The gene for hepatocyte nuclear factor (HNF)-4α is activated by glucocorticoids and glucagon, and repressed by insulin in rat liver

Seiichi Oyadomari[a,b], Fumihiko Matsuno[a], Shoaib Chowdhury[a], Tatsuya Kimura[a], Katsuro Iwase[a,c], Eiichi Araki[b], Motoaki Shichiri[b], Masataka Mori[a], Masaki Takiguchi[a,c,*]

[a] *Department of Molecular Genetics, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860-0811, Japan*
[b] *Department of Metabolic Medicine, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto 860-0811, Japan*
[c] *Department of Biochemistry, Chiba University School of Medicine, Inohana 1-8-1, Chiba 260-8670, Japan*

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Abstract The gene for a transcription factor hepatocyte nuclear factor-4α (HNF-4α) is responsible for maturity-onset diabetes of the young, type 1. We examined hormonal regulation of the HNF-4α gene in the liver. Stimulation of primary-cultured rat hepatocytes with dexamethasone or glucagon led to induction of HNF-4α mRNA, being antagonized by insulin. In the liver of streptozotocin-induced diabetic rat, mRNA and protein levels for HNF-4α were elevated, and were normalized by insulin treatment. Therefore, HNF-4α in the liver is likely to be involved in the regulation of glucose metabolism in response to these hormones.

Key words: Transcription factor; Gene regulation; Hormone; Primary hepatocyte; Dexamethasone; Streptozotocin

1. Introduction

Maturity-onset diabetes of the young (MODY) is a monogenic and heterogeneous form of diabetes characterized by early-onset (usually before 25 years of age) and autosomal dominant inheritance. Five genes have been shown or suggested to be associated with different forms of MODY. These genes include the hepatocyte nuclear factor (HNF)-4α/MODY1 gene on chromosome 20q [1], the glucokinase/MODY2 gene on chromosome 7p [2], the HNF-1α/MODY3 gene on chromosome 12q [3], the insulin promoter factor (IPF)-1/MODY4 gene on chromosome 13q [4], and the HNF-1β/MODY5 gene on chromosome 17q [5].

HNF-4α is a member of the steroid/thyroid nuclear receptor superfamily of transcription factors [6], and was first identified as a factor binding to cis regulatory regions of a number of liver selectively expressed genes [6]. In addition to the liver, HNF-4α is expressed abundantly also in the kidney and intestine [6], and at lower levels in pancreatic islets and insulinoma cells [7]. Targeted disruption of the HNF-4α gene in mice causes embryonic lethality associated with impaired gastrulation, indicating that it is essential for early development [8]. Fatty acyl-CoA thioesters have been shown to bind HNF-4α and modulate its transcriptional activity positively or negatively, depending on the chain length and the saturation degree of fatty acid portions [9]. Eight HNF-4α mutations associated with MODY and late-onset diabetes have been reported [1,10–16].

We have studied transcriptional regulation of genes for enzymes of the ornithine cycle [17], which is closely related to gluconeogenesis [18,19]. Ammonia and carbon skeletons derived from amino acids are converted to urea and glucose, respectively, through the ornithine cycle and gluconeogenic pathway in hepatocytes. The two metabolic pathways are regulated by hormones in a coordinated manner: glucocorticoids and glucagon activate, while insulin represses, the genes for enzymes of both pathways [17,20]. We demonstrated that HNF-4α activates the gene for the second enzyme of the ornithine cycle, named ornithine transcarbamylase, through binding to the enhancer region of the gene [21]. On the other hand, HNF-4α repressed the gene for the last enzyme arginase without apparent binding to regulatory regions [22]. HNF-4α was also reported to interact with the glucocorticoid response unit in the promoter region of the gene for phosphoenolpyruvate carboxykinase, a rate-limiting enzyme of the gluconeogenic pathway [23]. Therefore, HNF-4α is a candidate for the factor involved in hormonal regulation of both urea synthesis and gluconeogenesis. We examined whether expression of the HNF-4α gene in the liver changes in response to hormones controlling glucose homeostasis.

2. Materials and methods

2.1. Isolation and culture of primary hepatocytes

Parenchymal hepatocytes from male Wistar rats (140–160 g, 6 weeks) were isolated by in situ perfusion of livers with collagenase solution as described [24,25]. Cell viability was determined by 0.2% trypan blue exclusion and was greater than 90% in all cases. 5×10^6 cells were seeded into collagen-coated 10-cm dishes (Iwaki, Tokyo, Japan) with 10 ml of Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 200 μg/ml streptomycin and 100 U/ml penicillin G.

2.2. Streptozotocin (STZ)-induced diabetic rats

Male Wistar rats (90–100 g, 5 weeks) were divided into three groups (each containing three animals): normal control, diabetic, and insulin-treated diabetic animals. Diabetes was induced by intraperitoneal injection of STZ (100 mg/kg; Sigma, St. Louis, MO, USA) in 50 mM citrate, pH 4.5, containing 154 mM NaCl, on two consecutive days. Control rats were injected with the citrate buffer alone. Subcutaneous injection of neutral insulin (4.0 U/kg; Novo Nordisk, Copenhagen, Denmark) was done twice 24 and 6 h before killing on day 7. The

*Corresponding author. Fax: (81)-43-226 2037.
E-mail: mtak@med.m.chiba-u.ac.jp

Abbreviations: MODY, maturity-onset diabetes of the young; HNF, hepatocyte nuclear factor; STZ, streptozotocin

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animals of all three groups were subjected to liver excision and blood collection by cardiac puncture between 9:00 and 10:00 a.m.

Plasma glucose concentrations were measured by the mutarotase/glucose dehydrogenase method with a glucose photometer (HemoCue AB, Ängelholm, Sweden). Plasma insulin concentrations were determined by sandwich-type enzyme immunoassay using an insulin ELISA kit (Morinaga, Yokohama, Japan), and plasma glucagon concentrations by radioimmunoassay using a Daiichi kit (Daiichi Radioisotope Lab., Tokyo, Japan).

2.3. Northern blot analysis

Total RNA was isolated from hepatocytes and liver using the acid guanidinium thiocyanate–phenol–chloroform extraction procedure [26]. RNA (2 μg/lane) was electrophoresed in denaturing formaldehyde–agarose (1%) gels, and blotted on nylon membranes. A digoxigenin-labeled antisense RNA probe was synthesized from HNF-4α cDNA [27] (accession number D10554) under the control of the T7 promoter using a transcription kit (Roche Diagnostics, Tokyo, Japan). Hybridization followed by chemiluminescent detection on X-ray films was done as recommended by Roche Diagnostics. Densitometric quantification was done using the MacBas software (Fuji Photo Film, Tokyo, Japan).

2.4. Western blot analysis

Rabbit polyclonal antibody against a synthetic peptide corresponding to the 11 carboxyl-terminal residues of mouse HNF-4α protein was prepared essentially as described [6]. Protein extracts were prepared from liver nuclei purified in viscous sucrose solution as described [28]. The extracts (20 μg of protein) were subjected to SDS–polyacrylamide gel electrophoresis followed by electrotransfer to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham, Little Chalfont, UK). Densitometric quantification was done using the MacBas software.

2.5. Statistical analysis

Data were expressed as means ± S.E.M. Statistical significance of differences between groups was evaluated using unpaired Student's *t*-test. When the *P* value was less than 0.05, the difference was considered statistically significant.

3. Results

3.1. Increases in HNF-4α mRNA levels in response to dexamethasone and glucagon in primary-cultured hepatocytes

Rat hepatocytes were isolated by collagenase perfusion and plated on collagen-coated dishes in a medium containing 5% fetal bovine serum. Changes in HNF-4α mRNA levels during the culture were monitored by Northern analysis using total RNA prepared from the hepatocytes at various times after the plating (Fig. 1A). HNF-4α mRNA levels decreased by 24 h and reached a constant level, which was maintained during the succeeding culture up to 96 h. The following experiments were performed between 48 and 96 h of the culture.

Effects of glucocorticoids and glucagon on expression of the HNF-4α gene were examined by treating the primary hepatocytes with these hormones separately or in combination (Fig. 1B). Hormones were added into the culture medium twice 24 and 6 h prior to harvesting the cells. A synthetic glucocorticoid, dexamethasone (10^{-6} M), and glucagon (3×10^{-8} M) raised HNF-4α mRNA levels 5.3- and 3.5-fold, respectively. A combination of these hormones caused an 8.9-fold increase.

Various concentrations of dexamethasone and glucagon were tested for their effects on HNF-4α mRNA levels (Fig. 2A). Dexamethasone raised HNF-4α mRNA levels in a dose-dependent manner. The concentration for half-maximum effect (EC_{50}) was between 10^{-9} and 10^{-8} M, which was concordant with the K_d of 7×10^{-9} M [29] and EC_{50} of 3×10^{-9} M [30] measured with cloned glucocorticoid receptors. Glucagon also raised the levels dose-dependently with EC_{50} of about 10^{-10} M, which was comparable with physiological glucagon concentrations around 10^{-10} M in the rat portal vein [31] and lower than EC_{50} of 3.7×10^{-10} M measured for calcium flux with the cloned rat glucagon receptor [32].

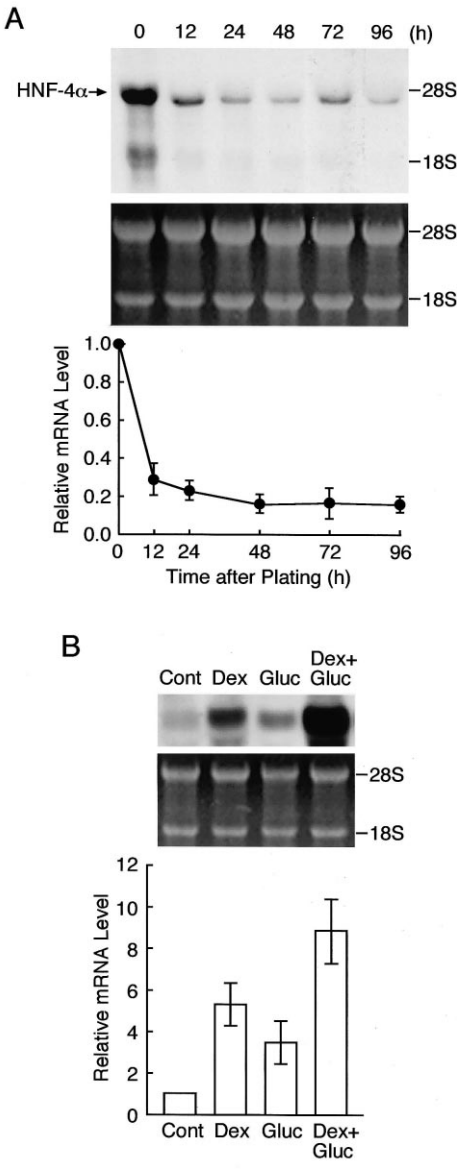

Fig. 1. Increases in HNF-4α mRNA levels by dexamethasone and/or glucagon in primary-cultured rat hepatocytes. A: HNF-4α mRNA levels in hepatocytes during the culture without hormones. Isolated hepatocytes were plated into Dulbecco's modified Eagle's medium containing 5% fetal bovine serum but no supplemented hormones, and cultured for indicated periods. The medium was changed at 24 and 72 h of the culture. B: Dexamethasone (10^{-6} M, Dex) and/or glucagon (3×10^{-8} M, Gluc) were added into the culture medium twice, i.e. 24 h and 6 h prior to harvesting at 72 h of the culture. Total RNA was isolated from the cells and subjected to blot analysis for detection of HNF-4α mRNA. Representative chemiluminograms are shown with ethidium bromide staining of rRNAs. At the bottom of each panel, quantified results of the chemiluminogram are presented as means ± S.E.M. for three independent experiments.

Time course of changes in HNF-4α mRNA levels in response to dexamethasone or glucagon was monitored (Fig. 2B). With each hormone, increases in HNF-4α mRNA were

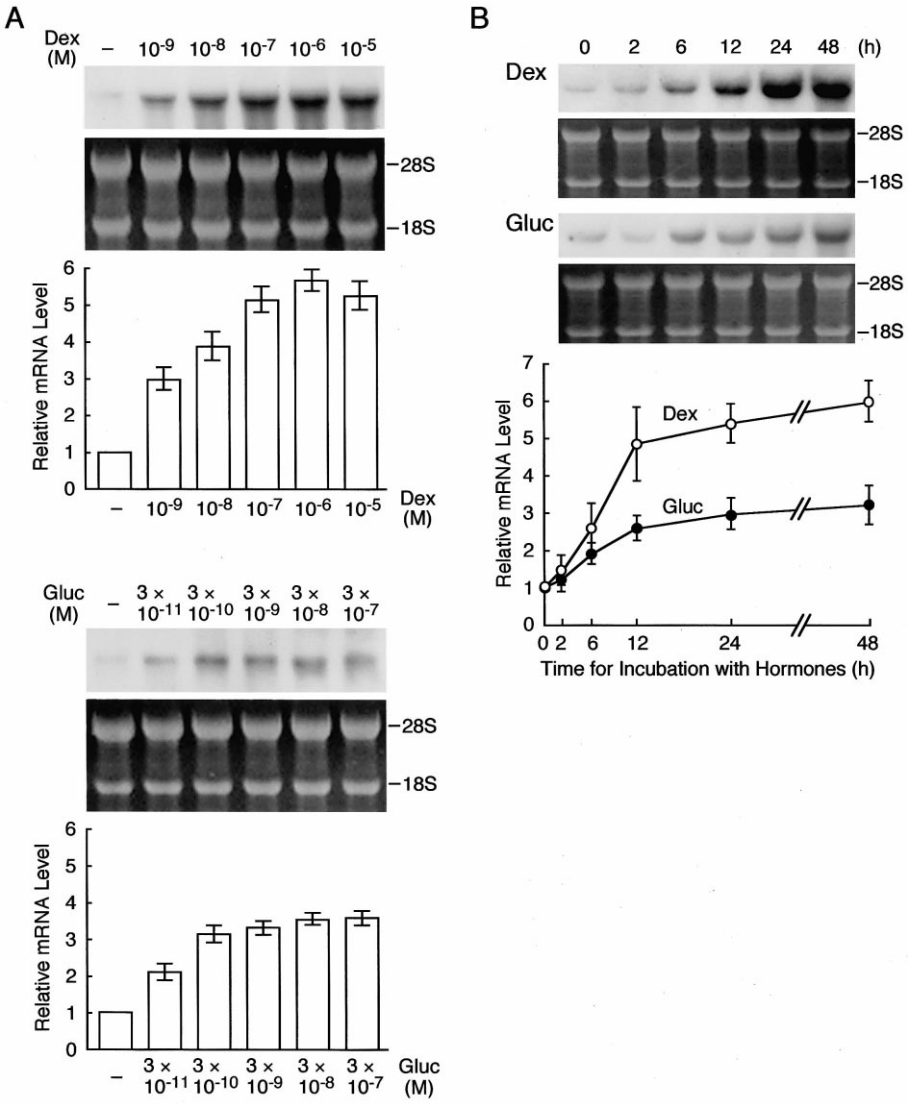

Fig. 2. Characterization of increases in HNF-4α mRNA levels by dexamethasone or glucagon. A: Dose-dependency. Various concentrations of dexamethasone (Dex) or glucagon (Gluc) were added into the culture medium of primary hepatocytes twice, i.e. 24 h and 6 h prior to harvesting. B: Time course. Dexamethasone (10^{-6} M, Dex) or glucagon (3×10^{-8} M, Gluc) were added into the culture medium of primary hepatocytes at indicated times before harvesting. Total RNA was isolated from the cells and subjected to blot analysis for detection of HNF-4α mRNA. Quantified results are presented as means ± S.E.M. for three independent experiments.

apparent at 6 h after addition of each hormone, reached plateau levels around 24 h, and were sustained by 48 h.

3.2. *Antagonistic repression by insulin of dexamethasone- or glucagon-stimulated HNF-4α mRNA induction*

Effects of insulin, which antagonizes glucocorticoids and glucagon in a number of liver functions, were examined by treating the hepatocytes with various concentrations of insulin in combination with a fixed amount of dexamethasone (10^{-6} M) or glucagon (3×10^{-8} M) (Fig. 3). Basal HNF-4α mRNA levels without dexamethasone nor glucagon were too low to monitor further down-regulation by insulin, if any, quantitatively. HNF-4α mRNA levels raised by dexamethasone (Fig. 3A) or glucagon (Fig. 3B) were decreased by insulin dose-

Table 1
Metabolic parameters of control, STZ-diabetic (STZ), and insulin-treated STZ-diabetic (STZ+Ins) rats

Group	Body weight (g)	Plasma glucose (mM)	Plasma insulin (pM)	Plasma glucagon (pg/ml)
Control	159.3 ± 1.2	10.4 ± 0.4	156.4 ± 34.8	215.9 ± 25.6
STZ	102.7 ± 4.69[a]	38.3 ± 2.9[a]	55.2 ± 0.6[b]	231.0 ± 52.0
STZ+Ins	114.3 ± 6.44	14.7 ± 1.5[c]	153.5 ± 36.3[d]	217.8 ± 36.3

Data are means ± S.E.M. of $n = 3$ per group. Significant differences were evaluated by the Student's *t*-test.
[a] $P < 0.001$ vs. control.
[b] $P < 0.05$ vs. control.
[c] $P < 0.01$ vs. STZ.
[d] $P < 0.05$ vs. STZ.

dependently with EC_{50} of 10^{-8} to 10^{-7} M, which was somewhat higher than the maximum insulin concentration of 5×10^{-9} M in the portal vein [31]. Effects of insulin against lower and physiological concentrations of glucocorticoids and glucagon remain to be examined.

3.3. Regulation of HNF-4α expression in the liver of the STZ-diabetic rats

In vivo effects of insulin on expression of HNF-4α were examined by using STZ-diabetic rats. As shown in Table 1, at day 7 after initiation of STZ treatment, the rats exhibited diabetic profiles with decreased body weights, hyperglycemia and hypoinsulinemia. Supplementation of insulin to the diabetic rats significantly lowered plasma glucose concentrations. Plasma glucagon concentrations were almost constant in any condition. HNF-4α mRNA (Fig. 4A) and protein (Fig. 4B) were increased in livers of the STZ-diabetic rats compared to control rats. Insulin administration normalized the increased mRNA and protein levels completely and partially, respectively, to the control levels. Therefore, expression of HNF-4α in the liver is repressed by insulin also in vivo.

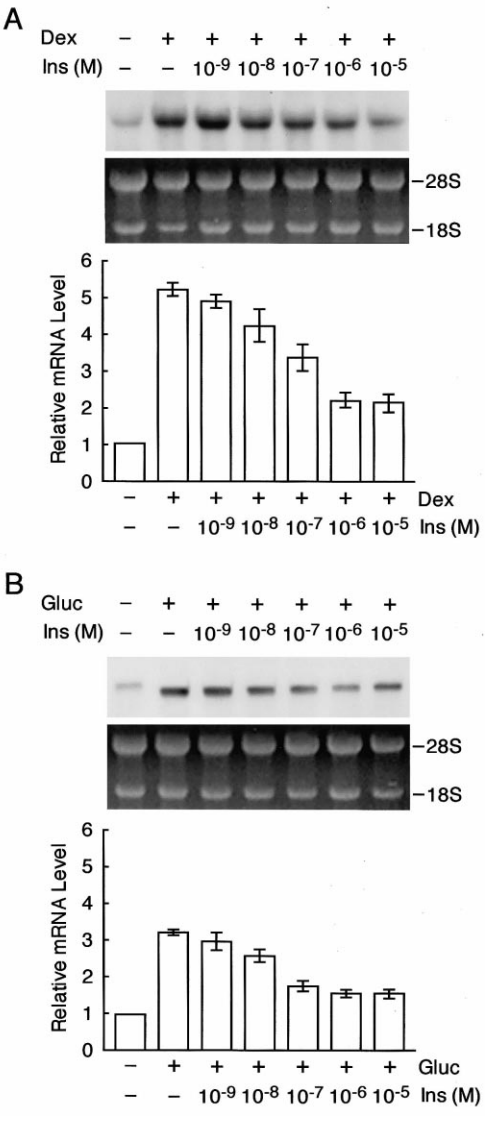

Fig. 3. Antagonistic effects of insulin on dexamethasone- or glucagon-stimulated increases in HNF-4α mRNA levels. Various concentrations of insulin in combination with dexamethasone (10^{-6} M, Dex) (A) or glucagon (3×10^{-8} M, Gluc) (B) were added into the culture medium of primary hepatocytes twice, i.e. 24 h and 6 h prior to harvesting, and RNA blot analysis for HNF-4α was carried out. Quantified results are presented as means ± S.E.M. for three independent experiments.

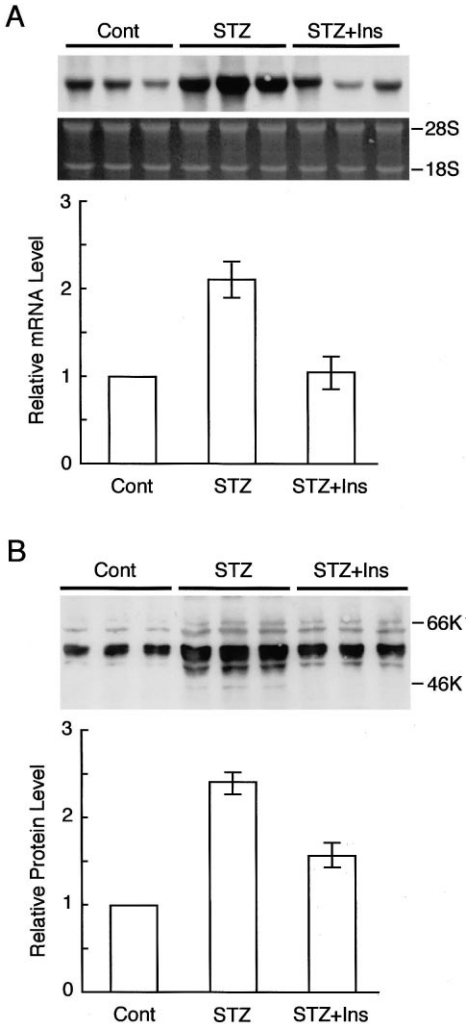

Fig. 4. Changes in HNF-4α mRNA (A) and protein (B) levels in STZ diabetes with or without insulin. Livers were excised from the normal control (Cont), STZ-diabetic (STZ), and insulin-supplemented STZ-diabetic (STZ+Ins) rats, each in triplicate. Total RNAs and tissue extracts were prepared from the livers, and subjected to Northern (A) and Western (B) analysis, respectively. Below the Northern chemiluminogram, ethidium bromide staining of rRNAs is shown. At the bottom of each panel, quantified results of the chemiluminograms are presented. Bars represent means ± S.E.M.

4. Discussion

In this study, we demonstrated that expression of HNF-4α in rat hepatocytes was increased by dexamethasone and glucagon, and decreased by insulin. These results suggest that hepatic HNF-4α is involved in regulation of blood glucose levels. A straightforward interpretation is that HNF-4α mediates up-regulation of blood glucose levels in response to glucocorticoids and glucagon, and down-regulation in response to insulin. Relatively slow inductions of HNF-4α mRNA by dexamethasone or glucagon (Fig. 2) are concordant with the

notion that induced HNF-4α mediates prolonged effects of the hormones for maintenance of increased blood glucose. However, formally, even a completely opposite interpretation cannot be ruled out: HNF-4α rather mediates rebound from initial effects of the hormones and down-regulates blood glucose levels.

HNF-4α has been reported to regulate a number of genes involved in glucose metabolism. A study on the visceral endoderm developed in vitro from embryonic stem cells deficient in HNF-4α revealed that this factor is critical for expression of genes for glucose transporter 2 and glycolytic enzymes such as aldolase B, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase [33]. HNF-4α binds to a DNA element of the glucocorticoid response unit in the promoter region of the gene for phosphoenolpyruvate carboxykinase, a key regulatory enzyme of gluconeogenesis [23], and seems to mediate repression of the gene by fatty acyl-CoAs, the ligands of HNF-4α [34]. Finally, the gene for another liver-enriched transcription factor HNF-1α, which is responsible for MODY3 [3], is under the control of HNF-4α [35]. One MODY3 pedigree was reported to result from a mutation in the HNF-4α-binding site in the HNF-1α gene promoter [36]. HNF-1α is expressed abundantly in endocrine pancreas [7] as well as in hepatocytes, and implicated in transcriptional regulation of the insulin gene [37]. These observations suggest that HNF-4α lowers blood glucose levels through stimulating insulin synthesis and secretion in β-cells, and through inhibiting gluconeogenesis and activating glucose uptake and glycolysis in hepatocytes. Concordant with this notion, phosphorylation of HNF-4α by protein kinase A, which mediates effects of glucagon/cAMP, results in repression of DNA-binding activity, presumably leading to attenuation of both inhibitor and activator activities in hepatocytes in response to the hormone [38].

Although previous studies [10,39] have attributed the primary defect in MODY1 mainly to β-cell failure, HNF-4α dysfunction in the liver potentially can also contribute to abnormalities of glucose metabolism. While many possibilities remain to be examined, one of the most feasible working hypotheses for the liver-oriented etiology for MODY1 is as follows: normal hepatic HNF-4α is a regulator lowering the blood glucose levels by mediating the rebound from initial effects of the hormones, and mutations in the HNF-4α gene result in derepression of genes involved in hepatic glucose production, and/or inactivation of genes involved in hepatic glucose utilization. To take an example for cell-differential roles of MODY genes, Postic et al. [40] recently demonstrated that disruption of the glucokinase gene, which is responsible for MODY2 [2], in β-cells and hepatocytes of mice led to severe and mild hyperglycemia, respectively. Such conditional knockout studies will be also helpful to examine cell-differential roles of HNF-4α.

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