Insulin Regulates Hepatic Apolipoprotein B Production Independent of the Mass or Activity of Akt1/PKBα

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Insulin is known to be a downregulator of apolipoprotein B (apoB) via the phosphatidylinositol 3-kinase (PI3K) pathway. Akt, also known as protein kinase B (PKB), is a serine/threonine kinase downstream target of PI3K. Recent studies in the fructose-fed hamster model of insulin resistance have shown that hepatic very-low-density lipoprotein (VLDL) secretion is associated with reduced phosphorylation of Akt, suggesting a potential link between Akt expression and/or activity and apoB production in hepatocytes. We hypothesized that overexpression of Akt1 downregulates apoB production. An expression vector with a constitutively active form of Akt1 was transfected in the rat hepatoma McArdle cells (McA RH-7777), McA cells stably expressing human apoB-15 and apoB-48 (15% and 48% of total apoB length), and human hepatoma HepG2. The overexpressed Akt1 was phosphorylated at Ser473 independent of acute insulin stimulation, suggesting that it was catalytically active. Despite dosage-dependent overexpression of Akt1 in both McA and HepG2 cells, neither intracellular nor secreted protein mass of intact apoB or transfected human apoB-15/apoB-48 was significantly affected by high intracellular levels of Akt1. Radiolabeling experiments also yielded no difference in the amount of newly synthesized apoB when comparing transfected and mock-transfected cells. Transfection in conjunction with high-dose insulin did not significantly decrease the secretion of either apoB-100 or apoB-48 in McA cells, or apoB-100 in HepG2 cells. HepG2 cells were more sensitive to the inhibitory effects of insulin on apoB secretion compared to McA cells, but neither model responded to Akt1. Overall, the data suggest that acute insulin-mediated inhibition of apoB may not be mediated by Akt1 and that insulin signaling molecules upstream of Akt1 may be more important in mediating control of apoB secretion.

APOLIPOPROTEIN B (apoB) is an essential component of the highly atherogenic very-low-density lipoproteins (VLDL) (reviewed in).[1] In fact, apoB and VLDL are assembled in a 1:1 ratio,[2] and therefore the amount of apoB is directly correlated with the level of atherosclerotic particles in circulation. It has been well established that insulin is a key regulator of apoB in various models,[3-10] although the exact mechanism remains unknown. It is believed that apoB is not regulated to a large extent at the transcriptional level as the cellular levels of apoB mRNA seemed unaffected by various stimuli including insulin.[8,11] Studies in rat hepatocytes first shed light on the mechanism of insulin inhibition of apoB secretion. Using the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin, the insulin effect on apoB secretion was abolished, suggesting that insulin inhibits apoB secretion via the PI3K pathway.[12] In addition, insulin increased PI3K activity associated with insulin receptor substrate-1 (IRS-1) and significantly increased the amount of PI3K present in endoplasmic reticulum (ER), the location of apoB biogenesis.[13] Furthermore, brefeldin A, an inhibitor of protein transport from ER to Golgi, inhibited apoB secretion, suggesting that the export of apoB from ER was required in the insulin effect.[12]

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase downstream target of PI3K.[14,15] This kinase has multiple roles in the cell, including the mediation of insulin response and cell survival signalling.[16] Three isoforms have been identified thus far in the mammalian cells: Akt1,[17,18] Akt2,[19,20] and Akt3.[21,22] All three isoforms are expressed ubiquitously but differentially in tissues and during developmental stages.[16] Activation pattern of the isoforms also differs depending on the tissue.[23] At present, the biological significance of these isoforms is unclear.

At the N-terminal region of Akt there is a pleckstrin homology (PH) domain, which serves as a membrane-targeting module.[24] The PH domain has been shown to bind to phospholipids,[25,26] specifically phosphatidylinositol-3,4,5-trisphosphate [PtdIn(3,4,5)P_3][27] and phosphatidylinositol-3,4-bisphosphate [PtdIn(3,4)P_2].[28-30] For Akt1, phosphorylation at 2 residues, Thr308 and Ser473, must occur to activate the kinase.[31] The first step towards activation is the translocation of Akt to the plasma membrane in a 3′ phosphoinositide lipid-dependent manner, such as that elicited by insulin-like growth factor-1 (IGF-1)[24] or insulin.[32,33] The translocation to the plasma membrane promotes Ser473 phosphorylation, which is in turn necessary for PDK1-mediated phosphorylation of Thr308.[34] The necessity for translocation to occur has been exploited in construction of a constitutively active Akt, which contains a src myristoylation sequence directing the kinase to the plasma membrane.[32,35]

Many studies support the idea that Akt plays an essential role in insulin signaling via the PI3K pathway. In 3T3-L1 adipocytes, a constitutively active Akt could stimulate glucose uptake and translocation of GLUT4.[32,35] Single-gene knockout mice deficient in Akt2 are impaired in many aspects of insulin signaling, exemplified by high blood glucose despite high serum insulin, low glucose disposal, and decreased rate of glucose uptake.[36]

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In insulin resistance, a pathological state in which response to physiological insulin levels is perturbed, there are apparent defects in signaling including decreases in insulin receptor mass and kinase activity, and in the amount and phosphorylation of IRS-1 and -2, PI3K activity, glucose transporter translocation, and the activity of intracellular enzymes.[37] In the fructose-fed hamster, an animal model of insulin resistance and dyslipidemia, hepatic Akt phosphorylation for both Ser473 and Thr308 was significantly reduced relative to controls upon insulin stimulation, while the protein expression was increased due to compensation.[38] Reduced Akt phosphorylation in the fructose-fed hamster hepatocytes was accompanied by a significant increase in VLDL–apoB secretion.[38] Decreased Akt activity has also been found in the insulin-resistant C57BL/Ksl-Lepr *db/db* mice[39] and glucose-induced insulin resistance appears to be associated with defective Akt activation.[40] However, fatty acid–induced insulin resistance does not seem to affect Akt phosphorylation.[41]

Given the evidence that Akt is important in insulin signaling via the PI3K pathway and that the regulation of apoB by insulin is also PI3K-dependent, it is feasible that Akt activation may mediate the insulin modulation of apoB. As Akt1 is the most studied isoform, and evidence suggests that incubation of rat hepatocytes with insulin activates primarily Akt1 with very small effects on Akt2 and no effect on Akt3,[23] Akt1 was chosen as the isoform for the present study. Therefore, the primary goal of this study was to overexpress a constitutively active Akt1 and determine the effects on apoB production. We hypothesized that overexpression of Akt1 would downregulate apoB synthesis and secretion in the rat hepatoma McArdle (McA) RH-7777, as well as in human hepatoma HepG2 cells.

MATERIALS AND METHODS

McA RH-7777 and HepG2 cells were obtained from ATCC (Manassas, VA). Alpha minimal essential medium (αMEM) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Wisent (Montreal, Canada). DMEM and αMEM without methionine, cysteine or glutamine were bought from Sigma (Oakville, Canada). Certified grade fetal bovine serum (FBS), G418 antibiotic, and Lipofectamine reagent were supplied by Invitrogen Life Technologies (Burlington, Canada). Bovine serum albumin, phenylmethyl sulfonyl fluoride (PMSF), and trypsin (tissue culture grade) were from Sigma. Trasylol (aprotinin) was acquired from Bayer (Etobicoke, Canada). [^{35}S]methionine protein labeling mixtures were purchased from PerkinElmer Life Sciences (Woodbridge, Canada). Prestained protein standards (rainbow markers) and Amplify were from Amersham (Baie d'Urfe, Canada). All chemicals used for sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE) were from BioRad (Mississauga, Canada). Polyclonal apoB antibodies were obtained from Midland Bio-Products (Boone, IA). Zysorbin was from Zymed Laboratories (Markham, Canada). Rabbit anti-mouse Akt and rabbit anti-phosphoserine 473 Akt were obtained from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit IgG–horse radish peroxidase (HRP) secondary antibody was purchased from Sigma. Polyvinylidene difluoride (PVDF) membrane was obtained from PerkinElmer Life Science.

Cell Culture

McA RH-7777 and stably transfected McA RH-7777 cells were grown in culture flasks at 37°C, 5% CO_2 in DMEM supplemented with 20% FBS and 100 U penicillin and 100 U streptomycin. For the stably transfected cells, 250 μg/mL of G418 was also added to the media. Cells were passaged every 3 to 4 days. HepG2 cells were grown in culture flasks at 37°C, 5% CO_2 in αMEM supplemented with 10% FBS and 100 U penicillin and 100 U streptomycin.

Transfection With Akt1 Construct

For experiments involving the transfection of cells with the Akt1 construct, cells were seeded at a density of 5×10^5 per 35-mm well in a 6-well plate the day before transfection. For each transfection, 1 to 4 μg of plasmid DNA in 100 μL of media was mixed with 15 μL of Lipofectamine reagent in 100 μL media and incubated for 30 minutes at room temperature. During that time, the media for the cells were changed to serum-free media. After the 30-minute incubation, the mixture was added to the cells and incubated for 4 to 5 hours at 37°C. The media was then changed back to serum-containing media with the appropriate antibiotics and allowed to grow to 100% confluency (48 hours).

SDS-PAGE and Immunoblotting

For experiments involving insulin treatment, 100 nmol/L of insulin was added directly into the well and incubated for the designated period of time. If blotting for proteins other than phospho-Ser473-Akt, cells were lysed in cell solubilizing buffer (phosphate-buffered saline containing 1% Nonidet P-40, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L PMSF, 10 μg/mL aprotinin). For phosphorylated Akt, cells were lysed in special solubilization buffer containing a phosphatase inhibitor mixture (150 mmol/L NaCl, 10 mmol/L tris [hydroxymethyl]aminomethane pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L PMSF, 10 μg/mL aprotinin, 100 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, and 2 mmol/L sodium vanadate).

The mini-gels (8×5 cm) were composed of 4% stacking and 8% resolving gels. Following electrophoresis, proteins were transferred electrophoretically onto PVDF membranes using a BioRad Wet Transfer System. The membranes were blocked with 5% fat-free dry milk powder solution and then incubated with primary antibody. After washing, the membranes were then incubated with a secondary antibody conjugated to peroxidase. After another round of washing, membranes were incubated in an enhanced chemiluminescence (ECL) detection reagent for 1 minutes and exposed to Kodak X-Omat Blue XB-1 film (Eastman Kodak, Rochester, NY). Films were developed and quantitative analysis was performed using densitometry. Determination of the molecular weight of electrophoretically separated proteins was based on comparison with prestained markers of known molecular weight.

Measurement of Akt1 Activity

Akt phosphotransferase activity of McA cells stimulated with insulin was measured using an immunoprecipitation kinase assay kit according to manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). McA cells were incubated for 2 minutes in complete (c)DMEM with and without added 100 nmol/L insulin prior to lysis in ice-cold buffer containing 1 μmol/L microcystin to ensure complete inactivation of phosphatases. As a positive control, L6 myotubes were stimulated for 2 minutes with 15 nmol/L IGF-1 prior to lysis. Akt1 was immunoprecipitated from each lysate by incubation with anti-Akt/PKB, PH domain SKB1. This antibody recognizes the PH of Akt1 and shows 30% cross-reactivity to the PH domain of Akt2. In parallel incubations, nonimmune mouse IgG was used to assess nonspecific binding. Active enzyme immunocomplexes were isolated by centrifugation and washed extensively followed by incubation with Akt/SGK-specific substrate peptide and [γ-^{32}P]adenosine triphosphate (ATP) ($\approx$3,000 Ci/mmol) for 10 minutes at 30°C with continuous shaking. Reactions were terminated by addition of trichloroacetic acid (TCA;

final 13.3% vol/vol), and phosphorylated substrate present in the supernatant was isolated by binding to phosphocellulose filters. After extensive washing, filters were radioassayed by β-scintillation counting. Nonspecific binding radioactivity was subtracted from each sample and results were expressed as fold increase in label incorporation of cells incubated with agonist versus no agonist.

Metabolic Labeling and Immunoprecipitation

For metabolic labeling and immunoprecipitation experiments, cells were preincubated in methionine-free media at 37°C for 1 hour and pulsed with 50 to 100 μCi/mL of [^{35}S]methionine for 15 minutes to 1 hour. For experiments involving oleate treatment, the preincubation media also contained 360 μmol/L oleate: bovine serum albumin (BSA) in 8:1 molar ratio. At various time points, the media were either collected or discarded. The cells were then washed twice with PBS. Chase media (20% FBS DMEM + 10 mmol/L methionine with 360 μmol/L oleate for McA cells or 10% FBS αMEM + 10 mmol/L methionine for HepG2 cells) were added at this time for pulse-chase experiments and incubated for 2 hours. The media were then collected and cells were lysed in cell solubilization buffer (PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L EGTA, 2 mmol/L PMSF, 10 μg/mL aprotinin). The lysates were clarified by centrifugation and supernatants were collected for immunoprecipitation.

Immunoprecipitation was performed by adding 5 μL of antibody to each sample and incubating overnight at 4°C. Zysorbin (50 μL) was then added to each sample and incubated for 1 hour. The pellets of immunoprecipitates were washed 3 times with wash buffer (10 mmol/L TRIS-HCl pH 7.4, 2 mmol/L EDTA, 0.1% SDS, 1% TritonX-100). Finally, the immunoprecipitates were prepared for SDS-PAGE by resuspending and boiling in SDS-PAGE sample buffer. The gels were composed of 4% stacking and 4.5% or 6% resolving gels. After electrophoresis, the gels were fixed and incubated in Amplify before being dried and exposed to Kodak X-Omat Blue XB-1 film at −80°C for 2 to 72 hours. To determine the radioactivity associated with apoB fragments, the corresponding bands were excised from the gel, digested, and quantified by liquid scintillation counting.

RESULTS

Transfection of Akt1 Construct in McA RH-7777 Resulted in Dosage-Dependent Overexpression

To test our hypothesis that overexpression of Akt1 can regulate apoB expression, we first established the possibility of introducing our Akt1 construct into McA RH-7777 cells by the liposomal method. To assess the success of transfection, we examined the level of Akt1 expression in McA RH-7777 cells. Because the Akt1 insert in the plasmid was tagged with c-myc, using an anti–c-myc antibody detected only the Akt1 that was produced as a result of the transfection. The empty vector pUSEamp(+) was used for mock transfections. Transfection with 0, 0.5, 1.0, or 2.0 μg of plasmid Akt1 showed a dosage-dependent expression of Akt1 after 48 hours, as determined by immunoblotting and densitometry (Fig 1). Relative to transfection with the empty vector for which no c-myc–containing protein was detected, 2 μg of Akt1 plasmid resulted in an expression that was 14-fold higher over gel background. Cell viability was not compromised at 2 μg of plasmid DNA as there was no change in cell morphology or cell number.

Insulin Increased Akt1 Activity in Wild-Type McA Cells

In wild-type McA cells, insulin stimulation resulted in a 12-fold (SD 6.4) increase in Akt1 activity relative to unstimulated cells, which is similar to the positive control of IGF-1–stimulated L6 myotubes that achieved a 11.4-fold (SD 4.1) increase in activity (data not shown). The assay was performed in 3 independent experiments. This established that McA cells were sensitive to insulin. Interestingly, Akt2 activity only increased by 30% upon insulin stimulation (data not shown).

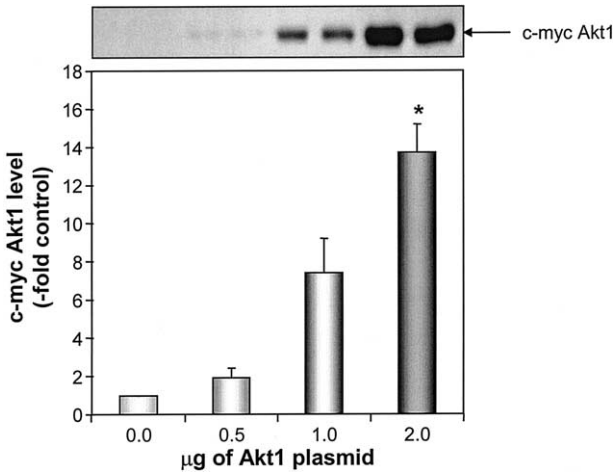

Fig 1. Transfection of Akt1 construct in McA RH-7777 resulted in dosage-dependent overexpression. McA-RH7777 cells were transfected with the c-myc Akt1 plasmid at the indicated concentration for 4 hours using Lipofectamine in DMEM, after which the media was replaced by 20% FBS in DMEM + 250 μg/mL G418. Cells were lysed 48 hours after transfection and subjected to 8% SDS-PAGE and transferred onto a PVDF membrane. Primary antibody used was mouse anti–c-myc (1:1,000). Secondary antibody used was goat anti-mouse IgG-HRP. Mean ± SEM values are from 3 independent experiments in duplicates. *$P < .05$ compared to mock transfection.

Overexpressed Akt1 Was Phosphorylated at Ser473 in Transfected McA Cells Independent of Acute Insulin Stimulation

Using an antibody that detected all isoforms of Akt, a band with a higher than expected apparent weight (perhaps due to the presence of the *c-src* sequence, the myc-tag, and/or the addition of myristic acid) was observed with Akt1 transfection. At 2 μg of the Akt1 construct, the protein level was increased by approximately 12-fold over the endogenous level independent of insulin stimulation (Fig 2A). Even though our Akt1 construct was myristoylated, we needed to determine whether the expressed Akt1 following the transfection was active. Using an anti-phospho-Ser473-Akt antibody, the band with the higher molecular weight was again observed in transfected cells. The endogenous level of phospho-Ser473 Akt was too low to be detected, but the level was 2–2.5 fold higher in the transfected cells over gel background ($P < .05$) (Fig 2B). The addition of 100 nmol/L insulin for 10 minutes did not significantly increase the phosphorylation in either the untransfected or transfected cells (Fig 2B). Data were normalized to micrograms of total protein in the sample.

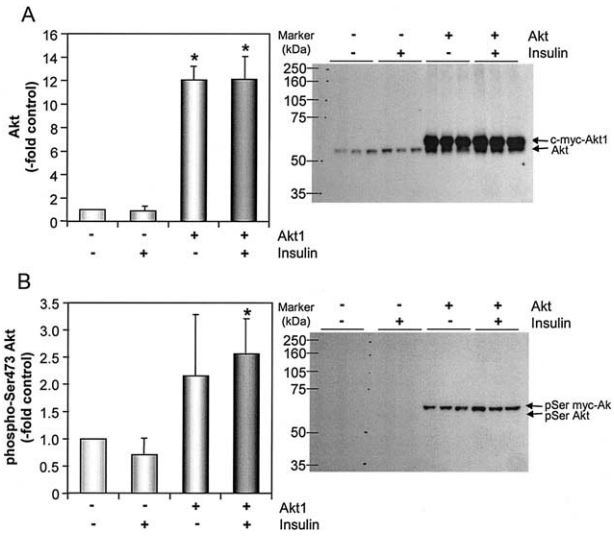

Fig 2. Overexpressed Akt1 is phosphorylated at Ser473 in transfected cells independent of acute insulin stimulation. McA RH-7777 cells were transfected with the empty vector or the c-myc Akt1 plasmid (2 μg of DNA) for 4 hours using Lipofectamine; 48 hours after transfection, 100 nmol/L insulin was added to cells for 10 minutes. Cells were then lysed and subjected to 8% SDS-PAGE and transferred onto a PVDF membrane. Primary antibodies used were rabbit anti-Akt (1:1,000) and rabbit anti-phospho-Ser473 Akt (1:1,000), for graphs on panels A and B, respectively. Secondary antibody used was goat anti-rabbit IgG-HRP. Mean ± SEM values are from 2 independent experiments in triplicates. *P < .05 compared to cells with mock transfection and without insulin treatment.

Overexpression of Akt1 in McA B15 and McA B48 Cells Did Not Affect the Amount of Cellular or Secreted ApoB

Using McARH-7777 cells that were stably expressing human apoB truncated fragments (McA B15 and McA B48, which expressed 15% and 48% of the total apoB DNA length, respectively), immunoblotting was performed to determine whether the overexpression of Akt1 affected the total amount of human apoB constructs produced by the cells. Using the mouse 1D1 antibody, which is specific to N-terminal region of human apoB but not rat apoB, no differences in the level of cellular or secreted apoB-15 (data not shown) or apoB-48 were detected despite dosage-dependent expression of activated Akt1 48 hours after transfection (Fig 3). Data were normalized to total cellular protein in each sample.

Overexpression of Akt1 in McA B15 and McA B48 Cells Did Not Affect the Amount of Newly Synthesized ApoB

Although the immunoblot could detect the total mass of the expressed apoB protein, it could not directly measure the synthesis and secretion of newly synthesized apoB. Insignificant differences in the total mass of apoB may mask small but significant changes in synthesis and/or degradation. Therefore metabolic labeling was performed to determine the status of newly synthesized apoB fragments in the cell and in the media. Lysates of McA B15 or McA B48 cells and their media were immunoprecipitated with goat anti-human apoB antibody after pulsing for 1 hour with 50 μCi/mL [^{35}S]methionine. No differences in the level of cellular or secreted apoB-15 (data not shown) or apoB-48 were detected despite dosage-dependent expression of activated Akt1 48 hours after transfection (Fig 4). Data were normalized to total radiolabeled protein determined by TCA precipitation.

Overexpression of Activated Akt1 in McA Cells With Overnight Insulin Treatment Did Not Affect ApoB Levels

It is known that the synthesis of apoB-100 requires approximately 14 minutes.[42] As such, short-term (10-minute) insulin treatment may not be long enough to affect apoB expression and therefore overnight treatment was attempted. Metabolic labeling was performed to compare the status of newly synthe-

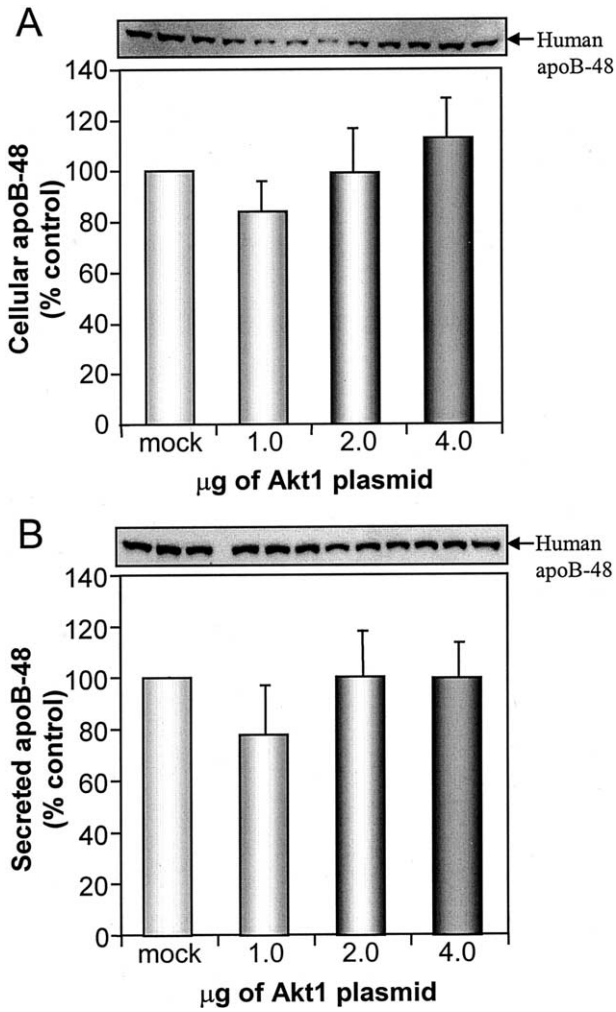

Fig 3. Overexpression of Akt1 in McA B48 cells does not affect the protein mass of cellular or secreted apoB in the absence of insulin. McA B48 cells were transfected with the empty vector or the c-myc Akt1 plasmid at the indicated concentration for 4 hours using Lipofectamine. Cells were lysed 48 hours after transfection and subjected to 6% SDS-PAGE, and transferred onto a PVDF membrane. Primary antibody was mouse 1D1 for apoB. Secondary antibody used was goat anti-mouse IgG-HRP. (A) Cellular human apoB-48. (B) secreted human apoB-48. Mean ± SEM values are from 4 independent experiments in duplicates.

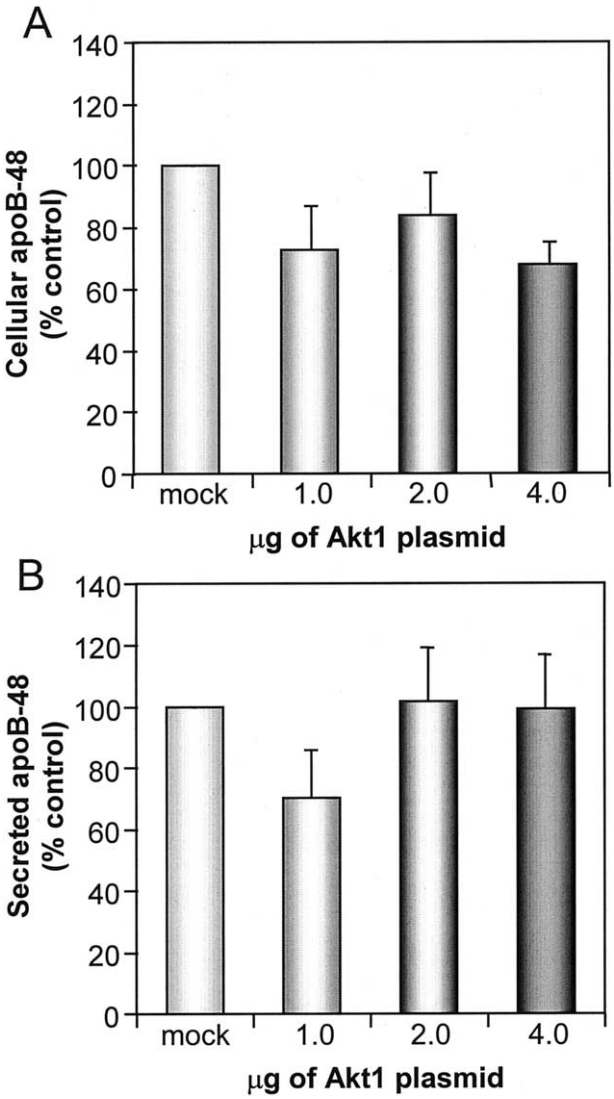

Fig 4. Overexpression of Akt1 in McA B48 cells does not significantly affect the amount of newly synthesized apoB in the absence of insulin. McA B48 cells were transfected with the empty vector or the c-myc Akt1 plasmid at the indicated concentration for 4 hours using Lipofectamine; 48 hours after transfection, cells were placed in media lacking methionine and cysteine for 1 hour. Cells were then pulsed with 50 μCi/mL [^{35}S]methionine for 1 hour, after which both the media and cells were harvested. Goat anti-human apoB antibody was added to each sample and rotated overnight. Samples were immunoprecipitated for 1 hour. After washing, samples were subjected to 8% SDS-PAGE and autoradiography. Bands were quantified by liquid scintillation counting. (A) ApoB-48 in cells; (B) apoB-48 in media. Mean ± SEM values are from 3 independent experiments in triplicates.

sized apoB with or without Akt1 transfection and with or without insulin treatment (Fig 5). Overnight high-dose insulin treatment (100 nmol/L) alone decreased the secretion of both apoB-100 and apoB-48 by approximately 15% and 25%, respectively; however, neither of these reached statistical significance. For apoB-100, all combinations of treatments yielded approximately the same amount apoB (Fig 5A). There was a trend towards a decrease in secretion of apoB-48 in McA RH-7777 ($P = .06$) with the combined insulin/Akt1 treatment; however, this may be due to the insulin effect alone (Fig 5B).

Overexpression of Activated Akt1 in HepG2 With Overnight Insulin Treatment Did Not Affect ApoB Levels

After having observed that the level of secreted apoB was not significantly decreased with high-dose insulin in McA RH-7777 cells, we performed a similar metabolic labeling experiment in HepG2 cells, as apoB secretion in this cell line is known to respond to insulin treatment. Overnight high-dose

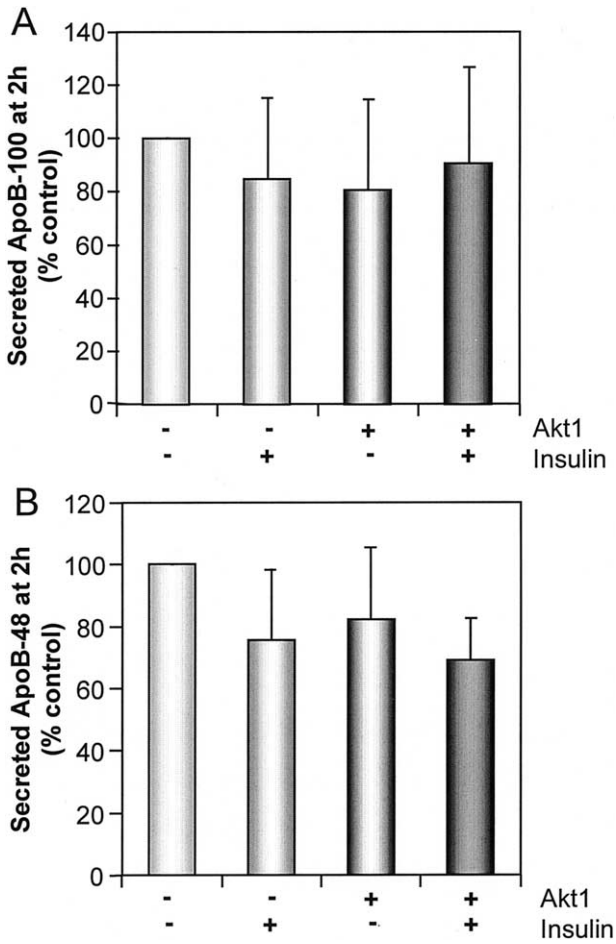

Fig 5. ApoB-100 and apoB-48 secretion by McA RH-7777 transfected with Akt1 and treated overnight with high insulin levels. McA RH-7777 cells were transfected with the empty vector or the c-myc Akt1 plasmid (2 μg of DNA) for 4 hours using Lipofectamine. After 24 hours, cells were incubated with 100 nmol/L insulin overnight. The next day, cells were placed in 20% FBS DMEM lacking methionine and cysteine supplemented with 360 μmol/L oleate for 1 hour. Cells were then pulsed with 50 μCi/mL [^{35}S]methionine for 30 minutes and chased with 10 mmol/L cold methionine for 2 hours, after which both the media and cells were harvested. Rabbit anti-rat apoB antibody was added to each sample and rotated overnight. Samples were immunoprecipitated for 1 hour. After washing, samples were subjected to 5.5% SDS-PAGE and autoradiography. Bands were quantified by liquid scintillation counting. (A) ApoB-100 in media; (B) apoB-48 in media. Mean ± SEM values are from 3 independent experiments in triplicates.

insulin treatment (100 nmol/L) alone significantly decreased the secretion of apoB-100 by approximately 41% ($P < .0001$) (Fig 6). Akt1 transfection alone did not decrease the secretion of apoB-100, but in fact appeared to increase apoB secretion by about 40% but failed to reach statistical significance ($P = .09$). However, dual treatment with insulin and Akt1 decreased apoB by approximately 25% relative to untransfected and non–insulin-treated cells ($P = .02$). When comparing +Akt1/−insulin to +Akt1/+insulin, there was a 48% decrease in apoB secretion in Akt1-overexpressing cells treated with insulin. In agreement with results in rat hepatoma cells, the overexpression of Akt1 did not seem to attenuate the secretion of apoB-100 in HepG2 cells.

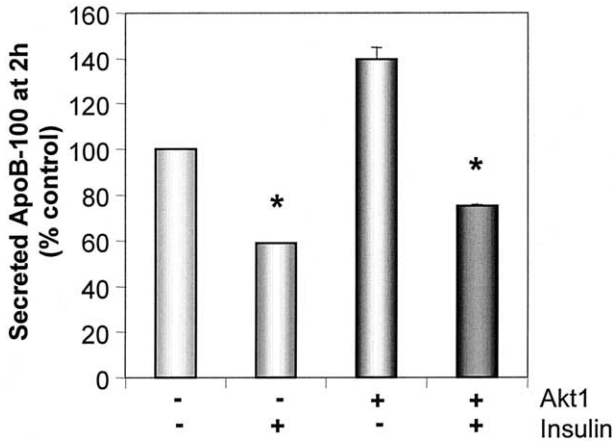

Fig 6. Overexpression of activated Akt in HepG2 with overnight insulin treatment did not affect apoB levels. HepG2 cells were transfected with the empty vector or the c-myc Akt1 plasmid (4 μg of DNA) for 4 hours using Lipofectamine. After 24 hours, cells were incubated with 100 nmol/L insulin overnight. The next day, cells were placed in αMEM lacking methionine and cysteine for 1 hour. Cells were then pulsed with 50 μCi/mL [^{35}S]methionine for 15 minutes and chased with 10 mmol/L cold methionine for 2 hours, after which both the media and cells were harvested. Goat anti-human apoB antibody was added to each sample and rotated overnight. Samples were immunoprecipitated for 1 hour. After washing, samples were subjected to 6% SDS-PAGE and autoradiography. Bands were quantified by liquid scintillation counting. Mean ± SEM values are from 2 independent experiments in triplicates. *$P < .05$ compared to conditions without insulin treatment.

DISCUSSION

In the present study, we tested the hypothesis that overexpression of Akt1 can regulate apoB expression. We first established that it was possible to transfect McA RH-7777 cells, a rat hepatoma cell line frequently used in apoB studies, with our Akt1 construct and achieve dosage-dependent overexpression. Despite overexpression of Akt1 in McA B15 and McA B48 cells, total cellular apoB or secreted apoB was not affected as determined by immunoblotting. Similarly, the amount of newly synthesized apoB was also unchanged with Akt1 overexpression. Wild-type McA cells were found to be responsive to insulin, as shown by a significant increase in Akt1 activity. Using an antibody specific for a phosphorylated Ser residue at position 473, we demonstrated that the overexpressed Akt1 was phosphorylated, implying that the kinase was active. In fact, it is likely that the activity was already at a maximal level since acute, high-dose insulin stimulation did not further increase phosphorylation. Overnight high-dose insulin treatment alone did not significantly decrease the secretion of either apoB-100 or apoB-48. No change was detected in the amount of secreted apoB-100 despite different combinations of insulin and/or Akt1 treatments. However, there was a trend towards a decrease in apoB-48 secretion in McA RH-7777 with combined insulin/Akt1 treatment, but this could be attributed to the insulin inhibition of apoB alone. These findings suggest that overexpression of Akt1 in McA RH-7777 cells does not affect apoB synthesis or secretion. Transfection was also performed in HepG2 cells with similar results, supporting the observations in McA cells.

In wild-type McA cells, Akt1 activity was significantly increased upon insulin stimulation, demonstrating that this cell line was sensitive to insulin stimulation and that the pathway leading to Akt was intact. The use of a constitutively active Akt has proven to be useful in determining its function.[32,35] However, in any overexpression experiment, there is the question of whether the overexpressed protein is active. For Akt1, phosphorylation at Thr308 and Ser473 is required for activation.[31] We detected high levels of phosphorylated Akt1 in transfected cells using an antibody specific for phosphorylated serine residue at position 473. The presence of protein bands with higher than expected molecular weight is probably indicative of the added length due to the *c-src* sequence, the myc-tag, and/or myristoylated status of the kinase. The functionality of the overexpressed Akt1 was assumed due to its phosphorylation status.

We also found that overexpression of Akt1 in McA B15 and McA B48 cells did not affect the total amount of cellular or secreted apoB, nor did it affect the amount of newly synthesized apoB. With cellular apoB, our observations are not surprising since insulin itself does not seem to affect the intracellular pool of apoB.[7] However, it was expected that secreted apoB would be affected given the ample support for insulin inhibition of apoB secretion in various systems.[3-10] One explanation is that apoB secretion in McA RH-7777 cells may be less sensitive to insulin than other cell types.[43] To eliminate this possibility, another system, the human hepatoma HepG2, was used to confirm our results. In agreement with our data in McA cells, despite sensitivity of apoB secretion to insulin-mediated inhibition, Akt1 overexpression still did not decrease apoB secretion in HepG2. In fact, a curious trend was observed in which Akt1 appeared to increase apoB secretion when comparing transfected versus untransfected cells (Akt1 *v* no Akt1 or Akt1 with insulin *v* insulin alone), even though this did not reach statistical significance (Fig 6). One possible explanation is that Akt has been suggested to inhibit Raf-1 of the mitogen-activated protein kinase (MAPK) pathway via cross-talk.[44] Preliminary results from MAPK pathway inhibition studies using chemical inhibitors from our group suggest that inhibition of MAP and ERK kinase (MEK) may affect apoB secretion in HepG2 cells (unpublished data).

The lack of inhibition of apoB by Akt1 can also be an indication that the insulin effect occurs upstream of Akt and only involves parts of the insulin cascade, perhaps only to the

point of PI3K. Consistent with this explanation, insulin regulation of apoB was found to be dependent on the activation and the number of insulin receptors.[4] Inhibition of PI3K with wortmannin abolished the insulin effect on apoB secretion, suggesting that PI3K is involved.[12] In addition, insulin increased PI3K activity associated with IRS-1 and increased the amount of PI3K present in the ER.[13] On the other hand, cellular Akt is mostly found in the cytoplasm (or plasma membrane when activated), and therefore may not be involved in the PI3K effect on apoB in the ER.

Another explanation for the current findings is the specific isoform of Akt used in the study. Mice deficient in Akt2 displayed many diabetes-like symptoms,[36] while Akt1 knockouts showed impaired growth with intact glucose homeostasis.[45] These knockout experiments seem to suggest that Akt isoforms have unique roles; however, one should keep in mind that the distribution of isoforms are also different. Although both Akt1 and Akt2 are present in the liver, Akt2 is the predominate isoforms in insulin-responsive tissues.[45] Therefore one cannot rule out the involvement of Akt1 in insulin signaling, but perhaps Akt1 activation alone is insufficient. Also, stimulation of rat hepatocytes with insulin induced the activation of Akt1 and Akt2 with similar kinetics.[23,46] Therefore, it is likely that Akt1 may have overlapping function with Akt2 when overexpressed in hepatocytes.

An alternative reason for the inability of Akt1 to downregulate apoB could be that PI3K is not acting via the insulin signaling pathway in the inhibition of apoB. The lipid product of PI3K, phosphoinositol-3,4,5-trisphosphate (PIP_3), can recruit other families of PH-containing signaling proteins in addition to protein serine-threonine kinases, including protein tyrosine kinases (Tec family), exchange factors for guanosine triphosphate (GTP)-binding proteins (Grp1 and Rac exchange factors), and adaptor proteins (GAB-1).[47] Therefore, it is feasible that other pathways may be involved.

In conclusion, we found that overexpression of active Akt1 alone did not significantly affect the total or newly synthesized levels of both cellular and secreted apoB in McA RH-7777 and in HepG2 cells. This may be an indication that even though insulin modulation of apoB involves PI3K, molecules upstream of Akt or in parallel pathways may be more important in the insulin-mediated inhibition of apoB secretion.

REFERENCES

1. Adeli K, Taghibiglou C, Van Iderstine SC, et al: Mechanisms of hepatic very low-density lipoprotein overproduction in insulin resistance. Trends Cardiovasc Med 11:170-176, 2001

2. Elovson J, Chatterton JE, Bell GT, et al: Plasma very low density lipoproteins contain a single molecule of apolipoprotein B. J Lipid Res 29:1461-1473, 1988

3. Patsch W, Franz S, Schonfeld G: Role of insulin in lipoprotein secretion by cultured rat hepatocytes. J Clin Invest 71:1161-1174, 1983

4. Patsch W, Gotto AM Jr, Patsch JR: Effects of insulin on lipoprotein secretion in rat hepatocyte cultures: The role of the insulin receptor. J Biol Chem 261:9603-9606, 1986

5. Sparks CE, Sparks JD, Bolognino M, et al: Insulin effects on apolipoprotein B lipoprotein synthesis and secretion by primary cultures of rat hepatocytes. Metabolism 35:1128-1136, 1986

6. Dashti N, Wolfbauer G: Secretion of lipids, apolipoproteins, and lipoproteins by human hepatoma cell line, HepG2: Effects of oleic acid and insulin. J Lipid Res 28:423-436, 1987

7. Sparks JD, Sparks CE: Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. J Biol Chem 265:8854-8862, 1990

8. Pullinger CR, North JD, Teng BB, et al: The apolipoprotein B gene is constitutively expressed in HepG2 cells: Regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. J Lipid Res 30:1065-1077, 1989

9. Adeli K, Sinkevitch C: Secretion of apolipoprotein B in serum-free cultures of human hepatoma cell line, HepG2. FEBS Lett 263: 345-348, 1990

10. Salhanick AI, Schwartz SI, Amatruda JM: Insulin inhibits apolipoprotein B secretion in isolated human hepatocytes. Metabolism 40:275-279, 1991

11. Dashti N, Williams DL, Alaupovic P: Effects of oleate and insulin on the production rates and cellular mRNA concentrations of apolipoproteins in HepG2 cells. J Lipid Res 30:1365-1373, 1989

12. Sparks JD, Phung TL, Bolognino M, et al: Insulin-mediated inhibition of apolipoprotein B secretion requires an intracellular trafficking event and phosphatidylinositol 3-kinase activation: Studies with brefeldin A and wortmannin in primary cultures of rat hepatocytes. Biochem J 313:567-574, 1996

13. Phung TL, Roncone A, Jensen KL, et al: Phosphoinositide 3-kinase activity is necessary for insulin-dependent inhibition of apolipoprotein B secretion by rat hepatocytes and localizes to the endoplasmic reticulum. J Biol Chem 272:30693-30702, 1997

14. Franke TF, Yang SI, Chan TO, et al: The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 81:727-736, 1995

15. Burgering BM, Coffer PJ: Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 376:599-602, 1995

16. Kandel ES, Hay N: The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res 253:210-229, 1999

17. Staal SP: Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: Amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci USA 84:5034-5037, 1987

18. Bellacosa A, Testa JR, Staal SP, et al: A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science 254:274-277, 1991

19. Jones PF, Jakubowicz T, Hemmings BA: Molecular cloning of a second form of rac protein kinase. Cell Regul 2:1001-1009, 1991

20. Altomare DA, Guo K, Cheng JQ, et al: Cloning, chromosomal localization and expression analysis of the mouse Akt2 oncogene. Oncogene 11:1055-1060, 1995

21. Nakatani K, Thompson DA, Barthel A, et al: Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. J Biol Chem 274:21528-21532, 1999

22. Brodbeck D, Cron P, Hemmings BA: A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain. J Biol Chem 274:9133-9136, 1999

23. Walker KS, Deak M, Paterson A, et al: Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: Comparison with protein kinase B alpha. Biochem J 331:299-308, 1998

24. Andjelkovic M, Alessi DR, Meier R, et al: Role of translocation in the activation and function of protein kinase B. J Biol Chem 272:31515-31524, 1997

25. Datta K, Franke TF, Chan TO, et al: AH/PH domain-mediated

interaction between Akt molecules and its potential role in Akt regulation. Mol Cell Biol 15:2304-2310, 1995

26. Klippel A, Kavanaugh WM, Pot D, et al: A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. Mol Cell Biol 17:338-344, 1997

27. Frech M, Andjelkovic M, Ingley E, et al: High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. J Biol Chem 272:8474-8481, 1997

28. James SR, Downes CP, Gigg R, et al: Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. Biochem J 315:709-713, 1996

29. Franke TF, Kaplan DR, Cantley LC, et al: Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science 275:665-668, 1997

30. Gagnon A, Chen CS, Sorisky A: Activation of protein kinase B and induction of adipogenesis by insulin in 3T3-L1 preadipocytes: Contribution of phosphoinositide-3,4,5-trisphosphate versus phosphoinositide-3,4-bisphosphate. Diabetes 48:691-698, 1999

31. Alessi DR, Andjelkovic M, Caudwell B, et al: Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J 15:6541-6551, 1996

32. Kohn AD, Summers SA, Bimbaum MJ, et al: Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 271:31372-31378, 1996

33. Goransson O, Wijkander J, Manganiello V, et al: Insulin-induced translocation of protein kinase B to the plasma membrane in rat adipocytes. Biochem Biophys Res Commun 246:249-254, 1998

34. Scheid MP, Marignani PA, Woodgett JR: Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. Mol Cell Biol 22:6247-6260, 2002

35. Kohn AD, Barthel A, Kovacina KS, et al: Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. J Biol Chem 273:11937-11943, 1998

36. Cho H, Mu J, Kim JK, et al: Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science 292:1728-1731, 2001

37. Pessin JE, Saltiel AR: Signaling pathways in insulin action: Molecular targets of insulin resistance. J Clin Invest 106:165-169, 2000

38. Taghibiglou C, Rashid-Kolvear F, Van Iderstine SC, et al: Hepatic very low density lipoprotein-ApoB overproduction is associated with attenuated hepatic insulin signaling and overexpression of protein-tyrosine phosphatase 1B in a fructose-fed hamster model of insulin resistance. J Biol Chem 277:793-803, 2002

39. Shao J, Yamashita H, Qiao L, et al: Decreased Akt kinase activity and insulin resistance in C57BL/KsJ-Leprdb/db mice. J Endocrinol 167:107-115, 2000

40. Nelson BA, Robinson KA, Buse MG: Defective Akt activation is associated with glucose-but not glucosamine-induced insulin resistance. Am J Physiol Endocrinol Metab 282:E497-E506, 2002

41. Kruszynska YT, Worrall DS, Ofrecio J, et al: Fatty acid-induced insulin resistance: Decreased muscle PI3K activation but unchanged Akt phosphorylation. J Clin Endocrinol Metab 87:226-234, 2002

42. Bostrom K, Wettesten M, Boren J, et al: Pulse-chase studies of the synthesis and intracellular transport of apolipoprotein B-100 in Hep G2 cells. J Biol Chem 261:13800-13806, 1986

43. Phung TL, Mooney RA, Kulas DT, et al: Suppression of the protein tyrosine phosphatase LAR reduces apolipoprotein B secretion by McA-RH7777 rat hepatoma cells. Biochem Biophys Res Commun 237:367-371, 1997

44. Moelling K, Schad K, Bosse M, et al: Regulation of Raf-Akt cross-talk. J Biol Chem 277:31099-31106, 2002

45. Cho H, Thorvaldsen JL, Chu Q, et al: Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem 276:38349-38352, 2001

46. Brozinick JT Jr, Roberts BR, Dohm GL: Defective signaling through Akt-2 and -3 but not Akt-1 in insulin-resistant human skeletal muscle: Potential role in insulin resistance. Diabetes 52:935-941, 2003

47. Cantley LC: The phosphoinositide 3-kinase pathway. Science 296:1655-1657, 2002