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Akt acutely activates the cholesterogenic transcription factor SREBP-2

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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

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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