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Akt Regulates the Survival of Vascular Smooth Muscle Cells via Inhibition of FoxO3a and GSK3*

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Apoptosis of vascular smooth muscle cells (VSMCs) may lead to atherosclerotic plaque instability and rupture, resulting in myocardial infarction, stroke, and sudden death. However, the molecular mechanisms mediating survival of VSMCs in atherosclerotic plaques remain unknown. Although plaque VSMCs exhibit increased susceptibility to apoptosis and reduced expression of the IGF1 receptor (IGF1R) when compared with normal VSMCs, a causative effect has not been established. Here we show that increased expression of the IGF1R can rescue plaque VSMCs from oxidative stress-induced apoptosis, demonstrating that IGF-1 signaling is a critical regulator of VSMC survival. Akt mediates the majority of the IGF1R survival signaling, and ectopic activation of Akt was sufficient to protect VSMCs *in vitro*. Both IGF1R and phospho-Akt expression were reduced in human plaque (intimal) VSMCs when compared with medial VSMCs, suggesting that Akt mediates survival signaling in atherosclerosis. Importantly, downstream targets of Akt were identified that mediate its protective effect as inhibition of FoxO3a or GSK3 by Akt-dependent phosphorylation protected VSMCs *in vitro*. We conclude that Akt and its downstream targets FoxO3a and GSK3 regulate a survival pathway in VSMCs and that their deregulation due to a reduction of IGF1R signaling may promote apoptosis in atherosclerosis.

Insulin-like growth factor 1 (IGF1)[2] is a ubiquitous factor exhibiting pleiotropic effects on different cell types. Stimulation of the IGF1 receptor (IGF1R) initiates signaling pathways involved in cell proliferation, differentiation, transformation, and survival. IGF1R-dependent signaling is crucial for the survival of many cell types including vascular smooth muscle cells (VSMCs). VSMCs are the principle source of collagen and extracellular matrix that maintain the tensile strength of atherosclerotic plaques, and VSMC loss induces multiple features of plaque instability (1). In humans, rupture or erosion of the atherosclerotic plaque underlie the majority of myocardial infarctions, stroke, and sudden death (2). We have previously shown that VSMCs derived from atherosclerotic plaques (pVSMCs) are more sensitive to apoptosis than cells derived from non-diseased vessels (3) and exhibit a defect in IGF1-dependent survival signaling (4). Oxidative stress is increasingly implicated in the development of atherosclerosis (5) and increased oxidative damage, and elevated levels of DNA strand breaks occur in human atherosclerotic plaques (6). Oxidative stress reduces IGF1R expression and induces VSMC apoptosis in culture (7–10). Reduced IGF1R expression is also seen within plaques, suggesting that IGF1R-dependent survival regulates apoptosis *in vivo* (11, 12). However, plaque VSMCs also show increased sensitivity to multiple proapoptotic stimuli, including the tumor suppressor gene *P53* and death receptor ligation (13, 14). It is therefore not known whether defective IGF1R expression alone is an important cause of reduced survival of pVSMCs.

A major downstream effector of IGF1R signaling is the serine/threonine kinase Akt (also known as Protein Kinase B (PKB)). Akt phosphorylates a large number of targets involved in glucose metabolism and cell differentiation, proliferation, and survival (reviewed in Ref. 15). We have previously shown that pVSMCs also exhibit reduced activation of Akt in response to IGF1 treatment (4), suggesting that Akt mediates IGF1R-dependent signaling in these cells. However, it is not known whether activation of Akt is necessary and/or sufficient for VSMC survival in response to apoptotic stimuli. Similarly, although Akt targets involved in survival have been identified in many cell types, Akt targets are frequently cell type-specific, and those important for VSMC survival have not been determined. Here we show that activation of Akt alone is sufficient to protect VSMCs from oxidative stress-induced apoptosis. Moreover, we demonstrate that Akt-dependent phosphorylation and subsequent inactivation of FoxO3a and GSK3 is important for VSMC survival.

EXPERIMENTAL PROCEDURES

Cell Culture—Human VSMCs were isolated from aortas of cardiac transplant patients or from atherosclerotic plaques following carotid endarterectomy with informed consent and approval of the Local Ethics Committee. Rat VSMCs were isolated from aortas of Wistar rats. Cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10 units/ml penicillin, 10 μg/ml streptomycin, 5 μg/ml L-glutamine, and 10% fetal calf serum. Cells were treated with 100 ng/ml recombinant human IGF1 (Peprotech), 100 nM 4-hy-

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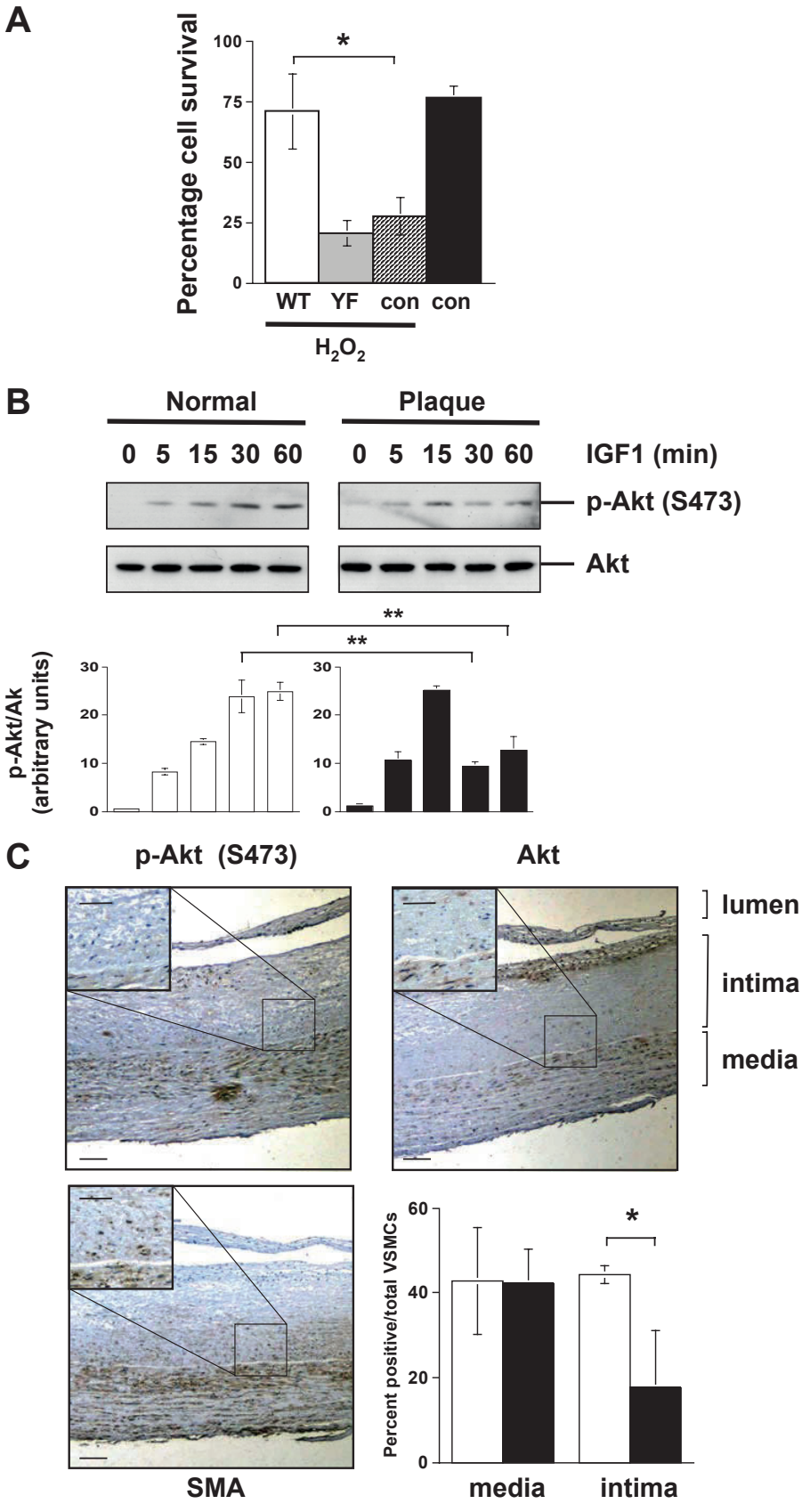
[2] The abbreviations used are: IGF1, insulin-like growth factor 1; IGF1R, IGF1 receptor; IGF1R YF, inactive point mutation of IGF1R; VSMC, vascular smooth muscle cell; pVSMC, atherosclerotic plaque-derived VSMC; HT, 4-hydroxytamoxifen; EGFP, enhanced green fluorescent protein; ER, estrogen receptor; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; DN, dominant negative.

droxytamoxifen (HT, Sigma), and 50 μM SB415286 (Sigma), as indicated. Since ongoing oxidative stress is a feature of atherosclerosis (5, 6) and is associated with VSMC apoptosis, we, like others, have used 25–50 μM hydrogen peroxide to induce VSMC apoptosis. Expression plasmids were transfected into rat VSMCs using SuperFect (Qiagen). Alternatively, infectious replication-deficient retrovirus was harvested from Bosc23 packaging cells and used to infect rat VSMCs in the presence of 8 μg/ml Polybrene (hexadimethrine bromide, Sigma). Rat VSMCs were selected and maintained in 5 μg/ml puromycin (Sigma) or 400 μg/ml G418 as appropriate. Human plaque-derived VSMCs were microinjected at 150 hectopascals for 0.1 s with expression plasmids at 1 mg/ml using Eppendorf Femtotips II and an Eppendorf Femtojet/Injectman 2. Cells expressing EGFP were counted using an Olympus IX51 microscope.

Plasmids—mAktER and A2 AktER (16) were cloned into the retroviral vector pBMN IRES puro (17). IGF1R wild type and YF mutant (18) were cloned into pBMN IRES puro (17). Dominant negative Akt (DN-Akt, T308A, S473A (19)) was cloned into pCDNA3, and the wild type and the A3 mutant of FoxO3a (20) were cloned into pCDNA3.1. pCDNA3 GSK3β wild type and S9A have been described (21).

Antibodies—Antibodies to the following proteins were used: Akt (Cell Signaling antibodies 9272 and 4691), phospho-Akt Ser-473 (Cell Signaling antibodies 9271 and 4060), GSK3α/β (Upstate Biotechnology antibody 05-412), GSK3α (AbCam antibody 28833), phospho-GSK3α/β Ser-21/Ser-9 (Cell Signaling antibody 9331), phospho-GSK3α Ser-21 (Cell Signaling antibody 9316), FoxO1 (Cell Signaling antibody 9462 and AbCam antibody 39670), FoxO3a (Santa Cruz Biotechnology antibody sc-11351 and Cell Signaling antibody 9467), phospho-FoxO1/phospho-FoxO3a



Thr-24/Thr-32 (Cell Signaling antibody 9464), Mcl1 (Santa Cruz Biotechnology antibody sc-819), Bad (Cell Signaling antibody 9292), phospho-Bad Ser-136 (Cell Signaling antibody 9295), Bim (Chemicon antibody AB17003), hemagglutinin (Sigma antibody H9658), phospho-Ser (antibody AbCam ab17465), β-actin (Sigma clone AC-15), and α-smooth muscle actin (Dako antibody 1A4).

Western Blot—Equal amounts of protein lysates prepared in Laemmli sample buffer were resolved on SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Millipore), and incubated with the indicated primary antibody. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

Cell Viability and Apoptosis—Cells were plated at the same density 16 h prior to each experiment. Proliferation and apoptosis were determined by time-lapse digital microscopy using an Olympus IX71 microscope controlled with Openlab software (Improvision). Apoptotic and dividing cells in two or more fields each containing 100 cells at the start of the experiment were counted. Apoptosis was verified by morphological changes, including nuclear condensation in the presence of Hoechst 33258 and membrane blebbing.

Immunohistochemistry—Paraffin-embedded sections of human carotid arteries were sectioned at 5-μm intervals. Specimens were dewaxed and microwaved in 120 mM sodium citrate buffer, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Sections were incubated in 10% bovine serum albumin for 1 h at room temperature and then with primary antibodies overnight at 4 °C followed by biotinylated secondary antibodies and horseradish peroxidase-conjugated streptavidin using the ABC kit (Vector Laboratories). Peroxidase activity was detected with diaminobenzidene. Sections were counterstained with hematoxylin. Cells expressing Akt, FoxO3a, GSK3α, or their phosphorylated species were counted in three or more random fields, each from three different plaque donors. Only cells whose staining exceeded a 125-pixel density were counted.

Statistical Analysis—Statistical analysis was performed using analysis of variance or Student's *t* test as appropriate. Significance was established when $p < 0.05$.

RESULTS

IGF1R Mediates Survival of VSMCs—Previously, we demonstrated that VSMCs derived from human plaques (pVSMCs) express lower levels of IGF1R when compared with VSMCs derived from normal human aortas (4, 7). pVSMCs also show reduced proliferation and increased sensitivity to apoptosis (3, 4). Ectopic expression of IGF1R in VSMCs increases the rate of proliferation and markedly reduces apoptosis (7, 22). Although there is a good correlation between reduced IGF1R expression and reduction in proliferation and survival in pVSMCs, a causative effect has not been established. To determine whether increased IGF1R expression could rescue the increased sensitivity of pVSMCs to apoptosis, we co-microinjected expression plasmids for either the wild-type IGF1R or the YF mutant along with EGFP into human pVSMCs. Co-injection with EGFP-expressing plasmids allowed tracking of injected cells within the population. When compared with plaque VSMCs microinjected with empty vector or IGF1R-YF, those injected with wild-type IGF1R showed increased protection against H_2O_2-induced apoptosis in response to IGF1 (Fig. 1*A*). This suggests that the level of IGF1R expression regulates the survival of VSMCs in response to oxidative stress.

IGF1R-dependent Survival of VSMCs Is Mediated by Akt—The serine/threonine kinase Akt is a major downstream effector of IGF1R signaling. Consistent with this, human pVSMCs demonstrated a transient increase in Akt phosphorylation (Ser-473) peaking at 15 min after IGF1 stimulation when compared with normal human VSMCs that demonstrated a more robust and persistent response (Fig. 1*B*). The reduction in the active phosphorylated form (Ser-473) of Akt was not due to reduced total Akt expression since this was not affected by IGF1 stimulation. Similar results were obtained from an additional two normal and two plaque-derived primary isolates of human VSMCs, indicating that reduced phospho-Akt was a common feature of plaque-derived cultures when compared with normal VSMCs (data not shown).

To examine whether the reduced phospho-Akt expression seen in pVSMCs *in vitro* was a valid representation of that seen in plaques *in vivo*, we examined expression of Akt and phospho-Akt in atherosclerotic plaques from three different patients where both the fibrous cap intimal cells and a relatively normal media could be examined in the same section. VSMCs (α-smooth muscle actin-positive, Fig. 1*C*) within the fibrous cap of the atherosclerotic plaque exhibited reduced phospho-Akt (Ser-473) expression *in vivo* when compared with medial cells (Fig. 1*C*). Again, this appeared to be due to a reduction in Akt activation (phosphorylation at Ser-473) rather than a reduction in total Akt expression.

To demonstrate directly that Akt mediates the effects of IGF1R activation in VSMCs, we expressed a dominant negative mutant of Akt (DN-Akt, T308A, S473A) (19) in rat VSMCs (Fig.

FIGURE 1. **IGF1R and Akt regulate survival of VSMCs.** *A*, human pVSMCs co-microinjected with an empty vector control (*con*) plasmid or with plasmids encoding either wild-type (*WT*) or the YF mutant of IGF1R and a plasmid expressing EGFP. 16 h after microinjection, the number of EGFP-expressing cells in the entire population was determined, and the cells were placed in serum-free medium for 6 h and then treated for 24 h with IGF1 (100 ng/ml) in the absence or presence of 25 μM H_2O_2. The graph shows the number of surviving EGFP-positive cells as a percentage of those originally injected. Results are mean ± S.D. ($n = 3$ different plaque VSMC isolates). * indicates $p < 0.05$. *B*, representative immunoblots for total and phospho-Akt (*p-Akt*) (Ser-473) in normal human carotid and plaque-derived VSMCs cultured in serum-free medium for 24 h and then stimulated with 100 ng/ml human IGF1. Similar results were obtained from two additional plaques and two additional normal VSMC isolates derived from different patients. Densitometry of the representative immunoblots is shown below as the ratio of phospho-Akt (Ser-473)/total Akt. Results are mean ± S.D. ($n = 3$ experimental replicates). ** indicates $p < 0.01$. *C*, immunohistochemistry for α-smooth muscle actin (*α-SMA*) and total and phospho-Akt (Ser-473) in sections of a single human carotid atherosclerotic plaque demonstrating reduced phospho-Akt (Ser-473) in intimal VSMCs when compared with healthy medial VSMCs. The *inset* shows a higher power magnification of the boxed area. *Scale bars* = 100 or 50 μm (*inset*). The proportion of Akt-positive (*white bars*) or phospho-Akt (Ser-473)-positive (*black bars*) VSMCs in the media or intima are quantified ($n = 3$, individual plaques from three different patients). * indicates $p < 0.05$.

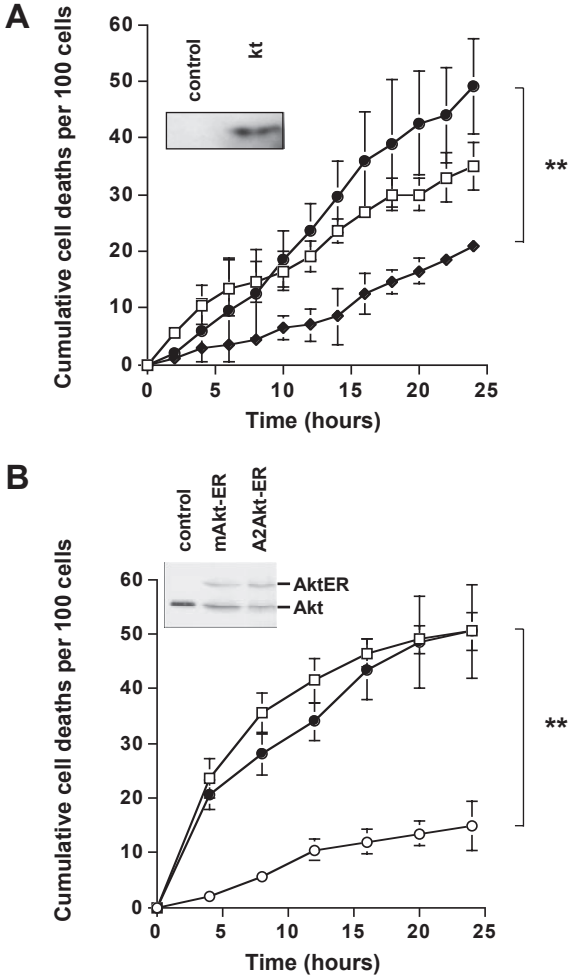


FIGURE 2. **IGF1-dependent survival of VSMCs is mediated by Akt.** *A*, time-lapse microscopic quantification of apoptosis in control rat VSMCs (□, ♦) or cells expressing a dominant negative Akt protein (●). Cells were cultured in serum-free conditions for 24 h and then treated with 50 μM H_2O_2 in the absence (□) or presence (♦, ●) of 100 ng/ml human IGF1. Results are mean ± S.D. (n = 3 different experiments). ** indicates $p < 0.01$. The *inset* shows expression of hemagglutinin-tagged DN-Akt by immunoblot. *B*, time-lapse microscopic quantification of apoptosis of rat VSMCs expressing mAktER (□, ○) or A2AktER (●). Cells were cultured in serum-free conditions for 24 h and then treated with 50 μM H_2O_2 in the presence of either 100 nM HT (○, ●) or an equivalent volume of the carrier, ethanol (□). Results are mean ± S.D. (n = 3 different experiments). ** indicates $p < 0.01$ *versus* both mAktER + ethanol and A2AktER + HT. The *inset* shows expression of both endogenous Akt and the AktER fusion proteins in rat VSMCs by immunoblot.

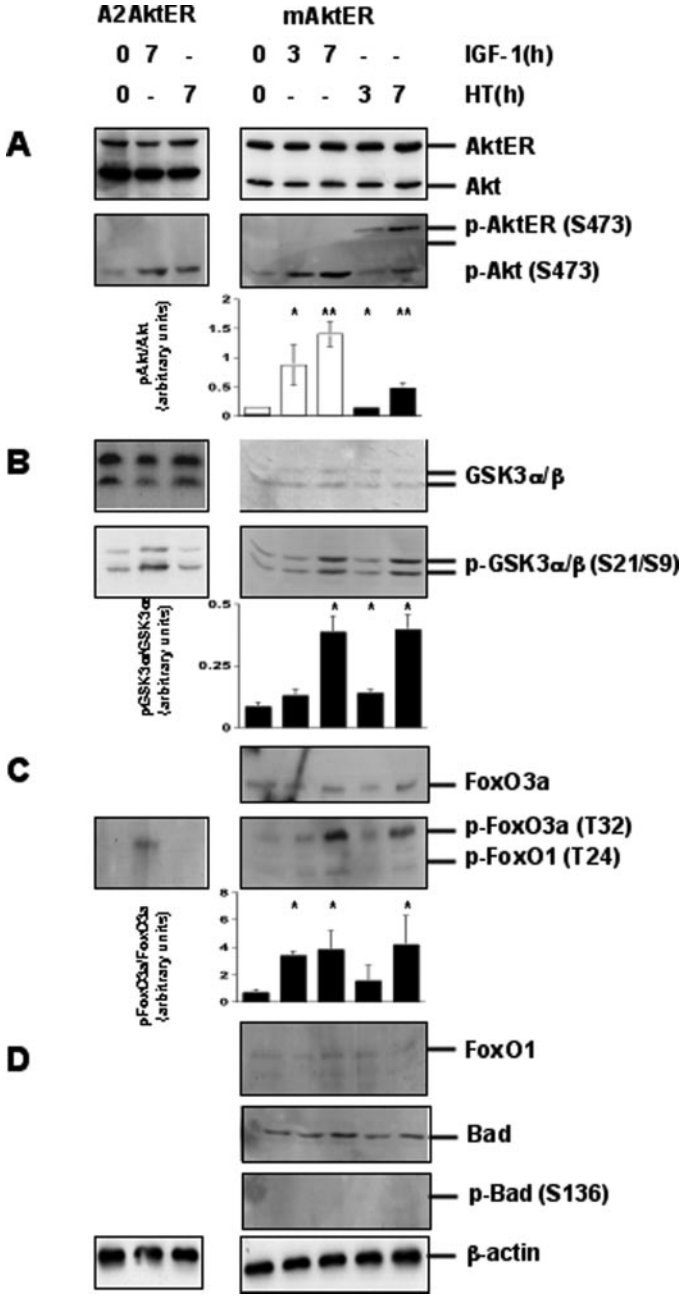


FIGURE 3. **Akt phosphorylates FoxO3a and GSK3 in VSMC.** Immunoblots of rat VSMCs expressing mAktER or A2AktER were cultured in serum-free conditions for 24 h and then treated with either 100 nM HT or 100 ng/ml IGF1 as indicated. Expression of β-actin is shown as a protein loading control. Densitometry is shown as the ratio of phosphorylated Akt (*p-Akt*, *white bars*) or AktER (*p-AktER*, *black bars*) relative to total Akt (*A*). Densitometry is also shown for the ratio of phosphorylated species of GSK3α (*p-GSK3*α, *B*) or FoxO3a (*p-FoxO3a*, *C*) relative to the total for each protein, respectively. Results are mean ± S.D. (n = 3 different experiments). * indicates $p < 0.05$, ** indicates $p < 0.01$. Immunoblots for FoxO1 (*p-FoxO1*), Bad (*p-Bad*), and phospho-Bad are also shown (*D*).

2*A*). DN-Akt markedly inhibited IGF1-stimulated survival (Fig. 2*A*), strongly suggesting that the major downstream effector of IGF1R-mediated survival in VSMCs is Akt. To examine whether Akt was sufficient to protect VSMCs from H_2O_2-induced apoptosis, we employed an ectopically inducible allele of Akt. Rat VSMCs were infected with retroviruses that express either a constitutively active Akt (myristoylated Akt, mAkt) or an allele containing an inactivating mutation in the myristoylation domain (A2Akt) fused to the ligand-binding domain of the estrogen receptor (mAktER and A2AktER, respectively; Fig. 2*B* (16)). These proteins are inactive in the absence of ligand (4-hydroxytamoxifen, HT), and the kinase activity of mAktER is only stimulated by the addition of HT to the culture medium (16). Consistent with this, phosphorylation of mAktER (Ser-473) was only observed following the addition of HT (Fig. 3*A*). In contrast, A2AktER was not phosphorylated at Ser-473 following the addition of HT and exhibited no kinase activity (see below). Note that a low level of endogenous phospho-Akt is observed in these cells even after 24 h in the absence of serum and that this was markedly increased by IGF1 treatment (Fig. 3*A*). Rat VSMCs cultured in the absence of IGF1 for 24 h (to suppress endogenous IGF1R signaling) showed ~35–60% apoptosis 24 h after the addition of 50 μM H_2O_2