Diabetologia (1999) 42: 887–891

Diabetologia
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Short communication

Overexpression of dominant negative mutant hepatocyte nuclear factor (HNF)-1α inhibits arginine-induced insulin secretion in MIN6 cells

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Abstract

Aims/hypothesis. To explain the mechanisms whereby mutations in the HNF-1α gene cause insulin secretory defects.
Methods. A truncated mutant HNF-1α (HNF-1α288t) was overexpressed in hepatoma cells (HepG2) and murine insulinoma cells (MIN6) using a recombinant adenovirus system and expression of the HNF-1α target genes and insulin secretion were examined.
Results. Expression of phenylalanine hydroxylase and α1-antitrypsin genes, the target genes of HNF-1α, was suppressed in HepG2 cells by overexpression of HNF-1α288t. In MIN6 cells, overexpression of HNF-1α288t did not change insulin secretion stimulated by glucose (5 mmol/l and 25 mmol/l) or leucine (20 mmol/l). Potentiation of insulin secretion by arginine (20 mmol/l, in the presence of 5 mmol/l or 25 mmol/l glucose) was, however, reduced ($p < 0.0001$ and $p = 0.027$, respectively). Similarly reduced responses were observed when stimulated with homoarginine. Expression of the cationic amino acid transporter-2 was not reduced and insulin secretory response to membrane depolarization by 50 mmol/l KCl was intact.
Conclusion/interpretation. The HNF-1α288t, which is structurally similar to the mutant HNF-1α expressed from the common MODY3 allele, P291fsinsC, exerts a dominant negative effect. Suppression of HNF-1α in MIN6 cells severely impaired potentiation of insulin secretion by arginine, whereas glucose-stimulated and leucine-stimulated insulin secretion was intact. Our findings delineate the complex nature of beta-cell failure in patients with MODY3. This cell model will be useful for further investigation of the mechanism of insulin secretory defects in these patients. [Diabetologia (1999) 42: 887–891]

Keywords MODY, hepatocyte nuclear factor-1α, recombinant adenovirus, MIN6 cells, dominant negative effect, arginine.

Received: 30 November 1998 and in revised form: 16 February 1999

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Abbreviations: MODY, Maturity onset diabetes of the young; HNF, hepatocyte nuclear factor; MIN6–288 t, MIN6 cells overexpressing HNF-1α288t; MIN6-*lacZ*, MIN6 cells overexpressing β-galactosidase; CAT, cationic amino acid transporter; PAH, phenylalanine hydroxylase; α1-AT, α1-antitrypsin; KRB-HB, HEPES-balanced Krebs-Ringer bicarbonate buffer, MOI, multiplicity of infection.

Mutations in the hepatocyte nuclear factor-1α (HNF-1α) gene and HNF-4α gene cause maturity onset diabetes of the young (MODY)-3 [1] and MODY1 [2], respectively. Hepatocyte nuclear factor-1α belongs to a family of homeodomain-containing transcription factors and its expression is regulated by HNF-4α [3]. Clinical studies have shown MODY1 and MODY3 are caused predominantly by insulin secretory failure [4, 5]. When insulin secretion was examined in stepped glucose infusion studies [4, 5], non-diabetic MODY1 and MODY3 mutation carriers showed reduced insulin secretory responses to glucose. Conversely, another report showed that in a non-diabetic carrier of the MODY3 mutation, the insulin secreto-

ry response to an intravenous glucose load was not impaired, but exaggerated [6]. It is also reported that in a non-diabetic MODY1 mutation carrier, insulin secretion in response to sustained hyperglycaemia (11.1 mmol/l) or bolus glucose infusion was intact [7]. These observations suggest that glucose-stimulated insulin secretion is not impaired in certain conditions in the carriers of MODY1 and MODY3 mutations. The mechanism underlying this apparent discrepancy has not been explained.

One common MODY3 mutation of the HNF-1α gene, P291fsinsC, apparently results in the expression of a truncated protein, which retains the dimerization and the DNA binding domains, but lacks the transcriptional activation domain. This mutant protein is likely to exert dominant negative effects on target gene transcriptional activation, since the mutant protein dimerizes with intact HNF-1α, or another related partner HNF-1β, producing a transactivationally inactive heterodimer [8]. Here, to produce a culture cell model and study the molecular mechanism of insulin secretory defects in HNF-1α deficient MODY3 beta cells, we constructed a recombinant adenovirus and efficiently expressed a mutant HNF-1α, HNF-1α288t, which was structurally similar to that expressed from the P291fsinsC MODY3 mutant allele, in culture cells. The effect of HNF-1α288t overexpression on target gene transcription and insulin secretion were studied using this model.

Materials and methods

Plasmid construction. Mouse HNF-1α cDNA (Genbank accession no. M57 966) and mouse cationic amino acid transporter-2 (mCAT2) cDNA [9] were obtained from MIN6 cells by reverse transcription (RT)-PCR. The mouse HNF-1α expression plasmid (pcDNA-mHNF-1α) was constructed by cloning a coding region of mouse HNF-1α cDNA into pcDNA3 (Invitrogen, Carlsbad Calif., USA). The expression plasmid for the truncated HNF-1α (pcDNA-mHNF-1α288t) was constructed by cloning a partial cDNA fragment with a termination codon after the 288th codon.

The reporter plasmid (pGL3-TK-βfib3) was designed to have three copies of HNF-1α binding sites (TGTCAAATATTAACTAAAGGG) in tandem in front of the herpes simplex virus thymidine kinase promoter, followed by the firefly luciferase gene.

Reporter assay. To HeLa cells, pcDNA-mHNF-1α or pcDNA-mHNF-1α288t was cotransfected with pGL3-TK-βfib3 and pCMVβ (Clontech, Palo Alto, Calif., USA) by the lipofection method using TransIT-LT1 (PanVera Corporation, Madison, Wis., USA) according to the supplier's protocols. Luciferase assay was carried out 48 h after transfection. Luciferase activity was normalized for transfection efficiency based on β-galactosidase activity.

Adenovirus mediated gene transduction. Recombinant adenoviruses AdexlCAmHNF-1α288t and AdexlCAlacZ, encoding *Escherichia coli* β-galactosidase, was constructed as described previously [10]. Adenovirus infection was done as described in [11] at a multiplicity of infection (MOI) of 10–20 pfu/cell, unless otherwise specified.

Semiquantification of α1-antitrypsin and phenylalanine hydroxylase mRNA. We extracted RNA from HepG2 cells 3 days after viral infection. The RT-PCR was carried out using specific primer sets (5′-GAA GAA GTT GGT GCA TTG GC-3′ + 5′-TTG GAT GGC TGT CTT CTC C-3′ for phenylalanine hydroxylase (PAH), 5′-AAT GCC GTC TTC TGT CTC G-3′ + 5′-CGC TCT TCA GAT CAT AGG TTC C-3′ for α1-antitrypsin (α1-AT), and 5′-ATG TGC AAG GCC GGC TTC GCG GGC GAC G-3′ + 5′-CAG CCA GGT CCA GAC GCA GGA TGG CAT G-3′ for β-actin) in the presence of [α-^{32}P] dCTP. Equal aliquots of PCR products were electrophoresed through non-denaturing 5% polyacrylamide gel. Radioactivities of the specific products were quantified using a BAS 2000 phosphor imager (Fuji Film, Tokyo, Japan).

Insulin release assay. Insulin secretion from MIN6 cells was measured by the static incubation method. After preincubation for 30 min in HEPES-balanced Krebs-Ringer bicarbonate buffer supplemented with 0.5% bovine serum albumin (KRB-HB) and 5 mmol/l glucose, the media were replaced with KRB-HB containing various secretagogues. After a further 2 h incubation at 37 °C, media were collected and immunoreactive insulin was measured by RIA using rat insulin as a standard. The amounts of insulin secreted were corrected by the amounts of cell protein in each well.

Statistical analysis. Data are presented as means ± SEM. The statistical significance of differences between mean values was assessed using the unpaired-Student's *t* test, or Mann Whitney's U-test whenever appropriate. *P*-values less than 0.05 were considered significant.

Results

Truncated HNF-1α exerts a dominant negative effect on target gene expression. The HNF-1α288t was defective in promoter activation. When pcDNA-mHNF-1α was cotransfected with pGL3-TK-βfib3, luciferase activity increased 6.9 ± 1.9 fold. In contrast, pcDNA-mHNF-1α288t increased luciferase activity only 1.3 ± 0.2 fold.

With the recombinant adenovirus AdexlCA-HNF-1α288t, the mutant HNF-1α was efficiently overexpressed in culture cells when examined by western blot analysis (data not shown). We then examined the effect on target gene expression in HepG2 cells. After 3 days of infection with AdexlCA-HNF-1α288t at MOI of 30, expression of the PAH and α1-AT genes, which is largely dependent on HNF-1α in hepatocytes [12], was suppressed to 10 and 25% of controls, respectively (Fig. 1). In contrast, under this condition, the expression of β-actin was not changed. Thus, overexpression of HNF-1α288t suppressed expression of PAH and α1-AT genes, via a dominant negative effect on intrinsic HNF-1α.

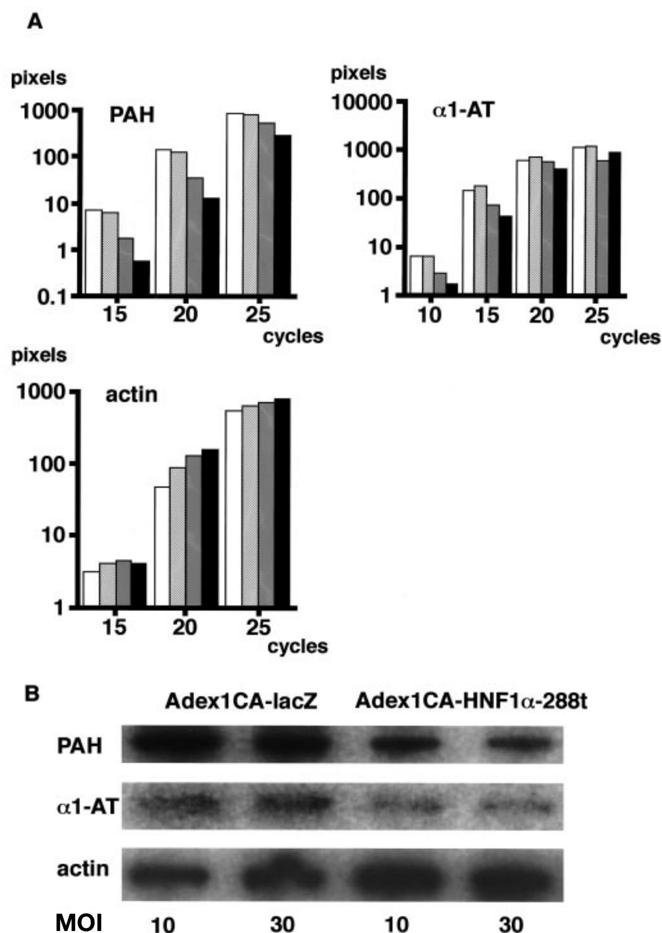

Fig. 1.A,B Dominant negative effect of HNF-1α288 t on target gene expression in HepG2 cells. Messenger RNA for phenylalanine hydroxylase (PAH), α1-antitrypsin (α1-AT) and actin were semiquantified by the RT-PCR method. The experiments were repeated twice and essentially the same results were obtained. Representative data are shown. **A** Amounts of RT-PCR products for each PCR cycle. **B** Representative autoradiogram. PAH: at 20 cycles, α1-AT: at 10 cycles, actin: at 20 cycles. □ Adex1CA-*lacZ* (MOI 10), ▨ Adex1CA-*lacZ* (MOI 30), ▩ Adex1CA-HNF1α-288 t (MOI 10), ■ Adex1CA-HNF1α-288 t (MOI 30)

Expression of dominant negative HNF-1α impairs arginine induced insulin secretion from MIN6 cells. We then overexpressed HNF-1α288 t in MIN6 cells. As shown in Fig. 2, at 5 mmol/l glucose, insulin secretion from MIN6 cells overexpressing truncated HNF-1α (MIN6–288 t) (105.5 ± 12.2 ng/ng protein) did not differ from that from MIN6 cells overexpressing β-galactosidase (MIN6-*lacZ*) (112.0 ± 11.2 ng/ng protein) ($p = 0.215$). Insulin secretion at 25 mmol/l glucose was increased by 11.8 ± 1.0 fold (MIN6–288 t) and 9.4 ± 0.7 fold (MIN6-*lacZ*) compared with that of MIN6-*lacZ* at 5 mmol/l glucose, without significant difference ($p = 0.060$). In the presence of 5 mmol/l glucose L-leucine (20 mmol/l) also increased insulin secretion to the same extent in both cells ($p = 0.505$).

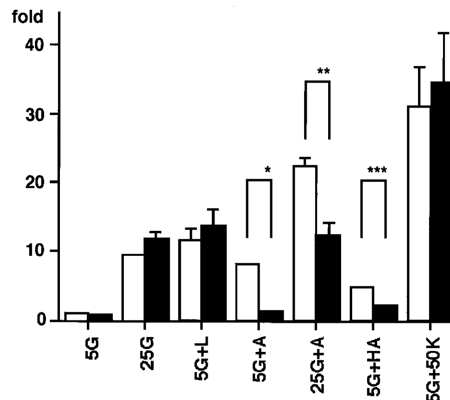

Fig. 2. Effects of HNF-1α288 t overexpression on insulin secretory responses of MIN6 cells. After 5 days of recombinant adenovirus infection, insulin secretion was measured by the static incubation method as described in Materials and methods. In each set of experiments, 5 mmol/l glucose stimulation was included and responses of MIN6-*lacZ* or MIN6–288 t cells to each secretagogue were expressed as fold increases from the values obtained from MIN6-*lacZ* cells with 5 mmol/l glucose stimulation (112.0 ± 11.2 ng/ng protein). Each assay was done in triplicate wells and values are means + SEM of at least 3 experiments. 5G: 5 mmol/l glucose, 25G: 25 mmol/l glucose, L: 20 mmol/l L-leucine, A: 20 mmol/l L-arginine, HA: 20 mmol/l homoarginine, 50 K: 50 mmol/l KCl. * $p < 0.0001$, ** $p = 0.027$, *** $p = 0.0024$. □ MIN6-*lacZ*, ■ MIN6–288t

In contrast to the responses to glucose and leucine, potentiation of insulin secretion by L-arginine was severely impaired. In the presence of 5 mmol/l glucose, 20 mmol/l L-arginine potentiated insulin secretion from MIN6-*lacZ* cells (8.08 ± 0.50 fold). This potentiation was, however, nearly abolished in MIN6–288 t cells. Similarly, diminished potentiation of insulin secretion was observed for arginine at 25 mmol/l glucose and for homoarginine, a non-metabolizable arginine analogue. One of the mechanisms by which arginine potentiates insulin secretion is direct depolarization of the beta-cell membrane potential through uptake of cationic amino acids into cells [13]. Thus, we examined the insulin secretory response to membrane depolarization by 50 mmol/l KCl, but found no statistically significant difference.

To exclude the possibility that the impairment of arginine potentiation of insulin secretion is due to adenovirus infection, we repeated the experiments at various MOIs. Potentiation of insulin secretion by arginine was severely impaired in cells infected with Ade × 1CA-HNF-1α288 t even at a MOI of 1.6, whereas it was not altered in cells infected with Ade × 1CA-*lacZ* even at a MOI as high as 36 (data not shown).

Expression of insulin, GLUT2 and cationic amino acid transporter mRNA in MIN6 cells expressing dominant negative HNF-1α. It has been suggested that HNF-1α affects the expression of insulin and

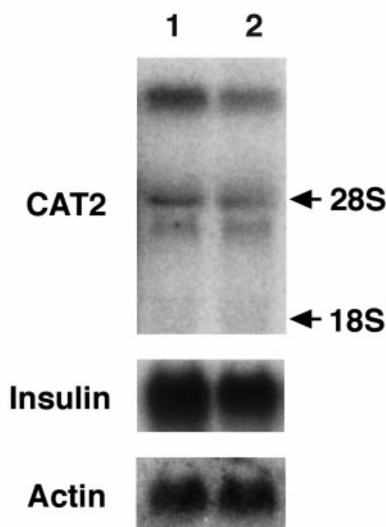

Fig. 3. Expression of CAT2 and insulin mRNA in MIN6–288t and MIN6-*lacZ* cells. After viral infection for 5 days, total cellular RNA was isolated. Twenty micrograms of RNA from MIN6–288t cells (lane 1) or MIN6-*lacZ* cells (lane 2) were electrophoresed, blotted onto a nylon membrane and probed sequentially with ^{32}P-labelled cDNA probes. Positions of ribosomal RNA are indicated. In addition to the 7–9 kb major CAT2 transcript, approximately 4 and 5 kb minor transcripts were also detected in MIN6 cells. Experiments were repeated three times independently and representative data are shown

GLUT2 genes [14, 15], but no differences in their mRNA expression was observed between MIN6–288t and MIN6-*lacZ* cells (Fig. 3 and data not shown). The cationic amino acid transporter CAT2 was suggested to be involved in the potentiation of insulin secretion by arginine [13]. No reduction in CAT2 mRNA was, however, observed in MIN6–288t cells (Fig. 3).

Discussion

We first showed that overexpression of the truncated HNF-1α, HNF-1α288t, suppressed target gene (PAH and α1-AT) expression in hepatoma cells. This effect is attributable to a dominant negative effect of the mutant HNF-1α on the intrinsic HNF-1α. We then studied the effects of decreased HNF-1α function on insulin secretion.

In MIN6–288t, impairment of arginine-stimulated insulin secretion was striking. Potentiation of insulin secretion by arginine was barely detectable at either the basal (5 mmol/l) or a high (25 mmol/l) glucose concentration. Arginine is a potent potentiator of insulin secretion in the presence of glucose, although the mechanism is still not clear. Nitric oxide, which is generated from arginine, was proposed as a mediator of insulin secretion [16] but this is controversial [13, 17]. A more favoured hypothesis is that the uptake of cationic amino acids directly depolarizes the beta-cell membrane and triggers insulin secretion [13, 18]. Recently, the high capacity, low affinity cationic amino acid transporter, CAT2 was suggested to mediate the potentiation of insulin secretion [13]. The expression of CAT2 mRNA in MIN6–288t cells was, however, not decreased, but increased compared with MIN6-*lacZ* cells (Fig. 3). Thus, impaired potentiation is not simply explained by decreased arginine transport by CAT2. Insulin secretion evoked by direct membrane depolarization by 50 mmol/l KCl was not impaired in MIN6–288t cells (Fig. 2), suggesting that the insulin secretory pathway was intact after depolarization of this membrane. These results indicate that undefined mechanisms other than direct membrane depolarization may account for the potentiation of insulin secretion by arginine, and the pathway is likely to be impaired in MIN6–288t cells.

In MIN6–288t cells, glucose-stimulated insulin secretion was not impaired under our experimental conditions, in which the glucose concentration was changed rapidly and sustained for 2 h. Leucine, which stimulates insulin secretion through intracellular metabolism and ATP generation, also stimulated insulin secretion to the same extent as control MIN6-*lacZ* cells. The results indicate that the basic machinery necessary for glucose-stimulated insulin secretion is intact. Our results were unexpected given those of earlier clinical studies [5] and pancreas perfusion and perifusion studies of islets from diabetic HNF-1α (–/–) mice [19]. Insulin secretory defects in human MODY3 patients seems not to be simple. Although insulin secretion was impaired in some patients with MODY3 [5], another report described a non-diabetic carrier of a MODY3 mutation, in whom the insulin response to an intravenous glucose load was somewhat exaggerated, despite the response to an oral glucose load being lower than in controls [6]. It is also noteworthy that in a non-diabetic MODY1 mutation carrier, insulin secretion in response to sustained hyperglycaemia (11.1 mmol/l) or bolus glucose infusion was not impaired [7]. Because the expression of HNF-1α is regulated by HNF-4α [3], mechanisms of beta-cell failure in MODY1 patients probably overlap those of MODY3 patients. These findings indicate that in certain circumstances, glucose-stimulated insulin secretion is intact in beta cells of MODY1/3 patients as in MIN6–288t cells under our experimental conditions. Insulin secretion from the islets of HNF-1α (±) mice was normal [19]. In the MIN6–288t cells, transcriptional activity of HNF-1α is likely to be suppressed by overexpression of the dominant negative mutant HNF-1α. One possible explanation for the normal glucose-stimulated insulin secretion in HNF-1α (±) mouse islets and MIN6–288t cells is that in murine beta cells, glucose-stimulated insulin secretion is less sensitive to the decreased HNF-1α activity than in human beta cells. A

changed expression pattern of the transcription regulators in the MIN6 insulinoma cells could also be the factor. It is also possible that the insulin secretory response from HNF-1α deficient beta cells remains relatively intact when there is a rapid increase in glucose concentration, or prolonged exposure to a high glucose concentration as under our experimental conditions (2 h at 25 mmol/l) but is impaired in response to a gradual increase in glucose concentration.

Clearly, the molecular mechanisms of beta-cell dysfunction in the MODY3 patients are complex. As a transcription factor, HNF-1α, regulates expression of the multiple genes in beta cells. Although the target genes are still not well defined, they are probably involved in multiple steps from secretagogue stimulation to insulin secretion and also possibly in insulin biosynthesis. The expression of two possible target genes, the GLUT2 and the insulin genes, was not impaired in MIN6–288t cells (Fig. 3 and data not shown) and HNF-1α (–/–) mice [19] and thus their expression is probably less dependent on HNF-1α in beta cells. Insulin secretion from MIN6–288t cells is clearly abnormal in respect to arginine potentiation. Insulin secretory response to arginine was also diminished in non-diabetic MODY1 subjects with a mutation in the HNF-4α gene [7] and in HNF-1α (–/–) mouse islets [19]. Therefore, MIN6–288t cells could represent at least a part of the beta cells in MODY3 patients. The molecular mechanism whereby the suppressed HNF-1α function leads to insulin secretory defects observed in MIN6–288t cells and in beta cells from MODY3 patients, is not fully understood. Elucidating the mechanisms underlying these unique insulin secretory defects and their link to the MODY3 phenotype would facilitate further understanding of the regulation and maintenance of normal beta-cell function. It may lead to the discovery of another key element in the regulation of beta-cell function, abnormalities of which play a role in the more common form of type 2 diabetes mellitus.

Acknowledgements. We thank Dr. N. Miura (Department of Biochemistry, Akita University School of Medicine, Akita, Japan) for anti-HNF-1α antiserum. We also thank Ms. Y. Kora-Miura and Ms. E. Kudo for their expert technical assistance. This study was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (10671074 to YT) and by Health Sciences Research Grants (Research on Human Genome and Gene Therapy) from the Ministry of Health and Welfare of Japan (to Y. Oka).

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