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A role for AMPK in the inhibition of glucose-6-phosphate dehydrogenase by polyunsaturated fatty acids

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ABSTRACT

Both polyunsaturated fatty acids and AMPK promote energy partitioning away from energy consuming processes, such as fatty acid synthesis, towards energy generating processes, such as β-oxidation. In this report, we demonstrate that arachidonic acid activates AMPK in primary rat hepatocytes, and that this effect is p38 MAPK-dependent. Activation of AMPK mimics the inhibition by arachidonic acid of the insulin-mediated induction of G6PD. Similar to intracellular signaling by arachidonic acid, AMPK decreases insulin signal transduction, increasing Ser^{307} phosphorylation of IRS-1 and a subsequent decrease in AKT phosphorylation. Overexpression of dominant-negative AMPK abolishes the effect of arachidonic acid on G6PD expression. These data suggest a role for AMPK in the inhibition of G6PD by polyunsaturated fatty acids.

Introduction

Polyunsaturated fatty acids have multiple regulatory actions within cells including activating cellular signaling pathways and regulating gene expression. The mechanism by which polyunsaturated fatty acids transduce their signal within cells is not completely understood. The inhibition of acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and spot 14 (S14) expression in rat liver by polyunsaturated fatty acids is associated with the activation of AMP-activated protein kinase (AMPK) [1–3]. Yet, the ability of dietary polyunsaturated fatty acids to activate AMPK in all tissues is not uniformly observed and a role for AMPK in regulating the transcription of glucose-responsive genes is controversial [4–7]. Therefore, we asked if a lipogenic gene regulated exclusively at a posttranscriptional level would involve AMPK activity in the inhibition of its expression by polyunsaturated fatty acids.

Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme in the pentose phosphate pathway. G6PD catalyzes the first reaction in this pathway by converting glucose-6-phosphate to 6-phosphogluconolactone [8–10]. NADPH is concomitantly produced during this reaction, providing 50–75% of the necessary reducing equivalents for fatty acid biosynthesis in liver [9]. G6PD expression is inhibited by polyunsaturated fat through a posttranscriptional mechanism involving decreases in the efficiency of splicing of the pre-mRNA [11]. The signaling pathway causing increased expression of G6PD involves insulin activation of the phosphatidylinositol 3-kinase (PI 3-kinase) pathway resulting in the accumulation of G6PD mRNA [12]. Polyunsaturated fatty acids such as arachidonic acid inhibit accumulation of G6PD mRNA by inhibiting insulin signaling through the activation of p38 mitogen activated protein kinase (p38 MAPK) and the subsequent Ser^{307} phosphorylation of insulin receptor substrate-1 (IRS-1) [12]. Upstream kinases stimulating the phosphorylation of p38 MAPK have remained elusive.

AMPK has been suggested to play a role in mediating the actions of fatty acids in cells. AMPK is a sensor of cellular energy because it is activated by an increase in the AMP/ATP ratio [13]. Activation of AMPK leads cells to abandon energy-consuming pathways (such as glycogenolysis and fatty acid synthesis) and to stimulate energy-producing pathways (such as β-oxidation and glycolysis). Polyunsaturated fatty acids have a similar activity within cells via the short-term inhibition of ACC activity that regulates both fatty acid synthesis and oxidation, as well as via long-term changes in the expression of genes in glycolysis, lipogenesis and fatty acid oxidation [14].

Due to the coinciding roles of both polyunsaturated fatty acids and AMPK in energy homeostasis, the role of AMPK in mediating the inhibitory effects of polyunsaturated fatty acids on lipogenic enzymes seems probable. In this report, we demonstrate that AMPK is an inhibitor of G6PD expression and that inhibition of AMPK activity abrogates the inhibitory action of polyunsaturated fatty acids on the accumulation of G6PD mRNA.

Materials and methods

Animal care and cell culture. Male Sprague–Dawley rats (150–200 g) were maintained on standard rodent chow under a normal

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light–dark cycle. Animal experiments were conducted in conformity with the Public Health Service policy on Humane Care and Use of Laboratory Animals, additionally; the Institutional Animal Care and Use Committee of the Division of Laboratory Animal Resources at West Virginia University approved all experimental procedures. Hepatocytes were isolated from the liver by a modification of the method of Seglen [15], as described previously [11]. Hepatocytes (3×10^6) were plated onto 60 mm collagen-coated plates in Hi/Wo/Ba medium (Waymouth MB752/1 plus 20 mM HEPES, pH 7.4, 0.5 mM serine, 0.5 mM alanine, 0.2% bovine serum albumin) plus 5% newborn calf serum to facilitate cell adhesion. Two hours post-isolation hepatocytes were washed twice with serum-free media, and were incubated overnight with serum-free media and 0.3 mg Matrigel per plate (BD PharMingen) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Treatments were added to the hepatocytes in fresh serum-free media, without Matrigel, after 20 h in culture (time zero). Hepatocytes not receiving 175 μM arachidonic acid (Nu-Check Prep: complexed with 4 mM bovine serum albumin and butylated-hydroxytoluene 0.1%) were treated with an equivalent volume of serum-free media containing 4 mM bovine serum albumin and butylated-hydroxytoluene 0.1% solution. All media contained supplemental a-tocopherol (5 μg/ml of medium). The AMPK activator, aminoimidazole carboxamide ribonucleotide (AICAR) was purchased from Toronto Research Chemicals Inc. The p38 MAP kinase inhibitor, SB203580 was purchased from Calbiochem.

Protein isolation and Western blot analysis. Preparation of cell lysates and western analysis was as described [12]. The primary antibodies against phosphorylated AKT (Ser^{473}), phosphorylated IRS-1 (Ser^{307}), phosphorylated p38 MAPK (Thr^{180}/Tyr^{182}), phosphorylated AMPK (Thr^{172}), phosphorylated ACC (Ser^{79}), phosphorylated PKC (pan, βII Ser^{660}), phosphorylated S6K-1, (Thr^{389}), phosphorylated MAP kinase kinase (MKK) 3/6 and total AKT, IRS-1, p38 MAPK, AMPK, ACC, tubulin were obtained from Cell Signaling Technology. Anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signaling Technology) was used as the secondary antibody, and the immunocomplexes were detected by enhanced chemiluminescence (Pierce). Images were visualized with film (Pierce) and quantified by densitometry using ImageQuant software (Amersham Biosciences).

Adenoviral vectors. Adenovirus expressing dominant-negative AMPK (kinase-dead due to K45R mutation in α2 subunit, with a 5′ myc tag; a gift from Dr. M. Birnbaum) [16] and adenovirus expressing green fluorescent protein (GFP; a gift from Dr. C.J. Rhodes) [17] were amplified in HEK 293 cells. Virus was purified by CsCl gradient in which the virus was layered over 1.25 g/ml CsCl in 100 mM Tris (pH 8.0) and 1.4 g/ml CsCl in 100 mM Tris (pH 8.0), and spun at 35,000 rpm for 1 h at 4 °C in a SW-41 rotor. The viral band was excised and overlayered with 1.34 g/ml CsCl in 100 mM Tris (pH 8.0) and spun at 60,000 rpm for 10 h at 4 °C in a VTi 65.2 rotor. The viral fraction was then desalted using a Sephadex G-25 column (Pharmacia). Viral titer was determined using the Adeno-X Rapid Titer kit (Clontech) per the manufacturer's instructions. The purified virus was stored in 30% glycerol in PBS at −80 °C.

Isolation of total RNA and quantitative real-time RT-PCR. Total RNA was isolated from 2 plates per treatment using Tri-Reagent (Ambion) according to manufacturer's instructions. Abundance of specific mRNA was measured using real-time reverse transcriptase polymerase chain reaction (RT-PCR). RNA (150–200 ng) was DNase I-treated and expression of all mRNAs was determined by real-time PCR (BioRad iCycler iQ) analysis. G6PD and RPL32 mRNAs were measured using TaqMan probes. ACC, SCD, FAS, S14, and cyclophilin B mRNAs were measured using Quantitect SYBR green (QIAGEN) according to the manufacturer's instructions. Sequences for primers and probes are as follows glucose 6-phosphate dehydrogenase, sense, 5′-TATGTCTATGGCAGCCGAGGT-3′, antisense, 5′-GCAGAGTGCAGATGGTGTAAG-3′; fatty acid synthase, sense, 5′-TGCAACTGTGCGTTAGCCACC-3′, antisense, 5′-TGTTTCAGGGGAGAAGAGACC-3′; stearoyl-coA desaturase, sense, 5′-AGCTCAGCCAAATGCTGTGTTGTC-3′, antisense, 5′-TGCCTTGATCAGTCACAGACACCT-3′; spot 14, sense, 5′-CAGGAGGTGACGCAGAAATAC-3′, antisense, 5′-GTGAGGTAAATACAGCGTCCC-3′; cyclophilin B, sense, 5′-CGTGGGCTCCGTTGTCTT-3′, antisense, 5′-TGACTTTAGGTCCCTTCTTCTTATC-3′; RPL32, sense, 5′-AAACTGGCGGAAACCCAGAG-3′; antisense, 5′-GCAGCACTTCCAGCTCCTTG-3′; G6PD probe, 5′-FAM/CCACAGAGGCAGATGAGCTGATGAAGAA/3BHQ-3′; RPL32 probe, 5′-/56-FAM/CCAGATCCTGATGCCCAACATTGC-3′. The relative amount of each mRNA was calculated using the comparative threshold cycle method. Expression of cyclophilin B or RPL32 was used as a control and the amount of each mRNA was calculated relative to this control.

Statistics. Statistics were performed using GraphPad Prism (version 4.0). Overall statistical significance was determined by one-way ANOVA; multiple comparisons were made using a Bonferroni post-test if the overall p-value after ANOVA was $p < 0.05$.

Results

Phosphorylation of AMPK at Thr^{172} is a measure of AMPK activation [13]. Incubation of primary rat hepatocytes with arachidonic acid induced AMPK phosphorylation compared to cells not treated with insulin or arachidonic acid (NA or I); total AMPK levels did not change (Fig. 1). The activation of AMPK in response to arachidonic acid was rapid and was detected as early as 10 min and retained through 60 min (data not shown). ACC phosphorylation at Ser^{79} was induced by treatment with arachidonic acid further indicating that arachidonic acid activates AMPK and its downstream targets in rat hepatocytes (Fig. 1) [18,19]. Coincident with the activation of AMPK, p38 MAPK and its upstream kinase MKK 3/6 were also activated (Fig. 1). The coincident activation of these

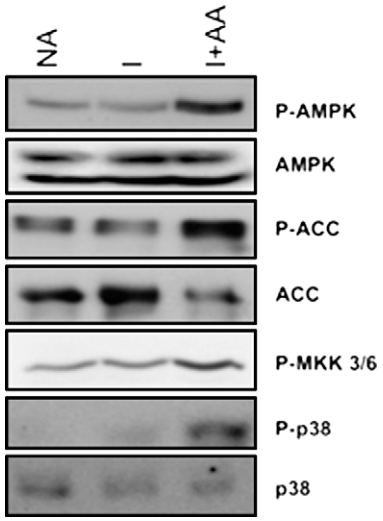

Fig. 1. Arachidonic acid induces activation of AMPK. Primary rat hepatocytes were plated on collagen coated dishes and incubated in Hi/Wo/Ba medium. After 20 h in culture, the medium was replaced with Hi/Wo/Ba with or without insulin (40 nM; I) and albumin-bound arachidonic acid (175 μM; AA). Total cell lysates were isolated 10 min after treatment. Western blot analysis was performed against phosphorylated AMPK (Thr^{172}) and total AMPK, phosphorylated ACC (Ser^{79}), and total ACC, phosphorylated MKK 3/6, and phosphorylated p38 MAPK (Thr^{180}/Tyr^{182}) and total p38 MAP kinase. A representative blot is shown. Repetition demonstrating the same results was as follows: $n = 3$ experiments for phosphorylated and total AMPK and ACC, $n = 2$ for P-MKK 3/6, $n = 7$ for phosphorylated and total p38 MAP kinase. NA, no addition; P-AMPK, phosphorylated AMPK; P-ACC, phosphorylated ACC; P-MKK 3/6, phosphorylated MKK 3/6; P-p38, phosphorylated p38 MAP kinase.

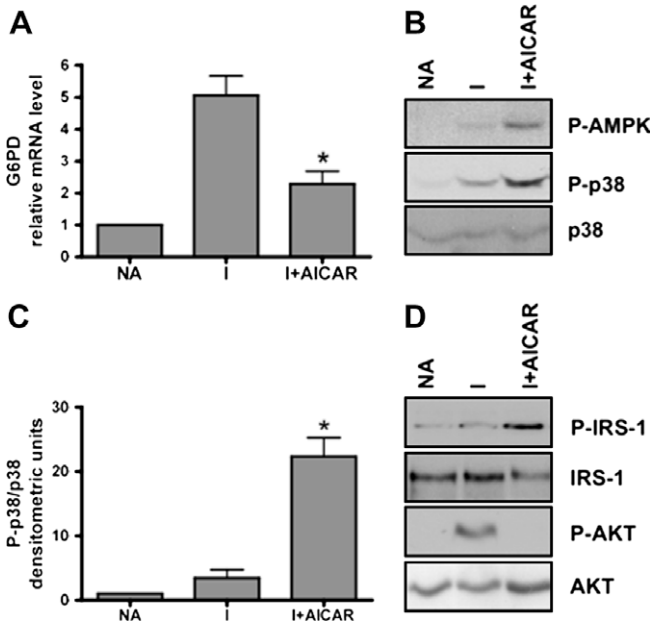

Fig. 2. Activation of AMPK inhibits the insulin-mediated induction of G6PD mRNA and insulin signal transduction. Primary rat hepatocytes were plated on collagen coated dishes and incubated in Hi/Wo/Ba medium. (A) After 20 h in culture, the medium was replaced with Hi/Wo/Ba with or without insulin (40 nM; I) and AICAR (0.5 mM). Media was replaced every 12 h with one of identical composition, and RNA was isolated after 24 h of treatment (2 plates/treatment). G6PD mRNA is calculated relative to RPL32 mRNA. Values are expressed as the fold-increase over no addition (NA). Each bar represents the mean ± SE of $n = 4$ independent primary hepatocyte isolations. (B) After 24 h in culture, the medium was replaced with Hi/Wo/Ba with or without insulin (40 nM; I) and AICAR (0.5 mM). Total cell lysates were isolated 10 min after treatments. Western blot analysis was performed against phosphorylated AMPK (Thr^{172}), and phosphorylated and total p38 MAPK. A representative blot is shown. (C) Densitometry was performed with ImageQuant software (Molecular Dynamics), and values were calculated as arbitrary densitometric units of phosphorylated/total protein. Values are expressed as the fold-increase over no addition (NA). Each bar represents the mean ± SE of $n = 4$ independent primary hepatocyte isolations. (D) Hepatocytes were treated with or without AICAR (0.5 mM) 24 h prior to the addition of insulin (40 nM; I). Total cell lysates were prepared 10 min after the addition of insulin. Western blot analysis was performed against total and phosphorylated IRS-1 (Ser^{307}) and AKT (Ser^{473}). A representative blot is shown and represents the mean ± SE of $n = 4$ independent primary hepatocyte isolations.

signaling molecules supports the hypothesis that the AMPK signaling pathway as well as the inhibitory p38 MAPK pathway are cooperating to inhibit G6PD expression.

To test for a role of AMPK signaling in the inhibition of G6PD expression, accumulation of G6PD mRNA was measured after treatment with AICAR, a pharmacological activator of AMPK [20]. Insulin-induced G6PD mRNA accumulation 5-fold compared to no addition, and the addition of AICAR to the hepatocytes inhibited this increase by 50% (Fig. 2A). This mimics the extent of inhibition of G6PD mRNA by arachidonic acid [12]. The abundance of G6PD mRNA is expressed relative to RPL32 mRNA abundance. In all experiments, RPL32 expression was not regulated by AICAR (data not shown). Hepatocytes were also treated with metformin, another pharmacological activator of AMPK, to test if AMPK activation by a different compound can also inhibit G6PD expression [21]. Like AICAR, metformin also inhibited the insulin-induction of G6PD mRNA by 50% or more (data not shown). As expected, AICAR enhanced AMPK phosphorylation in these cells (Fig. 2B). In addition, this stimulation of AMPK was accompanied by a 5-fold increase in p38 MAPK activation as compared to insulin alone (Fig. 2B and C). Thus, activation of AMPK by AICAR mimics the effect of arachidonic acid on G6PD expression and p38 MAPK activation.

Arachidonic acid activation of p38 MAPK is accompanied by phosphorylation of IRS-1 at Ser^{307}, which decreases insulin signal transduction and ultimately inhibits G6PD expression [12]. Incubation with AICAR also induced Ser^{307} phosphorylation of IRS-1 (Fig. 2D). In addition, the insulin-mediated activation of AKT, a measure of PI 3-kinase activity, was completely abolished in the presence of AICAR. These results suggest that like arachidonic acid, activation of AMPK with AICAR interferes with the PI 3-kinase pathway resulting in a decrease in the expression of G6PD mRNA.

In order to determine whether the activation of p38 MAPK by AICAR plays a role in the inhibition of G6PD, hepatocytes were treated with the p38 MAP kinase inhibitor SB203580. Insulin-induced G6PD mRNA accumulation by 3-fold despite the presence of SB203580; however, the AICAR-mediated inhibition of the G6PD mRNA expression was completely abolished (Fig. 3A). Thus, the AICAR-mediated inhibition of G6PD mRNA expression involves the p38 MAP kinase pathway.

The effect of AMPK activation by AICAR on the expression of other lipogenic genes was also determined. In primary rat hepatocytes, insulin stimulates the accumulation of mRNA for FAS, SCD, and S14 by 3- to 10-fold (Fig. 3B). The insulin stimulation of these genes was inhibited by AICAR, 53%, 55% and 59%, respectively. Treatment with the p38 MAP kinase inhibitor SB203580 blocked this effect of AICAR on these genes. The abundance of these mRNA is expressed relative to cyclophilin. AICAR and SB203580 did not affect the abundance of cyclophilin mRNA indicating the selectivity of these treatments (data not shown). Therefore, other lipogenic genes are inhibited when AMPK is activated, and this inhibition is attenuated when p38 MAPK activity is blocked.

To confirm a role of AMPK in the arachidonic acid-mediated inhibition of G6PD, hepatocytes were infected with adenovirus expressing dominant-negative AMPK. Hepatocytes not receiving dominant-negative AMPK were infected with adenovirus expressing green fluorescent protein (GFP). Infection with the dominant-negative AMPK dramatically decreased the activation of AMPK by arachidonic acid as measured by changes in ACC phosphorylation, a downstream target of AMPK (Fig. 4A). Virus expressing GFP did not alter AMPK activity. Western analysis against the Myc tag of the dominant-negative AMPK further confirmed expression of the adenovirus in the cells (data not shown). In hepatocytes infected with the control adenovirus, insulin treatment induced G6PD expression by 4-fold and treatment with arachidonic acid significantly inhibited this stimulation by 54% (Fig. 4B). Dominant-negative AMPK blocked the inhibition by arachidonic acid. This data provides evidence for a role of AMPK in the arachidonic acid-mediated inhibition of G6PD.

Discussion

Polyunsaturated fatty acids are potent regulators of cellular function and yet the signal transduction mechanisms that they use have remained elusive. In our previous studies, we have shown that arachidonic acid-mediated inhibition of insulin-induced G6PD expression involves the activation of p38 MAPK pathway, which in turn interferes with the PI 3-kinase pathway through the Ser^{307} phosphorylation of IRS-1, thus inhibiting the insulin-mediated induction of G6PD [12]. Fatty acids are also known to activate additional signaling pathways including AMPK [2]. In this report we present the following lines of evidence that arachidonic acid activation of AMPK is involved in the inhibition of G6PD expression. First, activation of AMPK, per se, inhibits the insulin induction of G6PD mRNA (Fig. 2A). Second, activation of AMPK by AICAR concomitantly activates p38 MAPK, results in Ser^{307} phosphorylation of IRS-1, and inhibits AKT phosphorylation similar to the action of arachidonic acid (Fig. 2B–D) [12]. Inhibition of p38 MAPK blocks

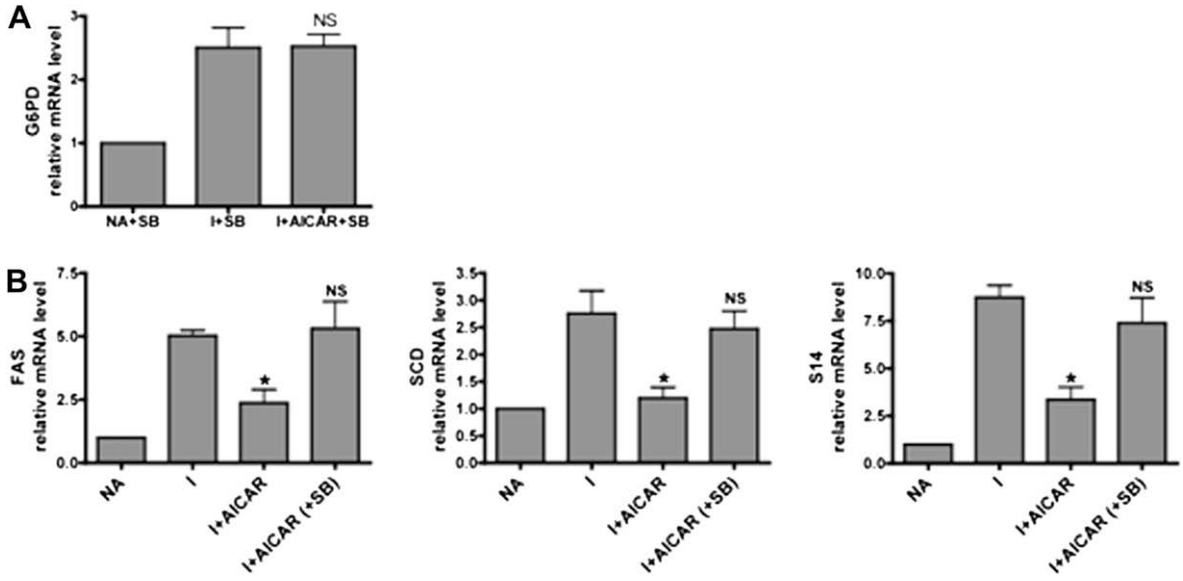

Fig. 3. Inhibition of lipogenic genes by polyunsaturated fatty acids by AMPK requires p38 MAPK. After 20 h in culture, the medium was replaced with Hi/Wo/Ba with or without the p38 MAP kinase inhibitor SB203580 (10 μM; SB) or AICAR (0.5 mM) for 2 h. Cells were then treated with or without insulin (40 nM; I). Media was replaced every 12 h with one of identical composition, and RNA was isolated after 24 h of treatment (2 plates/treatment). (A) G6PD mRNA is calculated relative to RPL32 mRNA. Values are expressed as the fold-increase over no addition (NA). Each bar represents the mean ± SE of $n = 4$ independent primary hepatocyte isolations. (B) FAS, SCD, and S14 mRNA abundance is calculated relative to rat cyclophilin B. Values are expressed as the fold-increase over no addition (NA). Each bar represents the mean ± SE of $n = 3$ independent primary hepatocyte isolations.

the effect of AICAR on accumulation of G6PD mRNA (Fig. 3A). These changes in the activity of signaling pathways and G6PD expression

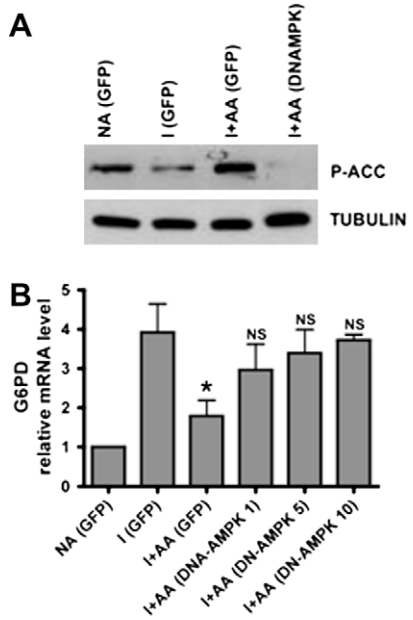

Fig. 4. Inhibition of AMPK activity blocks the arachidonic acid inhibition of G6PD expression. Primary rat hepatocytes were plated on collagen coated dishes and incubated in Hi/Wo/Ba medium. After 2 h in culture, hepatocytes were infected with 1, 5, and 10 infectious units/cell of either dominant-negative AMPK expressing adenovirus (DNAMPK) or control adenovirus expressing GFP. After 2 h, cells were washed twice, and media was replaced with one containing Matrigel. After 20 h in culture, the medium was replaced with Hi/Wo/Ba with or without insulin (40 nM; I) and arachidonic acid (175 μM; AA). (A) After 10 min of treatment, cell lysates were prepared and Western analysis was performed against phosphorylated ACC (Ser^{79}) and against tubulin as a loading control. (B) For isolation of mRNA, media was replaced every 12 h with one of identical composition, and RNA was isolated after 24 h of treatment (2 plates/treatment). G6PD mRNA abundance is calculated relative to rat cyclophilin B. Values are expressed as the fold-increase over no addition (NA). Each bar represents the mean ± SE of $n = 3$ independent primary hepatocyte isolations.

occur within the same time frame as arachidonic acid activation of AMPK and arachidonic acid inhibition of G6PD expression. Finally, arachidonic acid inhibition of G6PD mRNA requires active AMPK, as the expression of a dominant-negative AMPK blocks this inhibition (Fig. 4B). Thus, our data are consistent with the hypothesis that activation of AMPK by arachidonic acid enhances p38 MAP kinase activity resulting in the inhibition of insulin signal transduction and thereby G6PD expression.

The role of AMPK in mediating the effects of polyunsaturated fats on liver metabolism is controversial. Studies examining AMPK activation in response to dietary lipid are inconclusive [1,4,5,7]. These may reflect the difficulty in retaining the phosphorylation state of proteins during the preparation of tissue lysates. Alternatively, AMPK activation may not be a primary mechanism in regulation by dietary fatty acids. In this regard, incubation of rat hepatocytes with chylomicron remnants, the mode of delivery of dietary fat to liver, does not activate AMPK [22]. In contrast, liver is exposed to high concentrations of non-esterified fatty acids during times of enhanced lipolysis. AMPK activation occurs rapidly in response to free fatty acids (Fig. 1) and may play an important role in the partitioning of liver metabolism towards fatty acid oxidation and gluconeogenesis. It is not clear why activation of AMPK by fatty acids is observed by some laboratories (Fig. 1) [6] and not by others [4,5]. AMPK activation occurs rapidly and may require measurement at earlier time points or may be unique to specific fatty acids. Alternatively, procedural differences in hepatocyte culture and harvesting techniques may be responsible. Nonetheless, activation of AMPK by polyunsaturated fatty acids coincides with inhibition of G6PD expression.

The activities of the lipogenic enzymes, including G6PD, are coordinately regulated. Yet, the details of this regulation differ between the different lipogenic enzymes. G6PD is unique in that its regulation occurs primarily at a posttranscriptional step. The inhibition of G6PD and other lipogenic enzymes by AMPK suggests that this kinase is an important upstream signaling molecule impacting multiple steps in gene expression and altering the capacity of hepatocytes for fatty acid biosynthesis.

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