BIOCHEMICAL PHARMACOLOGY 75 (2008) 457–467

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AICAR positively regulate glycogen synthase activity and LDL receptor expression through Raf-1/MEK/p42/44^{MAPK}/p90^{RSK}/GSK-3 signaling cascade

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ARTICLE INFO

Article history:
Received 23 July 2007
Accepted 27 August 2007

Keywords:
AICAR
Raf-1
p90RSK
AMPK
Glycogen synthase
LDL receptor

ABSTRACT

5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is a commonly used pharmacological agent to study physiological effects which are similar to those of exercise. However, signal transduction pathways by which AICAR elicits downstream effects in liver are poorly understood. We report here that AICAR not only activated AMPK but also phosphorylated/deactivated glycogen synthase kinase-3 alpha/beta (GSK-3α/β) and dephosphorylated/activated glycogen synthase (GS) in a time-dependent manner in human hepatoma HepG2 cells. The signal connection between AICAR and GSK-3 is indirect and involves activation of Raf-1/MEK/p42/44^{MAPK}/p90^{RSK} signaling cascade as pharmacologic inhibition of MEK significantly reduced phosphorylation/deactivation of GSK-3 and consequent dephosphorylation/activation of GS. Moreover, silencing the expression of p90^{RSK}, a substrate of p42/44^{MAPK}, attenuated AICAR-dependent GSK-3 phosphorylation, implicating this kinase as a key mediator of AICAR signaling to GSK-3. Furthermore, consistent with the involvement of Raf-1 kinase cascade, AICAR-induced low-density lipoprotein (LDL) receptor expression in a p42/44^{MAPK}-dependent manner. Finally, AICAR requires AMPK-α2-dependent and -independent pathways to activate Raf-1 kinase cascade as suppression of AMPKα2 activity, and not of AMPKα1, partially blocked AICAR-dependent p42/44^{MAPK} activation and GSK-3 phosphorylation/deactivation. Collectively, these results highlight Raf-1 signaling cascade as the critical mediator of AICAR action on glucose and lipid metabolism in HepG2 cells.

1. 1. Introduction

Glycogen is the storage form of carbohydrate for virtually every organism from yeast to primates [1]. Most mammalian tissues store glucose as glycogen, with the major depots located in muscle and liver. Liver glycogen makes up 10% of total liver weight when fully replete, reflecting the importance of liver in glucose homeostasis. When plasma glucose levels rise after a meal, the liver clears glucose and stores it as glycogen. The crucial and rate-limiting step of UDP-glucose incorporation into glycogen is catalyzed by glycogen synthase (GS) [2]. GS is maintained in a low-activity state under basal conditions principally through the continual phosphorylation of site 3 by glycogen synthase kinase-3 alpha/beta (GSK-3) [3,4]. Insulin is believed to activate GS mainly through the deactivation of GSK-3 [5,6]; however, some level of regulation may involve glycogen-targeted protein phosphatases [7]. The mechanism leading to GSK-3 deactivation with insulin

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.08.028

involves phosphorylation of GSK-3 (Ser^{21} in β Ser and Ser^{9} in GSK-3β) by a phosphatidylinositol (PI) 3-kinase (PI-3K)- and protein kinase Akt (also known as protein kinase B)-dependent mechanism [8,9]. A number of other kinases have been identified that can phosphorylate GS *in vitro* [10], including AMPK, which can phosphorylate serine 7 of GS [11]. Phosphorylation of site 2, which can also be catalyzed by cAMP-dependent protein kinase (PKA), primes GS for further phosphorylation at site 2a by caseine kinase I, which in turn leads to a decrease in GS activity [12]. AMP-activated kinase (AMPK) is a metabolite-sensing enzyme that has been implicated in the mediation of exercise-induced glucose uptake and direct phosphorylation/deactivation of GS activity [13], although to date, little experimental evidence has attributed a role for AMPK in the regulation of GS activity *in vivo*. Pharmacologic activation of AMPK and a single amino acid mutation in the enzyme's γ subunit are associated with increased muscle glycogen content mainly in the fast-twitch muscles [14–16].

A commonly used pharmacological agent to induce activation of AMPK is the compound 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), which has frequently been used to characterize the effects of AMPK activation on glucose homeostasis in a variety of tissues [17]. In most cell types, this nucleoside is taken up and accumulates in the cytoplasm as the monophosphorylated nucleotide, ZMP, which activates AMPK without disturbing cellular adenine nucleotide ratios. AICAR treatment results in several adaptations that are similar to changes that occur during exercise. It has been repeatedly reported by *in vitro* and *in vivo* studies that AICAR causes an increase in glucose uptake in skeletal muscles [18,19], insulin sensitivity in obese rats [20] and human type 2 diabetics [21]. AICAR also affect expression of a number of glycolytic and lipogenic enzymes in the liver [22–25] that are normally under the control of nutrients and hormones, including insulin. Although there has been considerable progress in elucidating AICAR action in the skeletal muscle, there is little understanding of this process in the liver cells. This is particularly important given the fact that liver plays an important role in glucose and lipid homeostasis. Another important aspect that remains poorly explored is the signal transduction pathways through which AICAR may elicit its downstream effects on gene expression in the liver. Majority of AICAR action has been linked to phosphorylation/modulation of biosynthetic enzymes and transcription factors/coactivators by AMPK [26]. The experiments outlined in this investigation are designed to elucidate AICAR-induced major intracellular signaling events relevant to the regulation of glucose and lipid metabolism in the liver. Here we provide evidence that acute exposure of HepG2 cells to AICAR profoundly increases phosphorylation/activation of AMPK, Raf-1, extracellular-regulated kinase ($p42/44^{MAPK}$), p90 ribosomal S6 kinase ($p90^{RSK}$), phosphorylation/deactivation of GSK-3α/β, and dephosphorylation/activation of GS. The results presented here argue strongly in favor of a central role for Raf-1/MEK/$p42/44^{MAPK}$/$p90^{RSK}$ in mediating effects of AICAR on GSK-3 (and subsequently GS) and low-density lipoprotein (LDL) receptor. These studies were performed in hepatic cells that *in vivo* are a major target of lipid and glucose homeostasis.

2. Materials and methods

2.1. Materials

AICAR was purchased from Toronto Research Chemicals Inc. Metformin was purchased from Sigma–Aldrich. TRIzol and tissue culture supplies were purchased from Invitrogen Corp. PD98059, U0126, and SB202190 were purchased from Calbiochem. Phospho-specific antibodies to the activated forms of AMPK-α Thr^{172}, $p42/44^{MAPK}$ Thr^{202}/Tyr^{204}, $p38^{MAPK}$ Thr^{180}/Tyr^{182}, $p46/54^{JNK}$ Thr^{183}/Tyr^{185}, GSK-3α/β $Ser^{21/9}$, Raf-1 Ser^{259}, Raf-1Ser^{338}, $p90^{RSK}$ Ser^{380}, and Akt Thr^{308} purchased from Cell Signaling Technology Inc. Phospho-ACC Ser^{79}, nonphospho-ACC, phospho-MEK-1/2 $Ser^{219/221}$, nonphospho-AMPK-α1, and nonphospho-AMPK-α2 antibodies were purchased from Upstate Biotechnology. Phospho-GS $Ser^{641/645}$ was purchased from Upstate. Nonphospho-$p42/44^{MAPK}$, nonphospho-Raf-1, nonphospho GSK-3, and total actin were purchased from Santa Cruz Biotechnology. HRP-conjugated rabbit or mouse antibodies were purchased from Bio-Rad Laboratories Inc. Enhanced chemiluminescence detection kit was purchased from Amersham Pharmacia Biotech. Short-interfering RNA (siRNA) against AMPK-α1 and p90 ribosomal S6 kinase ($p90^{RSK}$) were purchased from Santa Cruz Biotechnology and Qiagen, respectively.

2.2. Cell culture

Human hepatoma HepG2 cells were maintained as monolayer cultures in Eagle's minimum essential medium (MEM, BioWhitaker Inc.), supplemented with 10% fetal bovine serum (FBS), L-glutamine (20 mM) and antibiotics (penicillin 200 U/mL and streptomycin 200 μg/mL) (Invitrogen Corp.). Cells were grown in a humidified 5% carbon dioxide-95% air atmosphere.

2.3. Glycogen synthesis assay

HepG2 cells were incubated for 3 h prior to assay in Krebs–Ringer bicarbonate buffer supplemented with 30 mM HEPES, pH 7.4, 0.5% BSA and 2.5 mM glucose. The cells were washed once with PBS and incubated in the above buffer without glucose. The cells were incubated for different periods with AICAR, and the reaction initiated by the addition of [^{14}C-(U)]glucose (2 μCi/sample) and glucose (5 mM final concentration). The assay was terminated after 1 h by washing with ice-cold PBS, and the cells were solubilized in 30% potassium hydroxide. The glucose incorporation into glycogen was determined as described previously [27].

2.4. siRNA studies

SiRNAs were transfected into HepG2 cells plated in six-well dishes using Dharmafect™4 (Dharmacon) according to the manufacturer's protocol [26]. In brief, AMPKα1, or nonsilencing control siRNA (20 or 40 nM) and Dharmafect™4 were mixed individually with serum free MEM to a total of 100 μl and incubated for 5 min prior to combined incubation of 20 min. The SiRNA mixtures were then added to phosphate-buffered saline washed HepG2 cells supplied with 1.8 ml of

MEM containing 10% serum. Transfected cells were cultured in the presence of siRNA for 48 h followed by 16 h serum starvation with MEM supplemented with 0.5% FBS. Treatments were carried out as indicated and analyzed by Western blotting. For knockdown of $p90^{RSK}$ expression, HepG2 cells were transfected with 50 or 100 nM of $p90^{RSK1}$, $p90^{RSK2}$, or nonsilencing control siRNA oligonucleotides using Oligofectamine as described by the manufacturer (Invitrogen). Approximately 24 h later, cells were trypsinized and seeded in 60-mm diameter plates. Cells were serum starved for 16 h and then stimulated for 3 h with 2 mM AICAR. Cells were lysed 72 h posttransfection, since optimal silencing of $p90^{RSK}$ was observed at this time point.

2.5. *Western blot analysis*

Equivalent amounts of lysates were fractionated by SDS-PAGE and transferred onto nitrocellulose membranes [27]. The membranes were blocked using 3% non-fat dried milk in phosphate buffered saline (PBS) or 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) depending on manufacturer's recommendations. Primary antibodies were diluted in either 3% non-fat dried milk in phosphate-buffered saline for Upstate Biotechnology antibodies, 5% non-fat dried milk in TBST for Santa Cruz Technology antibodies, or 5% bovine serum albumin in Tris-buffered saline for cell signaling technology antibodies and incubated at 4 °C overnight with gentle agitation. Membranes were washed and incubated for an additional hour with HRP conjugated secondary antibody. Immunoreactive proteins were visualized using enhanced chemiluminescence (Amersham Biosciences). Exposed films were scanned and quantitated with Quantity One software (BIO-RAD).

2.6. *Raf-1 kinase assay*

Raf-1 kinase assays were performed using a modification of the kit from Upstate Biotechnology Inc., using endogenous Raf-1 immunoprecipitated from 100-mm plates of HepG2 cells. Raf-1 kinase activity was measured in a kinase cascade reaction initiated by immunoprecipitated activated Raf-1. Instead of using radioactive ATP, activation of $p42/44^{MAPK}$ was determined by probing with anti-phospho-$p42/44^{MAPK}$ antibody.

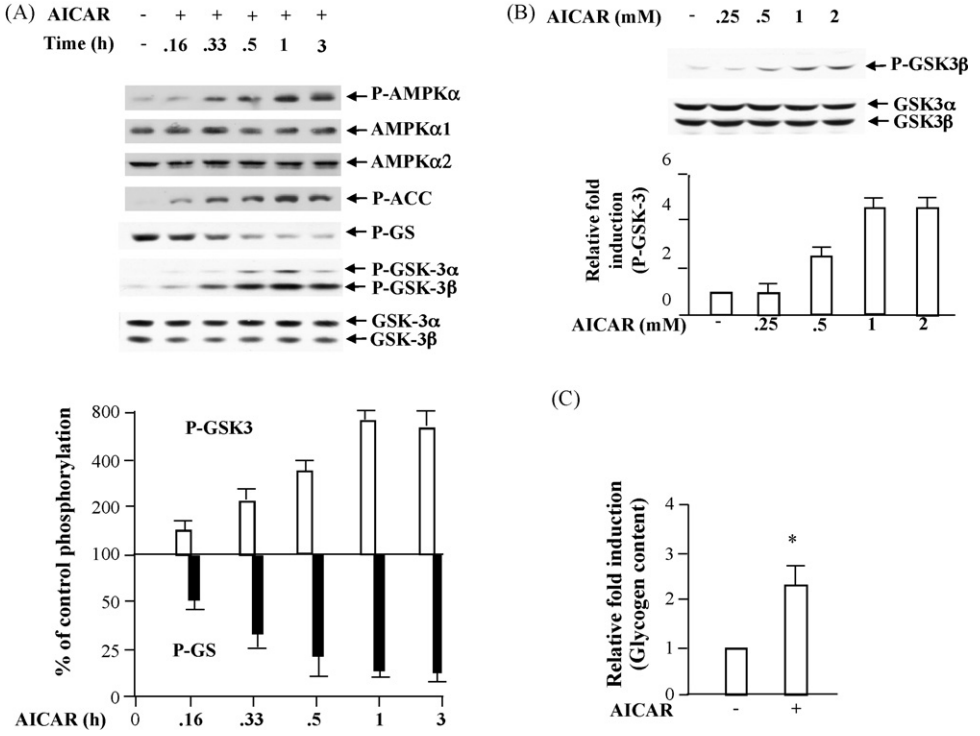

Fig. 1 – Treatment with AICAR activated AMPK and phosphorylated ACC and GSK-3 with simultaneous dephosphorylation of GS in a time- and dose-dependent manner in HepG2 cells. (A) Kinetics of changes in phosphorylation of AMPK, ACC, GSK-3 and GS by AICAR. HepG2 cells were treated with 2 mM AICAR for the indicated time periods, and cell lysates were immunoblotted for phospho-AMPK, total AMPK, phospho-ACC, phospho-GSK-3, total GSK-3, and phospho-GS. The phospho data are presented as relative to the untreated cells. Values shown are means ± S.E. of three separate experiments. (B) AICAR-induced phosphorylation of GSK-3 in a dose-dependent manner. HepG2 cells were treated with indicated concentrations of AICAR for 3 h to probe phosphorylation status of GSK-3. The phospho data are presented as relative to the untreated cells. Values shown are means ± S.E. of three separate experiments. (C) AICAR increases glycogen content in HepG2 cells. Cells were treated with AICAR for 15 min prior to the addition of [^{14}C-(U)]glucose. Following 1 h incubation in the presence of AICAR, total glycogen contents were determined by scintillation counting. ⁎, $p < 0.05$ relative to the value in the absence of AICAR, $n = 5$. Immunoblots shown are representative of three experiments which produced similar results. Values obtained from the control cells grown in the absence of AICAR were arbitrarily set at 1.

2.7. Quantitative PCR analysis

Total RNA was isolated from HepG2 cells by using TRIzol, and cDNA was synthesized from 1 μg of total RNA by using oligo(dT)$_{12–18}$ primer with the Superscript first-strand synthesis system (Invitrogen Corp.) [28–30]. The sequences of the primers used for quantitating the LDL receptor expression were 5′-AGGCTGTGGGCTCCA TCGCCTA-3′ and 5′-AGTCAGTC-CAGTACATGAAGCCA-3′, primers for SS were 5′-TGGAGTT-CGTGAAATGCCTTG-3′ and 5′-ACCGCCAGTCTGGTTGGTA-AAG-3′, primers for HMG-CoA reductase were 5′-GCATTG TCTTGTGGAGGA-3′ and 5′-AGGGAGAAGGTCAATTGCA-3′, primers for β-actin were 5′-ACTATGACTTAGTTGCGTTA-3′ and 5′-GGGCACGAAGGCTCATCATT-3′. [^{32}P]dCTP was incorporated into the PCR products for visualization and quantitation. The linear range for each primer set was determined empirically using different amounts of cDNA. Subsequent PCR analyses were carried out using the optimum cycle number determined for each primer set. The LDL receptor mRNA was normalized to β-actin mRNA, and the data for each point were plotted as the percentage of LDL receptor mRNA compared with controls.

3. Results

3.1. AICAR-induced phosphorylation/deactivation of GSK-3 and dephosphorylation/activation of GS in a time-dependent manner in HepG2 cells

Although AICAR has been used extensively to activate AMPK in various cell types, the efficacy and specificity is dependent on its uptake by the cell and on the accumulation of ZMP. To confirm that AICAR treatment resulted in AMPK activation in hepatic cells, we first examined levels of phosphorylated AMPK in HepG2 cells treated with AICAR for different periods of time. Lysates were subjected to immunoblot analysis using an antibody to the phosphoactivated form of AMPKα. The kinetic studies showed increased phosphorylation of AMPKα in a time-dependent manner (Fig. 1A), with maximal activation occurring at 3 h. Although phosphorylation at Thr^{172} is indicative of the activation state of AMPKα, we also performed an immunobl ot analysis with an antibody specific for phosphorylated form of acetyl CoA carboxylase (ACC) at Ser^{79} to ascertain whether altered phosphorylation of AMPKα had an effect on downstream AMPK target protein such as ACC. In accordance with the AMPK activation, ACC phosphorylation was also markedly elevated by AICAR relative to the untreated HepG2 cells (Fig. 1A). AICAR-induced increase in phospho-ACC was evident within 10 min of treatment and was maintained for 3 h. These results are consistent with several studies that have reported that phosphorylation of ACC is a sensitive marker of AMPK activity, often more sensitive even than increased levels of phospho-AMPKα [31,32], and verify that AICAR treatment activated AMPK in HepG2 cells.

To test if AICAR has a regulatory influence on GSK-3 and GS, cells were treated with AICAR and the regulatory phosphorylation of these kinases were measured using immunoblot analyses with phospho-specific antibodies to each of the two isoforms of GSK-3 or to GS. AICAR treatment caused a time-dependent increase in the serine$^{21/9}$ phosphorylation of both isoforms (Fig. 1A). Phosphorylation of GSK-3α followed a pattern similar to GSK-3β; however, the former did not appear to be affected to the same extent by AICAR. AICAR treatment did not alter the total level of GSK-3. Considering the increased phosphorylation of GSK-3 caused by AICAR treatment, we also examined the serine-phosphorylation of GS which is a known substrate for GSK-3. AICAR treatment caused a decrease in the inhibitory serine-phosphorylation of GS with a time course similar to the increased phosphorylation of GSK-3 (Fig. 1A). In accordance with the increased phosphorylation levels of GSK-3, the dephosphorylation of GS increased and the activity of GSK-3 decreased and GS increased following AICAR treatment (Fig. 1A), confirming that the phosphorylation levels of GSK-3 and GS reflect their enzymatic activities. Thus, AICAR treatment of HepG2 cells caused increased deactivation of GSK-3 and consequently activation of GS. To determine whether AICAR exhibited dose-dependent effect on GSK-3 phosphorylation, cells were also treated with increasing doses of AICAR for 3 h. We observed a gradual increase in GSK-3α/β

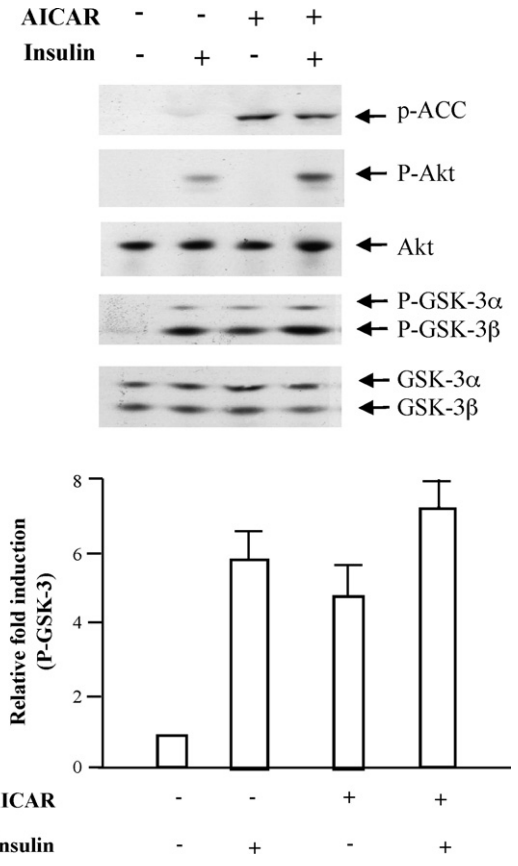

Fig. 2 – AICAR-induced GSK-3 phosphorylation is independent of Akt pathway. Cells incubated overnight in media with 0.5% serum were treated with 2 mM AICAR for 3 h, 1 ng/ml insulin for 10 min, or both. Cell lysates were immunoblotted for phospho-ACC, phospho-Akt, total Akt, phospho-GSK-3 and total GSK-3. Immunoblot shown are representative of three separate experiments which produced similar results. The phospho data is representative of three separate experiments and values shown are mean ± S.E.

phosphorylation occurring at concentrations of 1 mM and higher (Fig. 1B). As expected from the above studies, AICAR produced a significant increase in glycogen synthesis (Fig. 1C).

3.2. AICAR-induced GSK-3 phosphorylation/deactivation is possibly independent of Akt signaling pathway

Since it is well established that insulin regulates GSK-3 activity by increasing Akt phosphorylation/activation, we examined whether AICAR had similar effects on Akt phosphorylation and activation. Activation of Akt is mediated by dual phosphorylation on Thr^{308} and Ser^{473}. As expected, insulin increased Akt phosphorylation (Fig. 2) in agreement with previous reports [8,9]. In contrast, phosphorylation of Akt at Ser^{473} was unaffected by AICAR (Fig. 2). The above data shows clearly that Akt does not mediate phosphorylation/deactivation of GSK-3 by AICAR in HepG2 cells. We also observed a significant increase in GSK-3 phosphorylation on Ser^{473} in response to insulin that was further elevated by prior AICAR treatment. It is possible that insulin response is potentiated following AMPK activation due to convergence of Akt signaling with another signaling pathway on GSK-3.

3.3. AICAR-induced Raf-1/MEK/p42/44^{MAPK}/p90^{RSK} signaling cascade mediated AICAR-dependent phosphorylation/deactivation of GSK-3

We have previously demonstrated a cross-talk between stress-activated p38^{MAPK} and p42/44^{MAPK} [33]. To test if AICAR increased GSK-3 phosphorylation by activating intracellular signaling leading to its activation, changes in the levels of phosphoactivated forms of p42/44^{MAPK} and p38^{MAPK} were examined. AICAR treatment caused a significant increase in the phosphorylation of both p42/44^{MAPK} and p38^{MAPK} with a maximal (>10-fold) induction occurring at 3 h (Fig. 3A). The p42/44^{MAPK} activation was also accompanied by an increase in phosphorylation of its downstream substrate p90^{RSK} and an upstream kinase MEK in HepG2 cells. We then examined changes in the Raf-1 phosphorylation, since activation of MEK and p42/44^{MAPK} is under exquisite regulatory control by this kinase. In accordance with MEK phosphorylation, there is a coordinate increase in the phosphorylation of stimulatory Ser^{338} which reached a maximum at 3 h after stimulation, with a simultaneous decrease in an inhibitory Ser^{239} phosphorylation (Fig. 3B). The slightly lower electrophoretic mobility of the bands stained with antibody specific for nonphospho-Raf-1 in AICAR-treated cells reflects their phosphorylation. The changes in the phosphorylation of above kinases occurred without any significant changes in the protein levels.

Although kinetics of Raf-1 Ser^{338} phosphorylation paralleled an increase in p42/44^{MAPK} phosphorylation, levels of its phosphorylation do not always correlate with the kinase activity [34]. As a result, Ser^{338} phosphorylation may not be used as a surrogate marker of Raf-1 activation. We therefore examined Raf-1 kinase activity by an *in vitro* kinase assay. AICAR activated Raf-1 kinase activity (threefold) as compared to the untreated control (Fig. 3C). Thus, phosphorylation state

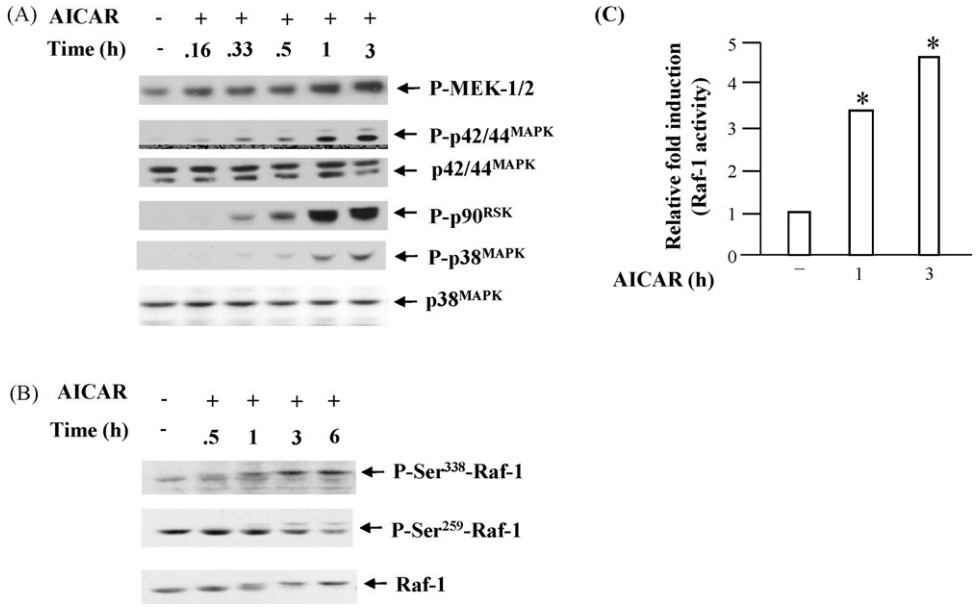

Fig. 3 – Activation of Raf-1/MEK/p42/44^{MAPK}/p90^{RSK} cascade by AICAR in HepG2 cells. (A) Kinetics of activation of MEK, p42/44^{MAPK}, p90^{RSK}, and p38^{MAPK} by AICAR. Cells treated with 2 mM AICAR for different time periods were immunoblotted for the indicated antibodies. Immunoblots shown are representative of three experiments, which produced similar results. (B) Kinetics of Raf-1 phosphorylation by AICAR. HepG2 cells treated with AICAR were probed for two different phospho-Raf-1 antibodies and total Raf-1. (C) Kinetics of increase in Raf-1 activity by AICAR treatment. HepG2 cells starved for 16 h were treated with 2 mM AICAR as indicated. Cells were lysed, and the supernatant of the lysates were used for Raf-1 immunoprecipitation and subsequent kinase assay. Phosphorylation of the Raf-1 substrate was analyzed by phosphoimaging and results are expressed as fold stimulation by AICAR as compared with untreated cells. ⁎, <0.05 relative to the value in the absence of AICAR, $n = 3$.

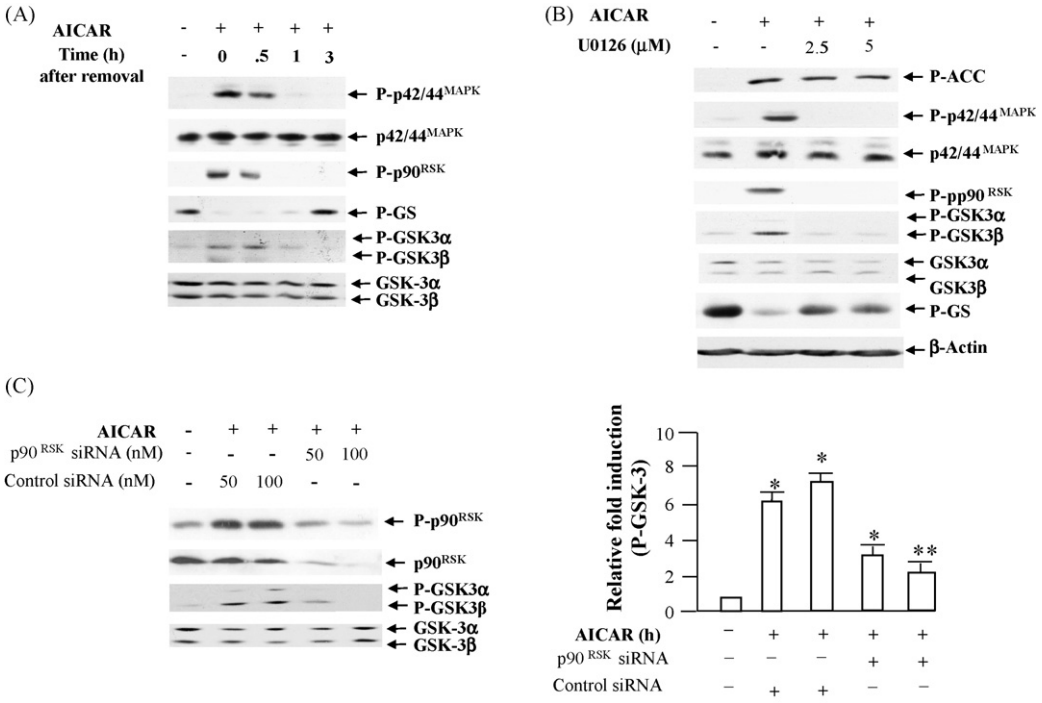

Fig. 4 – AICAR-dependent p42/44^{MAPK} activation is mediated through Raf-1/MEK cascade. (A) Correlation of the loss of phosphorylation of p42/44^{MAPK}, p90^{RSK}, P-GSK-3 with an increase in phosphorylation of GS upon removal of AICAR. HepG2 cells were incubated for 3 h with 2 mM AICAR and then switched to a medium lacking AICAR for the indicated time periods. Phosphorylation levels of the indicated proteins were detected by immunoblotting. Results shown are representative of three separate experiments. (B) Effect of inhibition of MEK on AICAR-induced p42/44^{MAPK} activation in HepG2 cells. Cells pretreated for 30 min with the indicated concentrations of U0126 were either untreated or treated with 2 mM AICAR for 3 h. Cell lysates were subjected to immunoblotting with the indicated antibodies. Immunoblot shown are representative of three experiments which produced similar results. (C) AICAR-induced GSK-3 phosphorylation requires p90^{RSK}. siRNA directed against p90^{RSK} significantly reduced endogenous levels of p90^{RSK} and also significantly reduced AICAR-induced GSK-3 phosphorylation. HepG2 cells cultured in six-well dishes were transfected with 50 or 100 nM of either control siRNA or p90^{RSK} siRNA. After 2 days, transfected cells starved for 16 h were subsequently treated for 3 h with 2 mM AICAR and processed for immunoblotting with the indicated antibodies. *, $p < 0.05$ relative to the value in the absence of AICAR and siRNA, $n = 3$; **, <0.01 relative to the value in the absence of AICAR and siRNA, $n = 3$.

of Raf-1 kinase paralleled changes in the enzymatic activity. Taken together, these results establish that the Raf-1/MEK/p42/44^{MAPK}/p90^{RSK} cascade is activated by AICAR in HepG2 cells.

To examine the relationship between phosphorylation levels of p42/44^{MAPK}, p90^{RSK}, and GSK-3 upon removal of AICAR, cells initially maintained for 3 h in a medium containing 2 mM AICAR were switched to a medium without AICAR for various time periods. Total cell extracts were subjected to Western blotting using the indicated antibodies. As shown in Fig. 4A, loss of phosphorylation of p42/44^{MAPK}, p90^{RSK} and GSK-3 correlated with AICAR removal. Phosphorylation of GS increased after AICAR was removed and by 3 h had reached the level in untreated cells, supporting the hypothesis that phosphorylation and activation of GS was a result of p90^{RSK} activation.

Next, we examined the role of Raf-1 cascade and its downstream effectors in mediating AICAR-induced phosphorylation/deactivation of GSK-3 and subsequent dephosphorylation/activation of GS by use of a selective inhibitor of MEK activation, PD98059. Fig. 4B shows that U0126 (either 2.5 or 5 μM) completely blocked AICAR-induced phosphorylation of p42/44^{MAPK} and GSK-3. Neither AICAR nor U0126 affected p42/44^{MAPK} protein expression, as indicated by immunoblotting of the same extracts with a phosphorylation-independent p42/44^{MAPK} antibody. Additionally, we performed siRNA knockdown strategy to silence the expression of p42/44^{MAPK} substrate, p90^{RSK} proteins, in HepG2 cells and evaluated AICAR-induced GSK-3 phosphorylation after transfection. p90^{RSK} protein levels and phosphorylation in response to AICAR were decreased by 60–80% in p90^{RSK} siRNA-transfected cells compared with nontransfected or control siRNA-transfected HepG2 cells (Fig. 4C). We found that AICAR-induced GSK-3 phosphorylation was highly reduced in p90^{RSK} SiRNA-transfected cells. Collectively, these results suggest that, AICAR-induced GSK-3 phosphorylation is mediated through Raf-1 kinase cascade and the downstream p90^{RSK} kinase is mainly responsible for GSK-3 phosphorylation by AICAR in HepG2 cells.

3.4. AICAR-induced LDL receptor expression in a p42/44^{MAPK}-dependent manner

We next examined functional role of AICAR's activation of Raf-1/MEK/p42/44^{MAPK} signaling pathway on gene expression. In considering potential targets of AMPK signaling, LDL receptor became an obvious candidate because we previously have demonstrated a correlation between p42/44^{MAPK} activation and LDL receptor induction under a variety of physiological conditions [35]. To test whether AMPK-mediated p42/44^{MAPK} activation indeed regulate gene expression, LDL receptor expression was measured following AICAR treatment for various time periods. As shown in Fig. 5A, LDL receptor expression was markedly increased by 3 h, with a maximal induction (approx. four- to fivefold) observed at 6 h. Interestingly, expression of other sterol-responsive genes involved in cholesterol homeostasis, such as 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, remained unaffected by AICAR (Fig. 5A), implying that LDL receptor induction is not due to depletion of the endogenous cholesterol level by AICAR. Furthermore, pretreatment of HepG2 cells with PD98059 abrogated AICAR-induced LDL receptor expression, suggesting a role for MEK-1/2 pathway in the induction process (Fig. 5B).

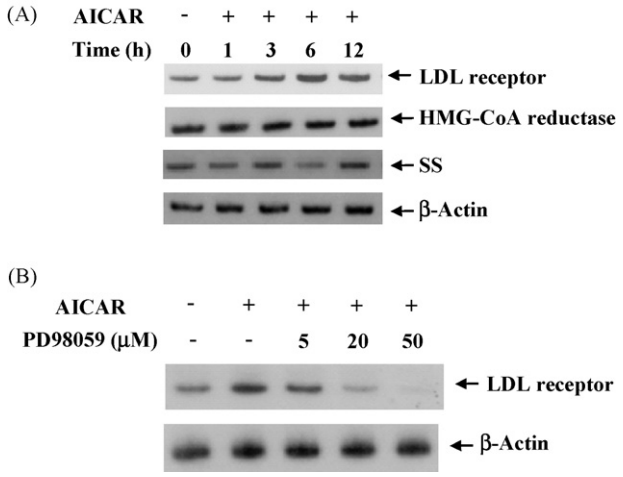

Fig. 5 – AICAR-induced LDL receptor expression in a p42/44^{MAPK}-dependent manner in HepG2 cells. (A) 2×10^5 cells were plated on day 0, and were refed with fresh medium on day 2. On day 3, cells were treated with 2 mM AICAR for the indicated time periods. Total RNA was subjected to RT-PCR to determine amounts of LDL receptor, HMG-CoA reductase, SS, and β-actin mRNAs. A representative autoradiogram is shown showing changes in expression of the above gene. (B) AICAR-induced LDL receptor is mediated through MEK-1/2 pathway. Cells pretreated for 30 min with the indicated concentrations of PD98059 were treated with 2 mM AICAR for 6 h. Total RNA from each treatment was subjected to RT-PCR analysis. Results shown are representative of three independent experiments. Autoradiograms were quantified densitometrically and RNA levels were normalized to β-actin levels. Densitometric analysis of each autoradiogram is expressed as means ± S.E. of three experiments performed in duplicate. Values obtained from control cells grown in the absence of AICAR or inhibitor were arbitrarily set at 1.

3.5. AICAR-induced p42/44^{MAPK} activation requires AMPKα2 and not AMPKα1 isoform

Since AICAR is often used to activate AMPK, we wondered if activation of Raf-1 and downstream kinases in HepG2 cells was due to AMPK activation. To determine the role of AMPK activation in AICAR-dependent p42/44^{MAPK} activation, HepG2 cells pretreated with Compound C [36], a selective inhibitor of AMPK, were treated with AICAR and assayed for the activation of AMPK and p42/44^{MAPK}. Pretreatment with Compound C (20 μM) significantly reduced AICAR-induced AMPKα phosphorylation but only partially attenuated AICAR-induced p42/44^{MAPK} phosphorylation (results not shown). Since HepG2 cells contained almost equal amounts of both AMPKα isoforms, we further examined which α isoform is required for AICAR-induced p42/44^{MAPK} activation. To determine the contribution of AMPKα1, HepG2 cells transfected with either siRNA-AMPKα1 (20 and 40 nM) or nonspecific siRNA control were treated with AICAR for 3 h and the effects on p42/44^{MAPK} activation were determined. As is evident in Fig. 6A, there is a highly significant decrease (~80%) in AMPKα1 level with specific siRNA, however it failed to block AICAR-dependent p42/44^{MAPK} activation, suggesting that this isoform is not involved in the activation process. We therefore examined the effect of reducing AMPKα2 on AICAR-dependent p42/44^{MAPK} activation. We were unable to reduce expression of this isoform using any of the commercially available siRNA. We therefore compared effect of infection of recombinant adenovirus encoding a dominant-negative (DN), myc-tagged mutant of AMPKα2 form (AMPKα2^{DN}) on p42/44^{MAPK} activation following stimulation with AICAR. Successful expression of the kinase was confirmed by probing lysates for antibodies to c-myc: a band with the expected size for AMPK^{DN} was evident in cells infected with an adenovirus encoding myc-tagged AMPK^{DN} but not in unifected cells or those infected with an adenovirus lacking AMPK (Fig. 6B). As expected, stimulation with AICAR resulted in p42/44^{MAPK} activation in Ad-Null-transduced HepG2 cells (Fig. 6B), whereas overexpression of AMPKα2^{DN} reduced AICAR-mediated effects as reflected by the loss of ACC phosphorylation and a parallel decrease in the phosphorylation of p42/44^{MAPK} (Fig. 6B). A slight but significant reduction in p42/44^{MAPK} activation was observed in response to AICAR in cells overexpressing AMPKα2^{DN} compare to cells transfected with the control plasmid. The reduced phosphorylation of AMPKα isoform is presumably due to mutant AMPKα2^{DM} form competing with the endogenous AMPKα2 for binding to the other subunits of AMPK.

4. Discussion

The results presented in this paper show for the first time, for any cell type, AICAR is an efficient activator of Raf-1/MEK/p42/44^{MAPK}/p90^{RSK}/GSK-3 signaling cascade and demonstrate requirement of this signaling cascade in AICAR-dependent dephosphorylation/activation of GS activity and LDL receptor induction in HepG2 cells. An increase in

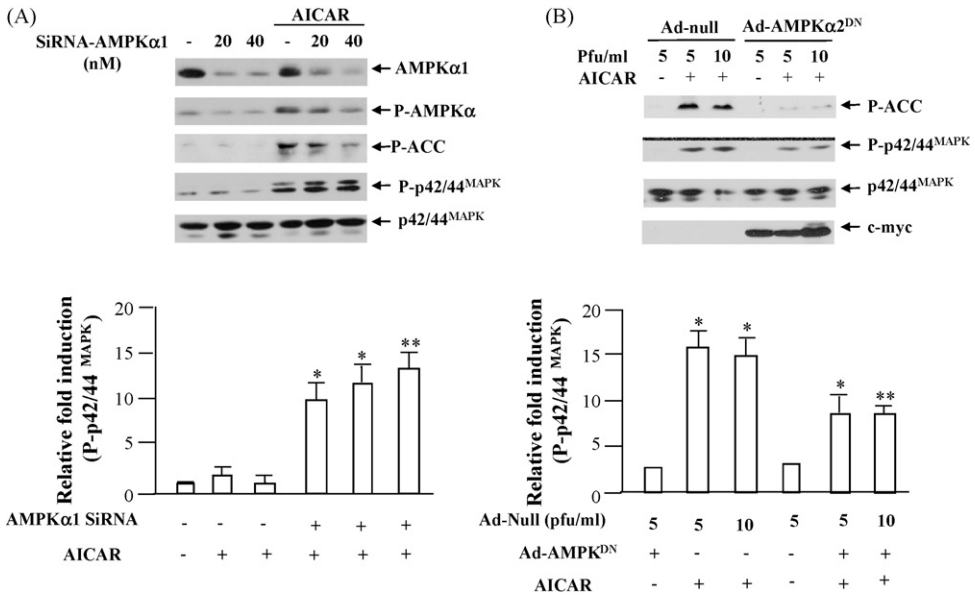

Fig. 6 – Partial requirement of AMPKα2, and not AMPKα1, for AICAR-induced p42/44^{MAPK} activation in HepG2 cells. (A) siRNA directed against AMPKα1 significantly reduced endogenous levels of this isoform, without significantly affecting p42/44^{MAPK} activation following treatment with 2 mM AICAR. HepG2 cells cultured in six-well dishes were transfected with 20 or 40 nM AMPKα1 siRNA. After 2 days, transfected cells starved for 16 h were treated for 1 h with 2 mM AICAR and processed for immunoblotting with the indicated antibodies. (B) Effect of adenovirus-mediated overexpression of AMPKα2^{DN} on AICAR-induced phosphorylation of ACC and p42/44^{MAPK}. HepG2 cells transduced with indicated doses of either Ad-Null or Ad-AMPKα2^{DN} virus for 2 days were starved for 16 h and then treated for 3 h with 2 mM AICAR to examine phosphorylation levels of ACC and p42/44^{MAPK}. Anti-c-myc was used to demonstrate overexpression of c-myc-tagged-AMPKα2^{DN}. The results shown are representative of three separate experiments. Signals were quantified with a phosphoimager and the results are expressed as fold stimulation by AICAR as compared to untreated cells (set at 1). Quantitative data are means ± S.E. from three separate experiments. ⁎, <0.05 relative to the value in the absence of AICAR and siRNA, $n = 4$; ⁎⁎, <0.01 relative to the value in the absence of AICAR and siRNA, $n = 4$.

glycogen biosynthesis, coupled with a selective increase in cholesterol uptake in liver, may underlie the ability of AICAR to improve glucose and lipid profiles through a common mechanism.

We found that AICAR decreases GSK-3 activity and the magnitude of GSK-3 deactivation is almost similar to that observed with insulin. In addition, our data also suggest that there are distinct mechanisms leading to GSK-3 deactivation in response to AICAR and insulin. For the insulin-induced deactivation of GSK-3, the findings are consistent with an Akt-dependent phosphorylation of GSK-3 on Ser$^{21/9}$ [8,9]. In contrast to insulin, AICAR treatment did not increase Akt phosphorylation. The inability of AICAR to elicit Akt phosphorylation, together with the lack of effect of wortmanin on AICAR-induced GSK-3 phosphorylation (Mehta et al., unpublished results), is consistent with the lack of any role for Akt in this process. There are other examples in the literature of an Akt-independent mechanism for deactivation of GSK-3. For example, numerous other stimuli also lead to inactivation of GSK-3 through Ser$^{21/9}$ phosphorylation in an Akt-independent manner, including growth factors such as epidermal growth factor and platelet-derived growth factor that stimulate the GSK-3-inactivating kinase pp90^{RSK} through MAP kinases [37,38], activators of p70 ribosomal S6 kinase (p70S6K) such as amino acids [39–41], activators of cAMP-activated protein kinase (PKA) [41–44] and PKC activators [45,46]. Interestingly, early studies have demonstrated that exercise does regulate GSK-3 activity independent of Akt [47,48]. Considering that exercise is shown to activate p42/44^{MAPK} and p90^{RSK} [49], it is possible that AICAR and exercise may work by a similar, Akt-independent mechanism for deactivation of GSK-3. Although the precise role for this level of regulation is not yet clear, such a mechanism may provide liver cells a means to synergize the action of insulin on glucose metabolism.

Another important observation is that AICAR not only activated AMPK, but also increased phosphorylation of Raf-1/MEK/p42/44^{MAPK}/p90^{RSK} cascade and GSK-3. Our SiRNA experiments suggested requirement of AMPK-α2 in this process, suggesting that parallel pathways are activated by AICAR which concomitantly require AMPK-α2 and another independent kinase. It is worth noting that in a recent study of transgenic mice where a dominant inhibitory AMPKα2 mutant was overexpressed in muscle, exercise-stimulated glucose uptake was only partially inhibited, thus implicating other AMPK-independent pathways in this process [50]. Early studies have also found AMPK-independent effects of AICAR in the liver cells [51,52]. It appears that our results add to the previous caveats concerning the use of AICAR to study the consequences of AMPK activation alone. Thus, actions ascribed to AMPK following AICAR treatment may be

influenced by the concomitant modulatory actions of AICAR on other kinases, such as Raf-1 kinase cascade. Interestingly, AMPK and p42/44^{MAPK} generally have opposing roles on cellular metabolism. AMPK is activated when AMP levels increases in conjunction with decreased ATP levels, and activated AMPK inhibits anabolic processes and promotes catabolism in order to minimize ATP utilization while promoting ATP production [53]. p42/44^{MAPK}, on the other hand, generally promotes anabolic cellular functions that utilize ATP, such as proliferation and cell growth [54]. The degree of activation of these kinases is expected to vary in a cell type-dependent manner, and may thus greatly influence cellular responses ascribed to AICAR. Compatible with this conclusion is the finding that AICAR can cause cell death [55–57] or protect cells from apoptosis [58–60] in a cell type-specific manner. Based on the above results, we propose that interplay between AMPK-dependent and -independent pathways probably play an important role in mediating AICAR action. These results emphasize a general point of significance, namely, that although AMPK activation may appear to dominate AICAR response, other parallel pathways can be critical, and thus should not be overlooked or ignored.

How does AICAR regulate LDL receptor transcription? The results presented here argue strongly in favor of a mechanistic link between AICAR, activation of Raf-1 kinase cascade, and LDL receptor induction. There are two potential mechanisms by which AICAR signaling may affect LDL receptor gene expression. First, AICAR-induced GSK-3 phosphorylation/deactivation may regulate sterol-response-element-binding proteins (SREBPs) function. Particularly relevant is an early finding that GSK-3 can phosphorylate SREBPs [61,62] that regulate expression of genes involved in fatty acid and cholesterol biosynthesis, including LDL receptor. The ability of AICAR to deactivate GSK-3 may function as an initial signaling event that results in changes in SREBPs stability, thus causing alterations in the lipid metabolism. Alternately, AICAR-induced p42/44^{MAPK} activity directly phosphorylates SREBPs and selectively potentiates transactivation potential [63]. These two mechanisms may operate in a synergistic fashion to modulate SREBPs function in response to AICAR. There is likely to be convergence of these signals at SREBPs, with integration of these signals may be critical in controlling SREBPs function and ultimately determining tissue and plasma lipid levels. Future studies will determine the contribution of SREBPs and specificity at the promoter region in selectively inducing LDL receptor expression by AICAR in HepG2 cells. It will also be interesting to examine whether activation of Raf-1 signaling cascade is the underlying mechanism for a recent observation showing that short-term overexpression of a constitutively active form of AMPKα2 in the mouse liver led to fatty liver [64].

In conclusion, results of this study suggest that AICAR may regulate glycogen metabolism primarily at the level of phosphorylation/deactivation of GSK-3 that leads to dephosphorylation/activation of GS in HepG2 cells. The mechanism leading to GSK-3 deactivation by AICAR treatment most likely involve Raf-1/MEK/p42/44^{MAPK}/p90^{RSK} cascade. The same signaling pathway is responsible for AICAR-induced LDL receptor expression. Elucidating the impact or interaction of AICAR initiated signaling will be crucial in understanding the molecular mechanisms regulating glucose and lipid metabolism in response to AICAR. Therapeutic intervention using AICAR will require further understanding of the signaling pathway components and the existence of cross-talk between them.

Acknowledgement

This work was supported by National Institutes of Health research grant HL079091.

REFERENCES

[1] Greenberg CC, Jurczak MJ, Danos AM, Brady MJ. Glycogen branches out: new perspectives on the role of glycogen metabolism in the integration of metabolic pathways. Am J Physiol Endocrinol Metab 2006;291:E1–8.
[2] Lawrence JC, Roach PJ. New insights into the role and mechanism of glycogen synthase activation by insulin. Diabetes 1997;46:541–7.
[3] Roach PJ. Control of glycogen synthase by hierarchal protein phosphorylation. FASEB J 1990;4:2961–8.
[4] Parker PJ, Caudwell FB, Cohen P. Glycogen synthase from rabbit skeletal muscle: effect of insulin on the state of phosphorylation of the seven phosphoserine residues in vivo. Eur J Biochem 1983;130:227–34.
[5] Welsh GI, Proud CG. Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. Biochem J 1993;294:625–9.
[6] Borthwick AC, Wells AM, Rochford JJ, Hurel SJ, Turnbull DM, Yeaman SJ. Inhibition of glycogen synthase kinase-3 by insulin in cultured human skeletal muscle myoblasts. Biochem Biophys Res Commun 1995;210:738–45.
[7] Dent P, Lavoinne A, Nakielny S, Caudwell FB, Watt P, Cohen P. The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. Nature 1990;348:302–8.
[8] Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995;378:785–9.
[9] van Weeren PC, de Bruyn KM, de Vries-Smits AM, van Lint J, Burgering BM. Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J Biol Chem 1998;273:13150–6.
[10] Cohen P. Dissection of the protein phosphorylation cascades involved in insulin and growth factor action. Biochem Soc Trans 1993;21:555–67.
[11] Carling D, Hardie DG. The substrate and sequence specificity of the AMP-activated protein kinase: phosphorylation of glycogen synthase and phosphorylase kinase. Biochim Biophys Acta 1989;1012:81–6.
[12] Zhang W, DePaoli-Roach AA, Roach PJ. Mechanisms of multisite phosphorylation and inactivation of rabbit muscle glycogen synthase. Arch Biochem Biophys 1993;304:219–25.
[13] Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, Goodyear LJ. Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. Diabetes 2000;49:527–31.
[14] Buhl ES, Jenssen N, Schmitz O, Pederson SB, Pederson O, Holman GD, et al. Chronic treatment with 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside

increases insulin-stimulated glucose uptake and GLUT4 translocation in rat skeletal muscles in a fiber type-specific manner. Diabetes 2001;50:12–7.
[15] Holmes BE, Kurth-Kraczek EJ, Winder WW. Chronic activation of 5′-AMP-activated protein kinase increases GLUT4, hexokinase, and glycogen in muscle. J App Physiol 1999;87:1990–5.
[16] Milan D, Jeon JT, Looft C, Amarger V, Robic A, Thelander M, et al. A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle. Science 2000;288:1248–51.
[17] Corton JM, Gillespie JG, Hawley SA, Hardie DG. 5-Aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem 1995;229:558–65.
[18] Merrill GF, Kurth EJ, Hardie DG, Winder WW. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol 1997;273:E1107–12.
[19] Iglesias MA, Furler SM, Cooney GJ, Kraegen EW, Ye JM. AMP-activated protein kinase activation by AICAR increases both muscle fatty acid and glucose uptake in white muscle of insulin-resistant rats in vivo. Diabetes 2004;53:1649–54.
[20] Pold R, Jensen LS, Jessen N, Buhl ES, Schmitz O, Flyvbjerg A, et al. Long-term AICAR administration and exercise prevents diabetes in ZDF rats. Diabetes 2005;54:928–34.
[21] Koistinen HA, Galuska D, Chibalin AV, Yang J, Zierath JR, Holman GD, et al. 5-Aminoimidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes 2003;52:1066–72.
[22] Leclerc I, Kahn A, Doiron B. The 5′-AMP-activated protein kinase inhibits the transcriptional stimulation by glucose in liver cells, acting through the glucose response complex. FEBS Lett 1998;431:180–4.
[23] Foretz M, Carling D, Guichard C, Ferre P, Foufelle F. AMP-activated protein kinase inhibits the glucose-activated expression of fatty acid synthase gene in rat hepatocytes. J Biol Chem 1998;273:14767–71.
[24] Hubert A, Husson A, Chedville A, Lavoinne A. AMP-activated protein kinase counteracted the inhibitory effect of glucose on the phosphoenolpyruvate carboxykinase gene expression in rat hepatocytes. FEBS Lett 2000;481: 209–12.
[25] Lochhead PA, Salt IP, Walker KS, Hardie DG, Sutherland C. 5-Aminoimidazole-4-carboxamide riboside mimics the effects of insulin on the expression of the 2 key gluconeogenic genes PEPCK and glucose-6-phosphatase. Diabetes 2000;49:896–903.
[26] Leff T. AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins. Biochem Soc Trans 2003;31(pt 1):224–7.
[27] Kumar KL, Marita AR. Troglitazone prevents and reverses dexamethasone induced insulin resistance on glycogen synthesis in 3T3 adipocytes. Br J Pharmacol 2000;130:351–8.
[28] Mehta KD, Chang R, Underwood J, Wise J, Kumar A. Identification of a novel *cis*-acting element participating in maximal induction of the human low density lipoprotein receptor gene expression in response to low cellular cholesterol levels. J Biol Chem 1996;271:33616–22.
[29] Huang W, Batra S, Korrapati S, Mishra V, Mehta KD. Selective repression of low density lipoprotein receptor expression by SP600125: coupling of histone H3-Ser10 phosphorylation and Sp1 occupancy. Mol Cell Biol 2006;26:1307–17.
[30] Huang W, Batra S, Atkins BA, Mishra V, Mehta KD. Increases in intracellular calcium dephosphorylate histone H3 at serine 10 in human hepatoma cells: potential role of protein phosphatase 2A-protein kinase Cbii complex. J Cell Physiol 2005;205:37–46.
[31] Al-Khalili L, Krook A, Zierath JR, Cartee GD. Prior serum- and AICAR-induced AMPK activation in primary human myocytes does not lead to subsequent increase in insulin-stimulated glucose uptake. Am J Physiol Endocrinol Metab 2004;287:E553–7.
[32] Pencek RR, Shearer J, Camacho RC, James FD, Lacy DB, Fueger PT, et al. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside causes acute hepatic insulin resistance in vivo. Diabetes 2005;54:355–60.
[33] Singh RP, Dhawan P, Golden C, Kapoor GS, Mehta KD. One-way cross-talk between $p38^{MAPK}$ and $p42/44^{MAPK}$. Inhibition of $p38^{MAPK}$ induces low density lipoprotein receptor expression through activation of the $p42/44^{MAPK}$ cascade. J Biol Chem 1999;274:19593–600.
[34] Chiloeches A, Mason CS, Marais R. S338 phosphorylation of Raf-1 is independent of phosphatidylinositol 3-kinase and Pak3. Mol Cell Biol 2001;21:2423–34.
[35] Mehta KD. Role of mitogen-activated protein kinases and protein kinase C in regulating low-density lipoprotein receptor expression. Gene Expr 2002;10:153–64.
[36] Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001;108:1167–74.
[37] Brady MJ, Bourbonais FJ, Saltiel AR. The activation of glycogen synthase by insulin switches from kinase inhibition to phosphatase activation during adipogenesis in 3T3-L1 cells. J Biol Chem 1998;273:14063–6.
[38] Saito Y, Vandenheede JR, Cohen P. The mechanism by which epidermal growth factor inhibits glycogen synthase kinase 3 in A431 cells. Biochem J 1994;303:27–31.
[39] Armstrong JL, Bonavaud SM, Toole BJ, Yeaman SJ. Regulation of glycogen synthase by amino acids in cultured human muscle cells. J Biol Chem 2001;276:952–6.
[40] Krause U, Bertrand L, Maisin L, Rosa M, Hue L. Signaling pathways and combinatory effects of insulin and amino acids in isolated rat hepatocytes. Eur J Biochem 2002;269:3742–50.
[41] Terruzzi I, Allibardi S, Bendinelli P, Maroni P, Piccoletti R, Vesco F, et al. Amino acid- and lipid-induced insulin resistance in rat heart: molecular mechanisms. Mol Cell Endocrinol 2002;190:135–45.
[42] Tanji C, Yamamoto H, Yorioka N, Kohno N, Kikuchi K, Kikuchi A. A-kinase anchoring protein AKAP220 binds to glycogen synthase kinase-3beta (GSK-3beta) and mediates protein kinase A-dependent inhibition of GSK-3beta. J Biol Chem 2002;277:36955–61.
[43] Li M, Wang X, Meintzer MK, Lsessig T, Birnbaum MJ, Heidenreich KA. Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3beta. Mol Cell Biol 2000;20:9356–63.
[44] Fang X, Yu SX, Lu Y, Bast RC, Woodgett JR, Mills GB. Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. Proc Natl Acad Sci USA 2000;97:11960–5.
[45] Ballou LM, Tian PY, Lin HY, Jiang YP, Lin RZ. Dual regulation of glycogen synthase kinase-3beta by the alpha1A-adrenergic receptor. J Biol Chem 2001;276:40910–6.
[46] Fang X, Yu S, Tanyi JL, Lu Y, Woodgett JR, Mills GB. Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. Mol Cell Biol 2002;22:2099–110.
[47] Markuns JF, Wojtaszewski JFP, Goodyear LJ. Insulin and exercise decrease glycogen synthase kinase-3 activity by different mechanisms in rat skeletal muscle. J Biol Chem 1999;274:24896–900.

[48] Price TB, Rothman DL, Taylor R, Avison MJ, Shulman GI, Shulman RG. Human muscle glycogen resynthesis after exercise: insulin-dependent and -independent phases. J Appl Physiol 1994;76:104–11.
[49] Yu M, Stepto NK, Chibalin AV, Fryer LG, Carling D, Krook A, et al. Metabolic and mitogenic signal transduction in human skeletal muscle after intense cycling exercise. J Physiol 2003;546:327–35.
[50] Jorgensen SB, Viollet B, Andreelli F, Frosig C, Birk JB, Schjerling P, et al. Knockout of the alpha2 but not alpha1 5′-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-β-4—ribofuranoside but not contraction-induced glucose uptake in skeletal muscle. J Biol Chem 2004;279:1070–9.
[51] Guigas B, Bertrand L, Taleux N, Foretz M, Wiernsperger N, Vertommen D, et al. 5-Aminoimidazole-4-carboxamide-1-b-ribofuranoside and metformin inhibit hepatic glucose phosphorylation by an AMP-activated protein kinase-independent effect on glukokinase translocation. Diabetes 2006;55:865–74.
[52] Guigas B, Viollet B, Taleux N, Foretz M, Vaulont S, Hue L. 5-Aminoimidazole-4-carboxamide ribonucleoside and metformin inhibit hepatic mitochondrial oxidative phosphorylation by two different AMPK-independent mechanism (Abstract). FEBS J 2005;272:A306.
[53] Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab 2005;1:15–25.
[54] Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient vs. sustained extracellular signal-regulated kinase activation. Cell 1995;80:179–85.
[55] Stefanelli C, Stanic I, Bonavita F, Flamigni F, Pignatti C, Guarnieri C, et al. Inhibition of glucocorticoid-induced apoptosis with 5-aminoimidazole-4-carboxamide ribonucleoside, a cell permeable activator of AMP-activated protein kinase. Biochem Biophys Res Commun 1998;243:821–6.
[56] Blazquez C, Geelen MJH, Velasco G, Guzman M. The AMP-activated protein kinase prevents ceramide synthesis de novo and apoptosis in astrocytes. FEBS Lett 2001;489:149–53.
[57] McCullough LD, Zeng Z, Li H, Landree LE, McFadden J, Ronnett GV. Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke. J Biol Chem 2005;280:20493–502.
[58] Rattan R, Giri S, Singh AK, Singh I. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside inhibits cancer cell proliferation in vitro and in vivo via AMP-activated protein kinase. J Biol Chem 2005;280:39582–93.
[59] Lopez JM, Santidrian AF, Campas C, Gil J. 5-Aminoimidazole-4-carboxamide riboside induces apoptosis in Jurkat cells, but the AMP-activated protein kinase is not involved. Biochem J 2003;370:1027–32.
[60] Kefas BA, Heimberg H, Vaulont S, Meisse D, Hue L, Pipeleers D, et al. AICA-riboside induces apoptosis of pancreatic beta cells through stimulation of AMP-activated protein kinase. Diabetologia 2003;46:250–4.
[61] Sundqvist A, Bengoechea-Alonso MT, Ye X, Lukiyanschuk V, Jin J, Harper JW, et al. Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCFFbw7. Cell Metab 2005;1:379–91.
[62] Kim KH, Song MJ, Yoo EJ, Choe SS, Park SD, Kim JB. Regulatory role of glycogen synthase kinase 3 for transcriptional activity of ADD1/SREBP-1c. J Biol Chem 2004;279:51999–2006.
[63] Roth G, Kotzka J, Kremer L, Lehr S, Lohaus C, Meyer HE, et al. MAP-kinases Erk1/2 phosphorylate sterol regulatory element binding protein (SREBP)-1a at serine 117 in vitro. J Biol Chem 2000;275:33302–7.
[64] Foretz M, Ancellin N, Andreelli F, Saintillan Y, Grondin P, Kahn A, et al. Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. Diabetes 2005;54:1331–9.