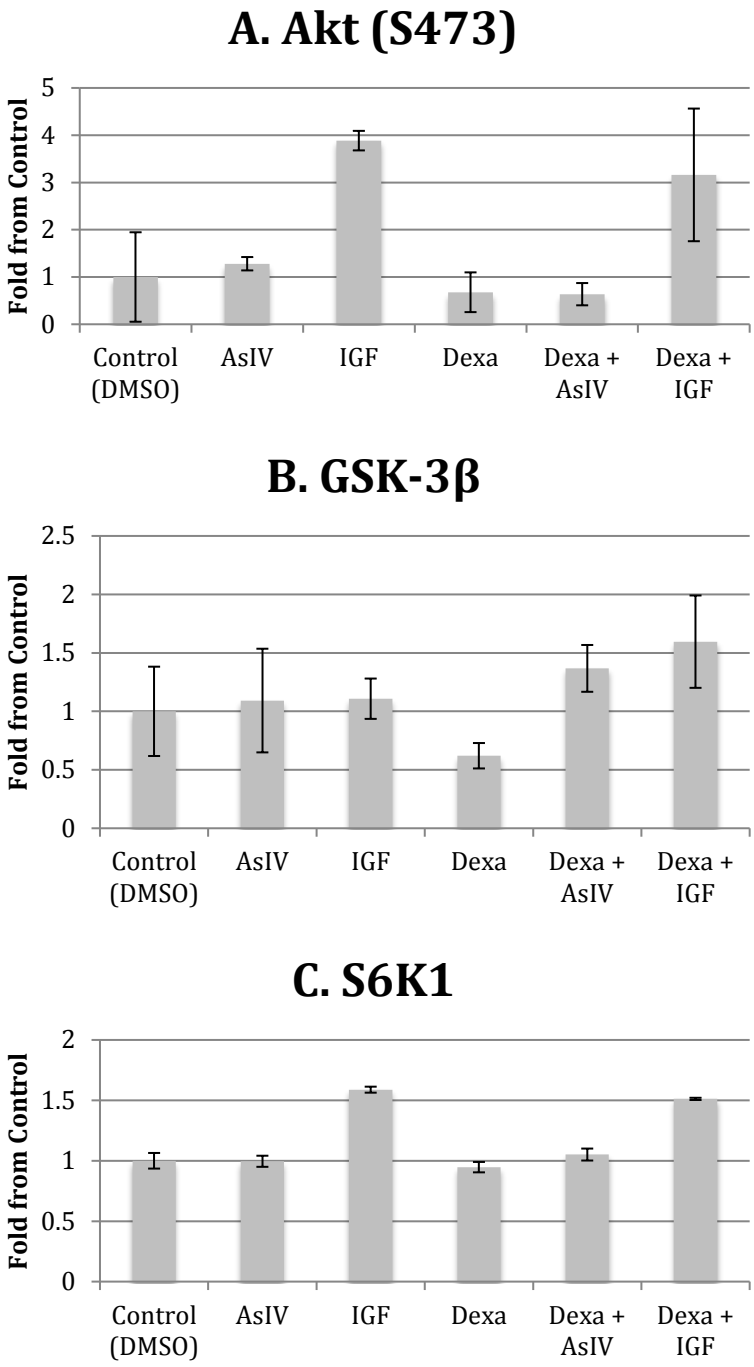


Figure 6. Cells treated in the presence of Horse Serum (n=3). Cells were maintained in horse serum during the entirety of treatment. This resulted in greater increases in both Akt and GSK-3β after AsIV treatment compared to controls. However, it appeared that S6K1 did not increase at the same rate. In addition, the downstream targets of GSK-3β and S6K1 were able to recover phosphorylation levels after treatment with Dexa, especially with GSK-3β.

Discussion

Skeletal muscle atrophy can occur as a result of aging, lack of physical activity, or as a debilitating component of many diseases. One potential strategy for combating muscle loss is by stimulating the cellular pathways responsible for protein synthesis. It has been shown that the Akt/mTOR signaling pathway is the mechanism controlling the balance between protein synthesis and degradation. Therefore, it is important to find substances with the potential to activate this signaling pathway to promote protein synthesis. One such compound is Astragaloside IV, a saponin phytochemical commonly found in *Astragalus membranaceus,* used in traditional Chinese medicine for glucose homeostasis. Because this saponin is capable of activating the Akt pathway to stimulate glucose metabolism and transport, our hypothesis is that it can also stimulate downstream activation of the protein synthetic pathway as well.

In the current study, we first looked to determine what length of AsIV treatment time would lead to the highest increases in Akt pathway phosphorylation, if any. We found the highest levels of phosphorylation after two hours of treatment for Akt, GSK-3β, and S6K1. These results suggest that two hours of treatment is optimal for Akt pathway activation in C2C12 muscle cells, so cultures were treated with AsIV for two hours in all subsequent experiments.

After two hours of treatment, phosphorylation generally declined in all target proteins. It is possible that is a result of the negative feedback loop between S6K1 and IRS. At sufficient quantities, activated S6K1 can act as an inhibitor of IRS by phosphorylating it at S270 (Zhang et al. 2008). This impairs the ability of the IRS to

stimulate glucose uptake and initiate protein synthesis. This could explain the decline we saw after two hours.

We then evaluated the effect of AsIV on myotubes subjected to serum-starved conditions two hours prior to treatment. There should theoretically be a decrease in basal protein synthesis activity in the absence of serum, which would lower the relative levels of Akt pathway activation. Subsequent treatment with AsIV or IGF would then provide an even more pronounced effect. Following serum starvation, we saw an increase in phosphorylation levels after IGF1 treatment alone and IGF1 treatment after Dexa pre-treatment. However, this was not the case for AsIV. We actually found decreases in phosphorylation along the Akt pathway compared to controls. When cells were treated with Dexa for 48 hours prior to AsIV treatment, there was a mild increase in phosphorylation. This effect was most pronounced with GSK-3β.

IGF was able to stimulate the Akt pathway to similar levels whether cultures were subjected to Dexa first or not. It was interesting to see the large effect that IGF was capable of producing in serum-starved conditions, compared to the decreases seen in AsIV cultures. Our hypothesis is that IGF is such a potent activator of the Akt pathway that the composition of the media does not make a considerable difference. It appears that IGF is still capable of activation in the absence of the growth factors provided by horse serum. Horse serum provides cells with growth factors, proteins, and trace elements for optimal growth and differentiation into myotubes. Although the absence of serum may drop baseline levels of Akt activation, it may also promote cell death due to the lack of essential elements for growth (Leicht et al. 2003; Simm et al. 1997). It is possible that

AsIV requires the presence of these growth factors and trace elements to enhance Akt pathway activation rather than activate from baseline levels.

We then repeated our experiments with horse serum continuously present until the cells were ready to harvest. Although we found modest increases in phosphorylation for Akt and GSK-3β after AsIV treatment, we found no increase in S6K1. This results is contradictory to the results seen in the time course experiments that showed over 4 times greater phosphorylation after 2 hours of AsIV treatment (See Figure 4). The one change in procedure between the two experiments was that the time course was performed in 100 mm culture plates, whereas the experimental groups thereafter were cultured in 60 mm plates. However, this should not cause such a change by itself. Similar to the serum-starved cultures, GSK-3β and S6K1 phosphorylation rose with AsIV treatment after initial incubation with Dexa for 48 hours. This effect was again most pronounced with GSK-3β, where Dexa + AsIV phosphorylation was nearly has high as IGF + Dexa.

It was interesting to see the profound increases in GSK-3β for the Dexa + AsIV group, the only trial that was comparable to IGF levels (See Figure 6). This may be related to role that GSK-3β also plays in cell survival and apoptosis. Although originally named for its role in glycogen metabolism, GSK-3β has since been shown to play roles in protein synthesis, cell proliferation, and cell division (Frame and Cohen 2001). Paradoxically, GSK-3β appears to play a role in cell death *and* cell survival. GSK-3β inhibition via PI3K can prevent cell death (Pap and Cooper 1998) and mice with a homozygous deletion of GSK-3β died before birth due to apoptotic liver degeneration (Hoeflich et al. 2000).

As discussed previously, AsIV can provide cardioprotective effects through Akt/GSK-3β phosphorylation. Through inhibition of GSK-3β, AsIV is capable of reducing cell death after ischemia/reperfusion injury to cardiac muscle cells *in vitro* (He et al. 2012) and minimizing cell damage *in vivo* (He et al. 2014). It is possible that Dexa, in addition to inhibition of protein synthesis, also led to some cell death. This may have been enough to stimulate AsIV to exert cell survival effects in muscle cells via GSK-3β inhibition.

It is entirely possible that AsIV can activate the Akt/GSK-3β pathway without subsequent protein synthesis activation. A recent study looked to determine if AsIV could protect retinal ganglion cells from oxidative injury (Hao et al. 2018). They found that AsIV reduced the level of reactive oxygen species (ROS) and mitigated ROS-induced changes to the mitochondria. This recent evidence is important for our study because ROS are extremely important for IGF1-induced hypertrophy in C2C12 muscle cells *in vitro* (Handayaningsih et al. 2011). IGF1 induced ROS production in C2C12 myocytes, and treatment with additional H_2O_2 enhanced IGF1-induced phosphorylation of the IGFR. This increase in phosphorylation was inhibited by the addition of antioxidants. Future studies should assess for ROS levels in AsIV-treated muscle cells, as these antioxidant properties may be suppressing protein synthesis and growth.

Future studies should continue to determine how AsIV affects skeletal muscles cells, especially in the context of protein synthesis. We hope to evaluate the downstream effects of Akt activation by measuring myotubes sizes before and after treatment, as well as using the SUnSET method to quantify the rate of protein synthesis. SUnSET (SUrface SEnsing of Translation) utilizes puromycin-labeled peptides and anti-puromycin antibodies to quantify the rate of protein synthesis between cell cultures via

immunodetection (Schmidt et al., 2009). Given the role that GSK-3β plays in cell apoptosis, it would also be interesting to track cell survival as a result of AsIV treatment.

Protein homeostasis is a constant balance between protein synthesis and degradation. Both are present, even in the presence of muscle growth. There is increases in both synthesis and degradation, but a comparatively greater increase in synthesis when there is growth. Conversly, in the presence of atrophy there will be increases in both again, but a greater rate of degradation than synthesis. In addition to markers of protein synthesis, further studies should therefore evaluate the effect of AsIV on markers of protein degradation.

IGF-1 has been shown to be sufficiently capable of stimulating protein synthesis on its own (DeVol et al. 1990), and the results of our study appear to confirm this. Although there is promise for IGF-1 to treat muscle wasting itself, IGF-1 receptors are ubiquitously expressed all over the body so any form of systemic treatment may affect other tissues as well (Gross 2003). It would interesting to see if AsIV can augment the effects of IGF-1, if muscle cells were treated with both concurrently.

Reference

Bhavsar SK, Föller, M, Gu S. Vir S. Shah MB, Bhutani KK, Santani DD, Lang F. 2009a. Involvement of the PI3K/AKT pathway in the hypoglycemic effects of saponins from Helicteres isora. J Ethnopharmacol. 126(3): 386-396. doi: 10.1016/j.jep.2009.09.027

Bhavsar SK, Singh S, Giri S, Jain MR, Santini DD. 2009b. Effect of saponins from Helicteres isora on lipid and glucose metabolism regulating genes expression. J Ethnopharmacol. (3):426

Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, et al. 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev. 15:2203-2208

Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ. 2001a. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem. 276:38349-38352

Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, Kaestner KH, Bartolomei MS, Shulman GI, Birnbaum MJ. 2001b. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science. 292:1728-1731

Coleman ME, DeMayo F, Yin KC, Lee HM, Geske R, Montgomery C, Schwartz RJ. 1995. Myogenic vector expression of insulin-like growth factor 1 stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice. J Biol Chem. 270:12109-12116

DeVol, DL, Rotwein P, Sadow JL, Novakofski J, Bechtel PJ. 1990. Activation of insulin-like growth factor gene expression during work-induced skeletal muscle growth. Am. J. Physiol. 259, E89–E95

Du Q, Zhang S, Li A, Mohammad IS, Liu B, Li Y. 2018. Astragaloside IV Inhibits Adipose Lipolysis and Reduces Hepatic Glucose Production via Akt Dependent PDE3B Expression in HFD-Fed Mice. Front Physiol. 9:15. doi:10.3389/fphys.2018.00015

Evans WJ, Morley JE, Argiles J, Bales C, Baracos V, Guttridge D, et al. 2008. Cachexia: a new definition. Clin Nutr. 27:793–799

Farkas J, von Haehling S, Kalantar-Zadeh K, Morley JE, Anker SD, Lainscak M. 2013. Cachexia as a major public health problem: frequent, costly, and deadly. J Cachexia Sarcopenia Muscle. doi:10.1007/s13539-013-0105-y

Fernandez AM, Dupont J, Farrar RP, Lee S, Stannard B, Le Roith D. 2002. Muscle specific inactivation of the IGF-I receptor induces compensatory hyperplasia in skeletal muscle. J Clin Invest. 109:347-355.

Frame S and Cohen P. 2001. GSK-3β takes centre stage more than 20 years after its discovery. Biochem J. 359(1):1-16

Francis G, Kerem Z, Makkar HPS, Becker K. 2002. The biological action of saponins in animal systems: a review. Br J Nutr. (6):587

Friedrichsen M, Birk JB, Richter EA, et al. 2012. Akt2 influences glycogen synthase activity in human skeletal muscle through regulation of NH_2-terminal (sites 2 + 2a) phosphorylation. Am J Physiol Endocrinol Metab. 304(6):E631-639

Glass DJ. 2003. Signalling pathways that mediate skeletal muscle hypertrophy and atrophy. Nat Cell Biol. 5(2):87-90. doi: 10.1038/ncb0203-87

Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N. 2001. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. Genes Dev. 15:1406-1418

Güçlü-Ustündağ O and Mazza G. 2007. Saponins: properties, applications and processing. Crit Rev Food Sci Nutr. 47:231–258

Handayaningsih AE, Iguchi G, Fukuoka H, Nishizawa H, Takahashi M, Yamamoto M, Herningtyas EH, Okimura Y, Kaji H, Chihara K, Seino S, Takahashi Y. 2011. Reactive Oxygen Species Play an Essential Role in IGF-I Signaling and IGF-I-Induced Myocyte Hypertrophy in C2C12 Myocytes. Endocrin. 152(3)912–921

Hao M, Liu Y, Chen P, Jiang H, Kuang HY. 2018. Astragaloside IV protects RGC-5 cells against oxidative stress. Neural Regen Res. 13(6):1081–1086. doi:10.4103/1673-5374.233452

He Y, Xi J, Zheng H, Zhang Y, Jin Y, Xu Z. 2012. Astragaloside IV inhibits oxidative stress-induced mitochondrial permeability transition pore opening by inactivating GSK-3β via nitric oxide in H9c2 cardiac cells. Oxid Med Cell Longev. 2012:935738. doi:10.1155/2012/935738

He Y, Li W, Yi H, Zheng H, Xi J. 2014. Mitochondrial mechanism of Astragaloside IV induced inhibition of GSK-3β in myocardial ischemia/reperfusion injury in rats. Chin Pharmacol Bulletin. 30(3):402-406

Heron-Milhavet L, Mamaeva D, LeRoith D, Lamb NJ, Fernandez A. 2010. Impaired muscle regeneration and myoblast differentiation in mice with a muscle-specific

KO of IGF-IR. J Cell Physiol. 225:1-6

Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. 2000. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature. 406:86–90. doi:10.1038/35017574

Lai KM, Gonzalez M, Poueymirou WT, Kline WO, Na E, Zlotchenko E, Stitt TN, Economides AN, Yancopoulos GD, Glass DJ. 2004. Conditional activation of akt in adult skeletal muscle induces rapid hypertrophy. Mol Cell Biol. 24:9295-9304

Latres E, Amini A, Amini A, Griffiths J, Martin F, Wei Y, Lin H, Yancopoulos G, Glass D. 2005. Insulin-like Growth Factor-1 (IGF-1) Inversely Regulates Atrophy-induced Genes via the Phosphatidylinositol 3 Kinase/Akt/Mammalian Target of Rapamycin (PI3K/Akt/mTOR) Pathway J. Biol. Chem. 280:2737-2744. doi:10.1074/jbc.M407517200

Lecker S, Goldberg A, Mitch W. 2006. Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. J Am Soc Nephrol. 17:1807–1819

Leicht M, Marx G, Karbach D, Gekle M, Köhler T, Zimmer HG. 2003. Mechanism of cell death of rat cardiac fibroblasts induced by serum depletion. Mol Cell Biochem. 251:119–126. doi:10.1023/A:1025446302759

Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell. 75:59-72

Matsuda H, Li YH, Murakami T, Yamahara J, Yoshikawa M. 1999. Structure-related inhibitory activity of oleanolic acid glycosides on gastric emptying in mice. Bioorg Med Chem. 7, 323–327

Miyazaki M, McCarthy JJ, Esser KA. 2010. Insulin like growth factor-1-induced phosphorylation and altered distribution of tuberous sclerosis complex (TSC)1/TSC2 in C2C12 myotubes. FEBS J. 277(9):2180–2191. doi:10.1111/j.1742-4658.2010.07635.x

Morrison WL, Gibson JN, Scrimgeour C, Rennie MJ.1988. Muscle wasting in emphysema. Clin Sci (Lond). 75(4):415-20.

Moses T, Papadopoulou KK, Osbourn A. 2014. Metabolic and functional diversity of saponins, biosynthetic intermediates and semi-synthetic derivatives. Crit Rev Biochem Mol Biol. 49(6):439–462. doi:10.3109/10409238.2014.953628

Navale AM and Paranjape AN. 2016. Glucose transporters: physiological and pathological roles. Biophys Rev. 8(1):5-9

Pap M and Cooper GM. 1998. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J Biol Chem. 273:19929-19932. doi:10.1074/jbc.273.32.19929

Peng XD, Xu PZ, Chen ML, Hahn-Windgassen A, Skeen J, Jacobs J, Sundararajan D, Chen WS, Crawford SE, Coleman KG, Hay N. 2003. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev. 17:1352-1365

Podolak I, Galanty A, Sobolewska D. 2010. Saponins as cytotoxic agents: a review. Phytochem Rev. 9(3):425–474. doi:10.1007/s11101-010-9183-z

Ren S, Zhang H, Mu Y, Sun M, Liu P. 2013. Pharmacological effects of astragaloside IV: a literature review. J Tradit Chin Med. 33(3): 413-416

Sandri M. 2011. New findings of lysosomal proteolysis in skeletal muscle. Curr Opin Clin Nutr and Metab Care. 14:223–229.

Sartorelli V and Fulco M. 2004. Molecular and cellular determinants of skeletal muscle atrophy and hypertrophy. Sci STKE. 244:pp. re11. doi:10.1126/stke.2442004re11

Schiaffino S and Mammucari C. 2011. Regulation of skeletal muscle growth by the IGF1 Akt/PKB pathway: insights from genetic models. Skelet Muscle. 1(1):4. doi: 10.1186/2044-5040-1-4

Schmidt EK, Clavarino G, Ceppi M, Pierre P. 2009. SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods. 6(4):275-277

Simm A, Bertsch G, Frank H, Zimmermann U, Hoppe J. 1997. Cell death of AKR-2B fibroblasts after serum removal: a process between apoptosis and necrosis. J Cell Sci. 110:819–828

Sitnick M, Bodine SC, Rutledge JC. 2009. Chronic high fat feeding attenuates load-induced hypertrophy in mice. J Physiol. 587(Pt 23):5753–5765. doi:10.1113/jphysiol.2009.180174

Sitnick M, Foley AM, Brown M, Spangenburg EE. 2006. Ovariectomy prevents the recovery of atrophied gastrocnemius skeletal muscle mass. J Appl Physiol. 100:286-293. doi:10.1152/japplphysiol.00869.2005

Swiderska E, Strycharz J, Wroblewski A, Szemrai J, Drzewoski J, Sliwinska A. 2018. Role of PI3K/AKT Pathway in Insulin-Mediated Glucose Uptake [Online First], IntechOpen, doi:10.5772/intechopen.80402. Available from: https://www.intechopen.com/online-first/role-of-pi3k-akt-pathway-in-insulin-mediated-glucose-uptake

von Haehling S, Anker SD. 2010. Cachexia as a major underestimated and unmet medical need: facts and numbers. J Cachexia Sarcopenia Muscle. 1(1):1–5. doi:10.1007/s13539-010-0002-6

Wang Z, Gu M, Li G. 2005. Surface Properties of Gleditsia Saponin and Synergisms of Its Binary System. J Disper Sci Technol. 26(3):341-347. doi:10.1081/DIS-200049604

Ward PS and Thompson CB. 2012. Signaling in control of cell growth and metabolism. Cold Spring Harb Perspect Biol. 4(7):a006783. doi:10.1101/cshperspect.a006783

Zhang L, Liu Q, Lu L, Zhao X, Gao X, Wang Y. 2011. Astragaloside IV stimulates angiogenesis and increases hypoxia-inducible factor-1α accumulation via phosphatidylinositol 3-kinase/Akt pathway. Pharmacol Exp Ther. 338(2):485-91. doi: 10.1124/jpet.111.180992

Zhang J, Gao Z, Yin J, Quon MJ, Ye J. 2008. S6K directly phosphorylates IRS-1 on Ser-270 to promote insulin resistance in response to TNF-(alpha) signaling through IKK2. J Biol Chem. 283(51):35375–35382. doi:10.1074/jbc.M806480200

Zhu R, Zheng J, Chen L, Gu B, Huang S. 2016. Astragaloside IV facilitates glucose transport in C2C12 myotubes through the IRS1/AKT pathway and suppresses the palmitate-induced activation of the IKK/IκBα pathway. Int J Mol Med. 37(6):1697-1705. doi:10.3892/ijmm.2016.2555