Abnormal Activation of Glycogen Synthesis in Fibroblasts from NIDDM Subjects

Evidence for an Abnormality Specific to Glucose Metabolism

ANN M. WELLS, IAIN C. SUTCLIFFE, ANDREW B. JOHNSON, AND ROY TAYLOR

To determine whether the tendency for NIDDM to run in families could relate to genetically determined defects in insulin stimulation of glycogen synthesis, skin fibroblasts from subjects with a strong family history of NIDDM were studied. Fibroblasts from nondiabetic subjects without any family history of NIDDM were studied as control subjects. The cells were studied after 7–16 passages in culture. Rates of glycogen synthesis were lower in fibroblasts from NIDDM subjects both basally and with maximal insulin stimulation (0.77 ± 0.11 vs. 0.46 ± 0.04 pmol · well^{-1} · h^{-1} [$P < 0.02$] and 1.49 ± 0.26 vs. 0.69 ± 0.05 pmol · well^{-1} · h^{-1} [$P < 0.01$]). Rates of glycogen synthesis were stimulated 1.9 ± 0.2-fold above basal in the control cells and 1.5 ± 0.1-fold above basal in the NIDDM cells ($P < 0.02$). Rates of [^{3}H]thymidine uptake were similar in control and NIDDM fibroblasts (basal, 28.3 ± 2.8 vs. 39.2 ± 8.0; maximum, 50.9 ± 7.2 vs. 69.3 ± 16.9 dpm × 10^{-3}, respectively). Rates of uptake increased similarly in control and NIDDM cells by 1.8 ± 0.1- and 1.7 ± 0.1-fold above basal. Maximum specific fibroblast insulin binding was similar for control and NIDDM subjects (194.0 ± 29.2 vs. 176.1 ± 24.9 fmol ^{125}I-labeled insulin bound/mg protein respectively). The tyrosine kinase activity of insulin receptors isolated from the control and NIDDM fibroblasts was similar (basal, 135 ± 30 vs. 149 ± 33; submaximal, 153 ± 28 vs. 155 ± 30; and maximal insulin, 191 ± 45 vs. 213 ± 48 dpm · mg protein^{-1} · min^{-1}). The observed abnormality was thus distal to the insulin receptor and did not involve all aspects of insulin signaling. These data provide evidence for a genetic influence on insulin control of glycogen synthesis in people with a strong family history of NIDDM. *Diabetes* 42:583–89, 1993

The tendency for NIDDM to run in families has long been recognized (1). Clearly, decreased tissue sensitivity to insulin and decreased insulin secretory capacity are implicated in the etiology of NIDDM, either or both of which could be genetically determined (2,3). Recent studies have demonstrated that first-degree relatives of NIDDM subjects tend to exhibit insulin resistance in pathways of glucose disposal, and this has been interpreted as evidence for a genetic basis of the insulin resistance of NIDDM (4–6). If this is correct, a genetic influence probably will be identified in either the process of insulin signal transmission across the cell membrane or the biochemical pathways involved in glucose uptake and metabolism. Once confirmed, further work could permit characterization of the responsible gene or genes.

Previous work on fibroblasts from NIDDM subjects did not take into account potential gene dose effects (7), because no selection was undertaken for subjects with a family history of NIDDM (8–12). As at least one third of NIDDM subjects have no family history of the condition and as NIDDM is suspected to be heterogeneous in nature, these studies may have overlooked genuine differences between fibroblasts from groups of NIDDM and control subjects. Thus, to mount a convincing investigation of possible genetic factors in cellular insulin action, subjects must be selected carefully. The subjects most likely to have a genetic tendency to decreased tissue insulin sensitivity are those with a family history of NIDDM.

To seek evidence for a genetic determination of insulin resistance, fibroblasts from NIDDM subjects with a

From the Human Metabolism Research Centre, University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom.

Address correspondence and reprint requests to Dr. Roy Taylor, Department of Medicine, Medical School, Framlington Place, Newcastle upon Tyne NE2 4LP, UK.

Received for publication 2 June 1992 and accepted in revised form 30 November 1992.

NIDDM, non-insulin-dependent diabetes mellitus; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; FCS, fetal calf serum; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; PMSF, phenylmethylsulphonyl flouride; WGA, wheat germ aglutinin; cpm, counts per minute; IGF-I, insulinlike growth factor I.

TABLE 1
Family histories of NIDDM in subjects studied

Subject no.	Sex	Affected parent	Affected sibling	Other relatives
1	Male	Mother	1 Sister	Grandmother (maternal)
2	Female	—	2 Sisters	—
3	Male	—	2 Sisters	—
4	Female	Mother	—	Grandparents (maternal) 3 Aunts (maternal) 4 Uncles (maternal)
5	Female	Mother	—	Grandmother (maternal) 1 Aunt (maternal) 8 Aunts (paternal)
6	Female	Mother	3 Sisters 3 Brothers	—
7	Male	Mother	2 Brothers	—

strong family history of NIDDM were compared with fibroblasts from a group of nondiabetic subjects with no family history of NIDDM. The response to insulin of glycogen synthesis and of thymidine uptake were examined to allow separation of a basic defect in insulin signal transmission from an effect solely on glucose metabolism. The tyrosine kinase activity of the insulin receptors was also examined.

RESEARCH DESIGN AND METHODS

Patients were selected from the clinic who had NIDDM >1 yr and had two or more close relatives with NIDDM (Table 1). Fasting blood glucose on treatment was 7.6 ± 0.8 mM. Control subjects had no metabolic disease and no family history of NIDDM. The age of the NIDDM and control subjects was similar (57.5 ± 3.2 vs. 50.7 ± 2.7 yr). The study was approved by the Newcastle upon Tyne Ethical Committee, and consent was obtained from subjects after full explanation of the procedure and its purpose.

Crystalline porcine insulin and porcine [^{125}I-tyr^{A14}] monoiodoinsulin (200–300 μCi/mg) were a gift from Novo. [^{32}P]ATP (300 Ci/mmol), [U-^{14}C]glucose (230 μCi/mmol), and 6-[^{3}H]thymidine (2 Ci/mmol) were obtained from Amersham, UK. Glu:Tyr 4:1 random copolymer, WGA insolubilized on 6% agarose macrobeads, HEPES, *N*-acetyl-D-glucosamine, aprotinin (10–20 trypsin inhibitor U/mg), ATP, bovine γ-globulins, BSA, polyethylene glycol 8000, and PMSF were all purchased from Sigma (London). The protein determination kit was obtained from Bio-Rad (Munich, Germany).

Cell culture. Cell cultures were established from 4-mm forearm skin-punch biopsies. A local anesthesia (1% lidocaine) was applied, but care was taken to infiltrate around and not into the area to be biopsied, because preliminary work showed that lidocaine inhibited establishment of cell culture. Fibroblasts were grown in monolayers in modified Eagle's medium containing glucose (5.5 mM) and supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% FCS. Cells were then subcultured in a 1:3 ratio using 0.05% trypsin solution in Ca^{2+}- and Mg^{2+}-free PBS. Fibroblasts were grown at 37°C in an atmosphere of 5% CO_2 and were used between the 6th and 16th passages. All experiments were conducted in quadruplicate on confluent cell monolayers grown in six-well trays. Before each experiment the cells were incubated in serum-free medium as detailed below.

Binding. Adherent fibroblasts grown in six-well trays were incubated in a HEPES binding buffer (100 mM HEPES, 120 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 15 mM $NaCH_3COOH$, 10 mM glucose, and 1% BSA, pH 7.65 [13]). Incubations were conducted in a total volume of 0.4 ml at 15°C for 4 h in the presence of ^{125}I-labeled insulin. Nonspecific binding was determined in the presence of an excess of unlabeled insulin (10^{-5} M). The reaction was stopped by rinsing three times with ice-cold PBS. The cells were solubilized with 20% KOH, and the cell radioactivity counted in a γ-counter. An aliquot was removed to estimate the protein content by the Bio-Rad protein assay method.

Glycogen synthesis. The rate of glycogen synthesis was estimated from the incorporation of [^{14}C]glucose into cellular glycogen during a 1-h incubation (13). After incubation for 16 h in serum-free medium, fibroblasts were preincubated for 15 min in 1 ml of culture medium with or without increasing concentrations of insulin. The concentration of glucose remained constant throughout all incubations at 5.5 mM. [^{14}C]Glucose (1.25 μCi, final concentration 5.4 μM) was added for 1 h. The incorporation was stopped by rapidly washing the cells three times with ice-cold PBS. Cells were solubilized in 20% KOH and transferred to separate tubes, where glycogen was precipitated with absolute alcohol and the radioactivity in glycogen was determined. Each cell line was studied in triplicate or quadruplicate.

Thymidine incorporation. The rate of thymidine incorporation into DNA was estimated after the fibroblasts had been preincubated for 12 h in serum-free medium and a further 24 h in I ml/well of serum-free medium with or without increasing concentrations of insulin (14). [^{3}H]Thymidine (1 μCi) was then added for 1 h. The reaction was stopped by rapid washing with ice-cold PBS. The monolayer was rinsed five times in 5% TCA and extracted with methanol, and the remaining cell constituents solubilized in 0.2 ml 20% KOH. The cell-associated radioactivity was counted. Each cell line was studied in triplicate or quadruplicate.

Purification of insulin receptors by WGA-agarose affinity chromatography. Confluent fibroblast monolayers (15–20 175-cm^2 flasks) were washed twice with ice-cold PBS, once with PBS containing 0.02% EDTA, and finally scraped into PBS containing 0.02% EDTA (5 ml/flask). Cell pellets obtained by centrifugation (typically 35 mg/flask) were resuspended into 50 mM HEPES and 150 mM NaCl (pH 7.6 at 4°C; buffer A) containing 0.1 mg/ml aprotinin and 2 mM PMSF and recentrifuged. The cell pellets were resuspended into 10 ml of Buffer A containing 0.1 mg/ml aprotinin, 2 mM PMSF, and 1% Triton X-100. After brief homogenization (30 s, Polytron homogenizer), the cell extract was solubilized by stirring for 60 min at 4°C and then centrifuged to remove insoluble material in a MSE Superspeed 65 ultracentrifuge (45 min, 150,000 *g*, 4°C). The supernatant crude receptor preparation was diluted 1:1 with buffer A containing 0.1%

Triton X-100 and mixed end-over with 1 ml of prewashed WGA-agarose in two batches (15). After overnight mixing at 4°C, the combined WGA-agarose was settled in a Pharmacia C10 column, and the nonglycoprotein fraction was run off. The column was washed extensively with a total of 75–100 ml of buffer A containing 0.1% Triton X-100, and then glycoprotein-enriched receptor fractions were eluted in 750-μl batches of buffer A supplemented with 0.1% Triton X-100 and 0.5M *N*-acetyl-D-glucosamine after equilibration for 20 min/batch. Three 10-μl aliquots of each receptor fraction were used for protein determination by the Bio-Rad dye-binding method using BSA as standard. Typically, the second fraction eluted was the fraction with the maximum protein concentration.

Insulin binding assay. WGA-purified receptor fractions (25 μl) were incubated in a final volume of 200 μl with ~20,000 cpm [^{125}I]monoiodoinsulin (50–100 pM) and competitive concentrations of unlabeled porcine insulin from 0 to 10^{-6} M in duplicates. The incubation buffer contained buffer A supplemented with 0.1% BSA and 1 mg/ml of bacitracin. Triton X-100 and *N*-acetyl-D-glucosamine in the receptor fractions were diluted in the assay to 0.01% and 62.5 mM, respectively. After incubation overnight at 4°C, receptor-bound insulin was separated by selective precipitation with polyethylene glycol 16; 50 μl 0.4% bovine γ-globulin carrier, and 250 μl 25% (wt/vol) polyethylene glycol in buffer A were added to the binding assay. After mixing, the assay tubes were stored on ice for 15 min and then centrifuged (5 min Beckman Microfuge B at 4°C; Beckman, Fullerton, CA). The supernatants were discarded, and the precipitated pellets were washed with 500 μl 12.5% (wt/vol) polyethylene glycol in buffer A. Bound tracer monoiodoinsulin was counted on a multiwell γ-counter.

Nonspecific binding determined in the presence of 10^{-6} M porcine insulin, which was always <5% of the total added counts, was subtracted from the percentage of binding at all other concentrations of insulin. For routine analysis of receptor fractions, only duplicate binding in the absence (maximum binding) or presence of 10^{-6} M porcine insulin (nonspecific binding) was determined. Specific binding by receptor fractions was expressed as femtomole of [^{125}I]monoiodoinsulin bound per milligram of protein.

Tyrosine kinase activity. WGA-purified receptor preparations (20 μl) were preincubated in triplicate in the absence or presence of 10^{-6} M insulin for 60 min at room temperature in a total volume of 50 μl 50 mM HEPES, 150 mM NaCl, 0.1 mg/ml BSA, and 19.2 mM $MgCl_2$ (pH 7.6 at room temperature). Submaximal stimulation of kinase activity was assayed by preincubation with 10^{-9} M insulin. After preincubation additions of substrate Glu:Tyr 4:1 copolymer (final concentration 2.5 mg/ml) were made over 10 min. Phosphorylation reaction was started by addition of 15 μl of $MnCl_2$-ATP mixture to give a final 80-μl reaction mixture of 50 mM HEPES, 150 mM NaCl, 12 mM $MgCl_2$, 4 mM $MnCl_2$, 0.6 mg/ml BSA, 50 μM ATP, and 5 μCi/tube [γ-^{32}P]ATP. After a 30-min incubation, the reaction was quenched by pipetting 2 × 35-μl aliquots from the reaction mixture onto filter paper discs (2 × 2-cm Whatman 3MM) and soaking in ice-cold 10% TCA containing 10 mM sodium pyrophosphate. The discs were extensively washed in three changes of ~400 ml of ice-cold 10% TCA and 10 mM sodium pyrophosphate over 3–4 h, during which the wash was continually stirred, and the discs were regularly agitated to prevent sticking together. After washing, the discs were dried by successively rinsing in 300 ml of ice-cold absolute ethanol and 300 ml of diethyl ether for 1 min each. The discs were then air dried for at least 30 min before counting for radioactivity in a liquid scintillation counter. Incorporation of ^{32}P into TCA-precipitable material was expressed as counts per minute per microgram of receptor preparation per minute after subtraction of counts per minute incorporated in the absence of substrate copolymer.

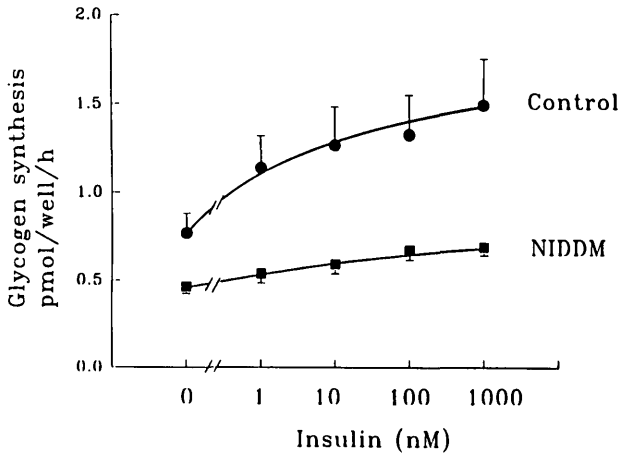

FIG. 1. Stimulation by insulin of [^{14}C]glucose incorporation into glycogen in fibroblasts from 7 NIDDM (■) and 6 control (●) subjects. Data are means ± SE.

Statistical analysis. All data are presented as means ± SE unless stated otherwise, and medians are given for data with skewed distribution. Statistical significance was calculated using Student's *t* test or Mann-Whitney U test as appropriate.

RESULTS

Glycogen synthesis. Rates of glycogen synthesis were lower in fibroblasts from NIDDM subjects both basally and with maximal insulin stimulation (0.77 ± 0.11 vs. 0.46 ± 0.04 pmol · well^{-1} · h^{-1}, [$P < 0.02$] and 1.49 ± 0.26 vs. 0.69 ± 0.05 pmol · well^{-1} · h^{-1} [$P < 0.01$]). The difference persisted at all insulin concentrations studies and was significant at each point (Fig. 1). The concentration of insulin required to bring about half-maximal stimulation was 4.2 ± 1.7 (median 2.0) × 10^{-9} M for the control group and 18.1 ± 11.9 (median 3.5) × 10^{-9}M for the NIDDM group (NS). Rates of glycogen synthesis stimulated 1.9 ± 0.2-fold above basal in the control cells and 1.5 ± 0.1-fold above basal in the NIDDM cells ($P < 0.02$). Within the NIDDM group, no relationship was found between the impairment of insulin responsiveness or sensitivity and the number of affected relatives.

Thymidine uptake. Rates of [^{3}H]thymidine uptake were similar in control and NIDDM fibroblasts (basal, 28.3 ± 2.8 vs. 39.2 ± 8.0; maximum, 50.9 ± 7.2 vs.

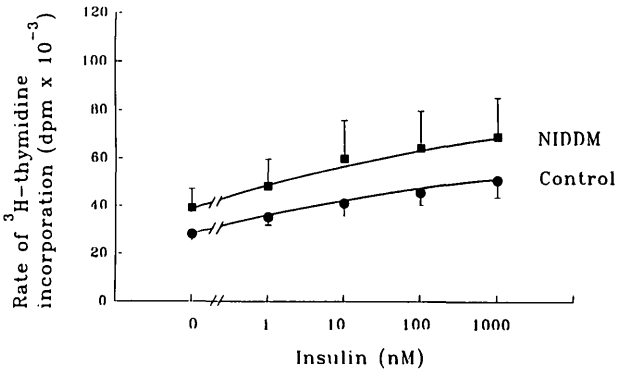

FIG. 2. Stimulation by insulin of [^{3}H]thymidine incorporation by fibroblasts from 7 NIDDM (■) and 6 control (●) subjects. Data are means ± SE.

69.3 ± 16.9 dpm × 10^{-3}, respectively). The insulin dose-response curves are shown in Fig. 2. The slightly higher mean rates observed in the NIDDM group were a consequence of two subjects with high rates, and the similarity between the groups is emphasized by consideration of median rates (basal, 30.7 vs. 30.9; maximum, 52.9 vs. 59.7 dpm × 10^{-3}, respectively). Half-maximal stimulation was observed at 4.8 ± 1.7 (median 2.8) vs. 10.1 ± 7.3 (median 3.0) × 10^{-9} M insulin (NS). Rates of uptake increased similarly in control and NIDDM cells by 1.8 ± 0.1- and 1.7 ± 0.1-fold above basal.

Insulin-receptor binding and tyrosine kinase activity toward exogenous substrate. Maximum specific insulin binding was similar for control and NIDDM subjects (194.0 ± 29.2 vs. 176.1 ± 24.9 fmol ^{125}I-labeled insulin bound/mg protein, respectively). The competitive binding curves for age-matched control subjects (n = 3, 6 experiments) and NIDDM subjects (n = 3, 7 experiments) were also comparable (Fig. 3). The concentration of unlabeled insulin necessary to produce a 50% reduction in tracer binding was 6.5 × 10^{-9} M for control subjects and 5.5 × 10^{-9} M for NIDDM subjects. Insulin

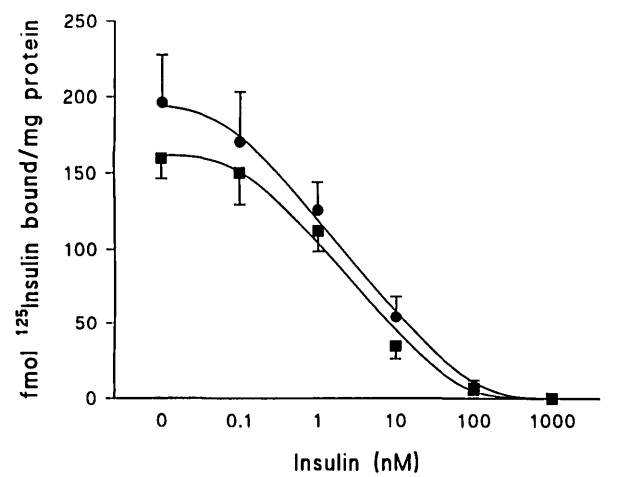

FIG. 3. Insulin-binding displacement curve for insulin receptors solubilized from fibroblasts of NIDDM (■) and control (●) subjects. Data are means ± SD of 7 studies using receptors solubilized from 3 individual NIDDM fibroblast cultures and 6 studies using receptors from 3 individual control fibroblasts.

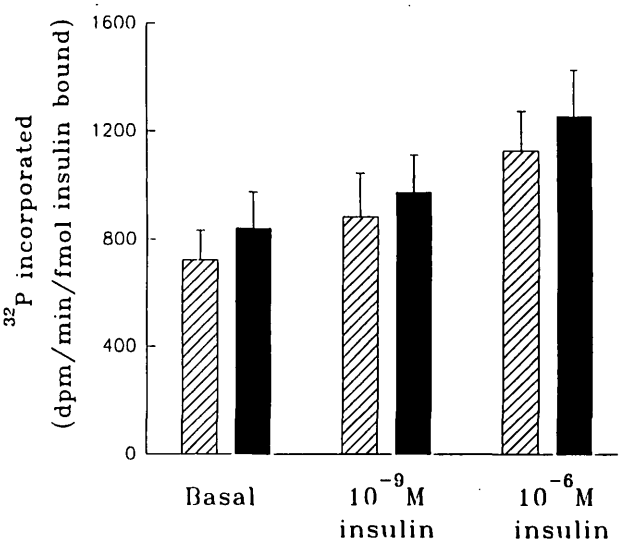

FIG. 4. Tyrosine-specific kinase activity of solubilized insulin receptors expressed as a function of insulin bound. Data are means ± SE of cultures from 2–4 experiments of 6 NIDDM (■) subjects and 6 control (▨) subjects.

stimulation of tyrosine kinase activity (Fig. 4) was significant both between basal and submaximal insulin stimulation (control subjects [n = 6], $P < 0.05$ and NIDDM subjects [n = 7], $P < 0.01$) and between submaximal and maximal stimulation (control [n = 6], $P < 0.05$ and NIDDM [n = 7] $P < 0.01$). The tyrosine kinase activity of insulin receptors isolated from the control and NIDDM fibroblasts was similar (basal, 135 ± 30 vs. 149 ± 33; submaximal, 153 ± 28 vs. 155 ± 30; and maximal insulin, 191 ± 45 vs. 213 ± 48 dpm · mg protein^{-1} · min^{-1}). This similarity persisted after correcting for insulin receptor number (basal, 723 ± 190 vs. 841 ± 133; submaximal insulin, 884 ± 226 vs. 973 ± 225; and maximal insulin, 1127 ± 253 vs. 1254 ± 313 dpm · fmol insulin bound^{-1} · min^{-1}; Fig. 4).

DISCUSSION

The findings of this study allow a more specific interpretation of previous observations of impaired glucose storage and impaired glycogen synthase activation in first-degree relatives of NIDDM subjects (4,17,18). In fibroblasts cultured in vitro for several passages, the biological behavior exhibited under standardized conditions will be determined by genetic factors (10,13). These results demonstrate a defect in the insulin response of glycogen synthesis in fibroblasts from NIDDM subjects with a strong family history for this condition. No significant alteration of insulin sensitivity as usually defined could be demonstrated, but the low responsiveness of the pathway in NIDDM cells made accurate assessment of the concentration of insulin to achieve half-maximal stimulation difficult to accurately determine. In absolute terms, the rate of glycogen synthesis was abnormally low at any insulin concentration. This metabolic pathway was studied because it is central to the control of glucose homeostasis by insulin and has consistently been shown to be abnormal in vivo (19–23). A second insulin-respon-

sive pathway not related to the control of glucose metabolism was also studied to be able to distinguish between abnormalities of transmembrane signaling and specific defects in metabolic control. Stimulation of thymidine uptake was found to be normal in the fibroblasts from NIDDM subjects, implying that transmission of the signal across the insulin receptor was not impaired. This deduction was confirmed by direct study of insulin receptor number and insulin activation of tyrosine kinase in solubilized receptors with the observation of no differences between receptors from NIDDM and control subjects.

Interpretation of the data on stimulation of thymidine uptake depends on the effects of insulin being mediated via the insulin receptor rather than the IGF-I receptor. If the latter played a major role, then normal function of the insulin receptor in intact cells could not be assumed. However, heterologous activation of receptors requires higher ligand concentration to achieve a given effect, and in the case of insulin and the IGF-I receptor the difference is at least two orders of magnitude (24,25). Figure 2 shows that insulin at 10^{-9} M was effective in stimulating thymidine uptake, and that effects on the cells from the NIDDM and control subjects were similar at this low concentration. The insulin effect on fibroblast growth has been shown to be additive to the IGF-I effect (26). Furthermore, blockade of IGF-I receptors by a specific monoclonal antibody does not change the stimulation of thymidine incorporation up to an insulin concentration of 10^{-7} M (27). We have previously characterized a panel of anti-insulin receptor antibodies that do not cross react with the IGF-I receptor (28,29). By use of the receptor blocking antibody, insulin-stimulated thymidine incorporation was blocked, whereas an insulinomimetic antibody brought about similar stimulation to insulin (A.M.W., R.T., M. Soos, K. Sidelle; unpublished observations). One reasonably may conclude that normal activation of the insulin receptor brings about similar stimulation of thymidine incorporation in cells from the two groups of subjects.

The insulin sensitivity of fibroblasts is lower than that of adipocytes (30) but not dissimilar to that estimated for whole-body glucose disposal (31) and human muscle in vitro (32) at around 9×10^{-10} M. At physiological insulin concentrations, the acute effect of insulin on fibroblast glucose uptake probably is small. However, this study was undertaken specifically to compare the insulin-signaling system in fibroblasts from diabetic and control subjects and not to allow extrapolation of results to major insulin-sensitive tissues. The ED_{50} for insulin binding to control cells in this study (6.5×10^{-9}) is similar to that observed in other recent studies (4×10^{-9} and 5.2×10^{-9} M) (13,25).

Early studies on fibroblasts from NIDDM subjects suggest that they may exhibit shorter replicative life spans in vitro (8,33). However, all more recent studies (9,12,34), including one repeat study by the same investigator (34), have demonstrated identical behavior in culture of fibroblasts from NIDDM and control subjects. Previous studies of glucose metabolism in NIDDM fibroblasts have not revealed abnormalities, although no attempt was made in those efforts to study cells carrying a genetic predisposition to insulin resistance by selecting subjects with a strong family history of NIDDM (8,9). In this study, the selection criteria used did not pick up any subjects with two diabetic parents, and detailed examination of the data for gene dose-response effects is not possible. Further work is required to elucidate this important question. Note that the only three subjects with both mother and at least one sibling affected included the two lowest responses to insulin stimulation of glycogen synthesis. The lack of paternal NIDDM in this study is likely to reflect the greater chance of men dying from other causes, including trauma, before NIDDM is diagnosed. The lack of abnormality in glucose metabolism observed in previous studies of NIDDM fibroblasts is significant in that the results of this study could not have been secondary to some persistent effect of the hyperglycemia in vivo. Indeed, the degree of hyperglycemia (mean fasting blood glucose on treatment 7.6 ± 0.8 mM) was not great in this study group.

Skeletal muscle glycogen synthase has been shown to be insensitive to insulin activation in both Caucasian NIDDM subjects and Pima Indians (35,36). Recently the same defect has been shown to occur in first-degree relatives of NIDDM patients (17). Treatment of newly presenting NIDDM patients with intensive diet therapy has no effect on muscle glycogen synthase activation, and addition of sulfonylurea therapy has been shown to have only a slight effect in increasing the enzyme activation (37). Metabolic factors such as higher concentrations of fatty acids that may alter in vivo glycogen synthase activity have been shown not to be relevant in this respect (38). These findings of decreased insulin responsiveness of glycogen synthesis in fibroblasts from subjects with a strong family history of NIDDM suggest that a genetically determined defect controls this pathway. It is unlikely that a condition such as NIDDM would involve a defect in the primary structure of major enzymes, and no reports of gene abnormality in NIDDM have followed the sequencing of the human glycogen synthase gene (39). Indeed, glycogen synthase from NIDDM muscle can be normally activated by high concentrations of glucose-6-phosphate in vitro (35,36). The activity of glycogen synthase in vivo is regulated by phosphorylation and dephosphorylation of the site 3 serine residues (40). The major effector of dephosphorylation is recognized to be protein phosphatase I, which in turn is activated by protein phosphatase 2A or B and deactivated by glycogen synthase kinase III (41,42). This network of control comprises only the final steps in insulin regulation of glycogen synthase activity, and unknown enzymes link these to the insulin receptor tyrosine kinase (43,44). Many potential candidate enzymes or cofactors in this pathway may be defective in NIDDM. The demonstration of defects in the control of glycogen synthase in fibroblasts of NIDDM subjects should expedite the task of identifying the affected step.

The observation that the characteristic defect manifest in adipose tissue and muscle of NIDDM subjects is also expressed in fibroblasts is of considerable theoretical interest. It implies that the defect is not solely expressed during differentiation of major metabolic tissues such as

skeletal muscle and must relate to basic cellular signaling mechanisms. Fibroblasts previously have been shown to reflect abnormalities in insulin signaling in states of extreme insulin resistance (13,45–48). Nonetheless, caution is required in extrapolating data acquired from one cell type to another. Once the defect in activation of the fibroblast glycogen synthesis is characterized, careful comparative work on freshly isolated human muscle will be required.

ACKNOWLEDGMENTS

This work was supported by Wellcome Trust Grant 017939/1.5.

We thank Dr. Jacqueline Capeau, Prof. K Siddle, and Dr. M. Soos for advice on fibroblast and tyrosine kinase methodology, and to NovoNordisk for supply of [^{125}I]monoiodoinsulin.

REFERENCES

1. Kobberling J, Tillil H: Empirical risk figures for first degree relatives of non-insulin dependent diabetics. In *The Genetics of Diabetes Mellitus*. Kobberling J, Tattersall, R, Eds. London, Academic, 1982, p. 201–209
2. Taylor R: Aetiology of non-insulin dependent diabetes. *Br Med Bull* 45:73–91, 1989
3. DeFronzo RA, Bonadonna RC, Ferrannini E: Pathogenesis of NIDDM: a balanced overview. *Diabetes Care* 15:318–68, 1992
4. Eriksson J, Fransilla-Kallunki A, Ekstrand A, Saloranta C, Widen E, Schalin C, Groop L: Early metabolic defects in persons at increased risk for non-insulin dependent diabetes mellitus. *N Engl J Med* 321:337–43, 1989
5. Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR: Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. *Ann Int Med* 113:909–15, 1990
6. Schalin-Jantti C, Harkonen M, Groop LC: Impaired activation of glycogen synthase in people at increased risk for developing NIDDM. *Diabetes* 41:598–604, 1992
7. O'Rahilly S, Wainscoat JS, Turner RC: Type 2 (non-insulin-dependent) diabetes mellitus: new genetics for old nightmares. *Diabetologia* 31:407–14, 1988
8. Goldstein S, Littlefield JW: Effect of insulin on the conversion of glucose C-14 to C-14-O2 by normal and diabetic fibroblasts in culture. *Diabetes* 18:545–49, 1969
9. Howard BV, Fields RM, Mott DM, Savage PJ, Nagulsparan M, Bennett PH: Diabetes and cell growth: lack of differences in growth characteristics of fibroblasts from diabetic and non-diabetic Pima Indians. *Diabetes* 29:119–24, 1980
10. Prince MJ, Tsai P, Olefsky JO: Insulin binding, internalization, and insulin receptor regulation in fibroblasts from type II, non-insulin-dependent diabetic subjects. *Diabetes* 30:596–600, 1981
11. Germinario RJ, Oliveira M, Manuel S, Taylor M: Characteristics of normal and maturity-onset diabetic (type II diabetes) cell cultures: life spans and DNA synthetic capabilities. *Gerontology* 32:148–57, 1986
12. Germinario RJ, Oliveira M, Manuel S, Taylor M: Hexose transport regulation in cultured fibroblasts derived from normal and type II diabetic patients. *Clin Invest Med* 10:295–302, 1987
13. Magre J, Reynet C, Capeau J, Blivet M-J, Picard J: In vitro studies of insulin resistance in patients with lipoatrophic diabetes: evidence for heterogenous postbinding defects. *Diabetes* 37:421–28, 1988
14. Hollenberg SA, Cuatrecasas P: Insulin stimulation of cell growth. *J Biol Chem* 250:3845–53, 1975
15. Le Marchand-Brustel Y, Ballotti R, van Oberghen E: Insulin receptor kinase activity in states with altered insulin action. In *Insulin Receptors Part A: Methods for the Study of Structure and Function*. Kahn CR, Harrison LC, Eds. New York, Liss, 1988, p. 163–79
16. Cuatrecasas P: Isolation of the insulin receptor of liver and fat cell membranes. *Proc Natl Acad Sci USA* 69:318–22, 1972
17. Vaag A, Henriksen JE, Beck-Nielsen H: Defective insulin activation of glycogen synthase in skeletal muscle in first-degree relatives to patients with type 2 (non-insulin dependent) diabetes mellitus. *Diabetologia* 34 (Suppl. 2):A70, 1991
18. Jackson RA, Blix PM, Matthews JA, Morgan LM, Rubenstein AH, Nabarro JDN: Comparison of peripheral glucose uptake after oral glucose loading and a mixed meal. *Metabolism* 32:706–10, 1983
19. Hollenbeck CB, Chen Y-DI, Reaven GM: A comparison of the relative effects of obesity and non-insulin dependent diabetes mellitus on in vivo insulin stimulated glucose utilization. *Diabetes* 33:622–26, 1984
20. Bogardus C, Lillioja S, Mott DM, Hollenbeck C, Reaven GM: Relationships between degree of obesity and in vivo insulin action in man. *Am J Physiol* 248:E286–91, 1985
21. Swislocki ALM, Donner CC, Fraze E, Chen Y-DI, Reaven GM: Can insulin resistance exist as a primary defect in non-insulin dependent diabetes mellitus? *J Clin Endocrinol Metab* 64:778–82, 1984
22. Campbell PJ, Mandarino LJ, Gerich J: Quantification of the relative impairment in actions of insulin on hepatic glucose production and peripheral glucose uptake in non-insulin dependent diabetes mellitus. *Metabolism* 37:14–21, 1988
23. Shulman GI, Rothman DL, Jue T, Stein P, DeFronzo RA, Shulman RG: Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin dependent diabetes by ^{13}C nuclear magnetic resonance spectroscopy. *N Engl J Med* 322:223–28, 1990
24. Zapf J, Schmid C, Froesch ER: Biological and immunological properties of insulin-like growth factors (IGF) I and II. *Clin Endocrinol Metab* 13:3–30, 1984
25. Sasaoki T, Kobayashi M, Takata Y, Ishibashi O, Iwasaki M, Shigeta Y, Goji K, Hisatomi A: Clarification of signaling pathways mediated by insulin and insulin-like growth factor I receptors in fibroblasts from patients with specific defect in insulin receptor. *Diabetes* 37:1515–23, 1988
26. Conover C, Hintz RL, Rosenfield RG: Comparative effects on somatomedin C and insulin on the metabolism and growth of cultured human fibroblasts. *J Cell Physiol* 122:133–41, 1985
27. Conover A, Hintz RL, Rosenfeld RG: Direct evidence that the insulin receptor mediates a mitogenic response in cultured human fibroblasts. *Horm Metab Res* 21:59–63, 1989
28. Taylor R, Soos M, Wells AM, Argyraki M, Siddle KS: Insulin-like and insulin inhibitory effects of monoclonal antibodies for different epitopes on the human insulin receptor. *Biochem J* 242:123–29, 1987
29. Siddle KS, Soos MA, O'Brian RM, Gandeston RH, Taylor R: Monoclonal antibodies as probes of the structute and function of insulin receptors. *Biochem Soc Trans* 15:47–51, 1987
30. Pedersen O, Hjollund E, Lindskof HO: Insulin binding and action on fat cells from young healthy females and males. *Am J Physiol* 243:E158–61, 1982
31. Kolterman OG, Gray RS, Griffin J, Burstein P, Insel J, Scarlett JA, Olefsky JM: Receptor and postreceptor defects contribute to the insulin resistance in non-insulin dependent diabetes mellitus. *J Clin Invest* 70:957–69, 1981
32. Argyraki M, Wright PD, Venables CW, Proud G, Taylor R: Study of human skeletal muscle in vitro: effect of NEFA supply on glucose storage. *Metabolism* 38:1183–87, 1989
33. Vracko R, Benditt EP: Manifestations of diabetes mellitus-their possible relationships to an underlying cell defect. *Am J Pathol* 75:204–21, 1974
34. Rowe DW, Starman BJ, Fujimoto WY, Williams RH: Abnormalities in proliferation and protein synthesis in skin fibroblast cultures from patients with diabetes mellitus. *Diabetes* 26:284–90, 1977
35. Bogardus C, Lillioja S, Stone K, Mott D: Correlation between muscle glycogen synthase activity and in vivo insulin action in man. *J Clin Invest* 73:1185–90, 1984
36. Johnson AB, Argyraki M, Thow JC, Jones IR, Broughton D, Miller M, Taylor R: Impaired activation of muscle glycogen synthase in non-insulin dependent diabetes mellitus is unrelated to the degree of obesity. *Metabolism* 40:252–60, 1991
37. Johnson AB, Argyraki M, Thow JC, Jones IR, Broughton DL, Miller M, Taylor R: The effect of sulphonylurea therapy on skeletal muscle glycogen synthase activity and insulin secretion in newly presenting non-insulin dependent diabetic patients. *Diabetic Med* 8:243–53, 1991
38. Johnson AB, Argyraki M, Thow JC, Cooper BG, Fulcher G, Taylor R: Effect of increased free fatty acid supply on glucose metabolism and skeletal muscle glycogen synthase acivity in normal man. *Clin Sci* 82:219–26, 1992
39. Browner MF, Nakano K, Bang AG, Fletterick RJ: Human muscle glycogen synthase cDNA sequence: a negatively charged protein with an asymmetric charge distribution. *Proc Natl Acad Sci USA* 86:1443–47, 1989
40. Cohen P, Parker PJ, Woodgett JR: The molecular mechanism by which insulin activates glycogen synthase in mammalian skeletal muscle. In Molecular Basis of Insulin Action, 1985. Gzech MP, New York, Plenum, p. 213–33
41. Dent P, Campbell DG, Hubbard MJ, Cohen P: Multisite activation of the glycogen binding subunit of protein phosphatase-1G by cyclic AMP-dependent protein kinase and glycogen synthase kinase-3.

Fed Eur Biochem Soc 248:67–72, 1989
42. Cohen P, Cohen PTW: Protein phosphatases come of age. *J Biol Chem* 264:21435–38, 1989
43. Czech MP, Klarlund JK, Yagaloff KA, Bradford AP, Lewis RE: Insulin receptor signaling: Activation of multiple serine kinases. *J Biol Chem* 263:11017–20, 1988
44. Klarlund JK, Bradford AP, Milla MG, Czech MP: Purification of a novel insulin-stimulated protein kinase from rat liver. *J Biol Chem* 265:227–34, 1990
45. Podskalny JM, Kahn CR: Cell culture studies on patients with extreme insulin resistance. II. Abnormal biological responses in cultured fibroblasts. *J Clin Endocrinol Metab* 54:269–75, 1982
46. Magre J, Grigorescu F, Reynet C, Caron M, Capony J-P, White MF, Picard J, Mirouze J, Capeau J: Tyrosine-kinase defect of the insulin receptor in cultured fibroblasts from patients with lipoatrophic diabetes. *J Clin Endocrinol Metab* 69:142-50, 1989
47. Longo N, Griffin LD, Shuster RC, Langley S, Elsas LJ: Increase glucose transport by human fibroblasts with a heritable defect in insulin binding. *Metabolism* 38:690–97, 1989
48. Longo N, Shuster RC, Griffin LD, Elsas LJ: Insulin receptor autophosphorylation and kinase activity are constitutively increased in fibroblasts cultured from a patient with heritable insulin resistance. *Biochem Biophys Res Commun* 167:1229–34, 1990