

Hepatocyte Nuclear Factor-4 α Involved in Type 1 Maturity-Onset Diabetes of the Young Is a Novel Target of AMP-Activated Protein Kinase

Isabelle Leclerc, Claudia Lenzner, Laurence Gourdon, Sophie Vaulont, Axel Kahn, and Benoît Viollet

Mutations in the *HNF4 α* gene are responsible for type 1 maturity-onset diabetes of the young (MODY1), which is characterized by a defect in insulin secretion. Hepatocyte nuclear factor (HNF)-4 α is a transcription factor that plays a critical role in the transcriptional regulation of genes involved in glucose metabolism in both hepatocytes and pancreatic β -cells. Recent evidence has implicated AMP-activated protein kinase (AMPK) in the modulation of both insulin secretion by pancreatic β -cells and the control of glucose-dependent gene expression in both hepatocytes and β -cells. Therefore, the question could be raised as to whether AMPK plays a role in these processes by modulating HNF-4 α function. In this study, we show that activation of AMPK by 5-amino-4-imidazolecarboxamide riboside (AICAR) in hepatocytes greatly diminished HNF-4 α protein levels and consequently downregulates the expression of HNF-4 α target genes. Quantitative evaluation of HNF-4 α target gene expression revealed diminished mRNA levels for HNF-1 α , GLUT2, L-type pyruvate kinase, aldolase B, apolipoprotein (apo)-B, and apoCIII. Our data clearly demonstrate that the MODY1/HNF-4 α transcription factor is a novel target of AMPK in hepatocytes. Accordingly, it can be suggested that in pancreatic β -cells, AMPK also acts by decreasing HNF-4 α protein level, and therefore insulin secretion. Hence, the possible role of AMPK in the physiopathology of type 2 diabetes should be considered. *Diabetes* 50:1515–1521, 2001

Maturity-onset diabetes of the young (MODY) is a monogenic form of type 2 diabetes characterized by autosomal-dominant inheritance and early onset, usually before 25 years of age. Heterozygous mutations in genes encoding the glycolytic enzyme glucokinase and five different transcription factors, hepatocyte nuclear factor (HNF)-1 α , HNF-1 β , HNF-4 α , insulin promoter factor-1, and NeuroD-1, have been shown to be associated with different forms of MODY (1). The gene for type 1 MODY (MODY1) is encoded by the liver-enriched transcription factor HNF-4 α that belongs to the nuclear receptors superfamily (2). Clinical studies have shown that MODY1 is characterized by a defect in nutrient-stimulated insulin secretion, suggesting that this disorder is caused by abnormal gene expression in pancreatic β -cells (3). HNF-4 α was first identified for its role in regulating liver-specific gene expression, but it has also been found in other tissues, including kidney, intestine, and endocrine pancreas (4,5). Genes activated by HNF-4 α encode serum proteins as well as enzymes involved in various metabolic pathways, including glucose, cholesterol, and fatty acid metabolism (4). Disruption of the murine *HNF4 α* gene by homologous recombination causes defective gastrulation resulting from dysfunction of the visceral endoderm, thus indicating its crucial role for early development (6). Using visceral endoderm derived from HNF-4 α -deficient embryonic stem cells, it has been shown that HNF-4 α is essential for regulating genes involved in glucose transport and glycolysis, e.g., genes for GLUT2 and glycolytic enzymes such as aldolase B and liver-type pyruvate kinase (L-PK) (7). Moreover, HNF-4 α is a key regulator of another liver-enriched transcription factor, HNF-1 α , which is also associated to MODY (8). The existence of a transcriptional hierarchy responsible for early-onset type 2 diabetes has been strengthened by the finding of a MODY3 mutation in the HNF-4 α binding site of the HNF-1 α promoter (9). Although it is likely that dysregulation of glucose homeostasis in MODY1 patients is mainly caused by β -cell dysfunction, we cannot exclude the possibility that HNF-4 α mutations have pleiotropic effects in other tissues expressing HNF-4 α , especially in the liver. Significant alterations in triglyceride metabolism have been observed in MODY1 patients and have been considered to be an effect of impaired hepatic expression

From the Cochin Institute of Molecular Genetics, Department of Genetics, Development, and Molecular Pathology, Institut National de la Santé et de la Recherche Médicale (INSERM) Unit 129, Paris, France.

Address correspondence and reprint requests to Corresponding author: Dr. Benoît Viollet, Institut Cochin de Génétique Moléculaire, Département de Génétique, Développement et Pathologie Moléculaire, Institut National de la Santé et de la Recherche Médicale (INSERM) Unit 129, 24 Rue du Faubourg Saint-Jacques, 75014 Paris, France. E-mail: viollet@cochin.inserm.fr.

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AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; apo, apolipoprotein; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assays; HNF, hepatocyte nuclear factor; L-PK, liver-type pyruvate kinase; MODY, maturity-onset diabetes of the young; MODY1, type 1 MODY; NF-Y, nuclear factor Y; PCR, polymerase chain reaction; PPI, preproinsulin; RT, reverse transcription; TBST, Tris-buffered saline with Tween; USF, upstream stimulatory factor.

of apolipoprotein (apo)-AII, apoB, and apoCIII (10,11). Very recently, the liver-specific knockout of *HNF4 α* has confirmed the central role of HNF-4 α in lipid metabolism via the control of apo gene expression (12).

The AMP-activated protein kinase (AMPK) has been defined as a metabolic master switch, phosphorylating key target enzymes that control flux through various metabolic pathways (13,14). It is the mammalian homologue of the sucrose nonfermenting-1 protein kinase, the key regulator of glucose-dependent gene repression in yeast (15–17). It is now well established that AMPK also plays an important role in glucose-dependent gene expression in mammalian cells (18,19). In hepatocytes, AMPK activation by 5-amino-4-imidazolecarboxamide riboside (AICAR) (18,20) or by adenoviral gene transfer of the AMPK catalytic subunit (21) inhibits the glucose transcriptional stimulation of several glycolytic and lipogenic genes, namely genes for L-PK, acetyl-CoA carboxylase, fatty acid synthase, and Spot 14. One recent report also suggests that AMPK activation in hepatocytes could stimulate the transcription of the gluconeogenic PEPCK gene (22), although this has been disputed (23). In pancreatic MIN6 β -cells, AMPK activation inhibits the activity of L-PK and preproinsulin (PPI) promoters in response to high glucose concentration, and, conversely, AMPK inhibition by microinjection of antibodies directed against AMPK stimulates the activity of L-PK and PPI promoters at low glucose concentrations (19). Recent evidence has also implicated AMPK in the modulation of insulin secretion (24,25).

Based on these observations, we hypothesized that AMPK could mediate the transduction signal that leads to the repression of glucose-activated gene expression (and possibly to insulin secretion) by modulating HNF-4 α function. Thus, we monitored the effect of AMPK activation on the functional properties of HNF-4 α . We found that AMPK activation in primary culture of hepatocytes resulted in the disappearance of the HNF-4 α protein. In addition, we demonstrate that AMPK activation dramatically decreased the transcription of various HNF-4 α target genes in hepatocytes. These genes include the transcription factor HNF-1 α , genes involved in glucose metabolism (e.g., genes for GLUT2, L-PK, and aldolase B), and genes encoding apoB and apoCIII. Consequently, our data suggest that the MODY1/HNF-4 α transcription factor is a novel target of AMPK. Accordingly, we can hypothesize that AMPK activation in pancreatic β -cells has a detrimental effect on insulin secretion by decreasing HNF-4 α protein levels and may contribute to the physiopathology of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Plasmids. The -183PK/Luci, (L3)₃-54PK/Luci, and (H4)₄-105TK/Luci constructs have been described elsewhere (26). pHNF4/Luci was obtained by polymerase chain reaction (PCR) amplification of murine HNF-4 α promoter (27,28) from mouse-tail genomic DNA with the following primers: 5'-TAC GGT TCA GCT GGA GCA ACC AAG-3' and 5'-ATG CCG GCA AGG GTT TTA GAG AGT-3'. It was subcloned into the pT-Adv (Clontech) by TA-cloning and then into the pGL3-Basic vector (Promega) at *Kpn*I-*Xho*I sites. All plasmids were constructed by standard DNA cloning procedures and verified by nucleotide sequencing.

Cell culture conditions. Hepatocytes from Sprague-Dawley rats (160–180 g) and from B6/CBA mice (3–5 months old) were isolated by the collagenase perfusion method (29), followed by low-speed iso-density Percoll centrifugation (30). Animals were fed ad libitum with standard laboratory diet. Hepatocytes were cultured as reported previously (26), using 199 medium (Gibco-BRL) containing 10% (vol/vol) fetal calf serum (Gibco-BRL), 5 mmol/l glucose,

20 nmol/l insulin, 1 μ mol/l triiodothyronine, and 1 μ mol/l dexamethasone, supplemented with penicillin and streptomycin.

Electrophoretic mobility shift assays. Rat hepatocytes were incubated with AICAR (Sigma), as indicated in the figure legends. A total of ~30 million cells were used for each assay. Nuclear extracts were prepared according to Hasegawa et al. (31). Electrophoretic mobility shift assays (EMSA) were performed with 1–5 μ g nuclear proteins incubated 20 min on ice with radiolabeled probe, 1 μ g poly(dI-dC) (Pharmacia), 10 mmol/l HEPES pH 7.9, 25 mmol/l KCl, 2.5 mmol/l dithiothreitol (DTT), 0.1 mmol/l EDTA, 0.25 mmol/l phenylmethylsulfonyl fluoride, and 5% (vol/vol) glycerol. Gels (5% polyacrylamide 19:1, Tris Borate EDTA 0.25 \times) were run at room temperature at 120–150 V, dried and autoradiographed.

Western blot analysis. Hepatocytes in primary culture were scrapped in 2 \times Laemmli's buffer [0.125 mol/l Tris-HCl, pH 6.8, 4% SDS, 10% β -mercaptoethanol, 20% glycerol, 0.004% bromophenol blue] or in reporter lysis buffer (Promega). DNA was disrupted by passing the lysate several times through a 22-gauge needle. Samples were boiled 10–15 min at 100°C and centrifuged (12,000g, 5 min). Protein concentration was determined on supernatant by Bradford assay (32). A total of 25–50 μ g total protein were resolved by 10% SDS-PAGE and transferred to Hybond-C nitrocellulose membrane (Amersham). The membrane was blocked by incubation in Tris-buffered saline with Tween (TBST) [10 mmol/l Tris-HCl pH 8.0, 150 mmol/l NaCl, 0.05% (vol/vol) Tween 20] supplemented with 5% skimmed milk powder for at least 1 h at room temperature. Polyclonal primary antibodies (HNF-4 α [33], kindly provided by Moshe Yaniv, Institut Pasteur, Paris; upstream stimulatory factor (USF) 2a [14]; and annexin V from Santa Cruz Biotechnology) were then added in the same buffer and incubated for 2 h at room temperature. Membranes were then washed three times for 15 min in TBST and incubated in TBST–5% skimmed milk with horseradish-peroxidase-linked anti-rabbit antibody (DAKO) for 45 min at room temperature. Finally, they were washed as above in TBST and revealed by enhanced chemiluminescence (Amersham).

Reverse transcriptase-PCR analysis. Total RNA was isolated from hepatocytes by lysis in guanidinium thiocyanate, followed by phenol extraction (34). For reverse transcription (RT)-PCR analysis, 50–100 μ g total RNA was first treated with 10 U RNase-free DNase I (ROCHE) in 7 mmol/l Tris, 0.14 mmol/l EDTA, 10 mmol/l MgCl₂, 1 mmol/l DTT, 40 U RNasin (Promega) in a final volume of 50 μ l for 1 h at 37°C. Then, 90 μ l of STOP buffer [10 mmol/l Tris, 1 mmol/l EDTA, 0.1% SDS, 0.3 mol/l NaCl] was added (final volume 140 μ l), and RNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol 25:24:1 (vol:vol) pH 8, ethanol-precipitated, and resuspended in a suitable volume of water. First-strand cDNA synthesis was performed as previously described (35). Oligonucleotide primer sequences used for PCR amplification were: L-PK: 5'-AGT CCG AGG TGG AAA TTG TGA AGG G-3', 5'-CCA GTC CCT CTG GGC CAA TTT TCC G-3'; GLUT2: 5'-CTG GGG CTT TCT GGA CAG AAG AGC AG-3', 5'-TGT CAG AAG ACA AGA TCA CCG GAA CC-3'; aldolase B: 5'-TGA TGG AGA CCA TGA CCT AGA GCA CT-3', 5'-CTT GGG CTG CCT GAC AGT TGG CCA-3'; HNF-1 α : 5'-TTC TAA GCT GAG CCA GCT GCA GAC G-3', 5'-GCT GAG GTT CTC CGG CTC TTT CAG A-3'; apoB: 5'-CTT CAG GGA ACA AAG CAG-3', 5'-TCA AGG GTG AGC TGA TTG-3'; apoCIII: CTC AAT AGC TGG AGT TGG-3', 5'-CTG AAG AGG TAG AGG GAT-3'; albumin: 5'-CTC CAG CAA ACT GCA GAC TTG CTG CG-3', 5'-TTT GGA ATC CAT ATT CTC CAA GCT TC-3'; glucokinase: 5'-CGA CTC TGG GGA CCG AAG GCA GAT C-3', 5'-CTC GGG TGC AGC TTG TAC ACG GAG C-3'; HNF-4 α : 5'-CTT CTT TCT TCA TGC CAG-3', 5'-ACA CGT CCC CAT CTG AAG-3'. Radioactive PCR was performed with 250 ng cDNA as template in 50 μ l final volume at different cycles to ensure PCR amplification in the linear range. Conditions were the same for each set of primers: 20–28 cycles of 30 s at 94°C, 30 s at 57°C, and 60 s at 72°C in PCR buffer 1 \times (Gibco-BRL), 3 mmol/l MgCl₂, 0.5 mmol/l dNTP, 0.5 μ Ci [α -³²P]dATP, 100–200 ng each primer, and 1 U *Taq* DNA polymerase (Gibco-BRL). Samples were loaded on 5% polyacrylamide gel and TBE 1 \times , then they were run, dried, and autoradiographed.

Transfection, luciferase, and β -galactosidase assays. For the next assay, 4 μ g of luciferase reporter constructs and 500 ng CMV- β -gal as internal transfection control were transfected with cationic liposomes in mouse hepatocytes using N-(2,3-dioleoyloxy) propyl)-N, N, N-trimethylammonium methylsulfate (DOTAP; Roche) according to the manufacturer's instructions. At 24 h after transfection, the medium containing the liposome-DNA complex was replaced with fresh medium with or without 200 μ mol/l AICAR for another 12–16 h. Cells were harvested 36 to 40 h after transfection in 400 μ l of reporter lysis buffer (Promega) according to manufacturer's instructions. Luciferase activity was performed on 20 μ l of cellular extract with a luciferase assay system (Promega). For β -galactosidase activity, 50 μ l of cellular extract was diluted in 1 ml of assay buffer (PBS, 1 mmol/l MgCl₂, 50 mmol/l β -mercaptoethanol). Then, 200 μ l o-nitrophenyl β -d-Galactopyranoside (onp; Sigma) in 4 mg/ml PBS was added, and the extract was incubated at 37°C for 1 h. The reaction was stopped with 500 μ l of Na₂CO₃ 1 mol/l. Optical density was read at 420 nm. Because we noted a consistent twofold diminution in

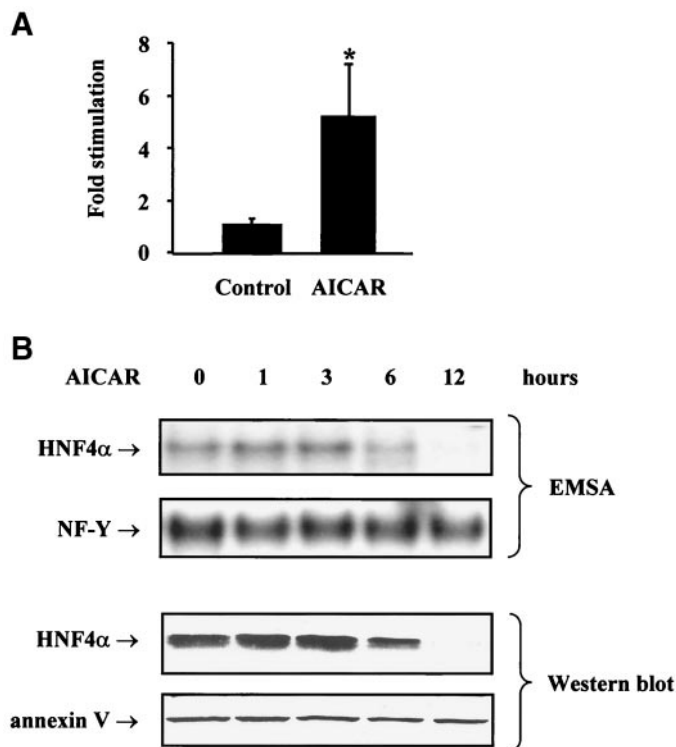


FIG. 1. AMPK activation decreases HNF-4 α protein levels. **A:** AMPK activity in hepatocytes in response to AICAR treatment. AMPK activity in hepatocytes was assayed in crude extracts against the synthetic peptide SAMS, as described in the RESEARCH DESIGN AND METHODS section. The hepatocytes were incubated overnight with 200 $\mu\text{mol/l}$ AICAR before cell lysis and protein extraction. * $P < 0.005$ for the effect of AICAR. **B:** HNF-4 α DNA-binding activity and protein levels after AMPK activation. Upper panel: EMSA were performed with 5 μg of nuclear extracts from rat hepatocytes incubated with 200 $\mu\text{mol/l}$ AICAR up to 12 h. HNF-4 α binding activity is shown on the HNF-4 α binding site from the rat L-PK promoter DNA probe (5'-TCGATCCTGGACTCTGGC-CCCAGT-3'). NF-Y binding activity is shown on the NF-Y binding site from the rat albumin promoter DNA probe (5'-GGGGTAGGAACCAAT-GAAATGAAAGGTTA-3'). The positions of HNF-4 α - and NF-Y-retarded complexes are indicated. Lower panel: Western blots were performed on whole cellular extracts from rat hepatocytes incubated with 200 $\mu\text{mol/l}$ AICAR up to 12 h. HNF-4 α and annexin V proteins are visualized with specific polyclonal antibodies and are indicated alongside the figure.

luciferase/ β -Gal units from pGL3-control vector (Promega) activity after the addition of AICAR, all promoter activities reported here were normalized with respect to those of pGL3-control vector.

AMPK assay. AMPK assays were performed essentially as described previously (19) on whole cell extracts using SAMS (the synthetic peptide HMR-SAMSGHLVKKRR) as substrate.

Data analysis. Data are given as the means \pm SE of at least three independent experiments. Comparisons between means were performed with Student's *t* test for paired data using Microsoft Excel software.

RESULTS

AMPK activation decreases HNF-4 α protein levels in a time-dependent manner. We have previously shown that AMPK activation by 200 $\mu\text{mol/l}$ AICAR in hepatocytes in primary culture inhibited glucose-dependent gene transcription (18). This result was confirmed by others using either AICAR (20) or a constitutively active form of the kinase (21), thereby indicating that the use of AICAR is relatively safe in terms of specificity for AMPK activation. Figure 1A shows that AMPK is efficiently activated in primary hepatocytes in response to 200 $\mu\text{mol/l}$ AICAR.

To test whether AMPK plays a role in transcriptional repression by modulating HNF-4 α function, we first as-

sessed HNF-4 α binding activity after AMPK activation in a primary culture of hepatocytes. For that purpose, we performed EMSAs using nuclear extracts prepared at different incubation times with 200 $\mu\text{mol/l}$ AICAR. As shown in Fig. 1B (upper panel), we observed a diminution of the HNF-4 α -retarded band after 6 h and a complete loss of HNF-4 α DNA-binding activity after 12 h of AICAR treatment. Similar results were obtained by using either the HNF-4 α binding site from the rat L-PK promoter (Fig. 1B) or the human α 1-antitrypsin gene promoter (not shown) as DNA probes. As a control, we monitored DNA-binding activity for the ubiquitously expressed transcription factor nuclear factor Y (NF-Y) and showed that it was not significantly affected by AICAR treatment over time (Fig. 1B). Competition studies using excess of unlabeled oligonucleotides corresponding to consensus HNF-4 α binding site and supershift assays using specific HNF-4 α antibodies were performed to confirm the identity of the HNF-4 α -retarded band (not shown).

The observed loss of the HNF-4 α -retarded band could be caused by changes in protein levels. To test this possibility, we performed Western blot analysis to monitor the level of HNF-4 α protein in response to AICAR treatment. As shown in Fig. 1B (lower panel), we observed that the HNF-4 α signal decreases after 6 h and practically disappears after 12 h of incubation with AICAR. In contrast, the level of the control protein annexin V was not affected by AICAR treatment over time. Thus, activation of AMPK in hepatocytes results in a complete loss of HNF-4 α DNA-binding activity that was strictly parallel to a pronounced time-dependent disappearance in HNF-4 α protein levels.

AMPK activation does not modify HNF-4 α mRNA levels. Considering the diminution of HNF-4 α protein after AMPK activation, it was tempting to speculate that the AMPK effect could be accounted for by suppression of *HNF4 α* gene transcription. To examine this issue, we evaluated HNF-4 α mRNA levels using a semiquantitative RT-PCR assay. As shown in Fig. 2A, HNF-4 α mRNA was constitutively expressed in the absence and presence of 200 $\mu\text{mol/l}$ AICAR, suggesting that AMPK activation did not affect transcription of the *HNF4 α* gene. As a control, we monitored the mRNA levels for the L-PK and albumin genes, which have previously been found to be sensitive and insensitive, respectively, to AMPK transcriptional repression (18,20). As expected, the expression of the L-PK gene was highly decreased in the presence of AICAR, whereas the expression of the albumin gene was not significantly affected by AICAR treatment (Fig. 2A).

In addition, to confirm that the AMPK effect was not mediated by direct transcriptional repression of the HNF-4 α gene promoter, we transiently transfected hepatocytes with a luciferase construct driven by the murine HNF-4 α promoter (27,28). Overnight incubation of these hepatocytes with 200 $\mu\text{mol/l}$ AICAR caused no changes in the luciferase reporter enzyme activity when compared with untreated hepatocytes (Fig. 2B). Therefore, the sensitivity of HNF-4 α protein abundance to AMPK activation was not related to a direct transcriptional effect on the HNF-4 α promoter.

AMPK activation represses transcription of HNF-4 α -dependent constructs. To determine whether HNF-4 α might be a bona fide transcriptional target of AMPK, we

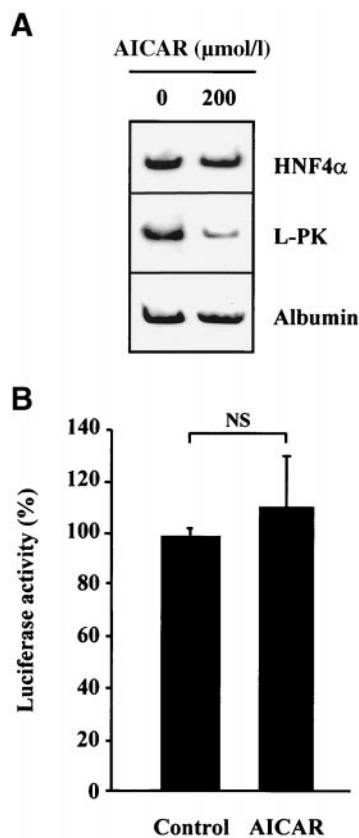


FIG. 2. Effect of AMPK activation on HNF-4 α gene transcription. **A:** Quantification of HNF-4 α mRNA levels after AMPK activation. Hepatocytes were incubated overnight with 200 μ mol/l AICAR, and mRNA levels for HNF-4 α , L-PK, and albumin were analyzed by semiquantitative RT-PCR analysis. **B:** Transcriptional activity of the HNF-4 α promoter in the presence of AICAR. Mouse hepatocytes were transfected with a luciferase construct driven by the murine HNF-4 α promoter, then 200 μ mol/l AICAR was added 24 h after transfection and left overnight before luciferase assay.

next monitored the response to AICAR treatment of several transiently transfected HNF-4 α -dependent constructs in primary culture of hepatocytes. For this purpose, we used reporter plasmids containing multimerized HNF-4 α binding site from either the rat L-PK promoter [(L3)₃-54PK/Luci] or the human α 1-antitrypsin promoter [(H4)₄-105TK/Luci]. In addition, we tested as a control transcriptional activity of the rat L-PK promoter regulatory sequences (183PK/Luci) previously identified to be HNF-4 α -dependent and AICAR-responsive (18,36). As shown in Fig. 3, in all cases, inhibition was always >70% after overnight incubation with 200 μ mol/l AICAR. Thus, our data indicate that AMPK can modulate HNF-4 α -dependent gene transcription.

AMPK activation downregulates the expression of HNF-4 α target genes in hepatocytes. To determine whether the observed effects on artificial promoter constructs were reflective of transcriptional regulation of HNF-4 α target genes in the context of native chromatin, we performed semiquantitative RT-PCR analysis of endogenous gene transcription in hepatocytes incubated with increasing amounts of AICAR, ranging from 0 to 200 μ mol/l. As shown in Fig. 4A, a frank diminution of known HNF-4 α -regulated genes (7,11,12), including L-PK, aldolase B, GLUT2, HNF-1 α , apoCIII, and apoB, was detected at an AICAR concentration of 150 μ mol/l. Interestingly,

the mRNA level of apoAI was not modified by AICAR treatment, nor was it diminished in HNF-4 α liver-specific knockout animals (12). For controls, we monitored albumin and glucokinase mRNA levels that were not significantly affected by AICAR treatment (Fig. 4A). In addition, we checked the abundance of the HNF-4 α and USF2a transcription factor proteins in hepatocytes incubated overnight with increasing AICAR concentrations. The amount of HNF-4 α protein declined for AICAR concentrations >150 μ mol/l, whereas the USF2a protein amount was not significantly modified under the same conditions (Fig. 4B). Hence, transcriptional decrease of the HNF-4 α -dependent gene observed after AMPK activation parallels the diminution of HNF-4 α protein levels, as revealed by Western blot analysis.

DISCUSSION

It is now well established that AMPK, in addition to its role in fuel partitioning (14), is also involved in the transcriptional control of glucose-regulated genes (18–22). The present study further elucidates the molecular targets involved in transcriptional repression by AMPK. Here, we demonstrated that the MODY1/HNF-4 α transcription factor is a novel target of AMPK. The extent of decrease in transcription rate induced by AICAR treatment essentially correlates with the decrease in HNF-4 α protein levels. This effect is most likely not related to a transcriptional repression of the *HNF4 α* gene promoter because no significant modification of HNF-4 α mRNA abundance can be detected by semiquantitative RT-PCR analysis (Fig. 2A) and the murine HNF-4 α promoter appears to be insensitive to AICAR treatment in transfection studies (Fig. 2B).

Cellular processes that limit the transcriptional activity, subcellular location, and abundance of transcription factors play an important role in regulating gene expression. Proteolysis is a prominent mechanism regulating transcription factor, which makes transcriptional activation and activator degradation closely coupled events (37,38). Several proteolytic processes have been implicated in transcription factor destruction, including ubiquitin-mediated proteolysis (39) and cleavage by calpains (40) and lysosomal proteases (41). In the case of transcription factor turnover by the ubiquitin-proteasome pathway, phosphorylation has often been associated as a positive signal

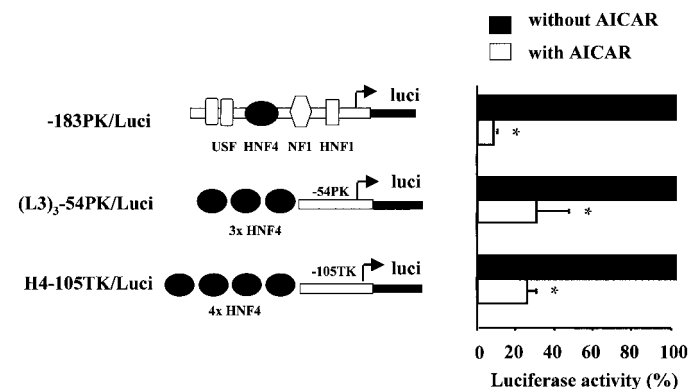


FIG. 3. The activity of HNF-4 α -dependent promoters after AMPK activation. Hepatocytes were transfected with the following constructs: -183PK/Luci, (L3)₃-54PK/Luci, or (H4)₄-105TK/Luci. At 24 h after transfection, 200 μ mol/l AICAR (white bars) was added and left overnight before luciferase assay. **P* < 0.05 for the effect of AICAR.

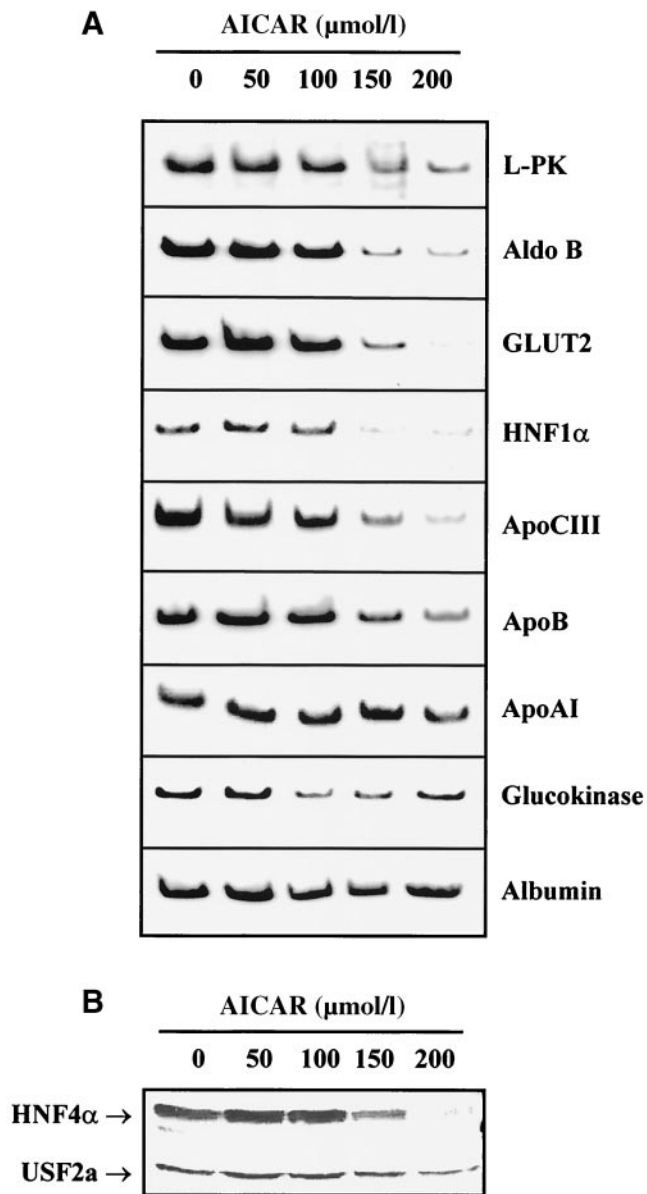


FIG. 4. AMPK activation downregulates HNF-4 α -regulated gene expression. **A:** Quantification of endogenous gene transcription after AMPK activation. Hepatocytes were incubated overnight with a dose-response of AICAR ranging from 0 to 200 $\mu\text{mol/l}$. mRNA levels were analyzed by semiquantitative RT-PCR analysis, as described in the RESEARCH DESIGN AND METHODS section. **B:** Western blot analysis of HNF-4 α and USF-2a protein abundance after AMPK activation. Hepatocytes were incubated overnight with a dose-response of AICAR ranging from 0 to 200 $\mu\text{mol/l}$ before total protein extraction and Western blotting.

for the ubiquitination and targeting of proteins to the 26S proteasome (42). Therefore, we verified that HNF-4 α is indeed a target of AMPK phosphorylation in cell-free extracts (not shown) and anticipated that this pathway would be involved in HNF-4 α degradation. To test this hypothesis, we treated hepatocytes concomitantly with AICAR and the proteasome inhibitor MG132. We observed that the diminution of HNF-4 α protein was not prevented under these conditions (not shown) suggesting that HNF-4 α disappearance was independent of the proteasome pathway. Alternatively, it is possible that phosphorylation of HNF-4 α by AMPK may introduce a conformational change that makes the protein more susceptible to degradation

resulting from proteolytic cleavage by cellular proteases. Efforts to identify the exact mechanisms implicated in this process are underway.

The rapid and controlled adjustment in the amount of transcription factors permits the attenuation of cellular responses to extracellular cues by rapidly modifying key signaling proteins. This provides a fail-safe mechanism against unbridled changes in the level of transcription and allows the transcriptional apparatus to be quickly reprogrammed in the case of unexpected change in the cellular state. AMPK is activated in response to ATP depletion and leads to the switching-off of ATP-consuming pathways and the switching-on of ATP-regenerating pathways. It plays a major role in lipid metabolism and in inhibiting fatty acid, cholesterol, and triglyceride synthesis; it also plays a major role in promoting fatty acid oxidation. This switch from anabolism to catabolism restores the energy status of the cell (25). By decreasing HNF-4 α protein levels (as in this study), AMPK decreases the apo synthesis and stops lipid export by the liver, thus preserving this carbon source for ATP repletion at the cellular level. Recently, HNF-4 α mutations have been associated with decreased levels of circulating apoAII, apoB, apoCIII, and triglycerides in MODY1 patients and in mice carrying a HNF-4 α -null mutation in the liver (10,11).

Genetics studies have shown that heterozygous mutations in HNF-4 α result in MODY1, an autosomal-dominant form of type 2 diabetes. MODY1 mutants included nonsense and frameshift mutations that give rise to truncated HNF-4 α proteins and missense mutations, which affect different domains of the HNF-4 α protein. These mutations impair protein activity by decreasing its binding to target DNA sequences or by affecting protein dimerization and/or the transactivation potential. Surprisingly, none of the MODY1 mutants described so far have exhibited any signs of a dominant-negative effect in functional studies (43). It is speculated that dominant-negative HNF-4 α mutants are not compatible with human development, as is the case with HNF4 α inactivation in mice (6). Hence, haploinsufficiency or reduced gene dosage may explain the mechanism leading to the MODY1 phenotype. Expression of HNF-4 α at a relatively low amount in endocrine pancreatic cells (5) appears to be a limiting factor for efficient transcription of HNF-4 α target genes. A 50% reduction in the amount of functional HNF-4 α protein would be deleterious in the context of pancreatic β -cells, whereas in hepatocytes it would have less dramatic effects on gene transcription.

The MODY1 phenotype is characterized by a defect in nutrient-induced insulin secretion by the pancreatic β -cells (3) and by a diminution in serum triglycerides, and it accounts for a diminution in apoAII, apoB, and apoCIII synthesis by the liver (10,11). Target genes of HNF-4 α in pancreatic β -cells include genes involved in glucose metabolism, e.g., GLUT2, L-PK, and aldolase B (44). Overexpression of wild-type HNF-4 α in INS1 β -cells is associated with an increase in insulin secretion in response to glucose, whereas the overexpression of a dominant-negative form of HNF-4 α causes a decrease in glucose-induced insulin secretion (44). Endogenous HNF-4 α protein levels in islet β -cells must therefore be tightly regulated to maintain normal insulin secretion and euglycemia. This observation suggests that reduced levels of HNF-4 α in the

pancreas could contribute to the development of β -cell dysfunction. It is noteworthy that AMPK activity is regulated by glucose in pancreatic β -cells and has been implicated in the control of glucose-stimulated insulin secretion (19,24). Whether HNF-4 α is also a target of AMPK in islet β -cells is of major interest because any inappropriate activation of the AMPK signaling pathway would down-regulate HNF-4 α protein levels. Consequently, this would have deleterious effects on glucose-induced insulin secretion and could contribute to the development of type 2 diabetes.

In conclusion, our results add the transcription factor MODY1/HNF-4 α to the list of AMPK targets. In addition to its role in the regulation of hepatic gene expression, HNF-4 α also regulates the expression of genes that are involved in glucose transport and glucose metabolism in the pancreatic β -cells. This suggests that the modulation of HNF-4 α protein levels by AMPK could have important metabolic effects. The potential importance of the loss of HNF-4 α protein after AMPK activation is reinforced by the fact that only a minor perturbation of HNF-4 α activity results in MODY1 (43). Therefore, we propose that modulation of HNF-4 α function by AMPK activity could be of importance in the pathophysiology of type 2 diabetes. This issue is an interesting area for future investigation.

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