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Research Article

Pyruvate dehydrogenase kinase 4 expression is synergistically induced by AMP-activated protein kinase and fatty acids

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Abstract. Organs are flexible as to which substrates they will use to maintain energy homeostasis. Under well-fed conditions, glucose is a preferred substrate for oxidation. During fasting, fatty acid oxidation will become a more important energy source. Glucose oxidation is decreased by fatty acids, a process in which the pyruvate dehydrogenase complex (PDH) and its regulator pyruvate dehydrogenase kinase 4 (PDK4) play important roles. It is currently unknown how energy status influences PDH activity. We show that AMP-activated protein kinase (AMPK) activation by hypoxia and AICAR treatment combined with fatty acid administration synergistically induce PDK4 expression. We provide evidence that AMPK activation modulates ligand-dependent activation of peroxisome proliferator-activated receptor. Finally, we show that this synergistic induction of PDK4 decreases cellular glucose oxidation. In conclusion, AMPK and fatty acids play a direct role in fuel selection in response to cellular energy status in order to spare glucose.

Keywords. Glucose metabolism, pyruvate dehydrogenase, pyruvate dehydrogenase kinase 4, AMP-activated protein kinase, fatty acids, hypoxia.

Introduction

The pyruvate dehydrogenase complex (PDH) catalyzes the oxidative decarboxylation of pyruvate (E1 component), the transfer of the resulting acetyl group to CoA (E2 component) and the regeneration of the oxidized lipoamide (E3 component). The reaction links glycolysis with oxidative metabolism because the product acetyl-CoA enters the citric acid cycle. The reaction at this critical branch point in metabolism is irreversible and therefore well controlled. The main regulation occurs by reversible phosphorylation of the E1 component of PDH, which abolishes enzyme activity. The PDH complex contains associated regulatory enzymes, including pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase. The activities of these enzymes determine the proportion of PDH in its active dephosphorylated state.

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The regulation of PDH activity plays an important role in the metabolic flexibility of organs, meaning the choice of which substrates an organ will use to maintain energy homeostasis. Under normal, well-fed conditions, glucose is the preferred substrate for oxidation. During fasting, however, mitochondrial fatty acid β-oxidation (FAO) will become a more important energy source as an alternative for glucose. This reciprocal relationship between FAO and glucose oxidation is known as the glucose-fatty acid cycle or Randle cycle [1]. In normal physiology, the main function of this cycle is glucose sparing during fasting when glucose supply is limited and plasma free fatty acids and FAO are elevated. The Randle cycle plays a crucial role in the pathophysiology of metabolic diseases such as type 2 diabetes.

The activity of PDK is regulated by both short- and long-term mechanisms. Short term regulation includes activation of PDK by the reaction products of PDH, i. e. acetyl-CoA and NADH. Thus, high acetyl-CoA/CoA and NADH/NAD^+ ratios activate PDK, leading to an inhibitory phosphorylation of PDH. PDK activity is inhibited by its substrate pyruvate, but also the therapeutic compound dichloroacetate. Long-term regulation of PDK occurs primarily at the transcriptional level. In mammals there are four PDKs, which have different biochemical properties and are expressed in a tissue-specific manner [2 – 5]. PDK1 was found almost exclusively in rat heart. Recently, it was described that PDK1 is a target for hypoxia inducible factor-1α (HIF-1α)[6, 7]. As such, PDK1 plays an indispensable role in the adaptation of cellular metabolism to hypoxia. PDK3 is abundantly expressed in rat testis, but its role is relatively unexplored. PDK2 and PDK4 seem to play adaptive roles in fasting and starvation. PDK2 is ubiquitously expressed and its expression increases significantly in liver and kidney upon fasting. PDK4 expression is highly responsive is tissues such as heart, skeletal muscle and kidney. The transcriptional regulation of PDK4 has been intensively studied over the last years and functional promoter elements have been identified for glucocorticoid receptor, peroxisome proliferator-activated receptor (PPAR), estrogen-related receptor and FOXO [8 – 10].

We now show that AMP-activated protein kinase (AMPK) activation and fatty acids synergistically induce PDK4 expression. AMPK is a sensor and regulator of cellular energy balance [11]. AMPK is activated by AMP, which is a signal for low energy status, but also by other signaling pathways, such as adiponectin signaling. Activated AMPK initiates a signaling cascade aimed at restoring cellular energy levels. A principal event is the phosphorylation of acetyl-CoA carboxylase (ACC), which converts acetyl-CoA in malonyl-CoA. Malonyl-CoA is an inhibitor of carnitine palmitoyltransferase 1 (CPT1), the first enzyme in the carnitine shuttle that is necessary for the import of acyl-CoAs into mitochondria. As such, CPT1 catalyzes a rate-limiting step in the FAO pathway. Therefore, AMPK activation aims to increase energy production by FAO. The crosstalk between fatty acid and energy status signaling in regulation of PDK4 expression is completely novel, and further increases the cells' reliance on fatty acids as energy source.

Materials and methods

Materials. The rabbit polyclonal anti-HIF-1α antibody was obtained from Novus Biologicals (NB100–449SS, Littleton, CO). A mouse monoclonal against phosphorylated acetyl-CoA carboxylase (ACC, Ser79) was obtained from Millipore (Upstate Biotechnology, #05–673, Amsterdam Zuidoost, The Netherlands). The rabbit polyclonal anti-ACC antibody was purchased from Cell Signaling Technology (Danvers, MA). Actinomycin D was obtained from MP Biomedicals (Eindhoven, The Netherlands) and dissolved in methanol. Compound C was purchased from Calbiochem (VWR International B.V., Amsterdam, The Netherlands) and dissolved in DMSO. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), oleate, creatine, L-carnitine, taurine and laminin were from Sigma (Zwijndrecht, The Netherlands). [U-^{14}C]-glucose was from GE healthcare (Diegem, Belgium). Expression plasmids (pcDNA3, Invitrogen) encoding AMPKα1, AMPKβ1 and AMPKγ1 were provided by Dr. D. Carling [12].

Cell culture. Fao cells (rat hepatoma cells, a gift from Dr. Latruffe, Université de Bourgogne, Dijon, France) were cultured in minimal essential medium (MEM) medium (+Earle's, +25mM HEPES, -L-glutamine) supplemented with 10% fetal bovine serum, L-glutamine, non-essential amino acids, penicillin, streptomycin and fungizone (all from Invitrogen, Breda, The Netherlands) and incubated in a humidified CO_2 incubator at 37 °C.

Cardiomyocyte isolation and culture. Cardiac myocytes were isolated from an adult rat heart as described by Luiken et. al [13]. Directly after isolation, cells were suspended in MKR-buffer (modified-Krebs-Ringer buffer in mM: 117 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 $NaHCO_3$, 10 HEPES) supplemented with 2 mM D-glucose and 0.45% fatty acid free BSA (MP Biomedicals) and plated on

laminin-coated (1 μg/ml) glass coverslips in six- well plates at a density of 10 000 cells/cm^2. Cells were left to attach for 1 hour at 37 °C and 5% CO_2. After attachment, cells were washed with MKR-buffer and treatments were started.

Treatments. In order to obtain a hypoxic culture environment, cells were placed in the Modular Incubator Chamber (MIC-101, Billups-Rothenberg, Del Mar, CA). The chamber was gassed with 95% N_2 and 5% CO_2 for 15 minutes at a constant pressure of 0.5 pounds per square inch (PSI). This equals a flow rate of approximately 31.7 L/min. Afterwards the chamber was sealed and placed at 37 °C. This procedure was repeated after 1 hour of incubation. At the end of the treatment, O_2 concentration in culture medium of water was assessed using a Clark type oxygen electrode. O_2 saturation measured was always between 25% and 19% of the maximal oxygen saturation at room temperature. The environment in the incubator is expected to be more hypoxic, since the transfer of the medium to the electrode allows oxygen diffusion.

For treatment of Fao cells, the cells received fresh MEM medium containing BSA, AICAR, oleate or their combination, at concentrations indicated in the figures. Oleate was prepared as a 2 mM stock solution MEM medium containing 2% BSA fatty acid free (Sigma).

For cardiomyocyte treatment, the cells received medium 199 + CCT (in mM: 5 creatine, 2 L-carnitine, 5 taurine) + 100 U/ml penicillin and 100 μg/ml streptomycin containing BSA, AICAR, oleate or their combination, at concentrations indicated in the figures. Oleate was prepared as a 2 mM stock solution in medium 199 containing 2% BSA fatty acid free (Sigma).

Expression analysis. Total RNA was extracted from frozen tissue samples or cells using Trizol reagent (Invitrogen). cDNA was synthesized from total RNA with the SuperScript First-Strand Synthesis System (Invitrogen) and random hexamer primers. The real-time PCR measurement of individual cDNAs was performed using SYBR green dye to measure duplex DNA formation with the Roche Lightcycler 480 system. The expression data were normalized by calculating the ratio with cyclophilin B (*Ppib*), a housekeeping gene. The sequences of the primer sets are available upon request.

Immunoblot analysis. Equal amounts of total Fao cell protein were separated by SDS-PAGE (7% for HIF-1α and 7% for ACC) and transferred onto nitrocellulose by semi-dry blotting. For immunoblotting of ACC, 7M ureum was added to the Laemmli sample buffer, the stacking and running gel.

Luciferase reporter assay for PPAR transactivation. The PPAR response element (PPRE) derived from rat *Acox1* was cloned three times in tandem in a pGL3 vector (Promega) that also encodes a TK minimal promoter by using *Sac*I and *Xho*I restriction sites [14, 15]. Fao cells plated in 48 well plates were transiently transfected with 0.6μg plasmid (0.3μg pGL3-PPRE3x with 0.3μg pCMV-β-galactosidase (Clontech) in Figure 4A – C; 0.15μg pGL3-PPRE3x combined with 0.15μg pcDNA3-AMPKα1, 0.15μg pcDNA3-AMPKβ1, 0.15μg pcDNA3-AMPKγ1 or 0.45μg pcDNA3 as the empty vector control in Figure 4D, E) in MEM medium without fetal bovine serum using lipofectamine 2000 (Invitrogen), according to the instructions of the manufacturer. Cells were incubated for 24 hours with MEM medium supplemented with 0.4% BSA and compounds at the concentrations indicated in the figure. Luciferase and β-galactosidase assays were performed as described [16].

Glucose oxidation. Glucose oxidation was assessed by measuring the production of $^{14}CO_2$ from [U-^{14}C]-glucose. Fao cells were seeded in a T25 culture flask. CO_2 production was measured during 24 hours of incubation in culture medium as described above with 1μCi of [U-^{14}C]-glucose as the tracer.

Statistics. Data are expressed as mean +/- SD. Differences between vehicle and treated cells were analyzed by a paired *t* test or oneway ANOVA followed by a Dunnett *post hoc* test (* denotes $P < 0.05$ and ** denotes $P < 0.01$) using SPSS. The Dunnett *post hoc* test was performed with the + AICAR + oleate or hypoxia + oleate condition as control category.

Results

Synergistic induction of PDK4 expression by hypoxia and fatty acids. To identify potential effects of cellular energy status and fatty acid supply on PDK4 expression, Fao cells were cultured under normoxic and hypoxic conditions in the presence and absence of oleate. The principal events during hypoxia are stabilization of HIF-1α protein and lactate production due to decreased mitochondrial respiration. Immunoblotting confirmed the presence of HIF-1α protein after the hypoxia treatment (Fig. 1A). The presence of oleate did not affect HIF-1α levels during normoxia and hypoxia (Fig. 1A). Hypoxia treatment also caused lactate production (Fig. 1B).

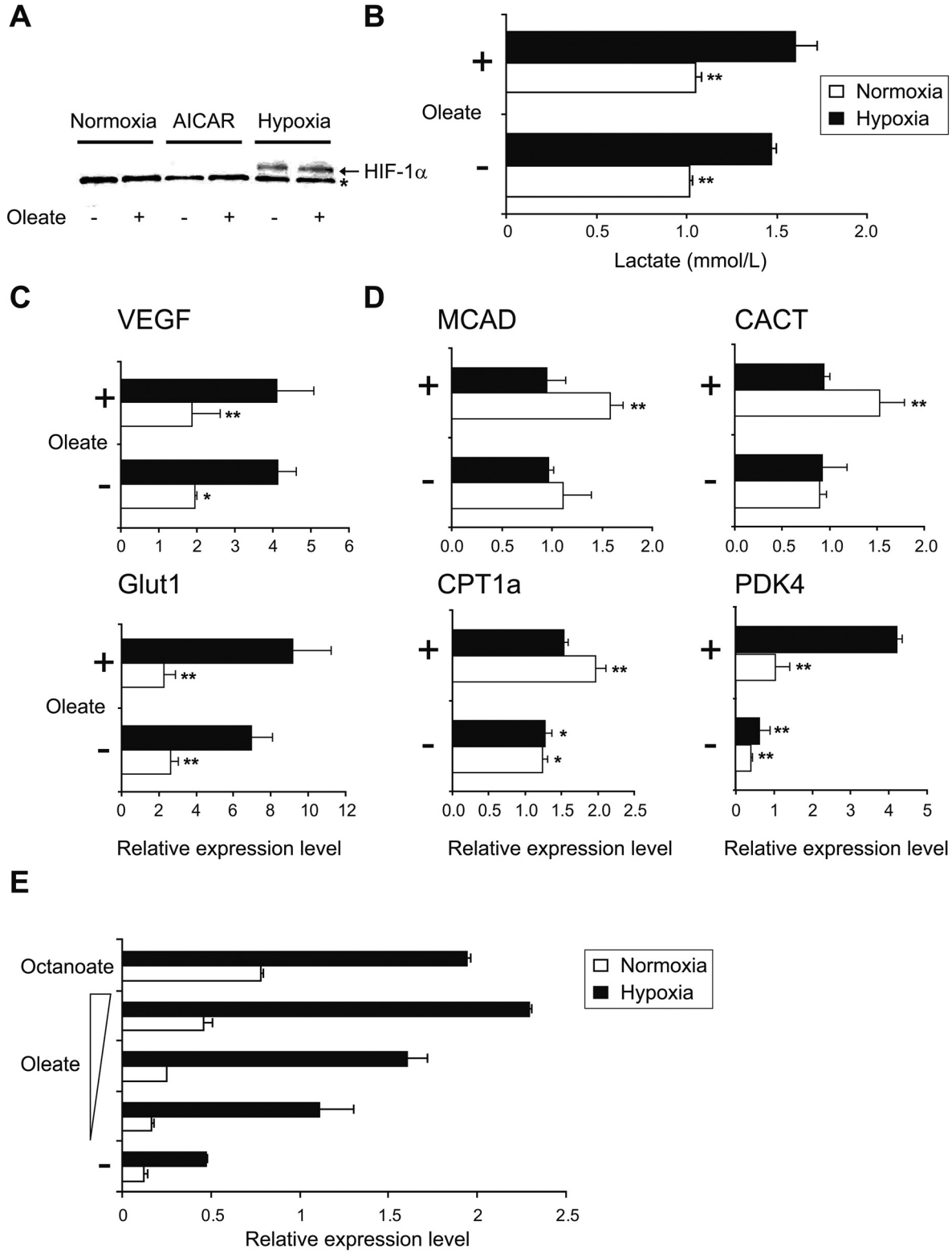

Figure 1. Synergistic induction of PDK4 expression by hypoxia and fatty acids. (*A*) Immunoblot analysis of Fao cell lysates using a HIF-1α antibody. Oleate concentration was 100μM. The * indicates an aspecific protein band. (*B*) Lactate concentration in the culture medium after 8 hours of incubation. Due to the FCS, initial lactate concentration in the medium was 1.19 mmol/L. Thus under normoxic condition Fao cells consume lactate, whereas during hypoxia there is lactate production. (*C*) Expression levels of selected HIF-1α target genes. (*D*) Expression levels of selected PPAR target genes. All incubations (*B, C* and *D*) were performed in triplicate. The data of one representative experiment are shown. (*E*) Dose response of the induction of PDK4 expression by oleate (25, 50 and 100μM) under normoxia and hypoxia. Induction of PDK4 by octanoate (400μM). All incubations were performed in duplicate.

We analyzed expression of genes involved in the hypoxia response and fatty acid metabolism. Hypoxia increased the expression of two classic markers of the hypoxic response, vascular endothelial growth factor (VEGF) and glucose transporter 1 (Glut1) (Fig. 1C). The expression of both genes was not affected by the presence of oleate. The expression of genes involved in FAO, medium chain acyl-CoA dehydrogenase (MCAD), CPT1a, carnitine acylcarnitine translocase (CACT) and acyl-CoA oxidase (ACOX1), was upregulated by the addition of oleate under normoxia (Fig. 1D and not shown). Many genes encoding enzymes of FAO, including CPT1a, MCAD and ACOX1, have functional PPAR response elements in their promoter [17 – 22]. The induction by oleate is therefore most likely caused by the ligand-dependent activation of PPAR by elevated free fatty acid levels. Hypoxia prevented the upregulation of the expression of these genes. Interestingly, the ligand-dependent induction of another PPAR target gene, PDK4, was strongly stimulated by hypoxia (Fig. 1D). Hypoxia alone did not affect PDK4 expression levels. The induction of PDK4 expression by hypoxia and oleate was dose dependent. Another fatty acid, octanoate, had a similar effect as oleate on PDK4 expression (Fig. 1E).

Hypoxia mimetics do not reproduce the effect of hypoxia on PDK4 expression. To define whether HIF-1α activation plays a role in the induction of PDK4 expression by fatty acids, we used $CoCl_2$. $CoCl_2$ mimics the hypoxia response by substituting iron, which inhibits the action of a prolyl hydroxylase and subsequently stabilizes HIF-1α. $CoCl_2$ indeed stabilized HIF-1α (Fig. 2A). $CoCl_2$ itself also increased PDK4 expression, but this induction was independent of the presence of oleate (Fig. 2B). Two other hypoxia mimetics, desferrioxamine (iron chelator) and dimethyloxalylglycine (2-ketoglutarate analog), also failed to reproduce the effect of hypoxia on PDK4 expression (data not shown). These results suggest that HIF-1α does not play a role in the induction of PDK4 by hypoxia and oleate.

AMPK activation mimics the effect hypoxia on PDK4 expression. Besides the stabilization of HIF-1α, hypoxia also decreases cellular energy status due to an inhibition of mitochondrial oxidative metabolism. As a result, AMP levels increase and AMPK is activated. To test whether AMPK activation could mediate the induction of PDK4 by hypoxia and oleate, we treated Fao cells with AICAR in the presence and absence of oleate. AICAR treatment did not stabilize HIF-1α (Fig. 1A). In contrast, both AICAR and hypoxia treatment did lead to phosphorylation of ACC, which is a downstream target of AMPK (Fig. 2C). The oleate-dependent induction of PDK4 expression was strongly stimulated by AICAR (Fig. 2D). In contrast, AICAR did not increase the ligand-dependent induction of mitochondrial thioesterase 1 (MTE1)[23], another PPAR target gene (Fig. 2D). AICAR alone did not affect PDK4 expression levels, thus perfectly reproducing the effects of hypoxia.

Most effects of AMPK activation are at the level of the proteome. To show that the effect of AICAR on PDK4 was transcriptional, we inhibited transcription using actinomycin D. Actinomycin D completely prevented the induction of PDK4 expression by AICAR and oleate, indicating that it is a transcriptional event (Fig. 2E). Actinomycin D itself had no effect on the expression of the housekeeping gene cyclophilin B, as demonstrated by a comparison of the Ct-values of treated and untreated samples (data not shown).

To add strength to the observation that AMPK mediates the effect of hypoxia on the induction of PDK4 expression by oleate, we used compound C, which is an AMPK inhibitor [24]. Compound C prevented the induction of PDK4 expression by hypoxia and oleate, indicating that AMPK mediates this response (Fig. 2F). A separate experiment showed that compound C was able to prevent AMPK signaling (data not shown).

Synergy of AMPK and fatty acids on PDK4 expression in primary cardiomyocytes. In order to reproduce our results obtained in Fao cells, we decided to make use of rat primary cardiomyocytes, since PDK4 is expressed abundantly in the (rat) heart. We treated cardiomyocytes with AICAR in the presence and absence of oleate and quantified the expression of PDK4, MTE1, MCAD and UCP3. The oleate-dependent increase in the transcription of PDK4 was strongly stimulated by AICAR (Fig. 3). In contrast, MTE1 and MCAD expression was not significantly stimulated by AICAR in the presence of oleate (Fig. 3). UCP3 expression in cardiomyocytes was increased in response to AICAR treatment, consistent with other reports on skeletal muscle [25]. The combination of AICAR and fatty acids increased expression even further. Interestingly, fatty acids alone had no effect. Our results in primary cardiomyocytes indicate that the synergy of AMPK activation and fatty acids on PDK4 expression is a physiologically relevant mechanism.

AMPK activation increases ligand-dependent PPAR activation. Although PDK4 is the only PPAR target gene that responded synergistically to hypoxia and

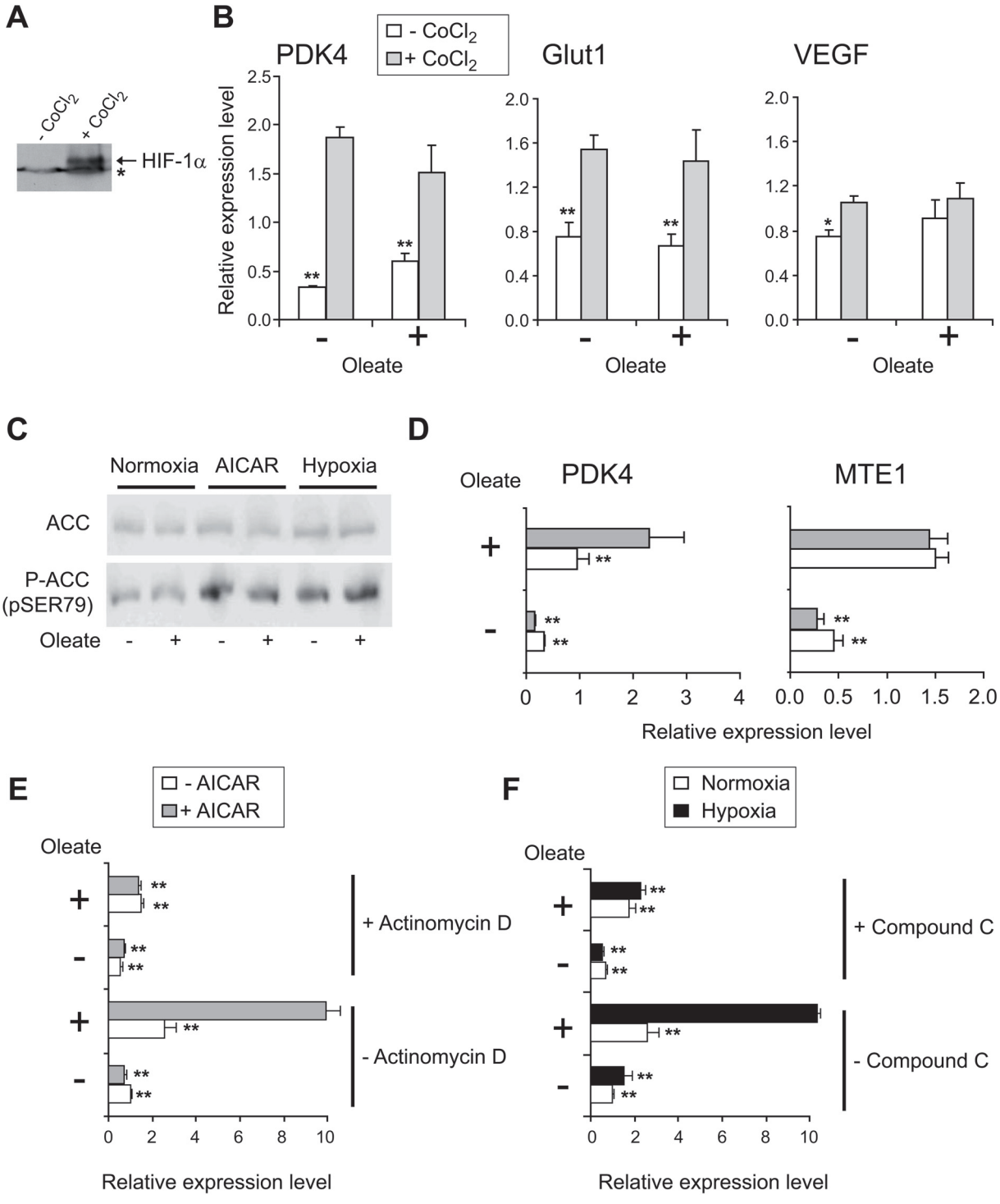

Figure 2. AMPK activation mimics the effect hypoxia on PDK4 expression. (*A*) Immunoblot of Fao cell lysates using a HIF-1α antibody. The hypoxia mimetic $CoCl_2$ was used at 100μM. Oleate concentration was 100μM. The * indicates an aspecific protein band. (*B*) Expression levels of PDK4 and HIF-1α target genes (Glut1 and VEGF). (*C*) Immunoblot analysis of Fao cell lysates using antibodies against ACC and phosphorylated ACC. The AICAR concentration was 2mM, the oleate concentration was 100μM. (*D*) Expression analysis of selected PPAR target genes (PDK4 and MTE1). (*E*) Expression analysis of PDK4 after inhibition of transcription using 1μg/mL actinomycin D. (F) Expression analysis of PDK4 after inhibition of AMPK activation by hypoxia using 12.5μM compound C. All incubations (*B, D, E* and *F*) were performed in triplicate.

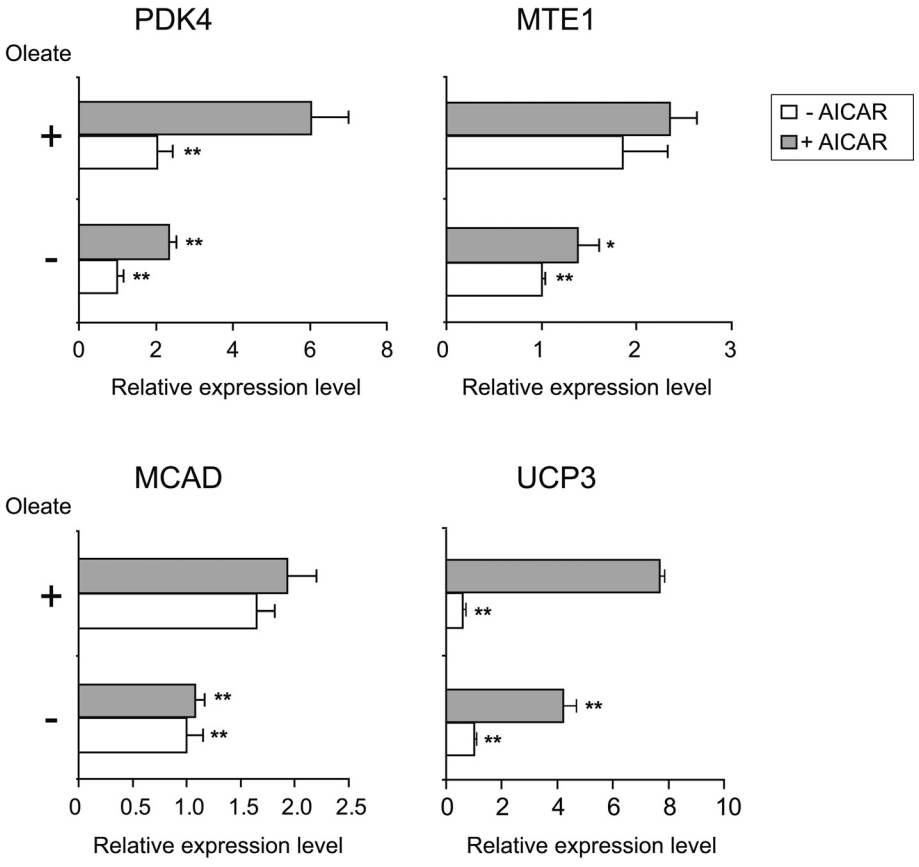

Figure 3. Synergy of AMPK and fatty acids on PDK4 expression in primary cardiomyocytes. Expression analysis of PDK4, MTE1, MCAD and UCP3. The AICAR concentration was 1mM, the oleate concentration was 100μM. All incubations were performed in triplicate.

AICAR, we reasoned that PPARα or PPARβ were the most likely candidate mediators of this effect. Fao cells express PPARα and at lower levels PPARβ, PPARγ is not expressed (data not shown and [26]). This explains why Fao cells respond better to Wy-14643, a specific PPARα ligand, when compared to GW501516, a specific PPARβ ligand (data not shown). To test if PPAR can mediate the effect AICAR, we employed a PPAR transactivation assay with a synthetic promoter consisting of the thymidine kinase minimal promoter and the PPRE derived from rat *Acox1* three times in tandem driving luciferase expression. This construct was transiently transfected to Fao cells. These Fao cells were subsequently treated with AICAR in the presence and absence of oleate. The ligand-dependent induction of luciferase was stimulated by AICAR (Fig. 4A). In contrast, AICAR alone did not increase luciferase activity (Fig. 4A), again perfectly mimicking the effects on PDK4 expression. To provide further evidence for a role of PPAR in this ligand-dependent induction, we used 2 synthetic PPAR agonists, bezafibrate, a PPAR pan-agonist (Fig. 4B) and Wy-14643 (Fig. 4C). AICAR is able to increase the ligand-dependent induction by both compounds (Fig. 4B, C). AICAR did not affect the ligand-dependent induction by GW501516 (data not shown), indicating that in Fao cells PPARα may primarily mediate the effects of AMPK activation.

Given that pharmacological activators and inhibitors can have non-specific or off-target effects, we further investigated the role of AMPK using molecular biological tools. We used the PPAR luciferase reporter system and transfected Fao cells with plasmids encoding AMPKα1, AMPKβ1 and AMPKγ1. Cotransfection of these AMPK subunits not only potentiated the effect of AICAR, but also increased basal luciferase reporter activity and the oleate- and bezafibrate-stimulated luciferase activity (Fig. 4D, E). These results prove that we are looking at specific effects of AMPK activity and not off-target AICAR effects. Similar results have been obtained after transfection of AMPKα2, β1 and γ1, AMPKα1 alone, and AMPKα2 alone (data not shown).

AMPK activation and oleate synergistically decrease glucose oxidation. We measured glucose oxidation rates in Fao cells after treatment of cells with AICAR and oleate. Oleate decreased glucose oxidation by 48% when compared with untreated cells (from 1.08 nmol/mg.h to 0.56 nmol/mg.h). AICAR, by itself, already decreased oleate oxidation by 79% (0.22 nmol/mg.h), but the combination of oleate and

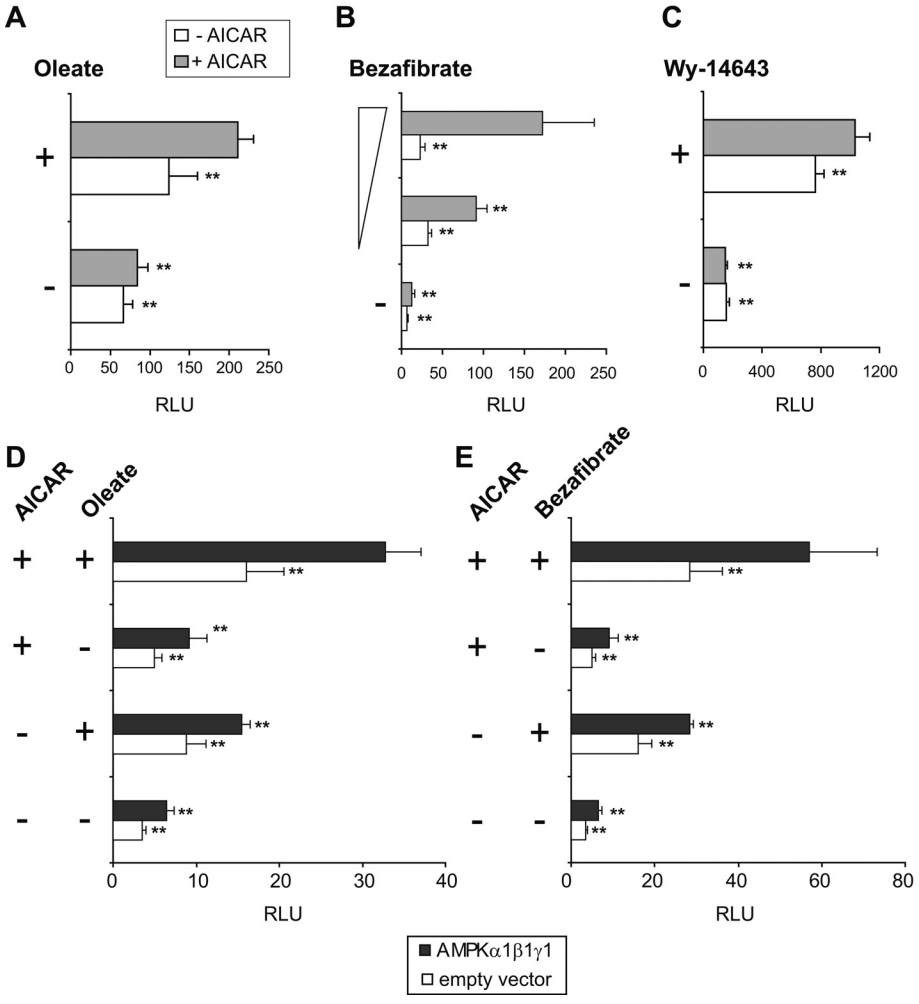

Figure 4. AMPK activation increases ligand-dependent PPAR activation. (*A*) Relative luciferase activity after transfection of a PPAR reporter plasmid and treatment with 0.5mM AICAR in the presence and absence of 100μM oleate. (*B*) Relative luciferase activity after transfection of a PPAR reporter plasmid and treatment with 0.5mM AICAR and a titration of bezafibrate (0, 50 and 200μM). (*C*) Relative luciferase activity after transfection of a PPAR reporter plasmid and treatment with 0.5mM AICAR in the presence and absence of 1μM Wy-14643. (*D*) Luciferase activity after transfection of a PPAR reporter plasmid and expression plasmids for AMPKα1, AMPKβ1 and AMPKγ1 as indicated in the figure. Cells were treated with 0.5mM AICAR in the presence and absence of 100μM oleate. (*E*). Luciferase activity after transfection of a PPAR reporter plasmid and expression plasmids for AMPKα1, AMPKβ1 and AMPKγ1 as indicated in the figure. Cells were treated with 0.5mM AICAR in the presence and absence of 50μM bezafibrate. All luciferase reporter assays were performed in quadruplicate.

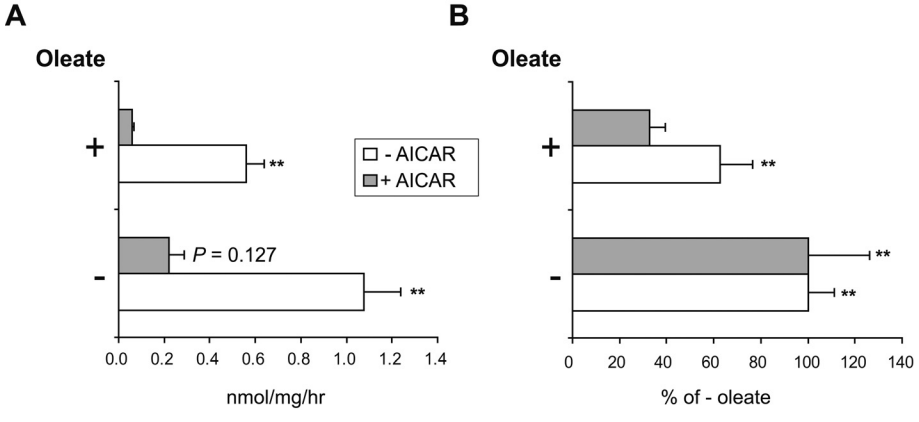

Figure 5. AICAR and oleate synergistically decrease glucose oxidation. (*A*) Glucose oxidation rate in Fao cells as measured by the production of $^{14}CO_2$ from [U-^{14}C]-glucose after treatment with 0.5mM AICAR in the presence and absence of 100μM oleate. All incubations were performed in triplicate. (*B*) Relative decrease of glucose oxidation by the addition of oleate. The data are the mean + SD of 3 independent experiments representing 8 to 10 incubations.

AICAR had the most pronounced effect, decreasing glucose oxidation by 94% (Fig. 5A, 0.06 nmol/mg.h). We combined the data of three independent experiments and normalized the data to the incubations without oleate (Fig. 5B). This shows that oleate alone decreased glucose oxidation by 37%, whereas in the combination of oleate with AICAR this effect was more than doubled (67%, Fig. 5B).

The strong decrease of glucose oxidation by AICAR alone might be explained by stimulation of mitochondrial fatty acid oxidation by AMPK-mediated ACC phosphorylation. An alternative explanation might be offered by recently reported AMPK-independent

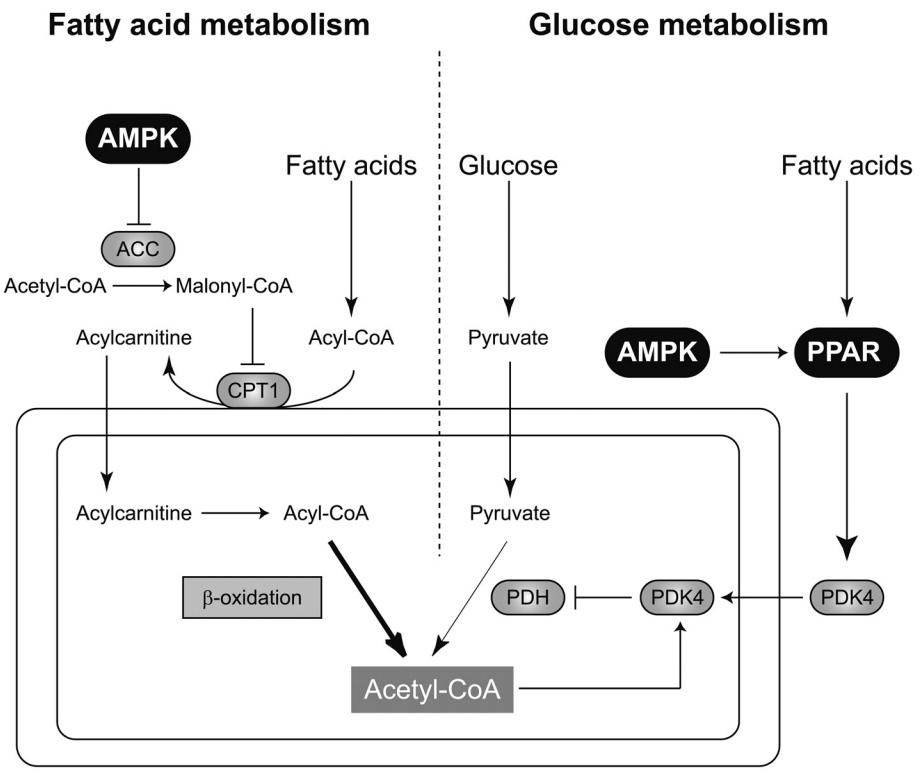

Figure 6. A scheme depicting major metabolic effects of AMPK activation and fatty acids. AMPK activation relieves repression of CPT1 by malonyl-CoA by phosphorylation and inactivation of ACC. At the same time, fatty acids and AMPK decrease glucose oxidation by a synergistic effect on the expression levels of PDK4.

effects of AICAR. AICAR is able to inhibit glucose phosphorylation and oxidative phosphorylation in hepatocytes [27, 28]. Our data, however, strongly suggest that fatty acids synergize with AMPK to decrease glucose oxidation.

Discussion

AMPK activation has a wide impact on cellular metabolism. One of the best-characterized effects is the decrease in the activity of cellular biosynthetic pathways. Principal targets for phosphorylation by AMPK are 3-hydroxy-3-methylglutaryl-CoA reductase and ACC, which leads to a decrease in the biosynthesis of sterols and fatty acids, respectively. More importantly, the decreased activity of ACC also leads to a decrease in cellular malonyl-CoA levels. Malonyl-CoA is an inhibitor of CPT1, the first enzyme in the carnitine shuttle that is necessary for the import of acyl-CoAs into mitochondria (Fig. 6). CPT1 catalyzes the rate-limiting step in the FAO pathway [29]. Therefore, the lower malonyl-CoA levels resulting from activated AMPK signaling promote increased FAO aimed to restore cellular energy status.

PPARs are other important mediators in tissue fuel selection. PPARs include PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3) and belong to the nuclear receptor family of transcription factors. PPARs function as ligand-activated transcription factors, which are activated by (dietary) fatty acids such as oleate. PPARα controls the expression of many enzymes of the FAO pathway and mediates the response to fasting [30, 31]. Many studies have shown that PDK4 is also a PPAR target gene. For example, PDK4 expression is upregulated by natural (fatty acids [9, 32]) and synthetic PPAR ligands (PPARα, Wy-14643 [9] and PPARβ/δ, GW501516 [33]). Importantly, the Wy-14643- and fasting-induced increase in PDK4 expression was absent in PPARα$^{-/-}$ mice [34] and the GW501516-mediated increase was absent in PPARβ$^{-/-}$ primary muscle cells [35]. More recently, a potential PPRE in the PDK4 promoter was characterized [36]. PDK4 is able to phosphorylate and inactivate PDH and therefore decrease glucose oxidation, which allows increased FAO. We now provide evidence that PDK4 expression is synergistically induced by AMPK and fatty acids (Fig. 6). AMPK activation by hypoxia or AICAR has no effect on PDK4 expression by itself, but increases the ligand-dependent activation of PDK4 by fatty acids (oleate but also octanoate, Fig. 1E).

We used a PPAR luciferase reporter assay to provide further evidence for a role for PPAR and AMPK. In this system, AICAR increased the ligand-dependent activation by the natural PPAR ligand oleate and by the synthetic PPAR agonists bezafibrate and Wy-14643. GW501516, a synthetic PPARβ agonist did not

have this effect, arguing that in Fao cells the effect of AMPK activation may be primarily mediated by PPARα. Moreover, cotransfection of the α, β and γ AMPK subunits potentiated the effect of AICAR, but also increased basal luciferase reporter activity and the oleate- and bezafibrate-stimulated luciferase activity. Although this does not provide a detailed molecular mechanism for the effect of AMPK activation on PPAR, we speculate that it might be mediated via direct or indirect PPAR phosphorylation. Many studies have shown that phosphorylation modulates PPARα activity (reviewed in [37]). N-terminal serine phosphorylation by ERK-MAPK, JNK, and p38 MAPK, increases ligand-dependent transcriptional activity. In one study, AMPK was activated in C2C12 myotubes by adiponectin [38]. This resulted in activated p38 MAPK, and consequently increased ligand-dependent PPARα activity. This effect was completely abolished in S6/12/21A mutant PPARα [38]. Another study showed that AICAR was able to stimulate FAO in skeletal muscle by activating PPARα [39]. Besides PPARα, PPARβ/δ is another possible candidate mediator for the effects of AMPK and fatty acids. As of this moment, PPARβ/δ phosphorylation has been poorly studied [37]. Recently, Narkar et al. [35] described molecular crosstalk between (exercise-) activated AMPK and PPARβ in skeletal muscle. Using luciferase reporter assays, they show that AMPK increase basal and ligand-dependent PPARβ activation. They furthermore show that AMPK activation does not lead to phosphorylation of PPARβ and that AMPK and PPARβ directly interact with each other. They conclude that AMPK may be present in a transcriptional complex with PPARβ, where it can potentiate receptor activity via direct protein-protein interaction and/or by phosphorylating coactivators such as PGC1α [35].

It is remarkable that PDK4 was the only one of the tested PPAR targets that responded to AMPK activation. The other tested PPAR targets were apparently unresponsive. Therefore, it seems that not all PPAR targets are as sensitive to changes in the ligand-dependent transactivation. One could argue that this is caused by specific properties of the PPREs present in each promoter. Our luciferase reporter assay using the PPRE from the unresponsive Acox1 would argue against this explanation. An alternative explanation could be that other elements in the promoter of non-responding genes antagonize the effects of AMPK on ligand-dependent PPAR activation.

An increase in PDK4 expression by itself is not sufficient to phosphorylate and inactivate PDH. PDKs are activated by acetyl-CoA, the product of the PDH reaction and FAO. Therefore, our results indicate that the synergistic induction of PDK4 expression by AMPK and fatty acids leads to a PDH complex that is much more sensitive to inhibition by acetyl-CoA produced by the oxidation of fatty acids. Indeed, we show that the decrease in glucose oxidation after the addition of fatty acids is greater when cells are also treated with AICAR. Combined, our results suggest that cells prefer to correct their cellular energy status by increasing FAO rather than glucose oxidation.

We show that the effect of AMPK activation on ligand-dependent activation of PDK4 expression is not only present in Fao cells, a rat hepatoma cell line, but also in primary cardiomyocytes. This suggests that it represents a physiologically relevant mechanism. AMPK activation has multiple effects on cardiac myocytes. As in skeletal myocytes, it leads to GLUT4 translocation to the sarcolemma, promoting glucose uptake. At the same time, fatty acid translocase (FAT)/CD36 also translocates to the sarcolemma, enhancing the uptake of fatty acids [40]. This furthermore demonstrates that AMPK regulates cardiac fatty acid use.

Our data might offer an explanation for the remarkable finding that, in heart, PDK4 is very often one of the genes that displays the largest changes in expression levels in response to metabolic disease or conditions that affect hepatic or cardiac metabolism. For example, PDK4 was elevated in animal models for physiological and pathological cardiac hypertrophy [41], diabetic cardiomyopathy [42, 43] and FAO defects [44, 45].

To summarize, AMPK and fatty acids synergistically induce PDK4 expression and decrease cellular glucose oxidation. We conclude that AMPK and fatty acids play a direct role in fuel selection in response to cellular energy status in order to spare glucose.

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1 Randle, P. J., Garland, P. B., Hales, C. N. and Newsholme, E. A. (1963) The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet 1, 785–789.
2 Bowker-Kinley, M. M., Davis, W. I., Wu, P., Harris, R. A. and Popov, K. M. (1998) Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex. Biochem. J. 329 (Pt 1), 191–196.
3 Patel, M. S. and Korotchkina, L. G. (2006) Regulation of the pyruvate dehydrogenase complex. Biochem. Soc. Trans. 34, 217–222.
4 Roche, T. E. and Hiromasa, Y. (2007) Pyruvate dehydrogenase kinase regulatory mechanisms and inhibition in treating diabetes, heart ischemia, and cancer. Cell Mol. Life Sci. 64, 830–849.

5 Sugden, M. C. and Holness, M. J. (2006) Mechanisms underlying regulation of the expression and activities of the mammalian pyruvate dehydrogenase kinases. Arch. Physiol. Biochem. 112, 139–149.
6 Kim, J. W., Tchernyshyov, I., Semenza, G. L. and Dang, C. V. (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab. 3, 177–185.
7 Papandreou, I., Cairns, R. A., Fontana, L., Lim, A. L. and Denko, N. C. (2006) HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab. 3, 187–197.
8 Araki, M. and Motojima, K. (2006) Identification of ERRalpha as a specific partner of PGC-1alpha for the activation of PDK4 gene expression in muscle. FEBS J. 273, 1669–1680.
9 Huang, B., Wu, P., Bowker-Kinley, M. M. and Harris, R. A. (2002) Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-alpha ligands, glucocorticoids, and insulin. Diabetes 51, 276–283.
10 Zhang, Y., Ma, K., Sadana, P., Chowdhury, F., Gaillard, S., Wang, F., McDonnell, D. P., Unterman, T. G., Elam, M. B. and Park, E. A. (2006) Estrogen-related receptors stimulate pyruvate dehydrogenase kinase isoform 4 gene expression. J. Biol. Chem. 281, 39897–39906.
11 Kahn, B. B., Alquier, T., Carling, D. and Hardie, D. G. (2005) AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. Cell Metab. 1, 15–25.
12 Stein, S. C., Woods, A., Jones, N. A., Davison, M. D. and Carling, D. (2000) The regulation of AMP-activated protein kinase by phosphorylation. Biochem. J. 345 Pt 3, 437–443.
13 Luiken, J. J., van Nieuwenhoven, F. A., America, G., van der Vusse, G. J. and Glatz, J. F. (1997) Uptake and metabolism of palmitate by isolated cardiac myocytes from adult rats: involvement of sarcolemmal proteins. J. Lipid Res. 38, 745–758.
14 Chegary, M., te Brinke, H., Doolaard, M., IJlst, L., Wijburg, F. A., Wanders, R. J. and Houten, S. M. (2008) Characterization of L-aminocarnitine, an inhibitor of fatty acid oxidation. Mol. Genet. Metab 93, 403–410.
15 Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A. and Evans, R. M. (1992) Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature 358, 771–774.
16 Sambrook,J. and Russell,D. W. (2001) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press.
17 Aoyama, T., Peters, J. M., Iritani, N., Nakajima, T., Furihata, K., Hashimoto, T. and Gonzalez, F. J. (1998) Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor alpha (PPARalpha). J. Biol. Chem. 273, 5678–5684.
18 Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. and Wahli, W. (1992) Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. Cell 68, 879–887.
19 Gulick, T., Cresci, S., Caira, T., Moore, D. D. and Kelly, D. P. (1994) The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. Proc. Natl. Acad. Sci. U.S.A 91, 11012–11016.
20 Napal, L., Marrero, P. F. and Haro, D. (2005) An intronic peroxisome proliferator-activated receptor-binding sequence mediates fatty acid induction of the human carnitine palmitoyltransferase 1A. J. Mol. Biol. 354, 751–759.
21 Osumi, T., Wen, J. K. and Hashimoto, T. (1991) Two cis-acting regulatory sequences in the peroxisome proliferator-responsive enhancer region of rat acyl-CoA oxidase gene. Biochem. Biophys. Res. Commun. 175, 866–871.
22 Tugwood, J. D., Issemann, I., Anderson, R. G., Bundell, K. R., McPheat, W. L. and Green, S. (1992) The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. EMBO J. 11, 433–439.
23 Stavinoha, M. A., RaySpellicy, J. W., Essop, M. F., Graveleau, C., Abel, E. D., Hart-Sailors, M. L., Mersmann, H. J., Bray, M. S. and Young, M. E. (2004) Evidence for mitochondrial thioesterase 1 as a peroxisome proliferator-activated receptor-alpha-regulated gene in cardiac and skeletal muscle. Am. J. Physiol Endocrinol. Metab 287, E888-E895.
24 Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J. and Moller, D. E. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. J. Clin. Invest 108, 1167–1174.
25 Stoppani, J., Hildebrandt, A. L., Sakamoto, K., Cameron-Smith, D., Goodyear, L. J. and Neufer, P. D. (2002) AMP-activated protein kinase activates transcription of the UCP3 and HKII genes in rat skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 283, E1239-E1248.
26 Poirier, H., Braissant, O., Niot, I., Wahli, W. and Besnard, P. (1997) 9-cis-retinoic acid enhances fatty acid-induced expression of the liver fatty acid-binding protein gene. FEBS Lett. 412, 480–484.
27 Guigas, B., Bertrand, L., Taleux, N., Foretz, M., Wiernsperger, N., Vertommen, D., Andreelli, F., Viollet, B. and Hue, L. (2006) 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside and metformin inhibit hepatic glucose phosphorylation by an AMP-activated protein kinase-independent effect on glucokinase translocation. Diabetes 55, 865–874.
28 Guigas, B., Taleux, N., Foretz, M., Detaille, D., Andreelli, F., Viollet, B. and Hue, L. (2007) AMP-activated protein kinase-independent inhibition of hepatic mitochondrial oxidative phosphorylation by AICA riboside. Biochem. J. 404, 499–507.
29 Eaton, S. (2002) Control of mitochondrial beta-oxidation flux. Prog. Lipid Res. 41, 197–239.
30 Forman, B. M., Chen, J. and Evans, R. M. (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc. Natl. Acad. Sci. U.S.A 94, 4312–4317.
31 Mandard, S., Muller, M. and Kersten, S. (2004) Peroxisome proliferator-activated receptor alpha target genes. Cell Mol. Life Sci. 61, 393–416.
32 Abbot, E. L., McCormack, J. G., Reynet, C., Hassall, D. G., Buchan, K. W. and Yeaman, S. J. (2005) Diverging regulation of pyruvate dehydrogenase kinase isoform gene expression in cultured human muscle cells. FEBS J. 272, 3004–3014.
33 Tanaka, T., Yamamoto, J., Iwasaki, S., Asaba, H., Hamura, H., Ikeda, Y., Watanabe, M., Magoori, K., Ioka, R. X., Tachibana, K., Watanabe, Y., Uchiyama, Y., Sumi, K., Iguchi, H., Ito, S., Doi, T., Hamakubo, T., Naito, M., Auwerx, J., Yanagisawa, M., Kodama, T. and Sakai, J. (2003) Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in skeletal muscle and attenuates metabolic syndrome. Proc. Natl. Acad. Sci. U S A 100, 15924–9.
34 Wu, P., Peters, J. M. and Harris, R. A. (2001) Adaptive increase in pyruvate dehydrogenase kinase 4 during starvation is mediated by peroxisome proliferator-activated receptor alpha. Biochem. Biophys. Res. Commun. 287, 391–396.
35 Narkar, V. A., Downes, M., Yu, R. T., Embler, E., Wang, Y. X., Banayo, E., Mihaylova, M. M., Nelson, M. C., Zou, Y., Juguilon, H., Kang, H., Shaw, R. J. and Evans, R. M. (2008) AMPK and PPARdelta agonists are exercise mimetics. Cell 134, 405–415.
36 Degenhardt, T., Saramaki, A., Malinen, M., Rieck, M., Vaisanen, S., Huotari, A., Herzig, K. H., Muller, R. and Carlberg, C. (2007) Three members of the human pyruvate dehydrogenase kinase gene family are direct targets of the peroxisome proliferator-activated receptor beta/delta. J. Mol. Biol. 372, 341–355.
37 Burns, K. A. and Vanden Heuvel, J. P. (2007) Modulation of PPAR activity via phosphorylation. Biochim. Biophys. Acta 1771, 952–960.
38 Yoon, M. J., Lee, G. Y., Chung, J. J., Ahn, Y. H., Hong, S. H. and Kim, J. B. (2006) Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated

protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha. Diabetes 55, 2562–2570.
39 Lee, W. J., Kim, M., Park, H. S., Kim, H. S., Jeon, M. J., Oh, K. S., Koh, E. H., Won, J. C., Kim, M. S., Oh, G. T., Yoon, M., Lee, K. U. and Park, J. Y. (2006) AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPARalpha and PGC-1. Biochem. Biophys. Res. Commun. 340, 291–295.
40 Luiken, J. J., Coort, S. L., Willems, J., Coumans, W. A., Bonen, A., van der Vusse, G. J. and Glatz, J. F. (2003) Contraction-induced fatty acid translocase/CD36 translocation in rat cardiac myocytes is mediated through AMP-activated protein kinase signaling. Diabetes 52, 1627–1634.
41 Kong, S. W., Bodyak, N., Yue, P., Liu, Z., Brown, J., Izumo, S. and Kang, P. M. (2005) Genetic expression profiles during physiological and pathological cardiac hypertrophy and heart failure in rats. Physiol. Genomics 21, 34–42.
42 Arikawa, E., Ma, R. C., Isshiki, K., Luptak, I., He, Z., Yasuda, Y., Maeno, Y., Patti, M. E., Weir, G. C., Harris, R. A., Zammit, V. A., Tian, R. and King, G. L. (2007) Effects of insulin replacements, inhibitors of angiotensin, and PKCbeta's actions to normalize cardiac gene expression and fuel metabolism in diabetic rats. Diabetes 56, 1410–1420.
43 Glyn-Jones, S., Song, S., Black, M. A., Phillips, A. R., Choong, S. Y. and Cooper, G. J. (2007) Transcriptomic analysis of the cardiac left ventricle in a rodent model of diabetic cardiomyopathy: molecular snapshot of a severe myocardial disease. Physiol. Genomics 28, 284–293.
44 Herrema, H., Derks, T. G., van Dijk, T. H., Bloks, V. W., Gerding, A., Havinga, R., Tietge, U. J., Muller, M., Smit, G. P., Kuipers, F. and Reijngoud, D. J. (2008) Disturbed hepatic carbohydrate management during high metabolic demand in medium-chain acyl-CoA dehydrogenase (MCAD)-deficient mice. Hepatology 47, 1894–1904.
45 Horiuchi, M., Kobayashi, K., Masuda, M., Terazono, H. and Saheki, T. (1999) Pyruvate dehydrogenase kinase 4 mRNA is increased in the hypertrophied ventricles of carnitine-deficient juvenile visceral steatosis (JVS) mice. Biofactors 10, 301–309.