Dominant-negative suppression of HNF-1alpha function results in defective insulin gene transcription and impaired metabolism-secretion coupling in a pancreatic beta-cell line

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Abstract

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Dominant-negative suppression of HNF-1α function results in defective insulin gene transcription and impaired metabolism–secretion coupling in a pancreatic β-cell line

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Mutations in the hepatocyte nuclear factor-1α (HNF-1α) have been linked to subtype 3 of maturity-onset diabetes of the young (MODY3), which is characterized by a primary defect in insulin secretion. The role of HNF-1α in the regulation of pancreatic β-cell function was investigated. Gene manipulation allowed graded overexpression of HNF-1α and controlled dominant-negative suppression of HNF-1α function in insulinoma INS-1 cells. We show that HNF-1α is essential for insulin gene transcription, as demonstrated by a pronounced decrease in insulin mRNA expression and in insulin promoter activity under dominant-negative conditions. The expression of genes involved in glucose transport and metabolism including glucose transporter-2 and L-type pyruvate kinase is also regulated by HNF-1α. Loss of HNF-1α function leads to severe defects in insulin secretory responses to glucose and leucine, resulting from impaired glucose utilization and mitochondrial oxidation. The nutrient-evoked ATP production and subsequent changes in plasma membrane potential and intracellular Ca2+ were diminished by suppression of HNF-1α function. These results suggest that HNF-1α function is essential for maintaining insulin storage and nutrient-evoked release. The defective mitochondrial oxidation of metabolic substrates causes impaired insulin secretion, indicating a molecular basis for the diabetic phenotype of MODY3 patients.

Keywords: HNF1α/insulin secretion/insulin transcription/mitochondria/MODY3

Introduction

Maturity-onset diabetes of the young (MODY) is an early-onset form of non-insulin-dependent diabetes mellitus, which is characterized by autosomal dominant inheritance. MODY phenotypes have now been linked to mutations in three genes, namely hepatocyte nuclear factor-4α (HNF-4α) on chromosome 20q for MODY1 (Yamagata et al., 1996a), glucokinase on chromosome 7p for MODY2 (Frooguel et al., 1993) and HNF-1α on chromosome 12q for MODY3 (Yamagata et al., 1996b). More recently, a MODY4 pedigree has been associated with mutations in a pancreatic homeodomain transcription factor PDX-1 (IPF-1, IDX-1, STF-1), which regulates the development of pancreas and the expression of insulin and other β-cell-specific genes (Stoffers et al., 1997). The transcription factor HNF-1α is known to be expressed in liver, kidney and intestine, and in the β-cells of the endocrine pancreas (Frain et al., 1989; Emens et al., 1992; Miquerol et al., 1994). The phenotypic analysis of MODY3 pedigrees, which revealed hyperglycaemia resulting from severe defects in glucose-stimulated insulin secretion, suggest that β-cell dysfunction plays an important pathophysiological role in the development of MODY3 (Byrne et al., 1996; Lehto et al., 1997).

The HNF-1α gene is comprised of 10 exons that span ~23 kb and codes for a 628-amino acid protein that is composed of three functional domains: a short N-terminal dimerization domain encoded by exon 1; a homeobox DNA-binding domain encoded by exons 2, 3 and 4; and a C-terminal transactivation domain encoded by exons 5–10 (Frain et al., 1989; Nicosia et al., 1990; Kaisaki et al., 1997). Mutations have been reported in MODY3 pedigrees affecting the dimerization domain, the DNA-binding domain or the transactivation domain (Yamagata et al., 1996b; Glucksmann et al., 1997). Mutations in any one of these three domains could lead to diminished amounts of functional HNF-1α by either haploinsufficiency or a dominant-negative mechanism. HNF-1α regulates the expression of many liver-specific genes by direct binding to their promoter and/or enhancer regions (Nicosia et al., 1990; Yanuka-Kashles et al., 1994). Some of these genes such as glucose transporter 2 (GLUT-2) and L-type pyruvate kinase (L-PK) are also expressed in pancreatic β-cells (Emens et al., 1992; Miquerol et al., 1994). In addition, a consensus binding site for HNF-1α has been identified in the promoter of the rat insulin I gene, and HNF-1α has been proposed to regulate the expression of the rat insulin I gene in concert with other transcription factors (Emens et al., 1992).

To explore the molecular basis by which HNF-1α deficiency causes β-cell dysfunction, we established the β-cell-derived insulinoma cell lines capable of overexpressing HNF-1α and its dominant-negative mutant, termed DNHNF-1α, under the tight control of a doxycycline-dependent transcriptional activator. DNHNF-1α, also referred to as SM6 by Nicosia et al. (1990), represents a substitution mutation of 83 amino acids in the HNF-1α DNA-binding domain and forms non-functional heterodimers with wild-type HNF-1α. The expression of HNF-1α, as well as DNHNF-1α, could be maximally induced to a level >100-fold that of endogenous HNF-1α, and the induction level could be pin-pointed within precise brackets by particular doxycycline doses and time intervals. We demonstrate that the levels of GLUT-2 and L-PK mRNA are rapidly reduced following the induction of DNHNF-1α. Maximal induction of DNHNF-1α resulted in a dramatic decrease in HNF-1α binding to double-
stranded oligonucleotides corresponding respectively to the FLAT element of the rat insulin-1 enhancer (Emens et al., 1992) and to the L1 element of the L-PK promoter (Miquerol et al., 1994). This dominant-negative suppression of HNF-1α function led to a pronounced reduction in the expression of insulin mRNA and the activity of the rat insulin-1 promoter. Remarkably, insulin biosynthesis and glucose- or leucine-stimulated insulin secretion are diminished in INS-1 cells overexpressing DNHNF-1α. The suppression of HNF-1α function in INS-1 cells also inhibited glucose and leucine metabolism, which resulted in reduced cellular ATP generation as well as decreased membrane potential depolarization and intracellular Ca2+ ([Ca2+]i) rises. These findings suggest that metabolism–secretion coupling is defective in β-cells deficient in HNF-1α function and elucidate the molecular basis of the impairment of insulin secretion in MODY3 patients.

Results

Establishment of insulinoma cells overexpressing HNF-1α and DNHNF-1α using an inducible system

The INS-1-derived INS-r3 cell line (Wang and Iynedjian, 1997), which carries the reverse tetracycline/doxycycline-dependent transactivator (Gossen et al., 1995) was secondarily transfected with plasmids encoding either the wild-type HNF-1α or its dominant-negative mutant DNHNF-1α driven by a minimal cytomegalovirus pro-
doses and time intervals. DNHNF-1α (SM6; Nicosia et al., 1990) represents a long-substitution mutation of HNF-1α, in which an essential part of the DNA-binding domain (amino acids 73–155) was replaced. Consequently, the mutant protein DNHNF-1α migrates slightly faster than the glycosylated wild-type protein (Lichtsteiner and Schibler, 1989). The mutant retains an intact N-terminal dimerization domain and C-terminal activation domain, as supported by reactions with antibodies against both N- and C-terminal sequences of HNF-1α (Figure 1B). DNHNF-1α is expected to heterodimerize with endogenous HNF-1α and to prevent its DNA binding.

**DNHNHF-1α prevents the binding of endogenous HNF-1α to its recognition site in the promoters of the rat insulin I and L-PK genes**

The high level of induction of DNHNF-1α by doxycycline may overcome the stabilization that occurs when the native transcription factor or factors (De Simone et al., 1991; Rey-Campos et al., 1991) bind(s) to DNA, thereby acting in a dominant-negative manner. We therefore used gel shift assays to analyze HNF-1α binding activity in nuclear extracts from HNF-1α15 and DNHNF-1α31 cells, cultured for 48 h in the presence or absence of doxycycline. The rat insulin I FLAT element (Emens et al., 1990) represents a long-substitution mutation of HNF-1α, which is involved in glucose-regulated gene expression. HNF-1α15 and DNHNF-1α31 cells were cultured with or without indicated doses of doxycycline for 14 or 48 h with 2.5 mM glucose, followed by an additional 8 h in culture medium with 2.5, 6, 12 and 24 mM glucose. One of two independent experiments is presented in Figure 3A and B. GLUT-2 transcripts which share strikingly similar expression patterns with L-PK mRNAs (Antoine et al., 1997) were responsive to glucose (Figure 3A). The glucose-stimulated expression of GLUT-2 and L-PK was not augmented in an additive manner by graded overexpression of HNF-1α. At low glucose (2.5 mM), however, when the glucose-responsive transcription factors were depressed, the expression of GLUT-2 and L-PK was enhanced by overexpression of HNF-1α. In comparison, the dominant-negative suppression of HNF-1α in DNHNF-1α31 cells resulted in reduced gene expression of GLUT-2 and L-PK (Figure 3B). At high glucose concentration (24 mM), on the other hand, the impact of repressed HNF-1α function on GLUT-2 and L-PK expression could be overcome by glucose-induced or -activated transcription (Figure 3B).

In conclusion, HNF-1α does not seem to mediate the glucose-responsiveness of these genes, but it plays an important part in the regulation of GLUT-2 and L-PK transcription in combination with other transcription factors. As reported (Marie et al., 1993), the β-cell-specific glucokinase gene (β-GK) was not responsive to the rise of glucose concentration in this β-cell-derived INS-1 cell line (Figure 3A and B). The mRNA levels of β-GK were not affected by overexpression of HNF-1α or DNHNF-1α. Note that even a slight increase in β-GK mRNA was seen in DNHNF-1α31 cells after 48 h induction (Figure 3B). This change was not specific as it also occurred in the expression of β-actin and of Beta-2/NeuroD, a transcription factor implicated in insulin gene transcription (Naya et al., 1997) (Figure 3B). Therefore, it can be concluded that the expression of β-GK is not regulated directly by HNF-1α, which is in agreement with previous analysis of the β-GK promoter (Shelton et al., 1992; Jetton et al., 1994).

The expression of the insulin gene in DNHNF-1α31 cells was decreased by >80% after dominant-negative suppression of HNF-1α for 48 h (Figure 3B). Recently, we have also established a similar cell line expressing a MODY3 mutant Pro291insC (Glucksmann et al., 1997) which has been proven to act in a dominant-negative manner (Yamagata et al., 1998). We found that overexpression of human mutant Pro291insC in INS-1 cells has similar consequences on gene expression of GLUT2, L-PK and insulin as the induction of DNHNF-1α (unpublished
Fig. 2. DNHNF-1α acts as a dominant-negative mutant by preventing the endogenous HNF-1α binding to DNA. (A) Gel shift assay with the oligonucleotide probes corresponding to the rat insulin I FLAT element and the rat L-PK L1 element, respectively. Eight micrograms of crude nuclear extracts from non-induced HNF-1α #15 and DNHNF-1α #31 cells and doxycycline-induced DNHNF-1α #31 cells were used in the binding reactions. To balance the signal, 2 µg of nuclear extract from doxycycline-induced HNF-1α #15 cells was used. 1 µl of undiluted antibody raised against the C terminus of HNF1-α was added to the reaction for supershift experiments. (B) Competition with unlabelled oligonucleotide probes. Formation of the HNF1-α binding complex was specifically competed for by an excess of cold probes (50-, 100- and 200-fold molar excess, respectively, from left to right). The retarded HNF1-α complex (marked by arrow), but not PDX-1 complex, formed by the 32P-labelled insulin probe was eliminated by an excess of the cold L-PK probe. The identity of the PDX-1 complex (see arrow) was confirmed by supershifting with anti-PDX-1. (C) Western blot analysis and quantification of the nuclear extracts. 20 µg of nuclear extract was loaded on each lane. The amount of ubiquitous TFIIE-α protein present in the nuclear extracts, as revealed by blotting with anti-TFIIE-α (top panel, indicated by arrow), was used to quantify the proteins in the nuclear extracts.

data). The mRNA level of PDX1, another transactivator regulating insulin gene transcription, was reduced by 14 h induction of DNHNF-1α, but recovered after 48 h (Figure 3B). As the expression patterns of the transcription factors PDX1 and Beta-2 did not match those of insulin (Figure 3B), they do not appear to be involved in the suppression of the insulin gene. The level of insulin transcripts was not altered in HNF-1α #15 cells by overexpression of wild-type HNF-1α protein (Figure 3A).

To confirm that HNF-1α regulates insulin gene transcription, we transiently transfected DNHNF-1α #31 cells with plasmid –410INSLuc encoding a luciferase reporter gene driven by the rat insulin I promoter containing the HNF-1α-binding sequence (German et al., 1992a; Sander and German, 1997). As shown in Figure 3C, the rat insulin I promoter activity was reduced by 5-fold after induction of the dominant-negative mutant for 48 h. In contrast, the maximal induction of wild-type HNF-1α led to a slight (1.7-fold) increase in the luciferase reporter enzyme activity in HNF-1α #15 cells transiently transfected with plasmid –410INSLuc.

In order to conclude that HNF-1α has a direct effect
on the rat insulin I promoter activity, HNF-1α15 and DNHNF-1α31 cells were transiently transfected with three luciferase constructs containing multimerized segments of the rat insulin I minihenhancer: wild-type (Far-FLA T<sub>wt</sub>), HNF-1α binding site-mutated (Far-FLATMF) and E-element-mutated (Far-FLATME) (German et al., 1992a,b). The rat insulin I promoter contains at least two regulatory elements, the Far element (−239 to −228) and the FLAT element (−222 to −208), which function synergistically to confer both tissue-specificity and glucose-responsiveness of the promoter (German et al., 1992a). The FLAT element itself is composed of two functionally distinct elements: a positive locus, FLAT-F that predominantly binds HNF-1α (Emens et al., 1992);
and the adjacent negative locus, FLAT-E that predominantly binds other homeodomain proteins (German et al., 1992a). As shown in Figure 3D, the induction of HNF-1α enhanced the luciferase activity by >100-fold in the HFN-1α515 cells transfected with Far-FLATwt plasmid. The HNF-1α activation requires an intact FLAT-F site since minienhancer with F element mutated (Far-FLATMF) is not activated by induction of HNF-1α (Figure 3D). It has been demonstrated that the very same mutation of this F element also abolished the HNF-1α binding activity (Emens et al., 1992). In contrast, the HNF-1α activation was well preserved in doxycycline-induced HFN-1α515 cells transfected with Far-FLATME that contains the E element mutation (Figure 3D). The basal luciferase activity was significantly higher in non-induced HFN-1α515 cells transfected with Far-FLATME than those transfected with Far-FLATwt or Far-FLATMF, which is in agreement with previous observations (German et al., 1992b). Therefore, HNF-1α is indeed capable of mediating transactivation of the rat insulin I promoter through the FLAT-F site, as proposed by Emens et al. (1992). The transient transfection experiments performed in DNHNHF-1α531 cells further supported the role of HNF-1α in the regulation of the rat insulin minienhancer. Induction of DNHNHF-1α resulted in 72.3 ± 25.2% and 90.3 ± 3% (n = 3) decreases of luciferase activity in DNHNHF-1α531 cells transfected with Far-FLATwt and Far-FLATME respectively, but had no inhibitory effects on the minienhancer with the HNF-1α site mutated (Far-FLATMF).

**Insulin secretory defect induced by loss of HNF-1α function**

Mutations in HNF-1α, which are linked to the MODY3 subtype, lead to hyperglycaemia because of severe derangement of glucose-stimulated insulin secretion from the pancreatic β-cells. We investigated the impact of controlled overexpression and dominant-negative suppression of HNF-1α on insulin secretory responses to glucose, leucine, and K+. These secretagogues were selected for their action at different and specific levels of the signal transduction cascade: glucose at the primary step, leucine at the mitochondria downstream of glycolysis, and K+ at the late depolarization event. Cells were cultured with or without doxycycline for either 14 h (Figure 4A and B) or 48 h (Figure 4C and D) in medium containing 2.5 mM glucose. Thereafter, insulin secretion was measured over a period of 30 min with indicated stimulators. Non-induced cells exhibited a typical insulin secretory response to 20 mM K+, which does not require generation of ATP and other metabolic coupling factors in contrast to the nutrient stimuli (Wollheim et al., 1996), was also decreased—albeit to a lesser extent when compared with the effects of glucose and leucine (Figure 4D). When secretion is related to insulin content rather than to DNA content, the leucine-stimulated insulin secretion fell from 3.7-fold in non-induced cells to 1.6-fold in induced cells, while the K+ stimulatory action was reduced from 2.6- to 1.9-fold under the same conditions. There is thus a marked inhibition of insulin secretion in response to glucose and leucine, which is not merely a reflection of the reduction of insulin content, but suggests impaired nutrient metabolism.

**Impaired glucose and leucine metabolism led to reduced ATP production in INS-1 cells deficient in HNF-1α function**

Glucose-stimulated insulin secretion is controlled by glucose metabolism comprising glycolysis and mitochondrial oxidation (Matschinsky, 1996). The glycolytic flux was investigated by following the production of [3H]water from D-[5-3H]glucose and the rate of oxidation was

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**Fig. 3.** HNF-1α is required for the gene expression of GLUT-2, L-PK and insulin. Northern blot analysis of gene expression in (A) HNF-1α515 and (B) DNHNHF-1α531 cells induced with indicated doses of doxycycline and cultured at given concentrations of glucose. Cells were cultured with indicated doses of doxycycline at 2.5 mM glucose for 14 or 48 h. Culture was continued for 8 h at 2.5 mM glucose or at the indicated glucose concentrations before cells were extracted for total RNA. RNA samples were analysed by hybridizing with the indicated cDNA probes. (C) Effect of HNF-1α dysfunction on rat insulin I promoter activity. DNHNHF-1α531 cells were transiently transfected with plasmid −410INSLuc by calcium phosphate–DNA co-precipitation. After 48 h culture with or without 500 ng/ml doxycycline, cells were collected, and 20 µg cytosolic protein was assayed for luciferase activity. Luciferase activity measured in non-induced cells transfected with plasmid Far-FLATwt was arbitrarily set at 1.0. Data are the mean ± SEM of six independent experiments. HNF-1α515 cells, which were subjected to the same procedures, served as controls to eliminate the possibility that the luciferase activity was affected non-specifically by doxycycline treatment. (D) HNF-1α activates the insulin promoter through the minienhancer FLAT-F element. HNF-1α515 cells were transiently transfected with three luciferase constructs containing multimerized segments of the insulin minienhancer: wild-type (Far-FLATwt), HNF-1α binding site-mutated (Far-FLATMF) and E-element-mutated (Far-FLATME). After 48 h culture with or without 500 ng/ml doxycycline, cells were collected, and 20 µg cytosolic protein was assayed for luciferase activity. Luciferase activity measured in non-induced cells transfected with plasmid Far-FLATwt was arbitrarily set at 1.0. Data are the mean ± SEM of three independent experiments.
Islet β-cell dysfunction caused by HNF-1α deficiency

Fig. 4. Effect of defective HNF-1α function on insulin secretory responses to stimulators. Cells were cultured at 2.5 mM glucose in the presence or absence of specified doses of doxycycline for 14 or 48 h as indicated. Cells were then incubated in KRBH with specified stimulators for 30 min. Insulin released in KRBH was quantified by radioimmunoassay and normalized by cellular DNA content [measured as described by Wang and Iynedjian (1997)]. (A) Glucose-stimulated insulin secretion in HNF-1α#15 cells induced with indicated doses of doxycycline for 14 h. Data represent the mean ± SEM of six separate experiments. Statistical significance between doxycycline-induced and non-induced cells was obtained at 2.5 and 6 mM glucose (P < 0.01, all data in the present study were analysed by unpaired Student's t-test). (B) Glucose-stimulated insulin secretion in DNHNF-1α#31 cells induced with 500 ng/ml doxycycline for 14 h. Data show the mean ± SEM of six independent experiments. Statistical significance between doxycycline-induced and non-induced cells was observed at 12 and 24 mM glucose (P < 0.01). (C) Glucose-stimulated insulin secretion in DNHNF-1α#31 cells induced with 500 ng/ml doxycycline for 48 h. Data are the mean ± SEM of six separate experiments. (D) Leucine- and KCl-stimulated insulin secretion in DNHNF-1α#31 cells induced with 500 ng/ml doxycycline for 48 h. Insulin secretion was measured during 30 min incubation with 20 mM leucine or 20 mM KCl in KRBH containing 2.5 mM glucose. Data represent the mean ± SEM of three independent experiments.

estimated from the conversion of D-[14C(U)]glucose and L-[1-14C]leucine to 14CO2. Cells of the HNF-1α#15 and DNHNF-1α#31 lines were cultured with or without 500 ng/ml doxycycline for 48 h in medium containing 2.5 mM glucose. The rate of glycolysis was measured over a time period of 30 min, at extracellular glucose concentrations of 2.5, 6, 12 and 24 mM. Under non-induced conditions, the glycolytic rate in both HNF-1α#15 and DNHNF-1α#31 cells increased as expected over the range of glucose concentrations (Figure 5A and B). Overexpression of HNF-1α had no significant impact on the glycolytic flux (Figure 5A). However, at 12 and 24 mM glucose concentrations, the dominant-negative suppression of HNF-1α resulted in a 40% reduction (P < 0.001, n = 6) in the rate of glycolysis (Figure 5B).

Glucose and leucine oxidation was then investigated in DNHNF-1α#31 cells. After the 48 h induction by doxycycline (500 ng/ml), formation of 14CO2 from 12.8 mM D-[14C(U)]glucose was found to be decreased by 45% (P < 0.02, n = 8) in cells overexpressing DNHNF-1α compared with non-induced cells (Figure 5C). As seen in Figure 5C, when L-[1-14C]leucine (20 mM) was provided as the 14C donor, this inhibition reached 74% (P < 0.01, n = 8), indicating that the mitochondrial metabolism was predominantly impaired.

This contention was borne out by measurements of cellular ATP levels (Figure 5D). In non-induced cells, both glucose and leucine caused 2-fold increases of ATP after 8 min incubation, whereas KCl had no effect. By contrast, the generation of ATP by glucose and leucine was completely abolished following suppression of HNF-1α function. It is noteworthy that the basal ATP levels at 2.5 mM glucose remain unaltered. These results further defined defective mitochondrial metabolism as the primary consequence of the loss of HNF-1α function.

Changes in plasma membrane potential and [Ca2+]i after suppression of HNF-1α function

To characterize further the mechanism underlying the impairment of insulin secretion, the effects of glucose and leucine on plasma membrane potential and [Ca2+]i were monitored in DNHNF-1α#31 cells. DNHNF-1α was induced by doxycycline (500 ng/ml) for 48 h prior to the spinner culture period for measurements on cell suspensions. The addition of 10 mM glucose (to yield a final concentration of 12.5 mM) evoked a clear depolariz-
Fig. 5. Glycolytic flux, mitochondrial oxidation and ATP levels after disruption of HNF-1α function. Cells were cultured with or without 500 ng/ml doxycycline at 2.5 mM glucose for 48 h before experiments. DNA content was used to normalize cell number variations between wells. The glycolytic flux in (A) HNF-1α#15 and (B) DNHNF-1α#31 cells was measured during 30 min incubation with indicated concentrations of glucose and a constant specific activity of tracer glucose. Data are mean ± SEM from six independent experiments. (C) Glucose and leucine oxidation: 14CO₂ formation during 1 h incubation with 12.8 mM D-[14C(U)]glucose or 20 mM L-[1-14C]leucine were both significantly inhibited by DNHNF-1α. (D) Mitochondrial ATP production measured for 8 min in DNHNF-1α#31 cells. Data are mean ± SEM from six separate experiments.

Discussion

Establishment of a cellular model for MODY3

β-cell dysfunction appears to be the primary cause of the diabetic syndrome in MODY3 patients. To investigate the consequences of impaired HNF-1α function, we have established β-cell-derived insulin-secreting cell lines, in which HNF-1α function could be suppressed in a controlled and dominant-negative manner. Many mutations in HNF-1α have been reported in MODY3 patients, and most of these are believed to act as dominant-negative mutants by forming non-functional heterodimers with the product of the normal HNF-1α allele. In our INS-1-derived stable cell line, DNHNF-1α, which is mutated in the DNA-binding domain, could be induced to extremely high levels capable of eliminating the function of endogenous HNF-1α through competitive dimerization. The dominant-negative effect was verified by the reduction of HNF-1α binding to its recognition sequences in the insulin enhancer and the L-PK promoter. Under non-induced conditions, DNHNF-1α is not detectable and the expression of HNF-1α represents the endogenous level of parental INS-1 cells and pancreatic islets. During the 48 h induction period, the cellular morphology and replication (DNA content) were unaltered (data not shown).

HNF-1β, which is closely related to HNF-1α and is able to heterodimerize with HNF-1α, overlaps the expression pattern of HNF-1α (De Simone et al., 1991; Rey-Campos et al., 1991; Pontoglio et al., 1996). Both proteins transactivate the targeted gene by forming a homodimer or heterodimer and binding to the same recognition sequences. Most HNF-1α mutant proteins identified in MODY3 pedigrees would result in loss of function of both HNF-1α and HNF-1β by a dominant-
Islet β-cell dysfunction caused by HNF-1α deficiency

Fig. 6. Plasma membrane potential and [Ca^{2+}]_{i} monitored in DNHNF-1α#31 cells. Bisoxonol fluorescence was used to measure plasma membrane potential. Glucose-induced membrane depolarization was inhibited in DNHNF-1α#31 (+Dox) cells compared with control cells (–Dox) (A). Similar effects were observed using l-leucine as nutrient stimulus (B). Rises in [Ca^{2+}]_{i} in response to glucose (C) or l-leucine (D) were also attenuated in DNHNF-1α cells and are analysed further in Table I.

Table I. Effects of glucose, leucine and KCl on [Ca^{2+}]_{i} (nM) in DNHNF-1α#31 cells

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<th>Basal</th>
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<td>95 ± 4</td>
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<td>+Dox</td>
<td>94 ± 5</td>
<td>119 ± 15</td>
<td>25 ± 5</td>
<td>136 ± 9</td>
<td>42 ± 4</td>
<td>303 ± 52</td>
<td>209 ± 14</td>
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DNHNF-1α was induced by doxycycline for 48 h prior to the spinner culture and Fura-2 AM loading. [Ca^{2+}]_{i} was then measured: just before the addition of substrates (basal); 5 min after the addition of 10 mM glucose (12.5 mM final) or 20 mM l-leucine; at the highest value after the addition of 20 mM KCl (KCl-peak); and 5 min after KCl (KCl-plateau). Values are mean ± SEM (see Figure 6C and D for representative traces).

strictly speaking, neither HNF-1α knockout mice nor cell lines overexpressing dominant-negative mutations reflect the 20-year period during which the MODY3 patients develop the diabetic syndrome. It must be assumed that moderate expression of dominant-negative mutants of HNF-1α will require long time periods before impact on β-cell function becomes apparent. Therefore, we chose high levels of overexpression of the dominant-negative DNHNF-1α to achieve rapid effects. However, the dominant-negative action is not the only mechanism leading to MODY3. Mutations in the promoter region (Gragnoli et al., 1997) and dimerization domain (Glucksmann et al., 1997) of HNF-1α, which result in reduced HNF-1α gene dosage, have also been associated with the MODY3 phenotype.

HNF-1α is necessary for insulin gene transcription

The nucleotide sequence of the insulin gene, as well as the deduced amino acid sequences, have been remarkably
conserved throughout evolution. Most species carry a single copy of the insulin gene, except the rat and mouse which functionally express two non-allelic insulin genes, termed insulin I and II. Evolutionarily conserved promoter sequences control the expression of the insulin gene that represents one of the hallmarks of β-cell differentiation in the developing pancreas. The exact length of the 5′-flanking region sufficient for basal and regulated transcriptional activity remains unclear, but sequences at least 4 kb upstream are proposed to regulate transcription (Fromont-Racine et al., 1990). The best-defined 400 bp of the insulin proximal promoter, which confers β-cell-specific expression, is composed of multiple cis-acting DNA elements interacting with distinct classes of transcription factors including PDX1, BETA2 and HNF-1α (reviewed by Sander and German, 1997).

Emens et al. (1992) reported that expression of HNF-1α cDNA in COS cells resulted in transactivation of a chloramphenicol acetyltransferase (CAT) reporter gene driven by the thymidine kinase promoter placed under the control of multimerized HNF-1α binding segments of the rat insulin I enhancer, and suggested that HNF-1α may be involved in cell-specific and physiological regulation of insulin gene transcription. A recent report by Lee et al. (1998) showed that the amount of immunohistochemically stained insulin peptide was significantly reduced in the pancreas of HNF-1α knockout mice. MODY3 patients exhibit diminished plasma insulin concentrations both under basal and glucose-stimulated conditions, and often require insulin replacement therapy (Byrne et al., 1996; Lehto et al., 1997). This suggests reduced expression of insulin in patients carrying a mutant allele of HNF-1α. To our knowledge, the present study provides the first demonstration at the molecular level that HNF-1α is essential for insulin gene transcription. There was a drastic reduction in HNF-1α binding to its recognition site in the rat insulin minienhancer when DNHNHF-1α was induced. Mutation of FLAT-F has been shown to eliminate the HNF-1α DNA-binding activity (Emens et al., 1992) and the present study clearly demonstrates that the same mutation also abolished its transactivation of luciferase reporter constructs containing multimerized segments of the rat insulin I minienhancer (Figure 3D). Furthermore, the dominant-negative suppression of HNF-1α function resulted in >80% decreases in both the expression of endogenous insulin mRNA and the activity of rat insulin I promoter. The marked time-dependent decrease in cellular insulin content is an expected consequence of the reduced insulin gene transcription. A 40% reduction in pancreatic insulin content (adjusted for pancreas weight) has also been reported by Pontoglio et al. (1998) in the HNF-1α knockout mice, which agrees with immunofluorescence studies on another transgenic mouse model (Lee et al., 1998). However, the unaltered insulin mRNA levels observed by Pontoglio et al. (1998) remain intriguing. In contrast to the altered insulin secretion and [Ca2+]i rises which were measured in islets of 2-month-old mice, Northern blotting of insulin was performed on 2-week-old animals. Nevertheless, the decreased insulin content does not explain the impaired insulin secretory response to nutrient stimuli in cells deficient in HNF-1α function.

HNF-1α regulates expression of genes involved in glucose transport and metabolism

Glucose is the predominant regulator of insulin secretion (Newgard and McGarry, 1995; Matschinsky, 1996; Prentki, 1996; Wollheim et al., 1996) and biosynthesis. In the latter case, the action on translation is acute, while it is more chronic on transcription and mRNA stability (Dumonteil and Philippe, 1996). All these effects require glucose metabolism via glycolysis; especially in the case of secretion, mitochondrial oxidation is of crucial importance (Prentki, 1996; Maechler et al., 1997). In β-cells and in INS-1 cells, expression of the high-capacity and low-affinity glucose transporter GLUT-2 and the high Km glucose-phosphorylating enzyme, glucokinase, are considered necessary for normal glucose sensing, although glucokinase rather than GLUT-2 constitutes the rate-limiting step for glycolytic flux (Sekine et al., 1994; Newgard and McGarry, 1995; Matschinsky, 1996; Wang and Lyndenjian, 1997). Furthermore, knockout of GLUT-2 only impairs the acute phase of glucose-stimulated insulin release (Guillam et al., 1997). It is known that the GLUT-2 promoter contains a putative HNF-1α-binding site (Emens et al., 1992), but the role of HNF-1α in the regulation of GLUT-2 gene expression has not been proven by experimental evidence until now. We demonstrate that defective HNF-1α function results in diminished expression of GLUT-2 transcripts. The reduced expression of GLUT-2 as shown by Northern blotting and by Western blotting, ~40% decrease; data not shown) may contribute in part to the diminished glycolysis, but is not sufficient to explain the impaired glucose metabolism. This conclusion is supported by the even more pronounced inhibition of leucine oxidation compared with glucose oxidation observed in DNNHF-1α-overexpressing cells. Leucine is a physiological stimulator of insulin secretion, which acts downstream of glycolysis and depends exclusively on mitochondrial oxidation for its signal generation (Prentki, 1996). The reduction of glycolytic flux following suppression of HNF-1α function is probably secondary to diminished mitochondrial oxidation, as it is well established that there is a tight coupling between aerobic and non-aerobic glucose metabolism in the β-cell (Sekine et al., 1994 and references therein).

We found that the expression of L-PK, which like GLUT-2 is a glucose-responsive gene (Marie et al., 1993; Antoine et al., 1997), is also regulated by HNF-1α. However, the activity of this enzyme is not flux-determining for glycolysis in the β-cell (MacDonald, 1995). In contrast, the glucose-sensing enzyme, glucokinase, is not affected by manipulations of HNF-1α function. Thus, alterations in glucokinase activity are unlikely to contribute to the severe impairment of insulin secretion in MODY3 patients (Byrne et al., 1994) in contrast to its involvement in the mild secretory defect in MODY2 patients (Froguel et al., 1993). The HNF-1α deficiency may also lead to defective liver glucose metabolism, since HNF-1α is known to regulate many genes important for liver function, such as GLUT2, phosphoenolpyruvate carboxykinase (Yanuka-Kashles et al., 1994). L-PK and insulin-like growth factor I (IGF-I; Lee et al., 1998). Gene knockout studies have shown that HNF-1α-deficient mice develop liver dysfunction (Pontoglio et al., 1996).
Defective mitochondrial function as an explanation for impaired nutrient-stimulated insulin secretion in β-cells deficient in HNF-1α function

Mitochondrial substrate oxidation in the β-cell plays a critical role in the generation of factors coupling glucose and leucine metabolism to insulin secretion (Prentki, 1996). This is most clearly demonstrated in patients with mutations in the mitochondrial genome who often suffer from a severe, maternally inherited diabetes syndrome characterized by impaired insulin secretion and neurosensory deafness (Maassen and Kadowaki, 1996). The mitochondrial DNA encodes 13 polypeptides which are subunits of the respiratory chain complexes, the majority of the subunits being encoded by nuclear DNA (Wallace et al., 1995). Decreased cytochrome c oxidase activity has been demonstrated in islets in such a diabetic patient post-mortem (Kobayashi et al., 1997). Elimination of mitochondrial DNA in cell culture has been shown to abolish completely glucose-stimulated insulin secretion (Soejima et al., 1996; Kennedy et al., 1998), whereas K⁺ depolarization, which also increases [Ca²⁺], continued to promote insulin secretion (Kennedy et al., 1998). Thus, functional mitochrondria are required for signal generation implicated in glucose-induced insulin secretion.

Suppression of HNF-1α function leads to profound inhibition of glucose- and leucine-induced insulin secretion. The underlying mechanism is revealed by the strong reduction of mitochondrial oxidation of these two nutrients. This is further emphasized by the abrogation of ATP production, which for glucose occurs predominantly and for leucine exclusively in the mitochondria. Mitochondrial ATP generation leads to membrane depolarization and voltage-dependent Ca²⁺ influx. Increased [Ca²⁺] triggers exocytosis of insulin (Wollheim et al., 1996). Mitochondrial metabolism also generates other coupling factors in a [Ca²⁺]-dependent manner (Maechler et al., 1997). Therefore, the inhibition of the membrane depolarization and [Ca²⁺] rises evoked by glucose and leucine suggests a primary defect in the production of ATP and other metabolic coupling factors by mitochondria. L-type Ca²⁺ channel function and the downstream process of insulin exocytosis (Wollheim et al., 1996) are not affected by loss of HNF-1α function, as K⁺-induced [Ca²⁺] rises and insulin secretion were largely preserved. In agreement with our results, Pontoglio et al. (1998) have recently reported that glucose-induced but not K⁺-evoked [Ca²⁺] increase was diminished in HNF-1α-deficient mice. In addition, insulin secretory responses to glucose and arginine in the perfused pancreas and perfused islets from these HNF-1α-null mice have also been shown to be dramatically reduced (Pontoglio et al., 1998).

In conclusion, the present findings demonstrate that suppression of HNF-1α activity has multiple and severe consequences for the β-cell. HNF-1α function is essential for insulin gene transcription and maintenance of cellular insulin content. Metabolism–secretion coupling is defective because of pronounced reduction of mitochondrial oxidation and ATP generation in response to the nutrient stimuli. These results propose a molecular basis for the impaired insulin secretion characteristic of the diabetic phenotype of MODY3 patients. The present study establishes a cell model for further exploration of the role of HNF-1α in transcription of the specific genes that are involved in mitochondrial oxidation and insulin secretion.

Materials and methods

Establishment of stable cell lines

The INS-1 cell line-derived clones were cultured in RPMI 1640 (11 mM glucose, unless indicated otherwise) supplemented with 10% fetal calf serum and other additions as given previously (Asfari et al., 1992). Stable transfection was performed using the calcium phosphate–DNA co-precipitation method followed by glycerol shock (Kingston et al., 1990). The establishment of the first-step stable clone INS-13 expressing the reverse tetracycline-dependent transactivator was described in detail previously (Wang and Iynedjian, 1997). The plasmids used in the second round of stable transfection were constructed by inserting either wild-type HNF-1α or DNHNF-1α cDNA (the latter corresponding to SM6; Nicosia et al., 1990; both kindly supplied by Dr R.Cortese, Istituto Di Ricerche Di Bisoglia Molecolare, Pomezia, Italy) downstream of the tetracycline operator–cytomegalovirus minimal promoter in plasmid PUH10-3 (Gossen et al., 1995). Plasmid pTK-hygro (a kind gift from Dr N.Quintinell) encoding the second selection marker, hygromycin resistance cassette, was co-transfected for selection by culture in the presence of 200 µg/ml of hygromycin. Resistant clones were picked individually with cloning rings and maintained in long-term culture with 150 µg/ml G418 and 100 µg/ml hygromycin. Immunoblotting with antibodies against hygromycin, which are provided against HNF-1α (N or C terminus, kindly provided by Dr R.Cortese) was used for screening clones with high-level expression of HNF-1α or DNHNF-1α protein after 24 h of cell culture with 1000 ng/ml doxycycline.

Immunoblot analysis

Immunoblotting procedures were performed as described previously (Wang and Iynedjian, 1997) using enhanced chemiluminescence ( Pierce) for detection. Nuclear extract from HNF-1α or DNHNF-1α cells was fractionated on a 9% SDS–polyacrylamide gel, transferred to nitrocellulose by electroboblotting, incubated in a sequential order with a blocking solution, primary antibody (used at the following dilutions: anti-HNF-1α antibodies from Dr R.Cortese, 1:1000; anti-TFIE-α antibodies from Santa Cruz Biotechnology, 1:3000; anti-PDX1 antibody from Dr T.Edlund, 1:5000); and HRP-conjugated secondary antibody (Pierce, 1:1000). Nuclear extracts from the non-transfected parent INS-1 cells and primary islets were used to demonstrate the endogenous HNF-1α level.

Nuclear extract preparation and electrophoretic mobility-shift assay (EMSA)

Cells in 10-cm dishes were cultured in complete INS-1 medium with or without 500 ng/ml doxycycline for 48 h. Thereafter, nuclear extracts were prepared according to Schreiber et al. (1988).

EMSA was performed as previously described by Philippe (1995) using the following double-stranded oligonucleotides: rat L-PK L1 elements (Miquel et al., 1994) 5'-TACGCTTGATACCATTACCAGGACTCGATCCGACAAATATGAAAATGGCTCAG; Rat insulin I FLA T 5'-CCGGTGACACTATCTTGCCTGAG; Rat insulin I FLAT element (Emens et al., 1992) 5'-GATTTGATATTACGTATTGACACCTATTAGATATTAGGG. The double-stranded oligonucleotides were end-labelled with [α-32P]dCTP and the Klenow fragment of Escherichia coli DNA polymerase I and used as probes. Binding reactions were conducted for 15 min at room temperature with 2 or 8 ng nuclear extract, 105 c.p.m. probe, 1 µg BSA, 2 mM DTT, 50 mM KCl, 6.25% glycerol, 20 mM HEPES pH 7.9, 5 mM MgCl₂, 0.5 mM EDTA, 1 µg poly(dI.dC) and 1 µg denatured salmon sperm DNA in a final volume of 20 µl. In competition experiments, the unlabelled competitor DNA (50-, 100- and 200-fold molar excess) was added simultaneously with the 32P-end labelled oligonucleotide duplex probe. For supershift experiments, 1 µl of the polyclonal rabbit anti serum was added to the reaction mixture after 7 min, and then continued for a further 8 min. The samples were subjected to electrophoretic separation on a 4% non-denaturing polyacrylamide gel. The gel was then dried, and labelled DNA–protein complexes were located by autoradiography.

Total RNA extraction and Northern blot analysis

Total RNA was extracted from cells cultured in 10-cm dishes by the guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). Total RNA (20 µg) was denatured with glyoxal and dimethylsulphoxide and separated on 1% agarose gels as described (McMaster and Carmichael, 1977). Resolved RNA was blotted to nylon
membranes by vacuum transfer (VacuGene™ XL, Pharmacia, Uppsala, Sweden), followed by UV cross-linking. The membranes were prehybridized and then hybridized to 32P-labeled random primer cDNA probes according to standard protocols (Sambrook et al., 1989). cDNA fragments used as probes for L-PK, GLUT-2, glucokinase, insulin, PDX1, Beta-2 and HNF-1α mRNA detection were obtained from corresponding expression vectors kindly given by Drs A.Kahn, B.Thorens, P.B.Iynedjian, J.Philippe, T.Edlund, M.-J.Tsai and R.Cortese.

Transient transfection and luciferase reporter enzyme assay

Cells from DNHHF-luc31 or HNF-1α-luc15 lines at 60–70% confluence were harvested from T75 flasks by trypsinization and seeded into 6-well culture dishes at a density of 1.5×10^5 cells per well. After culturing in complete medium for 18 h, cells in each well were replenished with 1 ml fresh medium 2 h before transfection. Plasmids (400ng) were electroporated into 10^5 HeLa cells at a constant specific activity of 0.313 nCi/nmol.

Measurement of cellular insulin content and insulin secretion

Insulin content was determined after extraction with acid ethanol as described previously (Asfari et al., 1992). Insulin secretion was measured over a period of 30 min, in Krebs-Ringer-bicarbonate-HEPES buffer (KRBH, 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH2PO4, 0.5 mM MgSO4. 1.5 mM CaCl2, 2 mM NaHCO3; 10 mM HEPES, 0.1% BSA) containing indicated stimulators. Insulin was detected by radioimmunoassay using rat insulin as standard and an antibody for rat insulin detection purchased from LINCO (St Louis, MO, USA).

Measurement of glycolysis, mitochondrial oxidation and intracellular ATP

Glucose utilization was measured and calculated as reported previously (Wang and Iynedjian, 1997). In brief, the production of 3H-labelled [3H]glucose medium with or without 500 ng/ml doxycycline for 48 h. Cells were lyzed in 1 ml of cell extract with 100 µl of cell extract with 10 µl luciferin–luciferase reagent (B.Ritz-Laser for helpful advice on the gel shift assays. This work was supported by the Swiss National Science Foundation (No. 32-49755/96).

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References


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