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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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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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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