Human Diabetes Associated with Defects in Nuclear Regulatory Proteins for the Insulin Receptor Gene

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Abstract

The control of gene transcription is mediated by sequence-specific DNA-binding proteins (trans-acting factors) that bind to upstream regulatory elements (cis elements). We have previously identified two DNA-binding proteins that specifically interact with two unique AT-rich sequences of the 5′ regulatory region of the insulin receptor gene which have in vivo promoter activity. Herein we have investigated the expression of these DNA-binding proteins in cells from two unrelated patients with insulin resistance and non–insulin-dependent diabetes mellitus. In these patients, the insulin receptor gene was normal. In EBV-transformed lymphoblasts from both patients, insulin receptor mRNA levels and insulin receptor expression were decreased. The expression of nuclear-binding proteins for the 5′ regulatory region of the insulin receptor gene was markedly reduced, and this defect paralleled the decrease in insulin receptor protein expression. These studies indicate that DNA-binding proteins to the regulatory region of the insulin receptor gene are important for expression of the insulin receptor. Further, they suggest that in affected individuals, defects in the expression of these proteins may cause decreased insulin receptor expression and insulin resistance. (J. Clin. Invest. 1996. 97:258–262.) Key words: insulin receptor • insulin resistance • non–insulin-dependent diabetes mellitus • gene transcription • trans-acting factors

Introduction

Resistance to insulin is an important feature of non–insulin-dependent diabetes mellitus (NIDDM) (1, 2). Recently, longitudinal studies of population with high prevalence of NIDDM indicate that insulin resistance antedates the onset of overt diabetes, and may be a predictive marker for this disease (3–6). However, in the majority of patients with NIDDM the cause of the insulin resistance is unknown (1–6). Insulin exerts its biological effects by interacting with its receptor which generates intracellular signals via a tyrosine kinase cascade (7–9). In many patients with NIDDM, insulin receptor expression is decreased (10). Studies of genetic syndromes of insulin resistance have been important for understanding the genetics of NIDDM (10–12). Although very rare, these syndromes show extremely severe insulin resistance and a simple mendelian pattern of inheritance (10). Thus, they represent models for investigating the mechanisms of insulin resistance in NIDDM.

Insulin resistant patients with reduced or absent insulin receptor expression in target cells have been reported (10). Many of these patients have point mutations or deletions in the coding sequence of the insulin receptor gene. However, receptor abnormalities due to defects in the generation of mRNA specific for the insulin receptor have been reported in patients with apparently normal insulin receptor genes, suggesting defects in gene regulation (13–15).

In mammalian cells, the initiation of mRNA synthesis is controlled by nuclear regulatory proteins (trans-acting factors) that modulate the transcription of genes and gene networks (16–18). During the last decade, unique DNA sequences that are involved in gene regulation (cis elements) have been identified, and this process has led to the detection and characterization of DNA regulatory proteins (18). To further understand the regulation of the insulin receptor, we and others have identified and analyzed the insulin receptor promoter region (19–29). This region extends over 1,800 bases 5′ upstream from the insulin receptor gene ATG codon. The insulin receptor promoter has no TATA or CAAT boxes, reflecting a feature common to the promoters of many constitutively expressed genes.

The insulin receptor is expressed at low levels in most cells and this expression appears to be driven in part by a series of GGGCGG repeats (in the region −400 to −650), which are putative binding sites for the mammalian transcription factor Sp1 (18). The insulin receptor is expressed at higher levels in differentiated target tissues such as muscle and fat (30). Recently, we have identified two nuclear-binding proteins (or a closely related family of proteins) that interact with two unique discrete regions of the insulin receptor gene promoter and increase during myocyte and adipocyte differentiation (29). DNase I footprinting localized nuclear binding to two unique
AT-rich regions of the insulin receptor promoter between nucleotides −782 to −800 and −1740 to −1775 (29). Reporter gene analysis of the DNA sequences that interacted with these proteins indicated that they had in vivo transcriptional activity and functioned primarily as promoter elements (29).

We report herein two patients with insulin resistance and NIDDM. In both, surface insulin receptors are decreased, and insulin receptor gene transcription is significantly impaired despite the fact that the insulin receptor genes are normal. In these patients we find that the expression of nuclear-binding proteins for the 5′ regulatory region of the insulin receptor gene is markedly reduced, suggesting that this defect may induce insulin resistance and NIDDM.

Methods

Patients. Two unrelated patients with insulin resistance and NIDDM were studied (Table I). Patient 1, an 11-yr-old Japanese boy, was diagnosed with NIDDM at 6 yr of age and had the male form of type A insulin resistance (13). In EBV-transformed lymphoblasts, 125I-labeled insulin binding to intact cells was decreased and levels of insulin receptor mRNA were very low, but the coding region of the insulin receptor gene had a normal nucleotide sequence (13).

Patient 2 was a nonobese 45-yr-old Italian man, with the usual features of NIDDM. He came to medical attention because of glycosuria on a routine urinalysis. At that time, he had fasting hyperglycemia (250 mg/dl) and hyperinsulinemia (34 μU/ml) that was not accompanied by other disorders such as acromegaly or glucocorticoid excess. He had been treated with sulfonylureas, but this drug was discontinued for two wk before this study. In EBV-transformed lymphoblasts, 125I-labeled insulin binding was reduced, and this reduction paralleled the decrease of the insulin receptor mRNA levels. Southern-blotting studies and cDNA analysis by direct sequencing of PCR-amplified cDNA (31, 32) revealed that both alleles of the insulin receptor gene were normal (unpublished data).

Cells. Cultured lymphoblast cell lines transformed with EBV were established according to standard techniques (33). Buffy-coat leukocytes from both patients were isolated from heparin-treated whole blood after the sedimentation of erythrocytes in the presence of one-fifth vol of 6% dextran 70 (Macrodex, Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Control EBV-transformed lymphoblasts from five nondiabetic individuals were obtained from normal volunteers and from American Type Tissue Culture, Rockville, MD. All lymphoblastoid cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS (13) in humidified atmosphere containing 5% CO2 at 37°C. Growth rates were similar in all cells.

Table I. Clinical Characteristics of Patients

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Patient 2</th>
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<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>11</td>
</tr>
<tr>
<td>Body-mass index</td>
<td>ND</td>
</tr>
<tr>
<td>Age at diagnosis (yr)</td>
<td>6</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td></td>
</tr>
<tr>
<td>fasting</td>
<td>100</td>
</tr>
<tr>
<td>2 h after 75 g glucose</td>
<td>310</td>
</tr>
<tr>
<td>Plasma insulin (μU/ml)</td>
<td></td>
</tr>
<tr>
<td>fasting</td>
<td>300</td>
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<tr>
<td>2 h after 75 g glucose</td>
<td>1845</td>
</tr>
<tr>
<td>Acanthosis nigricans</td>
<td>Yes</td>
</tr>
</tbody>
</table>

ND, not determined.

Insulin receptor mRNA and 125I-labeled insulin binding. Total cellular RNA was prepared with use of a guanidinium thiocyanate–cesium chloride technique (34), and insulin receptor mRNA abundance was determined in EBV-transformed lymphoblasts from both diabetic patients and all five nondiabetic control individuals using an RNase A protection assay as described (13, 31). Previously published procedures were used to measure the binding of 125I-labeled insulin to intact EBV-transformed lymphoblasts (33).

Nuclear protein extraction. Nuclear extracts were prepared from EBV-transformed lymphoblasts of both patients and all five normal individuals by the method of Dignam et al. (35). Equal number of nuclei were homogenized as previously described (29), and protein concentrations in the extracts were determined using the colorimetric assay of Bradford (36). Nuclear extracts were normalized by use of both the octamer (OCT-1) nuclear protein, a member of the OCT family which is apparently ubiquitous in mammalian cells (37), and the nuclear factor (NF)-κB nuclear protein, which binds to κ light–chain enhancer in B cells (38). Consensus oligonucleotides for both transcription factors were 5′ end labeled with (γ-32P)ATP and T4 polynucleotide kinase (Sigma Chemical Co., St. Louis, MO) and used for gel retardation assays under conditions suggested by the supplier (Promega Corp., Madison, WI).

Gel retardation assays of nuclear proteins for the insulin receptor gene and DNA fragments. Binding reactions were performed as previously described (29). Briefly, 4 μg of nuclear extracts from EBV-transformed lymphoblasts were incubated with 2 ng of either radiolabeled fragment C2 or E3 of the insulin receptor gene, in the presence of 1 μg poly(dI-dC) (Pharmacia) which was used as competitor DNA for nonspecific DNA-binding proteins in the nuclear extracts. After 30 min of incubation at 20°C reaction products were separated by electrophoresis through a nondenaturing 6% polyacrylamide gel, and free and bound DNA were detected by autoradiography.

The two sequences (C2 and E3) used in the binding assays were prepared as described (29). Probes for gel retardation analyses were obtained from a clone containing 1.8-kb of 5′ genomic sequence for the insulin receptor (29), after digestion with appropriate restriction endonucleases. Each probe was end labeled with (32P)dATP and (35P)dCTP using DNA polymerase I (31).

Figure 1. Protein-binding activity of consensus oligonucleotides containing DNA binding sites for the OCT-1 and NF-κB nuclear proteins. Nuclear extracts from EBV-transformed lymphoblasts of either normal or diabetic individuals were incubated with each radiolabeled probe and analyzed by gel retardation assays. Lanes: 1, probe alone; 2, probe plus nuclear extract from a nondiabetic control subject; 3, probe plus nuclear extract from a patient with NIDDM and normal insulin receptor expression; 4, probe plus nuclear extract from patient 1; 5, probe plus nuclear extract from patient 2. Arrows show the position of free (DNA) and bound (DNA-P) probe. A representative of three separated assays is shown.
Results

Detection of OCT-1 and NF-κB nuclear proteins in EBV-transformed lymphoblasts. In preliminary experiments, nuclear extracts from EBV-transformed lymphoblasts of either normal or diabetic individuals were tested in a gel retardation analysis using two different probes that interacted with the ubiquitous transcription factor OCT-1 and the NF-κB nuclear protein. This experiment was necessary to verify the quantity of nuclear protein in each nuclear extract preparation. By measuring the concentration of these two nuclear proteins, nuclear extracts were normalized. Fig. 1 indicates that OCT-1 and NF-κB nuclear proteins are expressed and present in a similar concentration in all of the nuclear extracts used for this study.

DNA binding activity to fragments C2 and E3 in EBV-transformed lymphoblasts. Nuclear extracts from EBV-transformed lymphoblasts were incubated with each radiolabeled fragment and tested for the presence of specific DNA-binding factors using gel retardation assays (29). We first studied the binding of DNA fragments to nuclear proteins from patient 1. DNA-binding activity of nuclear extracts from this patient was barely detectable with both probes C2 and E3 (Fig. 2A). Laser densitometric scanning of the autoradiograms revealed that nuclear extracts from patient 1 had 5–10% of C2 and E3 DNA-binding activity when compared to that of five nondiabetic control subjects (Table II).

We next determined binding of C2 and E3 DNA fragments to nuclear proteins from EBV-transformed lymphoblasts of patient 2. As with patient 1, the DNA-binding activity of nuclear extracts from patient 2 was also significantly reduced with both probes C2 and E3, although to a lesser extent than patient 1 (Fig. 2B). Laser densitometric scanning of the autoradiograms revealed that nuclear extracts from this patient had 20–25% of C2 and E3 DNA-binding activity when compared to that of nondiabetic control subjects (Table II).

Studies indicate that the vast majority of patients with the common form of NIDDM have normal insulin receptor levels (10). In preliminary studies of EBV-transformed lymphoblasts from several nonobese NIDDM individuals with normal expression of both the insulin receptor protein and insulin receptor mRNA, we have found that DNA-binding activity of nuclear extracts is similar to that of nondiabetic control subjects (Fig. 3).

Insulin receptor mRNA abundance in EBV-transformed lymphoblasts. Using an RNase A protection assay, insulin receptor mRNA abundance was determined in EBV-transformed lymphoblasts. In preliminary experiments, nuclear extracts from EBV-transformed lymphoblasts from either diabetic patients and nondiabetic control subjects. In EBV-transformed lymphoblasts from patient 1, the levels of insulin receptor mRNA were markedly reduced (5–10% of control) (Fig. 3). In cells from patient 2, the content of insulin receptor mRNA was also reduced compared to that from nondiabetic control subjects, although to a lesser extent than patient 1 (30–40% of control) (Fig. 3). The decrease in insulin receptor mRNA abundance in EBV-transformed lymphoblasts from patient 1 and patient 2 paralleled the decrease in DNA-binding activity by both C2 and E3 probes. Insulin receptor mRNA abundance was normal in EBV-transformed lymphoblasts from all NIDDM patients expressing normal levels of insulin receptors (Fig. 3).

\[ ^{125}\text{I}-labeled\text{ insulin binding to EBV-transformed lymphoblasts.} \]

An \(^{125}\text{I}-labeled\text{ insulin binding assay performed on intact EBV-transformed lymphoblasts from the two diabetic patients revealed a significant decrease in insulin binding compared to that from nondiabetic control subjects. With patient 1, ^{125}\text{I}-labeled insulin binding was almost undetectable (< 1% per 10^7 cells); with patient 2, ^{125}\text{I}-labeled insulin binding was decreased to ~35% of normal (Fig. 3). As with insulin receptor mRNA abundance, the decrease in ^{125}\text{I}-labeled insulin binding paralleled the decrease in DNA binding activity by both C2 and E3 probes. In contrast, cells from NIDDM patients with normal insulin receptor expression had normal ^{125}\text{I}-labeled insulin binding (Fig. 3).} \]

Discussion

Epidemiological evidence indicates that genetic factors contribute significantly to the etiology of insulin resistance and NIDDM (5). Recently, it has been estimated that a small but

Table II. DNA-binding Activity to Fragments C2 and E3 in EBV-transformed Lymphoblasts

<table>
<thead>
<tr>
<th></th>
<th>C2</th>
<th>E3</th>
</tr>
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<tbody>
<tr>
<td>Normal subjects (n = 5)</td>
<td>7.3±0.2</td>
<td>7.9±0.4</td>
</tr>
<tr>
<td>NIDDM patients (n = 6)*</td>
<td>7.0±0.3</td>
<td>7.3±0.4</td>
</tr>
<tr>
<td>Patient 1</td>
<td>0.6±0.1</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Patient 2</td>
<td>1.5±0.2</td>
<td>1.8±0.2</td>
</tr>
</tbody>
</table>

*, NIDDM patients with normal insulin receptor expression. Values represent arbitrary units as measured by laser densitometry. Results are the mean±SEM for three separate assays.

![Image 260](260_Brunetti_Brunetti_Foti_Accili_and_Goldfine.png)
significant fraction of patients with NIDDM have defects in insulin receptor function and/or expression (10, 14). Very insulin resistant patients with reduced or absent insulin receptor expression in target cells have been reported, and many of these patients have mutations or deletions in the coding sequence of the insulin receptor gene (10, 12). However, certain patients with apparently normal insulin receptor genes have reduced expression of both the insulin receptor protein and insulin receptor mRNA levels (13-15). In these patients it is possible that there are mutations in genes encoding trans-acting factors which regulate the level of insulin receptor gene expression.

Mutations in genes encoding transcription factors can give rise to diseases in humans and mice (39, 40). For instance, a non-sense mutation in the gene encoding the human pituitary tumor transforming gene arises in the offspring of diabetic parents. This work was supported in part by National Institutes of Health grant DK26667.

Acknowledgments

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References


Figure 3. Comparison of C2 and E3 DNA-binding activity to insulin receptor mRNA abundance and 125I-labeled insulin binding in EBV-transformed lymphoblasts. Values of DNA-binding activity and mRNA abundance represent arbitrary units as measured by laser densitometric scanning of the autoradiograms. Specific 125I-labeled insulin binding is expressed as percentage of total/100 cells. Results are the mean±SEM for three separate assays. IR, insulin receptor. □, normal subjects (n = 5); □, NIDDM patients with normal insulin receptor expression (n = 6); □, patient 2; □, patient 1.