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) 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 1-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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1 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 GSK3/β 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), FoxO3α (Santa Cruz Biotechnology antibody sc-11351 and Cell Signaling antibody 9467), phospho-FoxO1/phospho-FoxO3α

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**A** Percentage cell survival

**B** Normal Plaque Plaque

**C** p-Akt (S473) Akt
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IGF1R Protects Vascular Smooth Muscle Cells

IGF1R Mediates Survival of VSMCs—Previously, we demonstrated that VSMCs 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 H2O2-induced apoptosis in response to IGF1 (Fig. 1A). This suggests that the level of IGF1R expression regulates the survival of pVSMCs 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. 1B). 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. 1C) within the fibrous cap of the atherosclerotic plaque exhibited reduced phospho-Akt (Ser-473) expression in vivo when compared with medial cells (Fig. 1C). 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. 1D). This suggests that Akt represents a critical target of IGF1R-mediated signaling in VSMCs.
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DN-Akt markedly inhibited IGF1-stimulated survival (Fig. 2A), 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$_2$O$_2$-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. 2B (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 (Fig. 3A). 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. 3A). 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$_2$O$_2$.
ectopic activation of Akt with HT resulted in rapid phosphorylation following activation of Akt in cells (23), we were unable to demonstrate phosphorylation of IGF1 to cells deprived of serum for 24 h (Fig. 3). Although activation of mAktER or A2AktER by HT or by the addition of compared phosphorylation of putative Akt substrates after activation of Akt targets in some cells. Since we have shown that Akt is an important regulator of cell death in VSMCs, we sought to identify which targets of Akt mediate survival in these cells. We compared phosphorylation of putative Akt substrates after activation of mAktER or A2AktER by HT or by the addition of IGF1 to cells deprived of serum for 24 h (Fig. 3). Although phosphorylation of Bad by Akt confers survival in BALB/c 3T3 cells (23), we were unable to demonstrate phosphorylation of Bad following activation of Akt in vitro (Fig. 3D). In contrast, ectopic activation of Akt with HT resulted in rapid phosphorylation of FoxO3a and both α and β isoforms of GSK3 (Fig. 3, B and C). This was not observed in A2AktER-expressing control cells. Phosphorylation of FoxO3a and GSK3 α/β was also seen after stimulation with IGF1, consistent with the idea that Akt mediates signaling downstream of IGF1R. Although FoxO3a is phosphorylated by Akt in VSMCs, other members of the FoxO family are not. For example, FoxO1 is also expressed in these cells but was not phosphorylated in response to HT or IGF1 (Fig. 3, C and D).

FoxO3a is a transcription factor that activates many genes involved in cell death, including fas, bim, and puma (reviewed in Ref. 24). To determine whether inhibition of FoxO3a by Akt is important in the survival of VSMCs, we expressed a mutant FoxO3a allele (A3) in which all three potential Akt phosphorylation sites (Thr-32, Ser-253, and Ser-315) are changed to alanines (Fig. 4A) and which, therefore, cannot be phosphorylated and inhibited by Akt (20). The protection afforded by activation of Akt in H2O2-treated control rat VSMCs or those expressing wild-type FoxO3a was severely comprised by expression of FoxO3a-A3 (Fig. 4A). This indicates that inhibition of FoxO3a activity by Akt-dependent phosphorylation is required for survival of these cells.

To determine whether proapoptotic targets of FoxO3a were regulated after Akt activation, we examined the expression of Bim. Expression of Bim was reduced following activation of Akt in rat VSMCs expressing wild-type FoxO3a but not in cells expressing FoxO3a A3 (Fig. 4B).

To determine whether FoxO3a is a physiological target of Akt in human VSMCs as well as rat cells, we examined the expression of p-FoxO3a in lysates derived from normal human aorta or atherosclerotic plaque primary cultures (Fig. 4C). Reduced levels of phosphorylated Akt and FoxO3a were observed in the plaque-derived cultures when compared with normal aorta. Moreover, VSMCs within the fibrous cap of the atherosclerotic plaque exhibited reduced phospho-FoxO1/3a expression in vivo when compared with medial cells despite the presence of FoxO3a in these cells (Fig. 4D). The antibody used recognizes phosphorylated forms of both FoxO1 and FoxO3a, and we cannot be sure which protein is detected. However, we had not previously detected Akt-dependent phosphorylation of FoxO1 in rat VSMCs (Fig. 3), suggesting that the antibody is primarily detecting phosphorylated FoxO3a.

It is likely that Akt-dependent survival of VSMCs is mediated by more than one downstream effector. Indeed, GSK3 was also phosphorylated by Akt in VSMCs (Fig. 3), and recent data indicate that GSK3 may have a role in apoptosis regulation as well as glucose metabolism (25). To determine whether Akt-dependent inhibition of GSK3 is important for survival of VSMCs, we were treated in A2AktER-expressing control cells, nor in mAktER-expressing cells treated with vehicle (ethanol). These results demonstrate that IGF1R-dependent survival signaling in rat VSMCs is mediated primarily by Akt and that activation of Akt alone is sufficient to mimic the protective effects of IGF1R treatment.

Akt-dependent Survival of VSMCs Is Mediated by Inhibition of FoxO3a and GSK3—There are many reported substrates for Akt. For example, Bad, caspase 9, and members of the FoxO family are proapoptotic proteins that have been identified as Akt targets in some cells. Since we have shown that Akt is an important regulator of cell death in VSMCs, we sought to identify which targets of Akt mediate survival in these cells. We compared phosphorylation of putative Akt substrates after activation of mAktER or A2AktER by HT or by the addition of IGF1 to cells deprived of serum for 24 h (Fig. 3). Although phosphorylation of Bad by Akt confers survival in BALB/c 3T3 cells (23), we were unable to demonstrate phosphorylation of Bad following activation of Akt in vitro (Fig. 3D). In contrast, ectopic activation of Akt with HT resulted in rapid phosphorylation of FoxO3a and both α and β isoforms of GSK3 (Fig. 3, B and C). This was not observed in A2AktER-expressing control cells. Phosphorylation of FoxO3a and GSK3 α/β was also seen after stimulation with IGF1, consistent with the idea that Akt mediates signaling downstream of IGF1R. Although FoxO3a is phosphorylated by Akt in VSMCs, other members of the FoxO family are not. For example, FoxO1 is also expressed in these cells but was not phosphorylated in response to HT or IGF1 (Fig. 3, C and D).

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GSK3-mediated sensitivity to apoptosis of the interleukin-3-dependent murine prolymphocytic cell line FL5.12 occurs via phosphorylation of the antiapoptotic Bcl-2 family member Mcl1, resulting in its degradation via the lysosomal pathway (25). We therefore investigated whether Akt could inhibit the phosphorylation of Mcl1 by GSK3. Activation of AktER by HT or endogenous Akt by IGF1 increased Mcl1 protein levels (Fig. 5B), due to an increased half-life of Mcl1 (Fig. 5C). Moreover, the change in protein half-life in response to Akt activation correlated with phosphorylation of Mcl1 (Fig. 5D). Phosphorylation of Mcl1 in the absence of Akt activity was also suppressed by the GSK3 inhibitor SB415286 (Fig. 5D), suggesting that GSK3 is the primary kinase for Mcl1 in these cells. Taken together, these data suggest that phosphorylation and inhibition of GSK3 by Akt allows stabilization of Mcl1, thus promoting cell survival.

To determine whether GSK3 is a physiological target of Akt in human VSMCs as well as rat cells, we examined the expression of p-GSK3α (Ser-21) in human atherosclerotic plaques. VSMCs within the fibrous cap of the atherosclerotic plaque exhibited reduced phospho-GSK3 expression in vivo when compared with medial cells despite the presence of GSK3 in these cells (Fig. 5E). Consistent with this, we found reduced phospho-GSK3α/β in plaque VSMC primary cultures when compared with VSMCs from normal aorta (Fig. 4C).

DISCUSSION

Apoptosis of VSMCs within the atherosclerotic plaque is a critical determinant of plaque-dependent vascular events such as stroke and myocardial infarction. For example, apoptosis of VSMCs is increased in unstable versus stable plaques, suggesting that VSMC apoptosis promotes the loss of structural integrity of the fibrous cap. Consistent with this, induction of VSMC apoptosis in mice leads to features of vulnerable plaques (1). Nonetheless, the molecular mechanisms that govern VSMC survival have not been elucidated.

IGF1 is a potent inhibitor of apoptosis in human and rodent VSMCs (3, 4, 26). The lower level of IGF1R expression in pVSMCs in vitro (4, 7) and in intimal VSMCs in plaques (11) and oxidized low density lipoprotein-dependent down-regula-
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**A** Time-lapse microscopic quantification of apoptosis in rat VSMCs expressing mAktER and transfected with a control empty plasmid (C, □) or plasmids encoding either wild type (WT, ○) or the A3 mutant (●) of FoxO3a. Cells were cultured in serum-free conditions for 24 h and then treated with 100 nM HT and 50 μM H₂O₂. Apoptosis was determined by time-lapse microscopy. Results are mean ± S.D. (n = 3 different experiments). * indicates p < 0.05. The inset shows expression of hemagglutinin-tagged FoxO3a (HA-FoxO3a) by immunoblot. B, immunoblot for Bim in rat VSMCs expressing mAktER and either wild type or the A3 mutant of FoxO3a. Cells were cultured in serum-free conditions for 24 h and then treated with 100 nM HT for 8 h. β-actin is shown as a loading control. Densitometry is shown below as the ratio of Bim/β-actin. Results are mean ± S.D. (n = 3). * indicates p < 0.05, ** indicates p < 0.01. p-AktER, phosphorylated AktER. C, immunoblots of primary VSMC cultures from normal human aorta (N) or two different human plaques (P). Densitometry is shown as the ratio of phospho-Akt (p-Akt, white bars), phospho-FoxO3a (p-FoxO3a, gray bars) or phospho-GSK3β (p-GSK3β, black bars) relative to total protein for each species, respectively. Results are mean ± S.D. from three replicate immunoblots using the same VSMC isolates. D, representative immunohistochemistry images for total and phospho-FoxO3a in sections of a human carotid atherosclerotic plaque demonstrating reduced p-FoxO3a/3a 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 FoxO3a-positive (white bars) or phospho-FoxO3a/FoxO3a (p-FoxO3a/3a, Thr-32/Thr-24)-positive (black bars) VSMCs in the media or intima is quantified in the same individual plaques from three different patients shown in Fig. 1C. * indicates p < 0.05.

**B** FoxO3a

|      | WT | A3 |
|------|----|----|
| HT (h) | 0  | 8  |
| p-AktER (S473) | 0  | 8  |
| Bim   |    |    |
| β-actin |    |    |

**C** N P P

|      | p-Akt (S473) | p-FoxO3a (T32) | p-GSK3β (S21/S9) | β-actin |
|------|--------------|----------------|------------------|---------|

**D** FoxO3a p-FoxO1/3a

**FIGURE 4. Akt-dependent inhibition of apoptosis is mediated by phosphorylation of FoxO3a.** A, time-lapse microscopic quantification of apoptosis in rat VSMCs expressing mAktER and transfected with a control empty plasmid (C, □) or plasmids encoding either wild type (WT, ○) or the A3 mutant (●) of FoxO3a. Cells were cultured in serum-free conditions for 24 h and then treated with 100 nM HT and 50 μM H₂O₂. Apoptosis was determined by time-lapse microscopy. Results are mean ± S.D. (n = 3 different experiments). * indicates p < 0.05. The inset shows expression of hemagglutinin-tagged FoxO3a (HA-FoxO3a) by immunoblot. B, immunoblot for Bim in rat VSMCs expressing mAktER and either wild type or the A3 mutant of FoxO3a. Cells were cultured in serum-free conditions for 24 h and then treated with 100 nM HT for 8 h. β-actin is shown as a loading control. Densitometry is shown below as the ratio of Bim/β-actin. Results are mean ± S.D. (n = 3). * indicates p < 0.05, ** indicates p < 0.01. p-AktER, phosphorylated AktER. C, immunoblots of primary VSMC cultures from normal human aorta (N) or two different human plaques (P). Densitometry is shown as the ratio of phospho-Akt (p-Akt, white bars), phospho-FoxO3a (p-FoxO3a, gray bars) or phospho-GSK3β (p-GSK3β, black bars) relative to total protein for each species, respectively. Results are mean ± S.D. from three replicate immunoblots using the same VSMC isolates. D, representative immunohistochemistry images for total and phospho-FoxO3a in sections of a human carotid atherosclerotic plaque demonstrating reduced p-FoxO3a/3a 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 FoxO3a-positive (white bars) or phospho-FoxO3a/FoxO3a (p-FoxO3a/3a, Thr-32/Thr-24)-positive (black bars) VSMCs in the media or intima is quantified in the same individual plaques from three different patients shown in Fig. 1C. * indicates p < 0.05.

IGF1 (29) is, at least partly, due to increased survival of VSMCs, especially since loss of VSMC promotes features of advanced plaques (1). However, the downstream effectors of IGF1 signaling in VSMC survival are unknown. Here we show that the level of IGF1R is rate-limiting for IGF1-mediated survival in response to oxidative stress, a stimulus that is physiologically relevant to the plaque environment. Although activation of the IGF1R may result in activation of either or both the PI3K/Akt and the MAPK signaling pathways, survival of VSMCs is primarily dependent on activation of the Akt pathway, consistent with the requirement for intact PI3K activity (22, 26, 30, 31). Indeed, pVSMCs with reduced IGF1R expression also show impaired phosphorylation of Akt in response to IGF1 *in vitro* and in plaque intimal VSMCs *in vivo*.

Although our data implicate IGF1R/Akt as a critical regulatory pathway of pVSMC survival, the Akt targets that mediate survival have not been identified. In part, this is because previous studies have used high levels of constitutively active Akt that may not be physiologically relevant or a combination of growth factor stimulation and pharmacological agents to block downstream kinases such as PI3K, MAPK, and S6 kinase (S6K). We have employed an ectopically activated Akt molecule to study the direct effects of Akt activation in the absence of other signaling pathways emanating from the IGF1 receptor. We find that Akt activation is sufficient for VSMC survival in response to oxidative stress. Moreover, using this system, we have identified FoxO3a and GSK3 as *bona fide* Akt targets important for VSMC survival (Fig. 6).

A mutant FoxO3a that cannot be phosphorylated and therefore inhibited by Akt abrogates Akt-dependent survival in response to oxidative stress. Furthermore, expression of the FoxO3a transcriptional target, Bim, was reduced following Akt activation. Although this implicates Bim in mediating the proapoptotic effects of FoxO3a, FoxO3a has multiple
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Recently, GSK3 has been shown to regulate apoptosis of hematological cells following growth factor withdrawal, through the phosphorylation and subsequent ubiquitin-mediated degradation of the Bcl-2 family member Mcl1 (25, 44). The role of GSK3 and Mcl1 in VSMC survival is supported by several observations. First, GSK3 S9A, a mutant that cannot be phosphorylated and inhibited by Akt, partially abrogated Akt-dependent survival transcriptional targets (Fig. 6). For example, the Fas death receptor also induces VSMC apoptosis (14, 32), suggesting that inhibition of FasL expression may also be important (33). Similarly, FoxO3a-mediated repression of FLIP (FLICE-like inhibitory factor) may also be regulated by Akt and has been shown to contribute to survival of endothelial cells (34).

Despite the fact that the FoxO family of proteins have overlapping expression patterns and bind the same DNA sequence, they exert different effects in different cell types via modulation of distinct targets. FoxO proteins are known to regulate homeostasis of hematopoietic stem cells and endothelial cells (35–38), but less is known about their role in VSMCs. Although FoxO3a protects quiescent human colon carcinoma cells from oxidative stress via transcriptional induction of the antioxidant enzyme manganese superoxide dismutase (Li et al. (39), Kops et al. (50)) demonstrated that increased Akt activity in VSMCs from older rats was associated with increased phosphorylation of FoxO3a (at Ser-253) and reduced manganese superoxide dismutase expression. We observed no protective effect of FoxO3a in VSMCs, although it is possible that the protective effect of FoxO3a is only apparent in quiescent cells (40, 41). Although overexpression of FoxO3a itself inhibits VSMC proliferation in vitro and neointimal hyperplasia in a balloon carotid arterial injury model, this was associated with increased apoptosis (42, 43).

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Akt Protects Vascular Smooth Muscle Cells

![Diagram indicating a proposed model for the molecular pathways involved in Akt-dependent survival of VSMCs.](image)

of VSMCs following oxidative stress. Second, the constitutively active GSK3β S9A mutant also prevented Akt-dependent stabilization of Mcl1. Finally, increased levels of Mcl1 conferred protection against oxidative stress in VSMCs (data not shown). Consistent with this, Mcl1 is up-regulated by a number of growth factors and cytokines (45–48). Increased Bim and Puma expression (for example, in the absence of Akt signaling) might also inhibit Mcl1 function directly (49).

It is not possible to quantify the precise contribution of Akt-dependent FoxO3a and GSK3 inhibition to the survival of VSMCs, and it is likely that additional targets will be important. Although Akt may simultaneously suppress several proapoptotic proteins, failure to suppress an individual species will still result in a net decrease in protection and, thus, these effects are unlikely to be additive. Other targets may be relevant for survival during development or in response to other apoptotic triggers. For example, PI3K-dependent phosphorylation of the proapoptotic protein Bad is required for the survival of embryonic rat VSMCs following serum removal (26), although we found no evidence for Akt-dependent phosphorylation of Bad in adult rat VSMCs. It is possible that PI3K-dependent phosphorylation of Bad does not require Akt in these cells. Alternatively, phosphorylation of Bad, although important for survival in embryonic cells subject to serum withdrawal, may not be required for survival of adult-derived cells subject to oxidative stress. Indeed, the VSMCs used in these experiments did not undergo significant apoptosis in response to serum withdrawal.

In summary, our data demonstrate that vascular smooth muscle cell survival in response to a physiological death stimulus is dependent on IGF1R/Akt signaling and Akt-dependent inhibition of FoxO3a and GSK3. We show that plaque VSMCs exhibit defective IGF1R/Akt signaling in vitro and decreased expression of IGF1R and phospho-Akt in vivo. Although it is likely that other Akt targets also mediate VSMC survival, by dissecting these pathways, we have identified molecular mechanisms relevant to VSMC survival within the oxidative environment of the atherosclerotic plaque. Identification of the molecular pathways that govern survival of VSMCs may be useful in determining the fate of atherosclerotic plaques and, in particular, the likelihood of rupture.

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