Biochimica et Biophysica Acta 1823 (2012) 458–464

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

# Akt acutely activates the cholesterogenic transcription factor SREBP-2

Winnie Luu, Laura J. Sharpe, Julian Stevenson, Andrew J. Brown *

*School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia*

ARTICLE INFO

*Article history:*
Received 28 March 2011
Received in revised form 15 August 2011
Accepted 9 September 2011
Available online 8 October 2011

*Keywords:*
Akt
Cholesterol
IGF-1
PI3K
SREBP-2

ABSTRACT

Akt is an essential protein kinase for cell growth, proliferation, and survival. Perturbed Akt regulation is associated with a number of human diseases, such as cancer and diabetes. Recently, evidence has emerged that Akt is involved in the regulation of the sterol-regulatory element binding proteins, which are master transcriptional regulators of lipid metabolism. This offers a means by which synthesis of new membrane can be coordinated with cell growth and proliferation. However, the link between Akt and sterol-regulatory element binding protein-2, the major isoform participating in cholesterol regulation, is relatively unexplored. In the present study, we employed insulin-like growth factor-1 as an inducer of Akt signalling, and showed that it increased sterol-regulatory element binding protein-2 activation acutely (within 1 h). This insulin-like growth factor-1-induced sterol-regulatory element binding protein-2 activation was blunted when Akt was inhibited pharmacologically or molecularly with small interfering RNA. Furthermore, we employed a rapalog heterodimerisation system to specifically and rapidly activate Akt, and found that sterol-regulatory element binding protein-2 activation was increased in response to Akt activation. Together, this study provides compelling evidence that Akt contributes to the acute regulation of cholesterol metabolism through activating sterol-regulatory element binding protein-2.



## 1. Introduction

Akt (also known as protein kinase B) is a serine/threonine protein kinase downstream of phosphatidylinositol 3-kinase (PI3K). It is essential for regulating cell growth, proliferation, survival, and the interaction with environmental stimuli [1]. The PI3K/Akt pathway is initiated by ligands, such as insulin-like growth factor-1 (IGF-1), binding to and activating receptor tyrosine kinases (reviewed in [2]). These receptors are autophosphorylated, and this recruits PI3K to the membrane. PI3K is activated and generates phosphatidylinositol-3,4,5-trisphosphate from phosphatidylinositol-4,5-bisphosphate by phosphorylation. This in turn recruits inactive Akt via its Pleckstrin Homology domain, as well as two activating kinases, phosphoinositide-dependent kinase 1 (PDK1) [3] and mammalian Target of Rapamycin (mTOR) Complex 2 [4]. Once activated, Akt phosphorylates a large number of substrates, leading to their activation or inhibition.

Akt is implicated in many diseases, most notably in cancer and diabetes, due to aberrant Akt activity (for reviews, see [5,6]). Moreover, Akt has recently been implicated in a novel form of regulation, lipid metabolism, through the sterol-regulatory element binding proteins (SREBPs) [7].

SREBPs are master transcriptional regulators of lipid metabolism. There are three mammalian isoforms of the SREBPs; SREBP-1a, -1c, and -2 [8]. SREBP-1c regulates genes involved in fatty acid metabolism, and SREBP-2 regulates genes involved in cholesterol metabolism, while SREBP-1a targets both sets of genes. SREBPs are produced as a precursor bound to the endoplasmic reticulum (ER) membrane, complexed with SREBP cleavage-activating protein (Scap). Scap acts as an escort protein and senses sterol levels for end-product feedback regulation [9]. When sterol levels are sufficient, Scap adopts a conformation [10] which is held back by a retention protein called insulin-induced gene [11], preventing the activation of SREBP. When sterol levels are low, Scap escorts the SREBP precursor from the ER to the Golgi in coatomer protein II vesicles [12]. At the Golgi, SREBP is sequentially cleaved by two proteases, site-1 protease and site-2 protease to become activated. The mature SREBP fragment is transported to the nucleus to transcriptionally upregulate lipogenic target gene expression, which restores sterol homeostasis via lipid synthesis and uptake. This mature active form of SREBP-2 is thus the focus of this study.

There is emerging evidence implicating PI3K/Akt in the regulation of lipid metabolism through the SREBPs [7]. Coordination between

*Abbreviations:* 25HC, 25-hydroxycholesterol; CHO-7, Chinese hamster ovary-7; DN-Akt, dominant-negative Akt; FKBP, FK506-binding protein; FRB, FKBP-rapamycin binding; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IGF-1, insulin-like growth factor-1; LDLR, low density lipoprotein receptor; LPDS, lipoprotein-deficient serum; Myr, myristoylation; mTOR, mammalian Target of Rapamycin; pAkt, phosphorylated Akt; PBGD, porphobilinogen deaminase; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase; Scap, SREBP cleavage-activating protein; siRNA, small interfering RNA; SREBP, sterol-regulatory element binding protein

* Corresponding author at: BABS, School of Biotechnology and Biomolecular Sciences, Biological Sciences Building D26, University of New South Wales, Sydney, NSW 2052, Australia. Tel.: +61 2 9385 2005; fax: +61 2 9385 1483.
*E-mail address:* aj.brown@unsw.edu.au (A.J. Brown).

0167-4889/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2011.09.017

these pathways is logical considering that SREBPs are needed to produce the lipids for new membrane synthesis, which in turn is required for growing and proliferating cells [13]. Most work thus far has focussed on the SREBP-1c isoform, and these findings are mostly consistent [7]. The link between Akt and the SREBP-2 isoform, however, is relatively unexplored and is contentious. Our laboratory identified a novel input into SREBP-2 activation through the involvement of the PI3K/Akt pathway [14]. The ER-to-Golgi transport of Scap/SREBP-2 was inhibited by a potent inhibitor of PI3K, LY294002, and a dominant-negative form of Akt (DN-Akt). DN-Akt inhibits endogenous Akt activity by competing for upstream kinases that activate Akt [15], and this can prevent the activation of endogenous kinases other than Akt [15]. As LY294002 is an inhibitor of PI3K, an early component in the pathway, it can also inhibit downstream kinases other than Akt. Moreover, as with many pharmacological inhibitors, it is also reported to inhibit other targets, such as mTOR and casein kinase-2, with a similar potency as required for PI3K [16]. Thus, these approaches are susceptible to non-specific effects [7].

In the present study, we set out to investigate the link between Akt and SREBP-2 activation, using more selective tools than were available at the time of our previous study [14]. These include more direct approaches to reduce Akt activation than PI3K inhibitors, and more acute time-points to minimise indirect effects. In our previous work [14], statins were used to stimulate SREBP-2 activation, which is more related to cholesterol homeostasis than cell growth or proliferation. Here, we employed IGF-1, known to signal cell growth and proliferation via the Akt pathway [17], and a rapalog heterodimerisation system for a more specific and rapid induction of Akt activation, and thus explore the interaction between Akt signalling and SREBP-2 regulation.

## 2. Materials and methods

### 2.1. Materials

Chinese hamster ovary-7 (CHO-7) and CHO cells stably expressing green fluorescent protein fused to Scap (CHO/pGFP-Scap cells) [18] were generous gifts of Drs. Michael S. Brown and Joseph L. Goldstein (UT Southwestern Medical Center, Dallas, TX). Akt (pan) antibody and phosphorylated Akt (pAkt; S473) antibody were from Cell Signaling Technology (Beverly, MA). Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM/F12), newborn calf serum, Lipofectamine 2000, Lipofectamine LTX, Opti-MEM I reduced serum medium, ProLong Gold Antifade Reagent with DAPI, and Superscript III Reverse Transcriptase were from Invitrogen (Carlsbad, CA). Akt inhibitor IV, Akt inhibitor V (Triciribine), Akt inhibitor VIII (Akti-1/2), and PhosphoSafe Extraction Reagent were from Merck (Darmstadt, Germany). IGF-1 was from R&D Systems (Minneapolis, MN). α-tubulin antibody, bovine serum albumin (BSA), BSA (essentially fatty acid free), LY294002, LY303511, MG132, Protease Inhibitor Cocktail (contains AEBSF, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-64), TRI reagent, and Wortmannin were from Sigma-Aldrich (St. Louis, MO). 25-hydroxycholesterol (25HC) was from Steraloids (Newport, RI). Lipoprotein-deficient serum (LPDS) was prepared from newborn calf serum as previously described [19]. The Golgi marker plasmid, dsRed-Monomer-Golgi, encoding the N-terminal portion of human beta 1,4-galactosyltransferase which is targeted to the trans-medial region of the Golgi, was from Clontech (Mountain View, CA).

### 2.2. Methods

#### 2.2.1. Cell culture, pretreatments, and treatments

CHO-7 and CHO/pGFP-Scap cells were maintained in 5% (v/v) LPDS/DMEM/F12 and were serum-starved overnight in 0.1% (w/v) BSA (essentially fatty acid free) in DMEM/F12. HepG2 cells were maintained in 10% FCS/DMEM (low glucose), and serum-starved overnight in 0.1% BSA (essentially fatty acid free) in DMEM (low glucose). Where there were pretreatments, the cells were pretreated in fresh starvation media, and then treatments were added to the pretreatment media for the indicated length of time. Where there was no pretreatment, the cells were treated in fresh starvation media. The cells were pretreated and/or treated with various test agents [added in dimethylsulfoxide, ethanol, water, or 0.1% (w/v) BSA/phosphate-buffered saline (PBS)], as indicated in the figure legends. Within an experiment, the final concentrations of solvent were kept constant between conditions and did not exceed 0.3% (v/v).

#### 2.2.2. Harvesting protein for Western blot analysis

After treatment, cells were lysed in PhosphoSafe Extraction Reagent supplemented with 2% (w/v) SDS, protease inhibitor cocktail, and phosphatase inhibitor cocktail (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 10 mM β-glycerophosphate). For experiments where CHO-7 cells were transfected with siRNA or when the stable Flp-In cell-lines were tested, the cells were harvested in SDS lysis buffer (10 mM Tris–HCl (pH 7.6), 100 mM sodium chloride, 2% (w/v) SDS) with protease inhibitor cocktail and phosphatase inhibitor cocktail. Protein concentrations of the cell lysates were determined using the bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Equal amounts of protein were mixed with loading buffer (final conc.: 50 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) glycerol, 0.04% (w/v) bromophenol blue, and 1% (v/v) β-mercaptoethanol), boiled for 5 min, and subjected to SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane for analysis by Western blotting.

#### 2.2.3. Western blotting

Membranes were blocked with 5% (w/v) BSA/PBST [0.1% (v/v) Tween20 in PBS] ([or 5% (w/v) skim milk/PBST for 1D2]), and then incubated with primary antibody diluted in 5% (w/v) BSA/PBST. The following antibodies were used: Akt (pan), pAkt (S473), IgG-7D4 [a mouse mAb against the N-terminus of hamster SREBP-2 (amino acids 32–250) [20]; prepared in-house [21]], IgG-1D2 [a mouse mAb against the N-terminus of human SREBP-2 (amino acids 48–403) [22] (a generous gift from Dr Bao-Liang Song)], and α-tubulin. The membrane was then washed in PBST, incubated with secondary antibody in 5% (w/v) BSA/PBST ([or 1% (w/v) skim milk/PBST for 1D2]), and washed in PBST. The antibodies were visualised by the enhanced chemiluminescent detection system, and membranes were exposed to Hyperfilm. Proteins were identified by their predicted sizes (pAkt/Akt, 56 kDa; α-tubulin, 50 kDa; precursor SREBP-2, 124 kDa; mature SREBP-2, 52 kDa; FRB-Akt, ~75 kDa). Before reprobing, antibodies were removed with stripping buffer (25 mM glycine, 1.5% (w/v) SDS, pH 2).

#### 2.2.4. Densitometry

Protein band intensities from Western blots were quantified by densitometry using ImageJ (Version 1.42q) [23]. The bands corresponding to mature SREBP-2 were quantified to yield relative intensities (M), with the 1 h + IGF-1 or + rapalog condition set to 1 in each experiment.

#### 2.2.5. Fluorescence microscopy

CHO/pGFP-Scap cells were seeded on coverslips in duplicate wells per condition, transfected with dsRed-Monomer-Golgi using Lipofectamine LTX according to the manufacturer's instructions, and serum-starved overnight. The cells were refed with starvation media before they were pretreated with or without Akt inhibitor VIII for 1 h, and treated in the same media with IGF-1 for a further 4 h. Cells were fixed with 3% (v/v) formaldehyde/PBS and mounted on glass slides with ProLong Gold Antifade Reagent with DAPI. Images were obtained using an Olympus FV 1000 Confocal Inverted Microscope

(Olympus, Tokyo, Japan). The excitation maximum was 488 nm for GFP, 557 nm for dsRed, and 405 nm for DAPI.

*2.2.6. Quantitative RT-PCR*

CHO-7 or HepG2 cells were seeded in triplicate wells per condition and serum-starved overnight. Cells were refed starvation media containing pretreatments for 1 h, and then treated in the same media with IGF-1 for 2 h. Cells were harvested for total RNA using TRI reagent, essentially according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed to cDNA with Superscript III Reverse Transcriptase.

Quantitative real-time PCR (qRT-PCR) was performed using a Corbett Rotorgene 3000 and analysed using Rotor-Gene Version 6.0 (Build 27) (Corbett Research, Mortlake, NSW). Primers were used to amplify the cDNA of hamster [14] or human [24,25] *low density lipoprotein receptor* (*LDLR*), *3-hydroxy-3-methylglutaryl-coenzyme A reductase* (*HMGCR*), and the housekeeping control *porphobilinogen deaminase (PBGD)*. Changes in gene expression levels of *LDLR* and *HMGCR* were normalised to *PBGD* for each sample.

*2.2.7. siRNA transfection*

CHO-7 cells were transfected with 200 μM small interfering RNA (siRNA) [either SignalSilence® Akt siRNA I (#6211) or SignalSilence® Control siRNA Unconjugated (#6568) (Cell Signaling Technology, Beverly, MA)] using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions, with slight modifications. With the modified protocol, the cells were transfected in half the media volume, and refed culture medium every 24 h for 48 h without removing the siRNA complexes. The cells were then serum-starved overnight, and treated with IGF-1 in fresh starvation media for 1 h.

*2.2.8. Cloning the pBI-CMV-FRB-Akt-Myc-Myr-2xFKBP-HA-FRT vector, and creating a stable Flp-In cell-line*

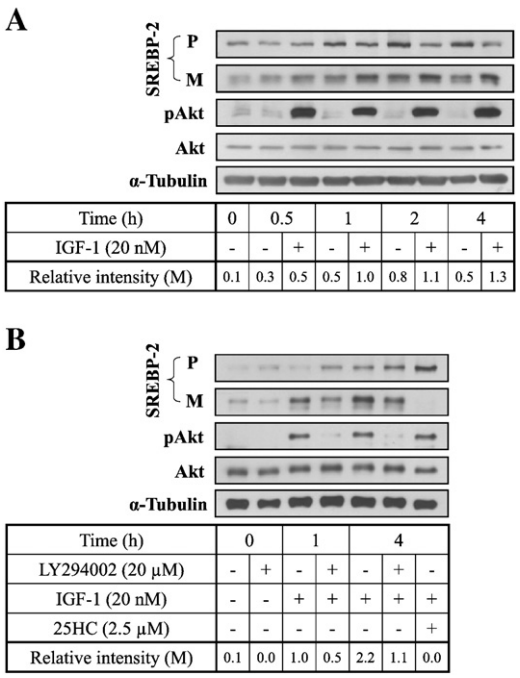
A plasmid containing a FRT recombination site and encoding myristoylated (Myr)-2xFK506-binding protein (FKBP)-HA and FKBP-rapamycin binding (FRB)-Akt-Myc driven by a bi-directional CMV promoter was created using polymerase incomplete primer extension [26]. Firstly, the bi-directional CMV promoter/enhancer was inserted into pcDNA5/FRT/TO to create pBI-CMV-FRT. Bovine Akt1 with a C-terminal Myc tag was amplified from pCMV-WT-Akt-Myc plasmid [27] and subcloned into the $pC_4$-$R_H$E plasmid encoding the FRB domain (ARIAD Pharmaceuticals, Cambridge, MA). The FRB-Akt-Myc was inserted into the destination plasmid, pBI-CMV-FRT. Myr-2xFKBP-HA from $pC_4$M-F2E (ARIAD Pharmaceuticals, Cambridge, MA) was similarly introduced into pBI-CMV-FRT in a second cloning step, yielding the complete expression vector. The resulting pBI-CMV-FRB-Akt-Myc-Myr-2xFKBP-HA-FRT construct was verified by sequencing and used to prepare CHO-7 stable cells generated in-house with the Flp-In system (Invitrogen, Carlsbad, CA), selecting for single colonies with 200 μg/mL hygromycin B. Empty vector stable cells were prepared using a pcDNA5/FRT/TO empty expression plasmid.

## 3. Results

*3.1. IGF-1 acutely increases SREBP-2 activation*

We determined if Akt activation induced by IGF-1 affects SREBP-2 activation within a 4 h time-course in Chinese hamster ovary (CHO)-7 cells, a cell-line commonly employed in cholesterol homeostasis studies [e.g. 28,29]. IGF-1 increased phosphorylated Akt (pAkt, the active form) levels within 30 min, and this was sustained for at least 4 h (Fig. 1A).

SREBP-2 activation results from ER-to-Golgi transport and proteolysis of precursor SREBP-2 to increase the mature (active) form of SREBP-2 [9]. This was monitored directly by Western blotting with an antibody that binds to the N-terminus of SREBP-2, and therefore detects both precursor and mature forms. With IGF-1 treatment, mature SREBP-2 was increased, indicating an increase in SREBP-2 activation (Fig. 1A). The earliest time IGF-1 stimulated a discernable effect on SREBP-2 activation was at 1 h.




| Time (h) | 0 | 0.5 | | 1 | | 2 | | 4 | |
|---|---|---|---|---|---|---|---|---|---|
| IGF-1 (20 nM) | - | - | + | - | + | - | + | - | + |
| Relative intensity (M) | 0.1 | 0.3 | 0.5 | 0.5 | 1.0 | 0.8 | 1.1 | 0.5 | 1.3 |

| Time (h) | 0 | | 1 | | 4 | | |
|---|---|---|---|---|---|---|---|
| LY294002 (20 μM) | - | + | - | + | - | + | - |
| IGF-1 (20 nM) | - | - | + | + | + | + | + |
| 25HC (2.5 μM) | - | - | - | - | - | - | + |
| Relative intensity (M) | 0.1 | 0.0 | 1.0 | 0.5 | 2.2 | 1.1 | 0.0 |

**Fig. 1.** IGF-1 stimulated SREBP-2 activation, which was reduced by a PI3K inhibitor. CHO-7 cells were seeded and serum-starved overnight. (A) The cells were then treated with or without IGF-1 (20 nM) for 0–4 h. (B) The cells were pretreated with or without LY294002 (20 μM) for 1 h, and then treated with IGF-1 (20 nM) for a further 0–4 h. 25HC (2.5 μM) served as a positive control. Whole cell lysates from (A) and (B) were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were probed for SREBP-2, pAkt, Akt, and α-tubulin. P and M denote the precursor and mature forms of SREBP-2, respectively. Western blots are representative of three separate experiments, and the relative intensity (M) value represents the densitometric quantification of the mature form of SREBP-2, where the 1 h + IGF-1 condition has been set to 1.

*3.2. PI3K inhibition blunts IGF-1-induced SREBP-2 activation*

To determine if IGF-1 stimulates SREBP-2 via PI3K within this time-frame, cells were pretreated with a pharmacological inhibitor of PI3K, LY294002 [30], for 1 h to repress basal PI3K activity before treating with IGF-1 for a further 0–4 h (Fig. 1B). An oxygenated sterol, 25HC, was included as a positive control as it is known to potently inhibit SREBP-2 activation [31]. LY294002 suppressed the IGF-1 stimulation of pAkt and SREBP-2 activation, while the inactive analogue, LY303511 [32], had no effect (Supplementary Fig. 1A). Another selective PI3K inhibitor, wortmannin, also effectively reduced both Akt and SREBP-2 activation (Supplementary Fig. 1B). These results demonstrate that IGF-1 increases SREBP-2 activation acutely via PI3K, potentially via Akt.

*3.3. A specific Akt inhibitor reduces SREBP-2 levels*

Although the major downstream effector of PI3K is Akt, LY294002 can also affect other targets downstream of PI3K [16]. To investigate the role of Akt in IGF-1-mediated SREBP-2 activation, a specific pharmacological inhibitor of Akt was utilised — Akt inhibitor VIII, isozyme-selective, also called Akti-1/2 [33]. This compound binds to the Pleckstrin Homology domain of Akt to prevent its activation, and has become one of the Akt inhibitors of choice [34]. Akt inhibitor VIII demonstrated a complete inhibition of Akt activation, and also decreased SREBP-2. Both the mature transcription factor as well as

the precursor were affected (Fig. 2A). Notably, SREBP-2 mRNA was unaffected by Akt inhibitor VIII treatment in this time frame (data not shown).

To determine if Akt inhibitor VIII increased SREBP-2 turnover, we inhibited proteasomal degradation with MG132. This did not rescue the precursor, but partially rescued mature SREBP-2, consistent with accelerated proteasomal degradation of the active form of SREBP-2 when Akt is inhibited (Fig. 2B).

As a complementary measurement of SREBP-2 transport from the ER to the Golgi, CHO cells stably expressing the SREBP escort protein, Scap, fused to GFP [18] were employed. These cells exhibit normal cholesterol homeostasis, and allow for convenient visualisation of the localisation of SREBP-2, which colocalises with Scap. When pretreated with Akt inhibitor VIII, cells tended to exhibit a diffuse, reticular pattern that is typical of ER localisation similar to the non-treated condition (Fig. 2C). This pattern is distinct from the characteristic Golgi juxtanuclear fluorescence of the IGF-1 alone treatment. This result is consistent with the inhibition of Akt disrupting ER-to-Golgi transport of SREBP-2 as seen in Fig. 2B, where there was a decrease in mature SREBP-2.

### 3.4. Additional Akt inhibitors also reduce SREBP-2 activation

It is recommended that the cellular effects of kinase inhibition should be observed with two structurally-unrelated kinase inhibitors [35]. Therefore, two additional Akt inhibitors were used to determine the correlation between acutely inhibiting Akt activity and SREBP-2 activity (1 h). Akt inhibitor IV and V were selected, as they do not affect PI3K, unlike other commercially available inhibitors such as Akt inhibitor I, II and III, which are analogues of phosphatidylinositol [36].

When used at previously published concentrations [37], Akt inhibitor IV, V, and VIII all decreased pAkt and mature SREBP-2 (Fig. 3A). Mature SREBP-2 protein levels mirrored SREBP-2 transcriptional activity, with Akt inhibitors IV and V also downregulating two SREBP-2 target genes, *LDLR* and *HMGCR* (Fig. 3B). Akt inhibitor VIII had a marginal effect, which approached statistical significance ($p = 0.077$ for *HMGCR*, student's paired *t*-test). Importantly, we confirmed these results in a human liver cell-line, HepG2, using the inhibitor with the greatest effect on Akt and SREBP-2 activation, Akt inhibitor IV (Supplementary Fig. 2).

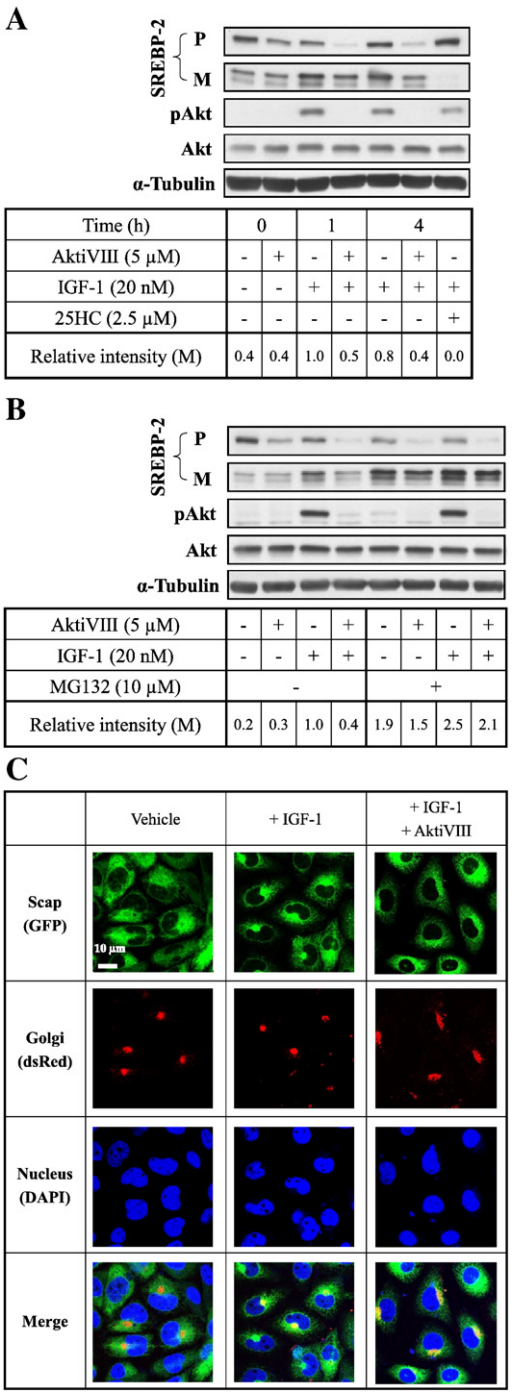
Overall, pharmacological inhibitors indicated that inhibiting Akt resulted in a concomitant reduction in mature SREBP-2 levels and downstream transcriptional activity.

### 3.5. Silencing Akt using siRNA decreases IGF-1-induced SREBP-2 activity

To complement our pharmacological inhibitors, we utilised a more specific molecular approach; gene silencing to knock down endogenous Akt expression. IGF-1-stimulated SREBP-2 activation was blunted when Akt was knocked down (Fig. 4A). Once again, this strengthens the link between Akt and SREBP-2 activation.

### 3.6. Rapid activation of Akt increases SREBP-2 activation

Our results thus far have focused on Akt inhibition approaches, and have relied on activating Akt with a growth factor, IGF-1, through a signalling pathway. Therefore, we employed a more specific and rapid system for activating Akt, similar to approaches used in previous studies [38,39]. Briefly, we cloned a bi-directional CMV-driven vector encoding FRB-Akt-Myc and myristoylated (Myr)-2xFKBP-HA (Supplementary Fig. 3A). This uses rapalog (a non-immunosuppressive derivative of rapamycin) to induce the heterodimerisation of the FRB and FKBP fragments. We stably expressed the construct in a CHO-7 Flp-In cell-line. Under basal conditions, FKBP is anchored to the plasma membrane by the Myr signal while FRB-Akt-Myc is cytoplasmic (Supplementary Fig. 3B). When rapalog is added, it binds to




| Time (h) | 0 | | 1 | | 4 | | |
|---|---|---|---|---|---|---|---|
| AktiVIII (5 μM) | - | + | - | + | - | + | - |
| IGF-1 (20 nM) | - | - | + | + | + | + | + |
| 25HC (2.5 μM) | - | - | - | - | - | - | + |
| Relative intensity (M) | 0.4 | 0.4 | 1.0 | 0.5 | 0.8 | 0.4 | 0.0 |

| AktiVIII (5 μM) | - | + | - | + | - | + | - | + |
|---|---|---|---|---|---|---|---|---|
| IGF-1 (20 nM) | - | - | + | + | - | - | + | + |
| MG132 (10 μM) | - | | | | + | | | |
| Relative intensity (M) | 0.2 | 0.3 | 1.0 | 0.4 | 1.9 | 1.5 | 2.5 | 2.1 |

**Fig. 2.** A specific Akt inhibitor decreased SREBP-2, and ER-to-Golgi trafficking of Scap. (A) and (B) CHO-7 cells were seeded and serum-starved overnight. (A) The cells were then pretreated with or without Akt inhibitor VIII (AktiVIII; 5 μM) for 1 h, and then treated with or without IGF-1 (20 nM) for a further 0–4 h. 25HC (2.5 μM) served as a positive control. (B) Cells were pretreated with or without AktiVIII (5 μM) and/or MG132 (10 μM) for 1 h, and then treated with or without IGF-1 (20 nM) for a further 1 h. Whole cell lysates from (A) and (B) were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were probed for SREBP-2, pAkt, Akt, and α-tubulin. P and M denote the precursor and mature forms of SREBP-2, respectively. Western blots are representative of three separate experiments, and the relative intensity (M) value represents the densitometric quantification of the mature form of SREBP-2, where the +IGF-1 condition has been set to 1. (C) CHO/pGFP-Scap cells were seeded and serum-starved overnight. Cells were then pretreated with or without AktiVIII (5 μM) for 1 h, followed by IGF-1 treatment (20 nM) for 4 h. Cells were fixed and mounted on slides with mounting buffer. Fluorescence microscopy was performed. Representative of at least three fields of view per condition.

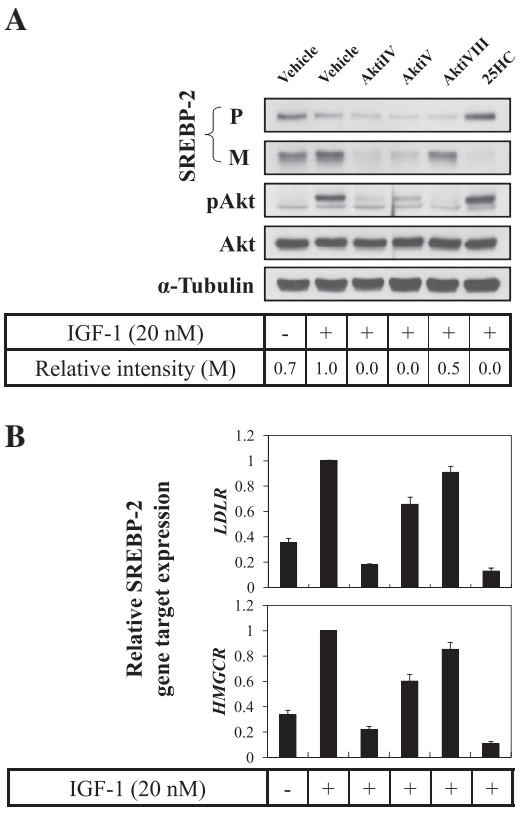



**Fig. 3.** Inhibitors of Akt decrease SREBP-2 activation and target gene expression. CHO-7 cells were seeded, serum-starved overnight, and then pretreated with the following inhibitors for 1 h: AktiIV (10 μM), AktiV (5 μM), AktiVIII (5 μM), or 25HC (2.5 μM). The cells were then treated with or without IGF-1 (20 nM) for a further 1 h (for protein harvest), or 2 h (for mRNA harvest). (A) Whole cell lysates were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were probed for SREBP-2, pAkt, Akt, and α-tubulin. P and M denote the precursor and mature forms of SREBP-2, respectively. Western blots are representative of three separate experiments, and the relative intensity (M) value represents the densitometric quantification of the mature form of SREBP-2, where the + IGF-1 condition has been set to 1. (B) Total RNA was harvested and reverse transcribed to cDNA, and gene expression levels of the *LDLR* and *HMGCR* were quantified using qRT-PCR, and normalised to the housekeeping gene, *PBGD*. Data are presented relative to the + IGF-1 condition which has been set to 1. Data were from 3 to 5 separate experiments, each performed in triplicate, and are presented as mean + SEM.

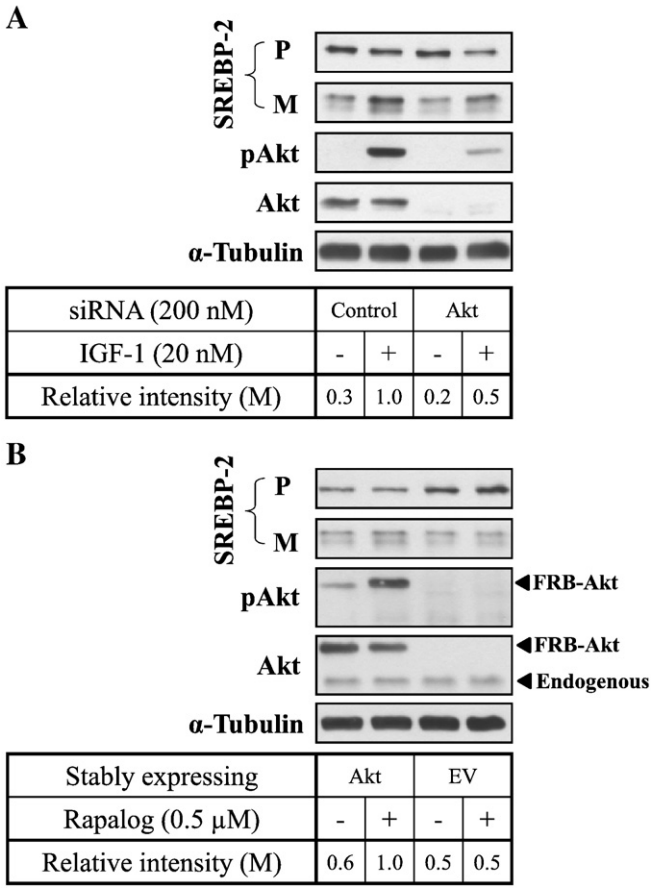



**Fig. 4.** Akt siRNA decreased IGF-1-induced SREBP-2 activation, and rapalog-activated Akt increased SREBP-2 activation. (A) CHO-7 cells were seeded, transfected with control siRNA (200 nM) or Akt siRNA (200 nM), fresh media was added every 24 h for 48 h, and then cells were serum-starved overnight. The cells were treated with or without IGF-1 (20 nM) for a further 1 h. (B) Stable cell-lines (expressing Akt or empty vector; EV) were seeded, serum-starved overnight, and then treated with or without rapalog (0.5 μM) for 1 h. Whole cell lysates from (A) and (B) were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were probed for SREBP-2, pAkt, Akt, α-tubulin. For (B), the bands corresponding to the 56 kDa endogenous pAkt/Akt and the ~75 kDa FRB-Akt are as indicated. P and M denote the precursor and mature forms of SREBP 2, respectively. Western blots are representative of at least two separate experiments, and the relative intensity (M) value represents the densitometric quantification of the mature form of SREBP-2, where the maximal condition (lane 2 for each panel) has been set to 1.

the FKBP that is anchored to the membrane, and FRB-Akt-Myc is brought to the membrane in close proximity to its activating proteins (PDK1 and mTOR complex 2), thereby activating Akt in a targeted manner.

Indeed, rapalog activated FRB-Akt-Myc in the stable cell-line (Fig. 4B). Importantly, SREBP-2 was also activated upon rapalog addition in the FRB-Akt-Myc stable cell-line and not the empty vector control cell-line. These results provide another line of evidence that Akt activates SREBP-2 acutely.

## 4. Discussion

Recent evidence suggests that PI3K/Akt activates the SREBPs, master transcriptional regulators of lipid metabolism [7]. Most studies have focused on SREBP-1c, involved in fatty acid metabolism. The link between PI3K/Akt and the predominant isoform involved in cholesterol metabolism, SREBP-2, is less well-defined. Therefore, this investigation aimed to expand our knowledge in this area, and here we have strengthened and extended previous studies (including our own [14]) in several ways. Firstly, instead of relying on proxy measures, we have determined mature SREBP-2 directly by Western blotting throughout. Secondly, we have used a key growth factor, IGF-1, which is well-documented to signal through Akt [17]. Thirdly, we have minimised the chances of pleiotropic effects, by studying acute time-points. Finally, we have used a multitude of pharmacological and molecular tools to induce and reduce Akt activation.

Our major finding is that activation of the Akt pathway positively modulates SREBP-2 activation acutely. Pharmacologically inhibiting PI3K (LY294002 and wortmannin) or Akt (Akt inhibitors IV, V and VIII) reduced IGF-1-induced SREBP-2 activation, indicating the involvement of the PI3K/Akt pathway (Figs. 1, 2, 3, and Supplementary Fig. 1). By using three Akt inhibitors that have distinct structures and differing modes of action, we have ensured that the effects we have observed are in fact due to Akt inhibition, and not artefactual. A genetic approach of silencing Akt with siRNA confirmed the correlation between Akt and SREBP-2 activation (Fig. 4A). Furthermore, the use of the rapalog heterodimerisation system further strengthened the finding that activating Akt causes SREBP-2 activation (Fig. 4B). As well as inhibiting the formation of mature SREBP-2, downstream gene targets were also regulated by Akt (Fig. 3B). The effects of Akt inhibitor on SREBP-2 mature protein levels mirrored the downregulation of

SREBP-2 target genes (*LDLR* and *HMGCR*), consistent with SREBP-2 activity being regulated by Akt.

By using multiple, independent lines of evidence we have comprehensively indicated that the Akt pathway upregulates the SREBP-2 pathway. The interplay between these two pathways is reasonable, given that Akt is involved in cell growth and proliferation, and SREBP-2 is needed for cholesterol production, which in turn is required for new membranes for cell growth. Akt is a positive effector which can amplify this process whereas sterols are the over-riding negative regulator. Accordingly, 25HC ablated SREBP-2 activation when Akt was stimulated by IGF-1 (Figs. 1B, 2A, 3, and Supplementary Fig. 2).

The molecular mechanism by which Akt activates SREBP-2 is controversial, as discussed elsewhere [7]. Proposed mechanisms include increased trafficking and processing [14] and reduced degradation of SREBP [40]. We have evidence for the involvement of ER-to-Golgi transport of SREBP-2, in that IGF-1-dependent traffic of the SREBP-2 escort protein Scap was impeded by Akt inhibition (Fig. 2C). While sterol addition effectively abolished mature SREBP-2 with an accompanying increase in SREBP-2 precursor, Akt inhibition generally reduced both precursor and mature forms. This suggests that Akt's effect, unlike that of sterols, is not solely due to reduced SREBP-2 processing. For example, there was some suggestion that proteasomal inhibition stabilised the mature form of SREBP-2 in response to Akt inhibition, consistent with reduced degradation (Fig. 2B), as observed for SREBP-1a and -1c [40].

The precise target(s) of Akt that affects SREBP-2 remains elusive. We have recently shown that the coatomer protein II cargo selection protein Sec24, involved in the transport of the SREBP-2/Scap complex from the ER to the Golgi [12] is phosphorylated by Akt [41]. However, we have been unable to demonstrate that Sec24 phosphorylation by Akt contributes to the increased SREBP-2 activation observed. A signalling hub downstream of Akt, mTOR Complex 1, is involved in SREBP-1c activation [42,43], but does not appear to mediate SREBP-2 activation, at least in this system, since the inhibitor of this complex, rapamycin, did not affect IGF-1 stimulated SREBP-2 processing in CHO cells [44].

Taken together, our data provide persuasive evidence that Akt influences SREBP-2 activation. Considering that Akt and lipids play crucial roles in a number of diseases, including diabetes, viral infections and cancer (reviewed in [7]), an Akt–SREBP-2 link may yield fresh perspectives into human health and disease. Further research is required to identify the Akt effector(s) and how they interact with SREBP-2 to influence its activity.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.09.017.

## Acknowledgements

We thank Gorjana Mitic for her initial work on the CHO-7 Flp-In cell-lines. Thanks also to the members of the Brown Lab for critically reviewing this manuscript. The Brown Lab is supported by grants from the National Health and Medical Research Council (568619), and the Prostate Cancer Foundation of Australia (PG2710).

## References

[1] B.D. Manning, L.C. Cantley, AKT/PKB signaling: navigating downstream, Cell 129 (2007) 1261–1274.
[2] D.P. Brazil, Z.Z. Yang, B.A. Hemmings, Advances in protein kinase B signalling: AKTion on multiple fronts, Trends Biochem. Sci. 29 (2004) 233–242.
[3] D.R. Alessi, M. Deak, A. Casamayor, F.B. Caudwell, N. Morrice, D.G. Norman, P. Gaffney, C.B. Reese, C.N. MacDougall, D. Harbison, A. Ashworth, M. Bownes, 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase, Curr. Biol. 7 (1997) 776–789.
[4] D.D. Sarbassov, D.A. Guertin, S.M. Ali, D.M. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex, Science 307 (2005) 1098–1101.
[5] A. Bellacosa, C.C. Kumar, A. Di Cristofano, J.R. Testa, Activation of AKT kinases in cancer: implications for therapeutic targeting, Adv. Cancer Res. 94 (2005) 29–86.
[6] T.F. Franke, PI3K/Akt: getting it right matters, Oncogene 27 (2008) 6473–6488.
[7] J.R. Krycer, L.J. Sharpe, W. Luu, A.J. Brown, The Akt-SREBP nexus: cell signaling meets lipid metabolism, Trends Endocrinol. Metab. 21 (2010) 268–276.
[8] J.D. Horton, N.A. Shah, J.A. Warrington, N.N. Anderson, S.W. Park, M.S. Brown, J.L. Goldstein, Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 12027–12032.
[9] J.L. Goldstein, R.A. DeBose-Boyd, M.S. Brown, Protein sensors for membrane sterols, Cell 124 (2006) 35–46.
[10] A.J. Brown, L. Sun, J.D. Feramisco, M.S. Brown, J.L. Goldstein, Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism, Mol. Cell 10 (2002) 237–245.
[11] T. Yang, P.J. Espenshade, M.E. Wright, D. Yabe, Y. Gong, R. Aebersold, J.L. Goldstein, M.S. Brown, Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER, Cell 110 (2002) 489–500.
[12] P.J. Espenshade, W.P. Li, D. Yabe, Sterols block binding of COPII proteins to SCAP, thereby controlling SCAP sorting in ER, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 11694–11699.
[13] A. Nohturfft, S.C. Zhang, Coordination of lipid metabolism in membrane biogenesis, Annu. Rev. Cell Dev. Biol. 25 (2009) 539–566.
[14] X. Du, I. Kristiana, J. Wong, A.J. Brown, Involvement of Akt in ER-to-Golgi transport of SCAP/SREBP: a link between a key cell proliferative pathway and membrane synthesis, Mol. Biol. Cell 17 (2006) 2735–2745.
[15] B. Vanhaesebroeck, D.R. Alessi, The PI3K-PDK1 connection: more than just a road to PKB, Biochem. J. 346 (Pt 3) (2000) 561–576.
[16] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, Biochem. J. 351 (2000) 95–105.
[17] D.R. Alessi, M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen, B.A. Hemmings, Mechanism of activation of protein kinase B by insulin and IGF-1, EMBO J. 15 (1996) 6541–6551.
[18] A. Nohturfft, D. Yabe, J.L. Goldstein, M.S. Brown, P.J. Espenshade, Regulated step in cholesterol feedback localized to budding of SCAP from ER membranes, Cell 102 (2000) 315–323.
[19] J.L. Goldstein, S.K. Basu, M.S. Brown, Receptor-mediated endocytosis of low-density lipoprotein in cultured cells, Methods Enzymol. 98 (1983) 241–260.
[20] J. Yang, M.S. Brown, Y.K. Ho, J.L. Goldstein, Three different rearrangements in a single intron truncate sterol regulatory element binding protein-2 and produce sterol-resistant phenotype in three cell lines. Role of introns in protein evolution, J. Biol. Chem. 270 (1995) 12152–12161.
[21] J. Wong, C.M. Quinn, I.C. Gelissen, A.J. Brown, Endogenous 24(S),25-epoxycholesterol fine-tunes acute control of cellular cholesterol homeostasis, J. Biol. Chem. 283 (2008) 700–707.
[22] D. Yabe, M.S. Brown, J.L. Goldstein, Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 12753–12758.
[23] M.D. Abramoff, P.J. Magalhaes, S.J. Ram, Image processing with ImageJ, Biophot. Int. 11 (2004) 36–42.
[24] D. Kielar, W. Dietmaier, T. Langmann, C. Aslanidis, M. Probst, M. Naruszewicz, G. Schmitz, Rapid quantification of human ABCA1 mRNA in various cell types and tissues by real-time reverse transcription-PCR, Clin. Chem. 47 (2001) 2089–2097.
[25] J. Wong, C.M. Quinn, A.J. Brown, Statins inhibit synthesis of an oxysterol ligand for the liver x receptor in human macrophages with consequences for cholesterol flux, Arterioscler. Thromb. Vasc. Biol. 24 (2004) 2365–2371.
[26] H.E. Klock, E.J. Koesema, M.W. Knuth, S.A. Lesley, Combining the polymerase incomplete primer extension method for cloning and mutagenesis with microscreening to accelerate structural genomics efforts, Proteins 71 (2008) 982–994.
[27] S. Dimmeler, I. Fleming, B. Fisslthaler, C. Hermann, R. Busse, A.M. Zeiher, Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation, Nature 399 (1999) 601–605.
[28] J.E. Metherall, J.L. Goldstein, K.L. Luskey, M.S. Brown, Loss of transcriptional repression of three sterol-regulated genes in mutant hamster cells, J. Biol. Chem. 264 (1989) 15634–15641.
[29] S. Gill, J. Stevenson, I. Kristiana, A.J. Brown, Cholesterol-dependent degradation of squalene monooxygenase, a control point in cholesterol synthesis beyond HMG-CoA reductase, Cell Metab. 13 (2011) 260–273.
[30] C.J. Vlahos, W.F. Matter, K.Y. Hui, R.F. Brown, A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), J. Biol. Chem. 269 (1994) 5241–5248.
[31] X. Du, Y.H. Pham, A.J. Brown, Effects of 25-hydroxycholesterol on cholesterol esterification and sterol regulatory element-binding protein processing are dissociable: implications for cholesterol movement to the regulatory pool in the endoplasmic reticulum, J. Biol. Chem. 279 (2004) 47010–47016.
[32] J. Ding, C.J. Vlahos, R. Liu, R.F. Brown, J.A. Badwey, Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils, J. Biol. Chem. 270 (1995) 11684–11691.
[33] S.F. Barnett, D. Defeo-Jones, S. Fu, P.J. Hancock, K.M. Haskell, R.E. Jones, J.A. Kahana, A.M. Kral, K. Leander, L.L. Lee, J. Malinowski, E.M. McAvoy, D.D. Nahas, R.G. Robinson, H.E. Huber, Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors, Biochem. J. 385 (2005) 399–408.
[34] J. Bain, L. Plater, M. Elliott, N. Shpiro, C.J. Hastie, H. McLauchlan, I. Klevernic, J.S. Arthur, D.R. Alessi, P. Cohen, The selectivity of protein kinase inhibitors: a further update, Biochem. J. 408 (2007) 297–315.

[35] P. Cohen, Guidelines for the effective use of chemical inhibitors of protein function to understand their roles in cell regulation, Biochem. J. 425 (2010) 53–54.
[36] Y. Hu, L. Qiao, S. Wang, S.B. Rong, E.J. Meuillet, M. Berggren, A. Gallegos, G. Powis, A.P. Kozikowski, Kozikowski, 3-(hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth, J. Med. Chem. 43 (2000) 3045–3051.
[37] J.C. Resendiz, M.H. Kroll, R. Lassila, Protease-activated receptor-induced Akt activation–regulation and possible function, J. Thromb. Haemost. 5 (2007) 2484–2493.
[38] Y. Ng, G. Ramm, J.A. Lopez, D.E. James, Rapid activation of Akt2 is sufficient to stimulate GLUT4 translocation in 3T3-L1 adipocytes, Cell Metab. 7 (2008) 348–356.
[39] M.P. Scheid, P.A. Marignani, J.R. Woodgett, Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B, Mol. Cell. Biol. 22 (2002) 6247–6260.
[40] A. Sundqvist, M.T. Bengoechea-Alonso, X. Ye, V. Lukiyanchuk, J. Jin, J.W. Harper, J. Ericsson, Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF(Fbw7), Cell Metab. 1 (2005) 379–391.
[41] L.J. Sharpe, W. Luu, A.J. Brown, Akt phosphorylates Sec24: new clues into the regulation of ER-to-Golgi trafficking, Traffic 12 (2011) 19–27.
[42] T. Porstmann, C.R. Santos, B. Griffiths, M. Cully, M. Wu, S. Leevers, J.R. Griffiths, Y.L. Chung, A. Schulze, SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth, Cell Metab. 8 (2008) 224–236.
[43] T.R. Peterson, S.S. Sengupta, T.E. Harris, A.E. Carmack, S.A. Kang, E. Balderas, D.A. Guertin, K.L. Madden, A.E. Carpenter, B.N. Finck, D.M. Sabatini, mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway, Cell 146 (2011) 408–420.
[44] L.J. Sharpe, A.J. Brown, Rapamycin down-regulates LDL-receptor expression independently of SREBP-2, Biochem. Biophys. Res. Commun. 373 (2008) 670–674.