In Skeletal Muscle, Glucose Storage and Oxidation Are Differentially Impaired by the IR\textsuperscript{1152} Mutant Receptor*

(Received for publication, July 19, 1996, and in revised form, December 3, 1996)

Matilde Caruso‡, Claudia Miele§§, Pietro Formisano‡, Gerolama Condorelli‡, Giuseppe Bifulco‡, Andrea Oliva‡, Renata Auricchio‡, Gabriele Riccardi§, Brunella Capaldo¶, and Francesco Beguinot‡¶

From the ‡Dipartimento di Biologia e Patologia Cellulare e Molecolare “L.Califano” and Centro di Endocrinologia ed Oncologia Sperimentale del C.N.R. and the ¶Dipartimento di Medicina Clinica e Sperimentale, “Federico II” University of Naples Medical School, 80131 Naples, Italy

L\textsubscript{6} myotubes expressing the constitutively active Arg\textsuperscript{1152}→Gln insulin receptor (L\textsubscript{6}Arg\textsuperscript{1152}) featured a 31% increased glucose consumption as compared with L\textsubscript{6} cells expressing wild-type receptors (L\textsubscript{6}WT). However, insulin treatment decreased glucose consumption of the mutant cells by 20% while increasing that of the L\textsubscript{6}WT by 30%. In the L\textsubscript{6}WT, insulin elicited a significant increase in glucose transport and GLUT1 and GLUT4 plasma membrane expression, while in the L\textsubscript{6}Arg\textsuperscript{1152}, all of these functions were constitutively activated and not further stimulated by insulin. Similarly, glycogen content and glycogen synthase activity were increased by 80 and 125%, respectively, in the L\textsubscript{6}Arg\textsuperscript{1152} versus the L\textsubscript{6}WT and unaffected by insulin (while a 2-fold increase was measured in insulin-exposed L\textsubscript{6}WT). Glucose oxidation and pyruvate dehydrogenase activity were also 25% higher in the mutant compared with the L\textsubscript{6}WT. However, in the L\textsubscript{6}Arg\textsuperscript{1152}, both functions decreased by 35% in response to insulin (while increasing by 60 and 80%, respectively, in the L\textsubscript{6}WT). Similarly as in the L\textsubscript{6}Arg\textsuperscript{1152}, in vivo, forearm glucose uptake in IR\textsuperscript{1152} patients was 2-fold higher than in control subjects. This difference was not accounted for by higher plasma glucose levels. We conclude that, in skeletal muscle, glucose storage and oxidation are differentially impaired by the expression of IR\textsuperscript{1152}, suggesting that their regulation by insulin involves divergent signaling pathways. Muscle expression of IR\textsuperscript{1152} may contribute to impairing glucose tolerance in IR\textsuperscript{1152} individuals.

Glucose tolerance is largely determined by insulin stimulation of glucose utilization by the liver and skeletal muscle and by insulin suppression of liver glucose output (1, 2). Decreased glucose utilization and increased glucose production by these tissues play an important causal role in generating hyperglycemia in non-insulin-dependent diabetes mellitus (NIDDM) (1, 3). However, the precise molecular mechanisms leading to these defects, as well as the relative contribution of muscle and liver to hyperglycemia in NIDDM are still unclear (1, 3).

A number of insulin receptor mutations have been described in NIDDM individuals (4, 5). Most of these defects have been studied either in cultured lymphocytes/skin fibroblasts from the patients or in transfected fibroblast-like cells. Therefore, the consequences of the mutant receptor expression in tissues relevant to in vivo glucose tolerance have received little attention until very recently. This is an important problem because (i) the expression of mutant insulin receptor alleles may occur in as much as 10% of the NIDDM individuals, and thus they might contribute to impaired glucose tolerance in a significant fraction of these patients (4, 5); and (ii) selective expression of defective receptors in liver or skeletal muscle may provide convenient models for investigating the relative contribution of these tissues to hyperglycemia in NIDDM.

In a family of individuals with NIDDM but no clinical features of the known genetic syndromes of severe insulin resistance, we have identified an insulin receptor point mutation leading to Arg\textsuperscript{1152}→Gln substitution in the regulatory domain of receptor \(\beta\)-subunits (IR\textsuperscript{1152}) (6). IR\textsuperscript{1152} patients exhibit marked skeletal muscle insulin resistance in glucose disposal as compared with most other NIDDM individuals but little change in insulin suppression of liver glucose production (6, 7). This suggested an important involvement of muscle in altering glucose tolerance in these patients. The Arg\textsuperscript{1152}→Gln substitution leads to constitutive activation of receptor kinase and signaling (8–10). In fibroblasts from the IR\textsuperscript{1152} patients and in NIH-3T3 cells expressing IR\textsuperscript{1152}, glucose and aminoacid transport across the plasma membrane and glycogen synthase activity exhibit high basal values and do not further increase in response to insulin (8–11). However, as in the case of most other naturally occurring insulin receptor mutants, the molecular mechanisms leading to IR\textsuperscript{1152} impairment of glucose disposal by skeletal muscle are unclear.

In the present work, this issue has been addressed by in vitro and in vivo studies. It has been shown that, at variance with all other mutations in the kinase domain of the insulin receptor, the Arg\textsuperscript{1152}→Gln substitution results in a gain of...
function enabling the receptor to signal inhibition of pyruvate dehydrogenase activity in response to insulin. Evidence was also obtained that insulin regulates glucose storage and oxidation in skeletal muscle cells through divergent signaling pathways.

**MATERIALS AND METHODS**

**General**—Preparation of plasmid DNA, agarose gel electrophoresis, restriction enzyme digestion, and DNA sequencing were performed by standard methods (12). Enzymes were from Boehringer Mannheim (Kivistegard, Denmark) or Pharmacia Biotech Inc. (Hillerod, Denmark). All radiochemicals as well as monoclonal IgG phosphotyrosine antibodies were from Amersham Corp. (Milano, Italy). mAb3 IR antibody was purchased from Oncogene Science (Manhasset, NY). The transfection reagent (DOTAP) was purchased from Boehringer Mannheim. Media and serum for tissue culture were from Life Technologies, Inc. (Grand Island, NY), electrophoresis and Western blot reagents were from Bio-Rad (Richmond, CA), and sulfo-N-hydroxyxysuccinimide long chain biontin was from Pierce. All other chemicals were from Sigma.

**Transfection, Cell Culture and Mutant Characterization, Insulin Binding, Insulin Receptor Metabolic Labeling, and Phosphorylation—**Wild-type and mutant insulin receptor constructs have been previously described (8). The L6 skeletal muscle cells were seeded (5–9 × 10^4 cells/cm^2) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2% fetal bovine serum as described previously (14). Under these cultural conditions, the myoblasts reach the confluence and then spontaneously differentiate into myotubes. Cationic liposome-mediated transfection of L6 myoblasts was performed according to Levine et al. (15) and to the DOTAP reagent manufacturer instructions. G-418 (Life Technologies, Inc.) was used at the effective dose of 0.8 mg/ml. Individual G-418-resistant clones were isolated and screened by ^125^I-insulin binding. Neither the cell clones expressing the wild-type or those expressing the mutant receptors exhibited significant change in morphology compared with the untransfected cells either at the myoblast or the myotube stage of differentiation. Based on fusion index (the relative proportion of nuclei in myotubes and in mononucleated cells (16)) and quantitation of creatine kinase activity, the ability of the L6 myoblasts to differentiate into myotubes as well as the differentiation time course were also unchanged upon transfection (Table I).

Unless differently specified, all of the biochemical studies shown in this work were performed with L6 cell clones (parental and transfected) at the myotube stage. In order to minimize the likelihood of nonspecific clonal variation however, control studies were also accomplished with the pools of transfected cells. Under no circumstance were discrepancies between the pool and the corresponding cell clones observed. ^125^I-insulin binding was studied as described previously (17) and analyzed according to Scatchard (18). Insulin receptor metabolic labeling, auto-phosphorylation, and kinase activity were studied as described earlier (8). CK activity was determined in the presence of diadenosine pentaphosphate using a previously described method (19).

**Glucose Disposal, 2-Deoxy-D-glucose and 3-0-methylglucose Uptake—**For glucose utilization studies, L6 myotubes were maintained in DMEM supplemented with 2% serum and 100 mg/dl glucose for 6 days. The cells were then incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air in an incubator. Glucose uptake was analyzed as described in Ref. 21. 3-O-methylglucose uptake was analyzed as described earlier (22). Detection of Glucose Transporters at the Cell Surface and in Total Cell Extracts—Cell surface expression of glucose transporters was analyzed according to Levy-Toledano, et al. (23). Briefly, myotubes were pre-incubated in serum-free DMEM supplemented with 0.25% BSA for 24 h and then further incubated in the absence or presence of 100 nM insulin for 1 h. The cells were rinsed and treated with 0.5 mg/ml N-hydroxysuccinimide long chain biontin in PBS containing 0.1 mm CaCl_2, 1 mm MgCl_2, pH 7.4 (biontinylation buffer), for 30 min at 4°C. The biontinylation reaction was quenched by adding 15 mm glucose in biotynilation buffer. The cells were then lysed in a solution containing 1% Triton X-100, 50 mm Hepes, pH 7.4, 10 mm Na_3PO_4, 100 mm NaF, 4 mm EDTA, 2 mm Na_3VO_4, 2 mm phenylmethylsulfonyl fluoride, and 0.2 mg/ml aprotinin, and proteins were precipitated with either GLUT1 or GLUT4 antibodies. Precipitated proteins were separated by 10% SDS-PAGE and transferred on nitrocellulose filters by blotting at 180 mA for 1 h (transfer buffer contained 25 mM Tris, 192 mm glycine, 20% methanol). Biotinylated transporters were finally revealed by incubation with peroxidased streptavidin and detection of chemiluminescence by autoradiography.

For determination of total transporter content, cell lysates (prepared as described above) were subjected to 10% SDS-PAGE and Western blotting as described above. Filters were incubated with GLUT1 or GLUT4 antibodies for 14 h at 4°C and then with peroxidase-anti-antibodies for 1 h at room temperature. Transporters were finally revealed by detection of chemiluminescence by autoradiography.

**Cell Glycogen Content and Glycogen Synthase Activity—**Glycogen content was determined as described by Keppler et al. (24). Briefly, myotubes were maintained in serum-free DMEM supplemented with 0.25% BSA in the absence or the presence of 100 nM insulin for 12 h. Cells were then collected in 0.6 N HClO_4, homogenized using a glass-teflon potter, and centrifuged at 1500 rpm for 10 min at 4°C. Aliquots of the homogenate were incubated with 0.033 m KHCO_3 and 9 mg/ml amyloglucosidase in 0.2 m acetate buffer, pH 4.8, for 2 h at 40°C. The reaction was stopped by addition of 0.6 N HClO_4 and centrifugation at 15,000 rpm for 4°C for 15 min. Glycose concentration was determined with a Beckman glucose analyzer.

Glycogen synthase activity was determined by a modification of the method of Thomas et al. (25). For this assay, myotubes were preincubated in Hepes buffer, pH 7.4, for 3 h at 37°C. The cells were then exposed to 100 nM insulin for 30 min at 37°C, rinsed with ice-cold 100 mM NaF, 10 mM EDTA, collected, and centrifuged at 2,000 rpm for 4°C for 10 min. The pellet was resuspended in 100 mM NaF and 10 mM EDTA, sonicated for 10 s at 300 watts at 4°C, and further centrifuged at 2,000 rpm for 10 min at 4°C. 20-μl aliquots of the supernatants were added to 60 μl of a reaction mixture containing 40 mM Tris-HCl, pH 7.8, 25 mM NaF, 20 mM EDTA, 10 mg/ml glycogen, 7.2 mg uridine 5'-diphosphate-glucose (UDPG), and 0.05 μM [14C]UDPG in the absence or the presence of 6.7 μM glucose 6-phosphate (final concentrations). The incubation was prolonged for 20 min at 30°C and the reaction terminated by precipitating 75 μl of the mixture on 2×2 cm squares of filter paper with cold ethanol. The filter papers were extensively washed with cold ethanol, and radioactivity was counted in a Beckman scintillation counter. Enzyme activity was expressed as percent of the glucose 6-phosphate-independent form.

**Glucose Oxidation and Pyruvate Dehydrogenase Activity—**Glucose oxidation was determined by a modification of the method of Lowe and coworkers (26). Briefly, for these experiments, cells were grown in 50-ml flasks and preincubated in serum-free DMEM for 18 h before the experiment. This medium was substituted with Joklik medium supplemented with 25 mM NaHCO_3, 5.55 mM [1-14C]glucose, 1.2 mM MgSO_4, 0.5 mM CaCl_2, 10 mM Hepes, pH 7.4, in the absence or the presence of 100 nM insulin, and the incubation was prolonged for 20 min at 30°C after capping the flasks with rubber stoppers containing a hanging well.
filled with rolled filter paper. 0.4 ml 1 m hyamine hydroxide in methanol was then injected through the rubber stoppers into the hanging wells followed by injection into the incubation medium of 0.4 ml 10% HClO4. The flasks were allowed to sit for 2 more h at 37°C. The filter papers were then removed and counted in a β-counter. Pyruvate dehydrogenase activity was assayed as the release of 14CO2 from (1-14C)pyruvic acid according to Seals and Jarett (27). For these assays, 100-mm cell dishes were incubated for 10 min at 37°C in the absence or the presence of 100 nM insulin in DMEM with 10 mM Hepes, pH 7.4, 0.2% BSA. This medium was supplemented with either 0.5 mM CaCl2, 10 mM MgCl2 (for determination of total pyruvate dehydrogenase (PDH) activity), or 10 mM dithiothreitol and 10 mM NaF (for determination of basal activity (28)). Cell extracts were obtained according to Benelli et al. (29), and 50-μl aliquots were added to 200 μl of 50 mM Tris-HCl, pH 7.4, 50 mM CaCl2, 50 mM MgCl2 in 17 × 100 mm polypropylene tubes and incubated. The assay was then initiated by addition of 1 mM dithiothreitol, 100 mM NaF, 50 mM KCl, 0.1 mM EDTA, and 10 μCi [1-14C]pyruvic acid (specific activity 9.8 mCi/mM), 0.5 mM β-NAD, 0.1 mM L-carnitine, and 1 mg/ml acetyl-CoA (final concentrations). The tubes were immediately capped with a rubber stopper through which was suspended a plastic column containing a small roll of filter paper. 14CO2 was collected for 1 h by injecting 0.2 ml of 1 m hyamine hydroxide through the rubber stopper onto the center wells. Radioactivity in this was determined in a scintillation counter. Blank values were determined by using boiled extracts under the same assay conditions as above and subtracted from all of the other values. Results were expressed in [14CO2] nCi/min/mg extract protein. Lactate concentration in the culture medium was determined as by Hohorst (30).

**TABLE II**

| Cell clone | Kd (nM) | Receptors/cell |
|------------|---------|----------------|
| L6         | 1.6     | 3,000          |
| WT1        | 1.8     | 32,000         |
| WT2        | 1.4     | 18,000         |
| WT3        | 1.5     | 9,000          |
| Mut1       | 2.0     | 30,000         |
| Mut2       | 1.2     | 19,000         |
| Mut3       | 1.6     | 10,000         |

**RESULTS**

The molecular mechanisms leading to the abnormal glucose disposal in muscle of the IR1152 individuals (6) were addressed by transfecting the mutant IR cDNA in the L6 cultured skeletal muscle cells. These cells maintain the ability to differentiate in culture and have been widely used as a cell model for studies on insulin action. Clonal cell lines were screened for expression of IR1152 by the method described in Materials and Methods. Results are given in Table I. Based on these analyses, all of these clones exhibited dissociation constants (Kd) for insulin between 1.2 and 2 nM. This is similar to the Kd values of the WT and mutant receptors expressed in other cells and to that of the endogenous IR measured in untransfected L6 cells. Thus, all transfected cell clones analyzed in this work exhibited normal insulin binding affinities in addition to comparable receptor levels.

To ensure that IR mutants were properly processed and transported to the cell surface, extracts were prepared from cells metabolically labeled with [35S]methionine. The radiolabeled IRs were then immunoprecipitated with anti-IR mAb3. In the two cell lines expressing the mutant IRs as well as in those expressing the WT IRs (Fig. 1, lanes B-G), these antibodies immunoprecipitated two proteins migrating at Mr = 130,000 and 95,000, which correspond to IR α and β-subunits, respectively. Based on laser densitometry, the intensity of these bands correlated well with the number of cell-surface receptors as measured by insulin binding, indicating that transfected receptors were normally synthesized and transported to the plasma membrane in the L6 cells. α and β-subunits were barely visible in untransfected L6 cells, which express a very low number of endogenous IRs (Fig. 1, lane A).

Based on pulse-chase experiments with [35S]methionine, there was no significant difference in the rate of IR biosynthesis between any of the transfected and the endogenous receptors (data not shown).

Upon in vitro autophosphorylation, receptor β-subunits migrated as 95 kDa bands on SDS gels (Fig. 2.). Phosphorylation increased by 2- to 5-fold upon insulin stimulation of untransfected and wild-type cells (Fig. 2, lanes A-F). In contrast, the mutant receptor exhibited almost no insulin-stimulated phosphorylation when expressed in L6 cells (Fig. 2 lanes G and K). In the absence of insulin, phosphorylation of the endogenous substrate IRS-1 was also very low in parental cells and in those expressing WT receptors while increasing by 2- to 5-fold, respectively, after insulin exposure. At variance, in the mutant cells, IRS-1 exhibited high basal phosphorylation and no increase after insulin exposure. Thus, as previously shown in human skin fibroblasts (11) and in other transfected cell types (8–10), in the L6 cells, IR1152 exhibited reduced autophosphorylation but constitutively increased kinase activity toward endogenous substrates.

To investigate the consequence of IR1152 expression on glucose disposal by the L6 cells, extraction of glucose from the culture medium was first quantified. Upon 12-h incubation in 100 mg/dl DMEM, untransfected cells extracted 0.85 ± 0.04 mg (value ± S.D.) of glucose/106 cells (Fig. 3, top, bar A). Basal glucose extraction by these cells did not significantly differ from that of the WT (Fig. 3, top, bars C, E, and G). During the same time period, however, the mutant cells had 1.02 ± 0.05 mg of glucose disposed (31% more than did the WT cells; difference was significant at the p < 0.01 level) (Fig. 3, top, bars F, I, and M). A 25% increase in basal glucose disposal was also measured with the mutant relative to the WT cell pools (data not shown). The presence of insulin into the culture medium increased glucose consumption by 20 and 30% in parental and wild-type cells, respectively (data not shown). Basal activity of glucose-6-phosphatase and incubation for 5 min at 37°C (31). Blank values were obtained by incubating the samples in the absence of glucose-6-phosphatase and subtracted from all of the other values.

In Vivo Studies—The subjects were studied in the postabsorptive state after a 15–17-h overnight fast. Glucose utilization in the forearm was calculated by subtracting the glucose concentration in a deep antecubital vein of the forearm (cannulated retrogradely) for glucose concentration measured in the brachial artery and multiplying this difference by the forearm flow measured by the dye technique (32).

To investigate the consequence of IR1152 expression on glucose disposal by the L6 cells, extraction of glucose from the culture medium was first quantified. Upon 12-h incubation in 100 mg/dl DMEM, untransfected cells extracted 0.85 ± 0.04 mg (value ± S.D.) of glucose/106 cells (Fig. 3, top, bar A). Basal glucose extraction by these cells did not significantly differ from that of the WT (Fig. 3, top, bars C, E, and G). During the same time period, however, the mutant cells had 1.02 ± 0.05 mg of glucose disposed (31% more than did the WT cells; difference was significant at the p < 0.01 level) (Fig. 3, top, bars F, I, and M). A 25% increase in basal glucose disposal was also measured with the mutant relative to the WT cell pools (data not shown). The presence of insulin into the culture medium increased glucose consumption by 20 and 30% in parental and wild-type cells, respectively (data not shown). Basal activity of glucose-6-phosphatase and incubation for 5 min at 37°C (31). Blank values were obtained by incubating the samples in the absence of glucose-6-phosphatase and subtracted from all of the other values.

In Vivo Studies—The subjects were studied in the postabsorptive state after a 15–17-h overnight fast. Glucose utilization in the forearm was calculated by subtracting the glucose concentration in a deep antecubital vein of the forearm (cannulated retrogradely) for glucose concentration measured in the brachial artery and multiplying this difference by the forearm flow measured by the dye technique (32).
glucose disposal by the L6 muscle cells but reverted normal insulin regulation.

In the basal state, the initial rate of 2-DG uptake was also very similar in untransfected and WT cells (1.2–1.3 nmol mg⁻¹ min⁻¹; Fig. 3, middle panel, bars A, C, E, and G). In comparison, the mutant cells exhibited a 54% increased uptake (bars I, M, and O; difference significant at the p < 0.001 level). The difference between WT and Mut cells was accounted for by an increase in the Vₘₐₓ of the transport in Mut cells, with no detectable change in Kₘ values (data not shown). Insulin induced a 61% increase in 2-DG uptake in the parental and wild-type cells (Fig. 3, middle panel, bars A, C, E, and G) while in the mutant, insulin-stimulated uptake exhibited only a small increase compared with the basal values (Fig. 3, middle panel, bars L, N, and P). Similar to 2-DG, the basal uptake of the non-phosphorylatable glucose analog 3-O-methylglucose was 2-fold increased in mutant relative to the WT cells and did not further increase in response to insulin (while increasing by 2.3-fold in insulin-stimulated WT cells; Fig. 3, bottom panel). It appeared, therefore, that glucose transport could contribute to the augmented non-insulin-dependent glucose disposal but not to the decrease measured upon exposure of the mutant cells to insulin.

This issue was further investigated by first analyzing total cellular content of GLUT4 and GLUT1 since these are important transporter isoforms well represented in the L6 cells. The cells were solubilized, and transporter content was studied by Western blot using specific GLUT1 and GLUT4 mAb. Based on laser densitometry, there was little difference in total transporter content between parental and WT cells (Fig. 4, lanes A-C). However, in four independent experiments with all of the clones reported in the present paper, mutant cells exhibited a 110 ± 5 and 61 ± 4% (values ± S.D.) decrease in GLUT1 and GLUT4, respectively, compared with the WT (Fig. 4, lanes D, and E; p < 0.01), suggesting that the constitutively active receptor down-regulated the transporters. Plasma membrane content of the two transporters was also markedly different in wild-type and mutant cells both in the absence and in the presence of insulin. Based on precipitation of biotinylated cell surface proteins with GLUT1 or GLUT4 mAb followed by streptavidin blotting and HRP detection, in five independent experiments, GLUT4 was almost absent from the plasma mem-
branched from basal parental or WT cells while GLUT1 was consistently revealed (Fig. 5 shows a representative experiment). In comparison, Mut cells exhibited 7 ± 0.8-fold increased membrane GLUT4 levels and a 50 ± 4% increase in that of GLUT1 compared with the WT/parental cells (difference between Mut and WT cells was significant at the p < 0.001 level). GLUT4 and GLUT1 membrane content increased by 7.6 ± 1 and 0.6 ± 0.08-fold, respectively, in both parental and WT cells after exposure to insulin (10^{-7} M, maximally effective concentration; difference relative to the basal was significant at the p < 0.001 level). No further membrane recruitment occurred upon insulin stimulation of the Mut cells either in the case of GLUT4 or of GLUT1 so that their relative surface amounts in WT versus the Mut cells were 1.08 and 1.20, respectively.

To further address the molecular basis of the abnormal glucose utilization by the IR^{152} cells, we have individually analyzed the major intracellular routes of glucose disposal. As shown in Fig. 6, top, basal glycogen contents in parental/WT cells and in the mutants were 1.2 and 2.2 μg/mg of protein, respectively (83% difference, significant at the p < 0.001 level). Insulin exposure of the cells for 12 h increased glycogen content in WT cells by 2-fold but did not result in any significant further glycogen accumulation by the Mut cells. Under basal conditions, [14C]glucose conversion into 14CO2 was also increased by 20% in the mutant as compared with the WT cells (Fig. 6, middle panel; p < 0.001). This increase in basal glucose oxidation was accompanied by a slight decrease in the ratio between the appearance of lactate into the culture medium and glucose disposal by the Mut cells (10%; p < 0.01; Fig. 6, bottom panel). Interestingly, insulin exposure increased 14CO2 production of the parental and WT cells by 35–65% (significant at the p < 0.001 level) but reduced that of the mutant cells by about 30% (p < 0.001) (25% decrease with the Mut cell pools; data not shown). This decrease was not due to receptor down-regulation of the mutant receptor since the mutant does not undergo insulin-induced internalization and down-regulation either in fibroblasts (9) or in the L6 cells (data not shown). While the ratio between the appearance of lactate into the culture medium and glucose disposal slightly decreased in parental and WT cells upon stimulation with insulin (10% decrease, significant at the p < 0.01 level), it increased by >20% in the Mut cells (p < 0.001).

The activity of glycogen synthase and PDH were also characterized. In parental and WT cells, glycogen synthase activity increased by 2.1- to 2.6-fold, respectively, in response to insulin (Fig. 7, top; p < 0.001). At variance, in Mut cell extracts, basal activity of the enzyme was comparable to that measured in maximally insulin-stimulated WT cells but did not exhibit further significant increase following insulin stimulation. Basal PDH activity in the Mut cells also appeared similar to that measured in insulin-stimulated parental/WT cells (Fig. 7, bottom). In parental/WT cells, insulin addition for 10 min elicited a 60–80% increase in the activity of PDH (significant at the p < 0.001 level). This acute effect was decreased to 30% above the basal activity when preceded by a 48-h preincubation of the cells with 100 nM insulin (significant at the p < 0.001 level; data not shown). At variance from the WT/parental myotubes, in the Mut cells, acute insulin exposure resulted in a 34% decrease in the activity of this enzyme (significant at the p < 0.01 level).

If the inhibition of PDH and glucose to CO2 conversion were responsible for the decreased glucose disposal occurring in the Mut cells after exposure to insulin, one would also predict glucose intracellular accumulation since the glucose transport system appears to be constitutively activated in these cells and glycogen accumulation exhibits no change. To verify this hypothesis, Glu-6-P intracellular concentrations were directly measured. Note, in Fig. 8, that basal Glu-6-P concentrations were 0.033 nmol/l in parental/WT cells and 0.023 nmol/l in the
mutants (difference significant at the p < 0.01 level) consistent with an accelerated glucose consumption rate in the latter cell type. However, after exposure to insulin, Glu-6-P concentrations increased to 0.037 nmol/l in the Mut cells (p = 0.001) but decreased to 0.022–0.025 in the parental and WT cells. 

To further address the role of IR1152 in altering glucose clearance by skeletal muscle, we have analyzed in vivo the forearm glucose uptake in the basal state in an IR1152 individual. In two independent studies, basal uptake was 1.85 ± 0.05 (value ± range; Table III) in this individual. This value was slightly higher than that measured in the same IR1152 individual during euglycemic hyperinsulinemic clamp (1.4 mg/l/min (6)). However, similarly as in the Mut cells, the basal glucose uptake in the IR1152 individual was ~120% higher than that in control subjects whether NIDDM or non-diabetic. Hyperglycemia could not account for this difference since the NIDDM control group was significantly more hyperglycemic than the IR1152 patient. It appeared therefore that, in vivo as well as in vitro, expression of IR1152 in skeletal muscle results in increased non-insulin-dependent glucose disposal and impaired glucose utilization in response to insulin.

**DISCUSSION**

As in the case of most other naturally occurring insulin receptor mutations, the role of IR1152 in impairing glucose tolerance at the level of each individual major insulin target tissue has not been investigated. In the present work, this issue has been addressed by studying the effect of IR1152 expression on glucose disposal by skeletal muscle in vitro and in vivo. The L6 skeletal muscle cells have been used as an in vitro model since, once differentiated into myotubes, these cells acquire several characteristics of adult skeletal muscle (17, 34). In addition, these cells have been widely used for studies on insulin action (14, 17, 35–38), and therefore they represent a well-characterized model system for these aims.

Similarly as in human fibroblasts and transfected NIH-3T3 cells (14, 10, 11), once expressed in the L6 myotubes, IR1152 exhibited no insulin-stimulated autophosphorylation but constitutively increased kinase activity toward the IRS-1 endogenous substrate. IR1152 expression also decreased total GLUT1 and GLUT4 glucose transporters into the cells suggesting down-regulation of the two transporter systems by the constitutively active IR1152 kinase. However, plasma membrane content of GLUT1 and GLUT4 was significantly increased in basal IR1152 cells (Mut) as compared with myotubes expressing WT receptors and did not further increase upon exposure to insulin.
IR1152-induced activation of the glucosetransport system of the depression of glucose metabolism in response to insulin; and (ii) addition, the activity of the synthase measured not either increase or decrease in the presence of insulin. In cells also appeared maximally activated, and its function did glucose consumption in the mutant cells did not appear to be tion. The intracellular route involved in insulin inhibition of tion but not for the inhibition occurring upon insulin stimula-

cellsthat accounts for the basal increase in glucose consump-
tion by the Mut cells truly reflects decreased glucose oxidation, and that (ii) insulin-induced inhibition of PDH through the IR1152 may be responsible for the observed impairment in glucose oxidation. Decreased glucose oxidation, in turn, could account for the decrease in glucose consumption occurring after insulin exposure of the Mut cells. If this hypothesis holds, one would also predict intracellular glucose accumulation when the mutants are exposed to insulin since the glucose transporter and glycolytic synthetic systems of these cells remained activated in the presence as well as in the absence of insulin. Consistently, Glu-6-P, the major intracellular form of free glucose, raised significantly when the Mut myotubes were exposed to insulin while decreasing in the wild-type and the untransfected cells.

A variety of mutations have been found or generated in the regulatory region of the insulin receptor (4, 5). To our know-
ledge, however, the Arg1152→Gln substitution is the first defect shown to result in a net gain of function by the receptor, i.e. the receptor acquires the ability to signal a novel effect (inhibition of PDH activity in response to insulin). This gain of function appears to be unique to the mutation rather than due to the chronic signaling by the constitutively active receptor since chronic exposure of WT cells to insulin does not allow a subse-
quent acute stimulation by insulin to inhibit PDH activity (data not shown). Interestingly, in the L6 muscle cells, IR1152 is capable of transducing an insulin signal on glucose oxidation but not on glucose storage. One might argue, therefore, that divergent signaling pathways control the two major pathways of glucose metabolism in the L6 cells so that each of the two is differentially affected by the mutant receptor activity. Due to both redundancy and/or complementation in signaling pathways (13, 33), the precise sequence of molecular events involved in insulin regulation of glucose storage and oxidation in muscle is still unclear. There is evidence, however, suggesting that activation of PKC is required for normal insulin stimulation of PDH activity (29). As previously shown, the mutation region in IR1152 is crucial for allowing PKC to phosphorylate the receptor (10). We also have preliminary evidence that IR1152-associated PKC as well as IR1152-associated PKC activity decrease below the basal levels after insulin stimulation of the receptor. 2 We suggest, therefore, that the inhibition of PDH activity below basal levels, occurring in the Mut cells when exposed to insulin, might depend on the abnormal interaction of the mutant receptor with PKC.

The relevance of these observations to IR1152 expression in skeletal muscle in vivo is also underlined by our findings on the forearm arterial-deep venous glucose differences in IR1152 pa-

2 P. Formisano, C. Miele, M. Caruso, and F. Beguinot, manuscript in preparation.
patients. Similarly as in the L6 myotubes, basal glucose disposal by the forearm was >2-fold greater in the patient than in control individuals. In addition, separate studies in these patients (6) have shown that forearm glucose uptake during hyperinsulinemia was even slightly smaller than that measured in the basal state although increasing by 10-fold in control individuals. We suggest, therefore, that similar mechanisms impair glucose metabolism in skeletal muscle expressing IR1152 in vitro and in vivo.

In summary, in the present paper, we have shown that the Arg1152→Gln substitution in the insulin receptor results in a gain of function for the receptor, i.e. the ability to signal PDH depression in response to insulin in vitro and in vivo. Analysis of IR1152 in skeletal muscle suggests that insulin regulation of glucose storage and oxidation involves divergent signaling pathways.

Acknowledgments—We are grateful to Dr. S. Gammeltoft (Bispebjerg Hospital, Copenhagen) for generously donating the WT IR cDNA and to Dr. L. Beguinot (DIBIT and Istituto di Neuroscienze e Bioimmagini H.S. Raffaele, Milan) for reviewing the manuscript. We also thank Drs. S.M. Aloj, E. Consiglio, G. Salvatore, and G.C. Vecchio (University of Naples Medical School) for continuous support and advice during the course of this work and Dr. D. Liguoro for technical help.

REFERENCES

1. Beck-Nielsen, H., Hother-Nielsen, O., Vaag, A., and Alford, F. (1994) Diabetologia 37, 217–221
2. Mitrakou, A., Kelley, D., Veneman, T., Jansen, T., Pangburn, T., Reilly, J., and Jerich, J. (1990) Diabetes 39, 1381–1390
3. Kahn, C. R. (1994) Diabetes 43, 1066–1084
4. Taylor, S., Cama, A., Accili, D., Barbetti, F., Quon, M. J., De La Luz Sierra, M., Suzuki, Y., Koller, E., Levy-Toledano, R., Wertheimer, E., Moncada, V. Y., Kadowaki, H., and Kadowaki, T. (1992) Endocr. Rev. 13, 28–57
5. Taylor, S., Wertheimer, E., Accili, D., Cama, A., Hone, J., Roach, P., Quon, M. J., Suzuki, Y., Levy-Toledano, R., Tsouls, M., De La Luz Sierra, M., Barbetti, F., and Gorden, P. (1994) Endocr. Rev. 2, 58–65
6. Coccozza, S., Porcellini, A., Riccardi, G., Mastroielli, A., Condorelli, G. L., Ferrara, A., Pianese, L., Miele, C., Capaldo, B., Beguinot, F., and Varrone, S. (1992) Diabetes 41, 521–526
7. Caruso, M., Miele, C., Capaldo, B., Palumbo, G., Bifulco, G., Oliva, A., Riccardi, G., and Beguinot, F. (1995) Proc. Endocrin. Soc., P 3–277A
8. Formisano, P., Sohn, K-J., Miele, C., Di Finizio, B., Petruzzello, A., Riccardi, G., Beguinot, L., and Beguinot, F. (1993) J. Biol. Chem. 268, 5241–5248
9. Formisano, P., DeNovelis, G., Miele, C., Tripedi, F., Caruso, M., Palumbo, G., Beguinot, L., and Beguinot, F. (1994) J. Biol. Chem. 269, 16242–16246
10. Miele, C., Formisano, P., Sohn, K-J., Caruso, M., Pianese, M., Palumbo, G., Beguinot, L., and Beguinot, F. (1995) J. Biol. Chem. 270, 15844–15852
11. Petruzzello, A., Formisano, P., Miele, C., Di Finizio, B., Riccardi, G., Ferrara, A., M., Beguinot, L., and Beguinot, F. (1995) J. Clin. Endocrinol. & Metab. 77, 409–412
12. Davis, L. A., Dibner, M. D., and Battey, J. F. (1986) Basic Methods in Molecular Biology, Elsevier/North Holland, New York
13. Taramento, H., Kadowaki, T., Toke, T., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Sato, T., Sekihara, H., Yoshioka, S., Horikoshi, H., Furuta, Y., Ikawa, Y., Kasuga, M., Yazaki, Y., and Aizawa, S. (1994) Nature 372, 182–186
14. Klop, A., and Ramlal, T. (1987) Biochem. J. 242, 131–136
15. Levine, A., Cantoni, G. L., and Razin, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10119–10123
16. Easton, T. G., and Reich, E. (1972) J. Biol. Chem. 247, 6420–6431
17. Beguinot, F., Kahn, C. R., Moses, A. C., and Smith, R. J. (1986) Endocrinology 118, 446–455
18. Scotter, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–675
19. Ingwall, J., and Fossel, E. T. (1983) in Perspectives in Cardiovascular Research (Alpert, N. R., ed.) Vol. 7, p. 601, Raven Press, Ltd., New York
20. Trinder, P. (1969) Anal. Biochem. 25, 486–499
21. Levy-Toledano, R., Caro, L. H. P., Hindman, N., and Taylor, S. (1993) J. Biol. Chem. 268, 15844–15852
22. Walker, P. S., Ramlal, T., Donovon, J. A., De La Luz Sierra, M., and Desbuquois, B. (1988) Metabolism 37, 1101–1106
23. Levy-Toledano, R., Caro, L. H. P., Hindman, N., and Taylor, S. (1993) Endocrinology 133, 1803–1808
24. Keppler, D., and Decker, K. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) 2nd Ed., Academic Press, New York
25. Thomas, J., Schlenker, K., Larner, J. (1968) Anal. Biochem. 25, 486–499
26. Abdel-Aleem, S., Li, X., Pessino, A., Beguinot, L., and Beguinot, F. (1993) J. Biol. Chem. 268, 15892–15898
27. Seals, J. R., and Jarett, L. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 71–81
28. Clot, J. P., Benelli, C., de Galli, B., Pastel Viny, M. C., Durant, D., and Desbuquois, B. (1988) Metabolism 37, 1101–1106
29. Benelli, C., Caron, M., de Galli, B., Fouque, F., Cherqui, G., and Clot, J. P. (1994) Metabolism 43, 1030–1034
30. Hohorst, H. J. (1957) Biochem. Z. 328, 509–521
31. Lang, G., and Michal, G. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) 2nd Ed., pp. 1238–1244, Academic Press, New York
32. Capaldo, B., Santoro, D., Riccardi, G., Perrotti, N., Saccà, L. (1986) J. Clin. Invest. 77, 1285–1290
33. Araki, E., Lipes, M. A., Patti, M. E., Bruning, J. C., Haag, R., Johnson, R. S., and Kahn, C. R. (1994) Nature 372, 186–190
34. Yaffe, D. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 467–471
35. Beguinot, F., Kahn, C. R., Moses, A. C., and Smith, R. J. (1985) J. Biol. Chem. 260, 15892–15898
36. Wang, P. H., Beguinot, F., and Smith, R. J. (1987) Diabetologia 30, 797–803
37. Beguinot, F., Kahn, C. R., Moses, A. C., White, M. F., and Smith, R. J. (1989) Endocrinology 150, 1609–1615
38. Beguinot, F., Smith, R. J., Kahn, C. R., Maron, R. Moses, A. C., and White, M. F. (1988) Biochemistry 27, 3222–3228
39. Bell, G. J., Burnot, C. P., Takeda, J., and Gould, G. W. (1993) J. Biol. Chem. 268, 19161–19164
40. Kuoivisto, U.-M., Martinez-Valdez, H., Bilan, P. J., Burdett, E., Ramlal, T., and Klop, A. (1991) J. Biol. Chem. 266, 2615–2621
41. Tanokura, M., Verdin, M., and Klop, A. (1994) J. Biol. Chem. 269, 29934–29942
42. Czech, M. P., Clancy, B. M., Pessino, A., Woon, C.-W., and Harrison, S. (1992) Trends Biochem. Sci. 17, 197–201
43. Mandarino, L. J., Wright, K. S., Verity, L. S., Nichols, J., Bell, J. M., Kolterman, O. G., and Beck-Nielsen, H. (1987) J. Clin. Invest. 80, 655–663