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# Role of Akt isoforms in IGF-I-mediated signaling and survival in myoblasts ☆

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ABSTRACT

Oxidative stress has been shown to induce apoptosis in a variety of tissues, while insulin-like growth factor-I (IGF-I) can oppose this effect. We found that $H_2O_2$ promoted cell death and apoptosis in C2C12 myoblasts, an effect that was completely prevented by exogenous IGF-I. One downstream mediator of IGF-I survival signaling is the serine/threonine kinase Akt, of which three isoforms have been identified in mammals. We found that Akt1 and Akt3 act on pro-apoptotic target molecules in an isoform-specific manner. Both Akt1 and Akt3 were responsible for phosphorylating FoxO3a at S253 and FoxO1 at T24, while Akt1 alone phosphorylated Bad at S136 and FoxO3a at T32. Our results provide evidence for IGF-I-stimulated isoform-specific actions of Akt on molecules involved in promoting apoptosis.


## Introduction

Cells under resting conditions possess endogenous antioxidant defenses that promote survival of the cell. Excessive reactive oxygen species such as those generated by hydrogen peroxide ($H_2O_2$) can cause damage to membrane lipids, essential proteins, and DNA, ultimately leading to cell death by necrosis or apoptosis [1,2]. Insulin-like growth-factor-I (IGF-I) is a peptide hormone that antagonizes apoptosis in multiple cell types [3–5]. Previous work has established that IGF-I exerts anti-apoptotic actions in part through a wortmannin- or LY294002-sensitive pathway in oxidatively stressed PC12, cardiac, and intestinal epithelial cells [4,8,9]; however, the exact mechanism whereby IGF-I promotes survival in response to $H_2O_2$ in C2C12 myoblasts has not been determined.

IGF-I signal transduction occurs through its cognate receptor and is mediated by several molecules, including phosphatidylinositol 3-kinase (PI3K) [6]. Interaction of class IA PI3K with the IGF-I receptor tyrosine kinases via adaptor molecules such as IRS, leads to activation of downstream effector molecules [7]. Generation of secondary lipid messengers by PI3K promotes activation of Akt (PKB), a serine/threonine kinase that plays a vital role in controlling cell survival and apoptosis by phosphorylating and inhibiting pro-apoptotic molecules such as Bad and FoxO3a [10–12]. Three Akt isoforms have been identified, and murine gene disruption models demonstrate that each isoform possesses a distinct function [13–16]. Although recent work suggests that redundant activity can exist [17], the potential differential contribution of each isoform in response to oxidative stress has not yet been elucidated in skeletal muscle. Here, we show that $H_2O_2$-induced apoptosis was prevented by IGF-I in myoblasts; furthermore, we show that pro-apoptotic Bad and FoxO3a are phosphorylated by Akt in an isoform-specific manner.

## Materials and methods

*Materials.* C2C12 murine myoblasts, initially obtained from ATCC (Manassas, VA), were a kind gift from Dr. John C. Lee (University of Texas Health Science Center at San Antonio). $H_2O_2$ and bovine serum albumin (fraction V) were purchased from Sigma (St. Loius, MO). rhIGF-I was purchased from Austral Biologicals (San Ramon, CA). All antibodies were obtained from Cell Signaling Technologies (Beverly, MA), and secondary anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz (Santa Cruz, CA).

*Cell culture conditions and siRNA transfections.* C2C12 myoblasts were maintained in high-glucose DMEM containing 10% FBS and antibiotics (growth medium), with medium being replenished after 24 h. Forty-eight hours after initial seeding cells were ~95% confluent and unless otherwise indicated, experiments were conducted under these conditions (in growth medium). Pre-designed Silencer Select siRNAs for mouse Akt1 (ID# s62215), Akt2 (ID# s62219), Akt3 (ID# s76463), Bad (ID# s222973), and negative controls (non-targeting siRNA ID# 4390843) were purchased from Ambion Inc. (Austin, TX). siRNA against FoxO3a was purchased from Dharmacon (Lafayette, CO) as "ON-TARGET plus SmartPool" (cat #L-040728-00). Cells ($1.8 \times 10^5$/well in 6-well culture dish) were reverse-transfected with double-stranded siRNA in antibiotic-free DMEM plus 10% FBS using Lipofectamine 2000 according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Twenty-

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four hours after reverse transfection, medium was changed to DMEM with 10% FBS containing antibiotics. Protein was isolated 48 h after transfection and the level of silencing determined by Western Blot as described later in this section.

*Plasmids.* Plasmids for wild-type HA-FoxO3a (plasmid # 1787) [11] and Bad (plasmid # 8778) [18] were obtained from Addgene (Cambridge, MA). Cells were reverse-transfected at 80% confluency using Lipofectamine 2000 and grown for 24 h until harvest.

*Cell counts.* Medium was removed from plates and monolayers detached with 0.05% trypsin–EDTA (Gibco). Cells were suspended in growth medium and counted in a hemacytometer chamber. For quantitation of cell nuclei and apoptosis, cells were analyzed by fluorescence microscopy after intravital staining with DAPI. A minimum of 800 nuclei were counted for each treatment, in a minimum of 10 fields.

*Protein extraction, immunoprecipitation, and Western immunoblot.* C2C12 cells were washed twice in ice-cold PBS and harvested in ice-cold lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM $Na_3VO_4$, 1 μg/ml Leupeptin, 1 mM PMSF, and 1:100 dilution of phosphatase inhibitor cocktails 1 and 2 (Sigma)). Homogenates were triturated through a small-bore needle, incubated on ice for 30 min, and then centrifuged at 14,000*g* for 10 min at 4 °C. Protein concentrations were determined by the method of Bradford [19]. For Western blotting, equal amounts of cell lysate proteins (typically 25 μg) were electrophoresed through denaturing SDS–PAGE. Proteins were transferred to PVDF membranes (Millipore Corp., Bedford MA). Membranes were incubated for 1 h in 5% dry milk solution in Tris-buffered saline plus 0.5% Tween-20 (TBST) and then incubated with the appropriate primary antibody overnight in 5% BSA in TBST. Membranes were washed three times in TBST followed by incubation with the appropriate secondary antibody and again washed three times. Membranes were incubated with enhanced chemiluminescence reagents (Thermo Fisher, Rockford, IL) and exposed to film.

*Statistics.* Data are presented as means ± SEM. Statistics were performed using one-way ANOVA and Dunnett's test *a posteriori*. A $P$-value < 0.05 was considered significant.

## Results

### *$H_2O_2$ increases, and IGF-I inhibits cell death in a dose-dependent manner*

To establish the susceptibility of C2C12 myoblasts to oxidative stress, ~95% confluent cells were treated with increasing concentrations of $H_2O_2$ for 24 h. A decrease in total cell number was ob-

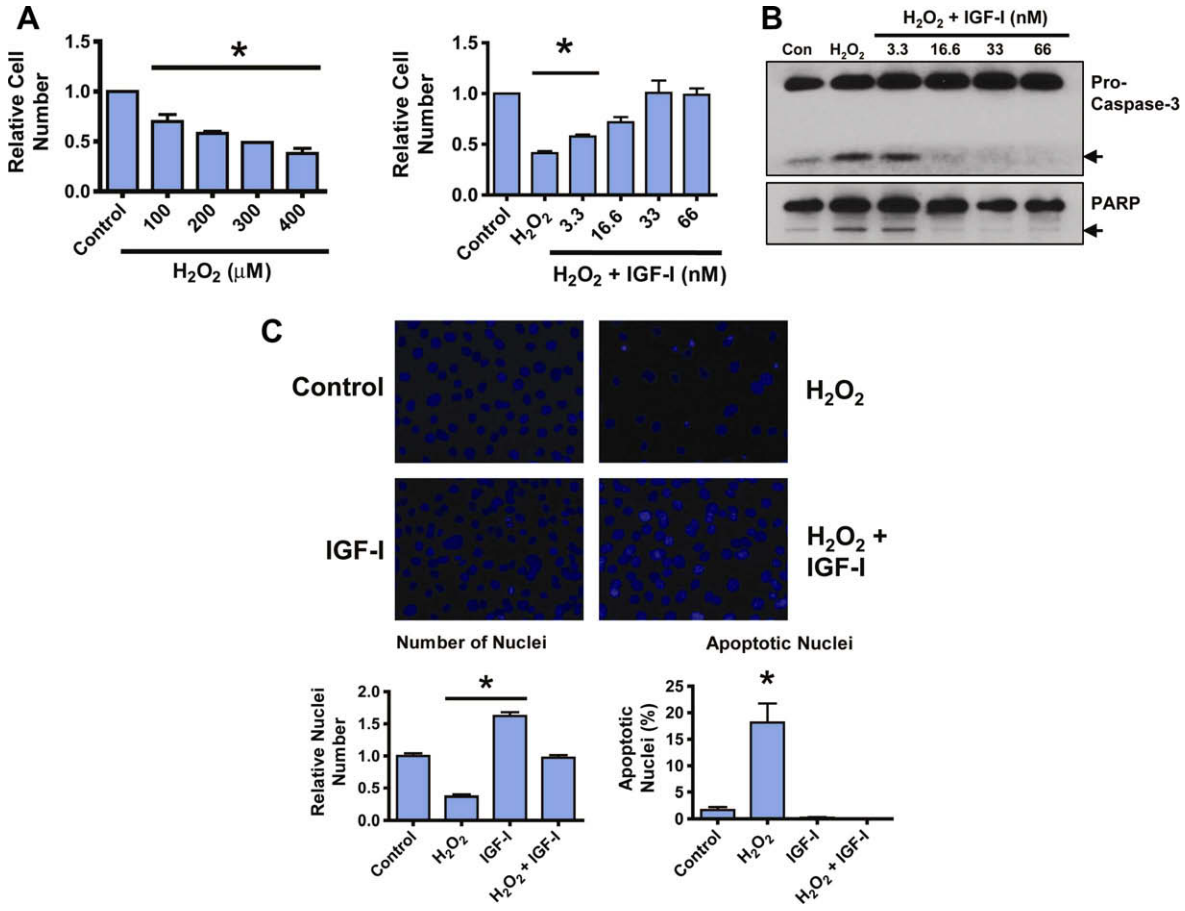


**Fig. 1.** $H_2O_2$ and IGF-I effects on cell death and survival. (A, left) 95% confluent C2C12 myoblasts were treated with increasing concentrations of $H_2O_2$ for 24 h, trypsinized, and adherent cells counted. Columns represent averages of three independent experiments and error bars indicate SEM. Asterisks indicate significant difference ($P < 0.05$) from control cell number, which was normalized to 1.0. (A, right) Cells were treated with the indicated concentrations of IGF-I 30 min prior to addition of 400 μM $H_2O_2$ for 24 h and then counted. Columns represent averages of two independent experiments and error bars indicate SEM. Horizontal bars span multiple treatments that statistically differed from control values, which were normalized to 1.0. Asterisks indicate significant difference ($P < 0.05$). (B) Cells were treated with the indicated concentrations of IGF-I 30 min prior to addition of 400 μM $H_2O_2$ for 4 h and then harvested for protein lysates. Cleavage of Caspase-3 and PARP (arrows) are indicated to the right of the blots. (C) Cells were treated with $H_2O_2$ and IGF-I as described in (A, right), followed by staining with DAPI as described in Materials and methods. Total nuclei and apoptotic nuclei (showing as condensed and brightly stained) were determined by counting. The percentage of apoptotic nuclei was obtained by expressing the number of apoptotic nuclei to the number of intact nuclei. Columns represent averages of two independent experiments and error bars indicate SEM. Asterisks indicate significant difference ($P < 0.05$) from control cell number, which was normalized to 1.0. A horizontal bar spans multiple treatments that statistically differed from IGF-I-treated control cells.

served with increasing concentrations of $H_2O_2$ (Fig. 1A, left). We next sought to determine the concentration of IGF-I that would oppose cell death induced by the highest $H_2O_2$ concentration tested (400 μM). Pre-treatment with IGF-I 30 min prior to addition of $H_2O_2$ was unable to prevent cell death at low concentration (3.3 nM), but at concentrations of 16.6 nM and above, IGF-I was able to prevent the loss in total cell number caused by 400 μM $H_2O_2$ (Fig. 1A, right). Because $H_2O_2$ has been shown previously to cause cell death at least partially through an apoptotic mechanism [20], we tested whether the reduction in cell number caused by $H_2O_2$ correlated with an elevation in the levels of biochemical markers of apoptosis and whether IGF-I opposed these effects. IGF-I prevented $H_2O_2$-induced cleavage of Caspase-3 and PARP in a dose-dependent fashion similar to the effect of IGF-I on cell numbers (Fig. 1B). Furthermore, we found that addition of 400 μM $H_2O_2$ for 24 h decreased C2C12 nuclei number by ~63%, and that $H_2O_2$ increased the number of apoptotic nuclei by ~18% ($P < 0.001$ for nuclei number and apoptotic nuclei, Fig. 1C). Pre-incubation with 16.6 nM IGF-I for 30-min eliminated apoptosis induced by $H_2O_2$ ($P < 0.001$). Taken together, these results suggest that 400 μM $H_2O_2$ causes cell death in ~95% confluent C2C12 myoblasts, and that pre-treatment with 16.6 nM IGF-I is associated with maintenance of cell number after 24 h and reduced indicators of apoptosis.

*FoxO3a and Bad promote apoptosis in myoblasts, and Akt-dependent phosphorylation of FoxO3a and Bad are mediated in an isoform-specific manner*

Both FoxO3a and Bad are known positive regulators of apoptosis [11,12]. To determine whether FoxO3a or Bad promote apoptosis in C2C12 myoblasts, cells were transfected with plasmids encoding WT FoxO3a and WT Bad. Overexpression of FoxO3a or Bad promoted cleavage of Caspase-3 and PARP (Fig. 2A), as well as caused widespread cell detachment when examined under microscopy (data not shown). Conversely, knockdown of FoxO3a and Bad reduced $H_2O_2$-induced Caspase-3 and PARP cleavage 48 h after transfection (Fig. 2B). Together, these data suggest that FoxO3a and Bad positively regulate apoptosis in myoblasts.

Akt lies downstream of IGF-IR and participates in cell survival by reducing activities of a number of pro-apoptotic molecules; additionally, recent evidence suggests that individual Akt isoforms can exhibit both overlapping and distinct functions [21]. To identify those Akt isoforms involved in phosphorylating pro-apoptotic target molecules, we employed siRNA technology (Fig. 3A). We found that Akt1, but not Akt3, was responsible for phosphorylating Bad at serine 136. Likewise, Akt1 was responsible for phosphorylating FoxO3a at threonine 32; however, both Akt1 and Akt3 contributed to phosphorylation of FoxO3a at serine 253. Additionally, threonine phosphorylation of FoxO1 at residue 24 was also co-regulated by Akt1 and Akt3 (Fig. 3B). A diagram of these actions is presented as Fig. 3C. These observations suggest that in myoblasts, Akt1 and Akt3 mediate IGF-I survival effects and perform both distinct and redundant functions on pro-apoptotic target molecules.

## Discussion

In this study, we found that IGF-I reduced $H_2O_2$-induced apoptosis, and that cells treated with IGF-I and $H_2O_2$ were similar in number to those of control cells after 24 h (Fig. 1). Hydrogen peroxide, a strong oxidant, can induce apoptosis, at least in part, through a mechanism involving the intrinsic mitochondrial death pathway [20,22].

One principle effector of IGF-I is Akt, which acts to suppress apoptosis in part by phosphorylating and inactivating pro-apoptotic molecules such as Bad [12] and FoxO transcription factors [11,23]. Because Akt exists as multiple isoforms and these isoforms possess both distinct and overlapping functions [14–17,21,24,25], we examined the responsiveness of Akt isoforms to IGF-I and tested the hypothesis that Akt acted on target pro-apoptotic molecules in an isoform-specific manner.

We observed that inhibition of Akt1 or Akt3 caused reductions in phosphorylation of FoxO3a at S253 and FoxO1 at T24, but that

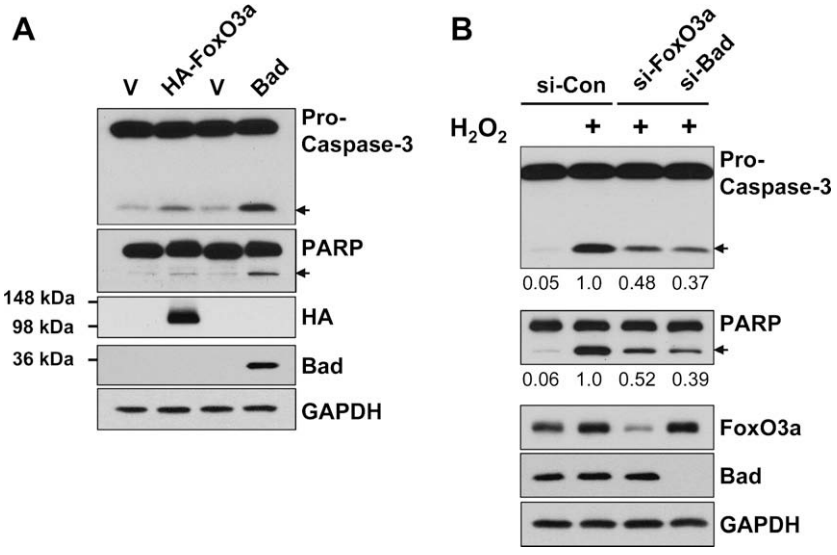


**Fig. 2.** Bad and FoxO3a are positive regulators of apoptosis in C2C12 myoblasts. (A) Myoblasts were transfected with pcDNA3 vector (V), WT pcDNA3-HA-FoxO3a, or WT pcDNA3-Bad for 24 h before harvest. Proteins detected by Western blot are indicated to the right of the images, and arrowheads indicate cleaved Caspase-3 and PARP. (B) Knockdown of FoxO3a or Bad reduces $H_2O_2$-induced apoptosis. Cells were reverse-transfected with 5 nM negative control siRNA (si-Con), 25 nM siRNA against FoxO3a (si-FoxO3a), or 5 nM siRNA against Bad (si-Bad). Cells were maintained for 48 h before addition of 400 μM $H_2O_2$ for 4 h. Effects of FoxO3a and Bad deficiency on $H_2O_2$-induced Caspase-3 and PARP cleavage are indicated by arrows on the right of the images. Efficacy of RNAi-mediated knockdown of FoxO3a and Bad was determined by Western blot. Quantification of cleaved Caspase-3 and PARP from a representative experiment is indicated below lanes; the amount of $H_2O_2$-induced cleaved Caspase-3 and PARP were set at 1.0. The experiment was performed twice.

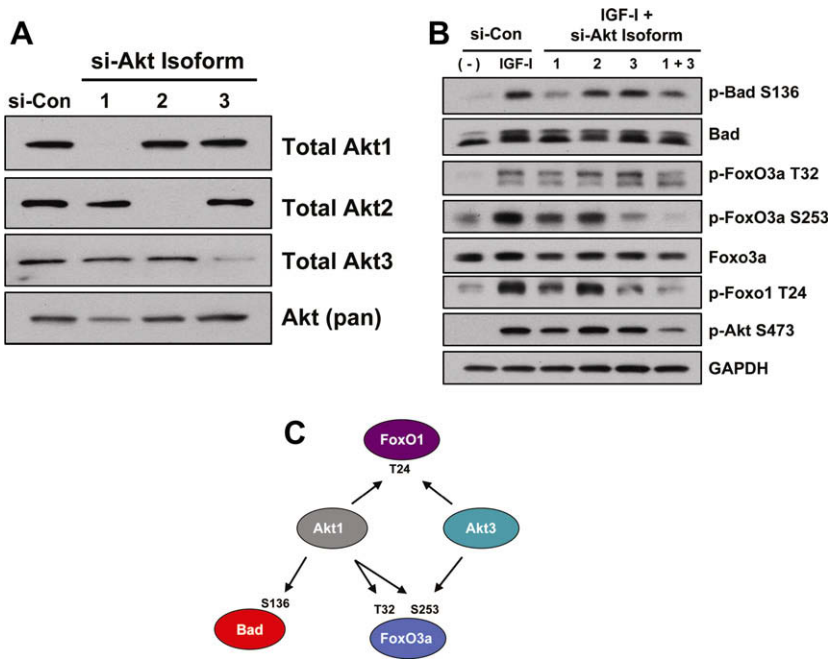


**Fig. 3.** Efficacy of si-RNA-mediated knockdown of Akt isoforms and IGF-I-stimulated Akt actions on target molecules. (A) Extent of RNAi-mediated knockdown of Akt isoforms was determined by Western blot. Cells were reverse-transfected with 5 nM negative control siRNA (si-Con), 5 nM siRNA against Akt1 (si-Akt1), 5 nM siRNA against Akt2 (si-Akt2), or 5 nM siRNA against Akt3 (si-Akt3). Akt isoforms detected by Western blot are indicated to the right of the images, and blots are representative of two independent experiments. (B) Reverse-transfections using siRNA directed against Akt isoforms were performed as described in (A), with the following siRNA concentrations: 10 nM negative control siRNA (si-Con), 5 nM siRNA against Akt1 (si-Akt1) plus 5 nM si-Con; 5 nM siRNA against Akt2 (si-Akt2) plus 5 nM si-Con; 5 nM siRNA against Akt3 (si-Akt3) plus 5 nM si-Con; or 5 nM si-Akt1 + 5 nM si-Akt3. Myoblasts were serum-starved for 2 h without (—) or with 16.6 nM IGF-I and harvested for protein lysates. Detected proteins are indicated to the right of the images, and blots are representative of two independent experiments. (C) Model of Akt isoform-specific actions on pro-apoptotic target molecules as determined from (B). Both Akt1 and Akt3 are involved in IGF-I-mediated phosphorylation of FoxO3a S253 and FoxO1 T24. Only Akt1 was found to be responsible for phosphorylating FoxO3a at T32 and Bad at S136.

Akt1 alone was responsible for phosphorylating Bad at S136 and FoxO3a at T32 (Fig. 3B and C). These findings offer novel insight into the tightly-controlled functions of Akt isoforms in promoting survival against pro-apoptotic molecules. One elusive property of Akt isoform regulation is the mechanism whereby specific Akt isozymes are regulated. One possibility is that Akt isoforms are subject to intracellular compartmentalization or localization [10]. Another possibility includes isoform-specific de-phosphorylation by phosphatases. Indeed, the identity of two such phosphatases (PH domain leucine-rich repeat protein phosphatase 1 and 2, abbreviated as PHLPP1 and PHLPP2) has recently been discovered [21,26], and experiments in human diabetic subjects suggest that PHLPP1 may contribute to regulation of glucose and lipid metabolism in skeletal muscle of diabetics [24]. Whether Akt isoforms are subject to differential intracellular localization or to de-phosphorylation by PHLPPs in myoblasts remains to be established.

In conclusion, we found that IGF-I prevented $H_2O_2$-induced apoptosis in myoblasts, and that pro-apoptotic targets of IGF-I-stimulated Akt are phosphorylated in an isoform-specific fashion.

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