Insulin receptor autophosphorylation in cultured myoblasts correlates to glucose disposal in Pima Indians

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1Department of Medicine, Division of Diabetes and Endocrine Research, Mount Zion Medical Center, University of California, San Francisco, California, 94143-1616; and 2Clinical Diabetes and Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Phoenix, Arizona 85016

Youngren, Jack F., Ira D. Goldfine, and Richard E. Pratley. Insulin receptor autophosphorylation in cultured myoblasts correlates to glucose disposal in Pima Indians. Am. J. Physiol. 276 (Endocrinol. Metab. 39): E990–E994, 1999.—In a previous study [Youngren, J. F., I. D. Goldfine, and R. E. Pratley. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E276–E283, 1997] of skeletal muscle biopsies from insulin-resistant, nondiabetic Pima Indians, we demonstrated that diminished insulin receptor (IR) autophosphorylation correlated with in vivo insulin resistance. In the present study, to determine whether decreased IR function is a primary trait of muscle, and not secondary to an altered in vivo environment, we cultured myoblasts from 17 nondiabetic Pima Indians in whom insulin-stimulated glucose disposal (M) was measured during hyperinsulinemic-euglycemic glucose clamps. Myoblast IR autophosphorylation was determined by a highly sensitive ELISA. IR autophosphorylation directly correlated with M (r = 0.56, P = 0.02) and inversely correlated with the fasting plasma insulin (r = −0.58, P < 0.05). The relationship between M and IR autophosphorylation remained significant after M was adjusted for the effects of percent body fat (partial r = 0.53, P < 0.04). The relationship between insulin resistance and the capacity for myoblast IR autophosphorylation in nondiabetic Pima Indians suggests that variations in IR-signaling capacity may be intrinsic characteristics of muscle that contribute to the genetic component determining insulin action in this population.

resistance. Over 50% of adult Pimas have the disease (12), making this an important group in which to study the causes of insulin resistance and its role in the development of type 2 diabetes. Prior studies in Pimas have indicated that insulin resistance occurs both in subjects with impaired glucose tolerance (14) and in many individuals with normal glucose tolerance (15). In normoglycemic Pimas, resistance to insulin is involved in the progression to impaired glucose tolerance and is the single strongest predictor of future development of type 2 diabetes (15). Both inherited factors and obesity, which is also highly prevalent in this group, contribute to insulin resistance in the Pima Indians (12, 13). Insulin resistance is a familial trait in Pimas (16) and also increases as a function of increasing body fat (2). The cellular mechanisms of insulin resistance in Pimas as well as other groups are unknown.

Cellular insulin action occurs after insulin binding to the extracellular α-subunits of the insulin receptor (IR), which results in autophosphorylation of specific tyrosine residues on the transmembrane β-subunits (20). This autophosphorylation activates the protein tyrosine kinase activity of the IR, which then phosphorylates key intracellular substrates and initiates the cellular mechanisms involved in enhancing glucose uptake, glycogen synthesis, and other responses to insulin (11). We have previously studied muscle biopsies from insulin-resistant, nondiabetic Pima Indians to investigate the biochemical mechanisms of insulin resistance in individuals without the hyperglycemia and other overt metabolic abnormalities associated with diabetes mellitus. We observed that IR autophosphorylation and protein tyrosine kinase activity were impaired in skeletal muscle tissue samples from insulin-resistant, nondiabetic Pima Indians and that the signaling capacity of muscle IR was strongly correlated with whole body insulin action (23). The relationship between decreased IR autophosphorylation and decreased in vivo insulin action was independent of obesity. This observation suggested that a low IR-signaling capacity may contribute to insulin resistance in this population both related to and independent of obesity.

To further understand whether insulin resistance is an intrinsic characteristic of muscle in these individuals, we have employed primary muscle cell cultures...
from nondiabetic Pima Indians to study insulin action in muscle removed from an altered physiological environment in vivo (22). In myoblasts from nondiabetic Pimas, impaired glucose incorporation into glycogen in culture is correlated with in vivo insulin resistance (22). If the range of IR-signaling capacities observed in muscle biopsies is also present in cultured myoblasts, an impairment in this first step of the insulin-signaling pathway could explain the insulin resistance observed in myoblasts. In the present study, we have examined IR autophosphorylation in myoblasts from nondiabetic Pima Indians to determine whether reduced IR function was an intrinsic characteristic of muscle in insulin-resistant individuals.

METHODS

Subjects. Seventeen Pima Indians (11 males, 6 females) participating in an ongoing longitudinal study of risk factors for type 2 diabetes mellitus agreed to undergo muscle biopsies for this study. All subjects provided written informed consent according to the guidelines of the National Institute of Diabetes and Digestive and Kidney Diseases Institutional Review Board and the Tribal Council of the Gila River Indian Community before participation. All subjects were healthy as judged by a medical history, a physical examination, and routine clinical laboratory tests. None of the subjects was taking medications that could affect plasma insulin concentrations or glucose metabolism. Percent body fat was estimated by dual-energy X-ray absorptiometry (21). Insulin action was measured in vivo with a hyperinsulinemic-euglycemic glucose clamp procedure (22). Briefly, 100- to 500-mg biopsies were plated in uncoated 25-cm² flasks and were finely minced, and the cells were dissociated by incubating with 0.25% trypsin, 0.1% type IV collagenase, and 0.1% BSA for 30 min. The cells were collected by centrifugation at 150 g and then were plated in uncoated 25-cm² flasks for 1 h at 37°C to remove fibroblasts. The residual cellular material was transferred to fresh 60-mm² plates coated with 0.01% type I collagen. Myoblasts were grown in DMEM supplemented with 25 mM HEPES, 10% fetal calf serum, 2 mM glutamine, 0.5% chick embryo extract, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. The 10% fetal calf serum contains 2 nM insulin and 5.5 mM glucose. Subcultured cells were plated at either 2.5 × 10⁵ cells in 25-cm² coated flasks, 6.4 × 10⁴ cells in six-well plates, or 1.6 × 10⁶ cells in 60-mm² coated flasks. Cultures used for experimental procedures are between the fourth and eighth population doubling. Before assay, myoblasts were serum starved for 18 h and then were solubilized under basal conditions or after exposure to 100 nM insulin for 5 min. Myoblasts were solubilized in 50 mM HEPES, pH 7.6, 150 mM NaCl, 1% Triton X-100, 2 mM Na₂VO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µM leupeptin, and 2 µM pepstatin A. Lysates were allowed to solubilize for 1 h at 4°C and then were centrifuged for 1 h at 150,000 g. Supernatants were collected and stored at -70°C for subsequent study. Protein content of cellular extracts was determined by the Bradford method (3). The differentiation state of myoblasts was determined by assaying extracts for creatine phosphokinase (CPK) activity (Sigma, St. Louis, MO; Refs. 10, 22). Assessment of myoblast CPK activity confirmed that each cell line was of a muscle-specific lineage. Undifferentiated myoblast morphology was confirmed visually by the absence of fused myotubes and a relatively low CPK activity (10, 22).

IR ELISA. IR content of myoblasts was determined by specific ELISA as described previously (8). Briefly, microtiter 96-well plates were coated with 2 µg/ml anti-IR antibody (MA-20) for 18 h at 4°C. After being washed, solubilized cellular extract containing 3 and 6 µg protein of each sample were diluted in binding buffer (50 mM HEPES, 150 mM NaCl, 0.1% BSA, 0.1% Triton X-100, 2 mM Na₂VO₄, 1 mM PMSF, 1 mg/ml bacitracin, 2 mM leupeptin, and 2 mM pepstatin, pH 7.6) and added to each well and allowed to bind overnight at 4°C. Readout of bound IR was accomplished with the sequential addition of biotinylated anti-IR antibody CT-1 in 100 µl buffer B (50 mM HEPES, 150 mM NaCl, 0.05% Tween-20, 2 mM Na₂VO₄, 1 mg/ml bacitracin, 1 mM PMSF, and 1% BSA, pH 7.6), peroxidase-conjugated streptavidin (Pierce, Rockford, IL) also in 100 µl buffer B, ELISA amplification system (NEN Research Products, Boston, MA) for signal enhancement, and 3,3′,5,5′-tetramethylbenzidine peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, MD) for color development. The absorption at 450 nm of each well was measured in a microtiter plate reader (Du Pont-NEN, Boston, MA). Myoblast autophosphorylation, E LISA. The autophosphorylation state of myoblast IR was determined with an ELISA specific for IR tyrosine phosphorylation (23). Lysates from cells harvested in the basal state or after exposure to insulin were added to 96-well microtiter plates precoated with MA-20 as per the IR ELISA. In the autophosphorylation ELISA, solubilized cellular extract containing 40 pg of IR was diluted in binding buffer and added to each well. After overnight binding, wells were washed, and immunocaptured IR was incubated with 30 ng biotinylated anti-phosphotyrosine antibody (UBI, Lake Placid, NY) in 100 µl buffer B. The remaining steps of the autophosphorylation were identical to those of the IR ELISA.

Statistical analysis. All data were analyzed with SAS (Cary, NC). Plasma insulin concentrations were log transformed to achieve a normal distribution before parametric analysis. Simple and multiple linear regression models with calculation of partial correlation coefficients were used to analyze the relationships among selected variables. Data are presented as means ± SE, and P < 0.05 was considered significant.

RESULTS

In myoblasts cultured from nondiabetic Pima Indians, IR content of myoblasts was determined by specific ELISA. IR content per milligram of protein was somewhat higher than for mature muscle tissue and was not...
related to any physiological measures of obesity or insulin action (data not shown). Data from the IR ELISA were employed to ensure that equivalent amounts of IR were used in the determination of IR autophosphorylation.

The autophosphorylation state of IR was determined by specific and highly sensitive tyrosine phosphorylation ELISA. In IR collected from serum-starved, non-insulin-stimulated cells, the autophosphorylation level was not distinguishable from background. In an initial insulin dose-response study of five cell lines, incubation of myoblasts with varying concentrations of insulin (0.1–100 nM) for 5 min led to a dose-dependent increase in IR autophosphorylation (Fig. 1). Maximal autophosphorylation occurred at 100 nM insulin, and half-maximal activation occurred at 2.5–3.0 nM insulin. These values are consistent with those reported for IR from biopsies of mature human muscle stimulated by insulin in vitro (23). Maximal in vivo IR autophosphorylation was thus determined at 100 nM insulin in the full set of samples.

In myoblasts from 17 subjects, maximal IR autophosphorylation positively correlated with insulin-mediated glucose disposal rates (M) during hyperinsulinemic-euglycemic clamps (r = 0.56, P = 0.02) and inversely correlated with fasting insulin levels (r = −0.58, P < 0.05; Fig. 2), demonstrating a relationship between IR function and whole body insulin action. Glucose disposal rates were also inversely related to percent body fat (r = −0.53, P < .024; data not shown). To examine the relationship between myoblast IR function and insulin resistance unrelated to obesity, regression analysis was performed to control for the negative effects of increased adiposity on whole body glucose disposal.

M values, adjusted for percent body fat, remained significantly correlated with myoblast IR autophosphorylation (partial r = 0.53, P < 0.04; Fig. 3).

In myoblasts from the same lineage, grown and stimulated with insulin on separate plates, the coeffi-
Discussion

In the present study, we have employed a highly sensitive ELISA to study IR autophosphorylation in cultured myoblasts from nondiabetic Pima Indians who had a wide range of obesity and insulin action. The small quantity of IR required for this technique (40 pg) enabled us to make the first determinations of IR function in human primary muscle cell cultures. We previously employed this IR tyrosine phosphorylation ELISA to study muscle biopsies from a similar population of Pima Indians (23). In that study, we found that IR autophosphorylation capacity in skeletal muscle biopsies decreased as a function of increasing obesity in the Pimas and was positively correlated with insulin-stimulated glucose disposal. In addition, we demonstrated that the degree of autophosphorylation of immunocaptured IR correlated strongly with the capacity of receptors to function as protein tyrosine kinases against exogenous substrates.

Numerous other studies have found evidence for impaired muscle IR tyrosine kinase activity in both obesity and type 2 diabetes mellitus (1, 4, 17). These consistent results have increased the likelihood that diminished IR signaling might play a role in the insulin resistance of these states, but they have not precluded the possibility that reductions in IR function arise secondarily to these conditions. However, several studies have suggested that muscle IR defects may be an early or primary event in the development of insulin resistance that precedes the altered metabolic environment associated with either obesity or hyperglycemia. Handberg et al. (9) reported that IR tyrosine kinase activity is decreased in muscle from lean, nondiabetic, first degree relatives of type 2 diabetes mellitus patients, who are themselves insulin resistant and at increased risk for developing type 2 diabetes mellitus. In our prior study (23) of nondiabetic Pima Indians, for whom there is a strong genetic component of insulin resistance, IR autophosphorylation capacity was significantly correlated with glucose disposal values even after adjustment for the effects of obesity. These studies have provided indirect evidence that in some populations, reduced muscle IR signaling is an intrinsic, heritable trait that plays a primary role in the pathogenesis of insulin resistance.

Recently, primary muscle cell cultures have been employed to study insulin action in human subjects (5, 10). This technique allows for the analysis of muscle from individuals removed from an altered in vivo environment associated with factors such as obesity, hyperglycemia, and hyperinsulinemia. We have previously studied insulin action in myoblasts from nondiabetic Pima Indians having a wide range of obesity and insulin sensitivity (22). These myoblasts, cultured in a manner identical to the present study, expressed a variety of muscle-specific proteins and mRNA (22). Decreased myoblast glucose incorporation into glycogen was correlated with decreased in vivo glucose disposal (22). In this model, the insulin-resistant phenotype was maintained through 9–10 population doublings.

The present study was undertaken to determine whether differences in IR autophosphorylation capacity exist in myoblasts cultured from insulin-resistant subjects, which would therefore offer a possible mechanism to explain insulin resistance in these cells. We observed a significant correlation between insulin-stimulated IR autophosphorylation in the myoblasts and insulin-stimulated glucose disposal in vivo. This relationship was independent of the negative impact of obesity on glucose disposal. Thus these results strongly suggest that there is a wide range in IR-signaling capacity between individuals, which may be an intrinsic characteristic of muscle. Furthermore, in some individuals, a low capacity for autophosphorylation in muscle IR may contribute to the manifestation of an inherited insulin resistance.

Whereas we observed a significant correlation between myoblast IR autophosphorylation and whole body glucose disposal, there are several factors that limit the ability to directly compare these variables. Glucose disposal during the hyperinsulinemic glucose clamp represents the biological response of muscle tissue to high physiological insulin levels. In contrast, myoblast IR autophosphorylation was determined after exposure of cells to a maximal dose of insulin. Our dose-response studies in whole cells indicated that differences in IR function between cell lines resulted from changes in the maximal responsiveness, not the sensitivity of IR to insulin. Without a right shift in the insulin dose-response curves of IR, an altered maximal responsiveness reflects changes in the response of IR to submaximal dose of insulin. Thus a diminished maximum responsiveness of IR could produce a diminished tissue response to submaximal insulin levels.

In addition, whole body glucose disposal values reported in this study may not fully reflect the capacity for insulin-stimulated glucose uptake. Studies of insulin-resistant obese subjects have suggested that glucose disposal during the hyperinsulinemic glucose clamp may not reach steady-state levels by 100 min, the time point used to calculate M in the present study (18). Thus insulin action may be underestimated in the more insulin-resistant subjects in this study, which would affect the relationship between myoblast IR autophosphorylation and glucose disposal.

However, the relationship between this myoblast IR autophosphorylation and in vivo glucose disposal could not be expected to be exact even if muscle IR function were a primary determinant of muscle insulin action. Obesity and numerous other acquired factors can influence whole body insulin action, and whether or not these variables act through modification of IR function, these factors would presumably be eliminated in cultured cells after several passages. Whereas we corrected M values for the effects of obesity, presumably these measurements were also influenced by many other factors distinct from the intrinsic characteristics of muscle in these individuals. This would account for
the scatter between M values and the autophosphorylation of myoblast IR in response to insulin. Thus, whereas a greater sample size and an improved statistical model of acquired determinants of whole body insulin action might improve our understanding of the relationship between myoblast IR function and muscle insulin response, the present study provides strong evidence that, in Pima Indians, insulin sensitivity is associated with an increased capacity for IR autophosphorylation, observable in cultured myoblasts, which is intrinsic to muscle.

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REFERENCES


