*Originals***Insulin-Stimulated Glucose Uptake, Leucine Incorporation into Protein, and Uridine Incorporation into RNA in Skin Fibroblast Cultures from Patients with Diabetes Mellitus**

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Summary. Since intrinsic cellular abnormalities have previously been reported in diabetes mellitus, skin fibroblasts from patients with insulin-dependent diabetes and non-insulin-dependent diabetes, and from age-matched young and old controls, were examined for stimulation by insulin (4–4000 ng/ml) of glucose uptake, leucine incorporation into protein, and uridine incorporation into RNA. No differences in insulin-stimulated glucose uptake were seen between donor types. At 40 and 400 ng/ml, insulin did not stimulate as much leucine incorporation into protein in insulin-dependent diabetics as in young controls ($p < 0.05$) and at 4000 ng/ml, less insulin-stimulated leucine incorporation was seen in insulin-dependent diabetics than in young controls or non-insulin dependent diabetics ($p < 0.01$). Lower insulin-stimulated uridine incorporation into RNA in old controls than in other cell lines appeared to be largely secondary to a two-fold increase in basal incorporation in these old controls. These results provide additional evidence for intrinsic cellular abnormalities in diabetes mellitus. Whether the differences in basal or insulin-stimulated response between fibroblasts of different donor types are attributable to alterations in protein or RNA synthesis, metabolite pool size or turnover have yet to be determined.

Key words: Diabetes mellitus, insulin-dependent diabetes, non-insulin-dependent diabetes, human fibroblasts, insulin, glucose uptake, RNA, protein, cellular abnormalities, cell culture, uridine incorporation, leucine incorporation

Alterations in plating efficiency, growth rate, population density at confluence, replicative lifespan and protein synthesis [1–7] in fibroblast cultures established from diabetic patients suggest that intrinsic cellular abnormalities do occur in diabetes. These changes are said to resemble aging and, therefore, may have pathogenic relevance to the complications

of diabetes [2]. If this is indeed the case, then the development of complications of diabetes may be related less to the degree of metabolic control than to the inherent genetic predisposition of the individual. Since this is an important distinction, and since the fibroblast is intimately involved in glycoprotein synthesis, alterations of which may be related to diabetic microangiopathy [8], the study of fibroblasts from diabetic patients may provide additional insight into these questions.

Human fibroblasts have been shown to respond to insulin. Increases in glucose uptake [9], uridine incorporation into RNA [9], and α -amino-isobutyrate uptake [10] occurred at physiological concentrations of insulin. Less sensitive indices of insulin action were the incorporation of leucine into protein [9], thymidine into DNA [10], and glucose oxidation [11]. Previous studies to determine whether fibroblasts from controls and diabetics might differ in responsiveness to insulin have examined only insulin-stimulated glucose oxidation. Goldstein and Littlefield, using extremely high concentrations of insulin (10 μ g/ml), failed to show differences between cells from diabetic and control donors [11].

In the present study, the responsiveness of cells from insulin-dependent diabetics (IDD), non-insulin-dependent diabetics (NIDD), and age-matched young and old controls (YC and OC, respectively), were examined for insulin-stimulated glucose uptake, leucine incorporation into protein and uridine incorporation into RNA.

Materials and Methods*Fibroblast Donors*

IDD patients (age 27 ± 3 years, mean $\pm$ SEM) had ketoacidosis-prone hyperglycaemia with acute onset of symptoms. NIDD patients (52 ± 5 years) had hyperglycaemia, gradual onset of symptoms after the age of 30, and absence of ketoacidosis. Control donors (YC 25 ± 4 , OC 62 ± 3 years) had normal levels of fasting plasma glucose and no diabetes among their first degree relatives.

Cultures

Fibroblasts were established in tissue culture from minced skin biopsies taken from the deltoid skin of human donors, using previously reported methods [6, 12]. All studies were performed on cells that had been stored in liquid nitrogen and had not undergone more than 17 in vitro passages (one passage = 1 to 2 split = one population doubling). Prior to liquid nitrogen storage, fibroblast cultures were examined for the presence of mycoplasmas by tissue culture [13] and uracil incorporation [14] techniques. No evidence of contamination was detected. For each type of donor, cell lines from 6 different donors were used. No significant differences in passage numbers were detected between experimental protocols.

Experimental Cultures

Fibroblasts were retrieved from freeze storage, rapidly thawed and maintained in 450 g glass prescription bottles in supplemented Eagle's minimal essential medium [12]. For individual experiments, cells were loosened with trypsin solution (0.25% trypsin in phosphate-buffered saline, w/v, pH 7.4), suspended in supplemented Eagle's minimal essential medium, centrifuged, resuspended in supplemented Eagle's minimal essential medium, counted in a haemocytometer and dispersed by micropipette (100 to 200 μ l aliquots) into sterile glass scintillation vials (Kimble), as previously described [12]. Initial cell density was 2 to 3 $\times 10^5$ cells/vial. Following overnight incubation at 37°, the culture medium was removed, the cells (in mid to late log phase of growth) were washed gently with phosphate-buffered saline and 2.0 ml of experimental medium was added. For glucose uptake studies, experimental medium consisted of Eagle's minimal essential medium supplemented with nonessential amino acids [15] and neomycin (50 μ g/ml) but without fetal bovine serum, and with glucose decreased to 40 mg/dl. Studies on leucine incorporation into protein and uridine incorporation into RNA were carried out with experimental medium consisting of Eagle's minimal essential medium supplemented with nonessential amino acids and neomycin, but without fetal bovine serum. After 24 h of incubation in the appropriate serum free experimental medium, mitotic activity was markedly inhibited [12]. At that time, fresh experimental medium supplemented with various amounts of porcine insulin (single peak, Eli Lilly Co., Indianapolis) was added, and incubation at 37° was continued for 60 min at which time the appropriate radio-labelled substrate was added. Glucose uptake, ^3H -leucine incorporation into protein, and ^{14}C -uridine incorporation into RNA were measured as described previously [9]. Incubation times were 30 min for glucose uptake and 60 min for both leucine and uridine incorporation into protein and RNA, respectively. The results are from single experiments on each donor using 4 replicates for each data point (SEM generally < 5% for each mean).

Isotopes

D-(U- ^{14}C) glucose (specific activity 56.3 $\mu\text{Ci}/\text{mMole}$), L-(4,5- ^3H) leucine (3.13 mCi/mMole) and (2- ^{14}C) uridine 52.7 $\mu\text{Ci}/\text{mMole}$ were obtained from New England Nuclear.

Statistics

The data were examined by the analysis of variance utilizing a split plot in space design [16]. Comparisons between the means for each donor type (calculated from the means of quadruplicate measurements for each donor within a donor type) for basal metabolite uptake or incorporation were analyzed by the Newman-Keul's multiple comparison procedure [17]. Glucose uptake, leucine incorporation into protein and uridine incorporation into RNA were expressed as a ratio of the insulin-stimulated effect over con-

trol, and this ratio has been designated as "insulin ratio". By this definition, the control ratio is 1.0. The comparison of the mean insulin ratio for combined donor types at each insulin concentration to the control ratio, and the comparison of insulin ratios at each insulin concentration between donor types were analyzed by Dunn's multiple comparison procedure (Tables 1, 2 and 3) [18].

Results

Basal uridine incorporation into RNA and mean insulin ratios for insulin-stimulated incorporation are shown in Table 1. For combined donor types, the effect of insulin on uridine incorporation into RNA was highly significant at each insulin concentration utilized ($p \leq 0.01$). When the differences in increments between the mean insulin ratios and control ratio were compared between donor types for each insulin concentration, a significantly greater stimulation of uridine incorporation was noted in NIDD than OC at 4.0 ng/ml insulin and in YC than OC at 40, 400 and 4000 ng/ml insulin (Table 1). When basal uridine incorporation into RNA was examined, however, OC showed a two-fold increase when compared to YC, IDD and NIDD. These differences in the basal uptake between OC and the other cell lines were significant ($p < 0.05$).

Basal leucine incorporation into protein and mean insulin ratios for insulin-stimulated incorporation are shown in Table 2. For combined donor types, significant increases were found at all insulin concentrations utilized ($p \leq 0.01$). Comparison between donor types of differences in increments between mean insulin ratios and control ratio showed a sig-

Table 1. Basal incorporation of uridine into RNA, and mean insulin ratios for insulin-stimulated incorporation

Donor type	Basal incorporation (mean cpm $\pm$ SEM)	Mean insulin ratios Insulin concentration (ng/ml)			
		4.0	40	400	4000
YC	492 $\pm$ 49	1.36	1.68	1.84	1.89
OC	1011 $\pm$ 230 ^a	1.13 ^b	1.24 ^b	1.44 ^b	1.42 ^b
IDD	500 $\pm$ 60	1.40	1.70	1.89	1.83
NIDD	507 $\pm$ 54	1.44	1.53	1.67	1.66
Mean insulin ratios for combined donor types		1.33 ^c	1.54 ^c	1.71 ^c	1.70 ^c

^a $p < 0.05$ when compared to each of the other donor types

^b For comparison of differences in insulin effects between donor types, the least significant differences used for Dunn's multiple comparison procedure were calculated to be 0.31 ($p = 0.05$) and 0.36 ($p = 0.01$). Based upon these, OC vs. NIDD (insulin 4.0 ng/ml), $p < 0.05$; and OC vs. YC (insulin 40, 400 and 4000 ng/ml), $p < 0.01$

^c Least significant differences were calculated to be 0.08 ($p = 0.05$) and 0.10 ($p = 0.01$). Thus, all of these ratios are significantly different from control ($p \leq 0.01$)

Table 2. Basal incorporation of leucine into protein, and mean insulin ratios for insulin-stimulated incorporation

Donor type	Basal incorporation (mean cpm $\pm$ SEM)	Mean insulin ratios Insulin concentration (ng/ml)		
		40	400	4000
YC	385 $\pm$ 50	1.35	1.44	1.64
OC	1134 $\pm$ 245	1.29	1.43	1.49
IDD	880 $\pm$ 240	1.15 ^a	1.23 ^a	1.29 ^a
NIDD	1154 $\pm$ 246	1.24	1.38	1.52
Mean insulin ratios for combined donor types		1.26 ^b	1.37 ^b	1.49 ^b

^a For comparison of differences in insulin effects between donor types, the least significant differences used for Dunn's multiple comparison procedure were calculated to be 0.20 ($p = 0.05$) and 0.23 ($p = 0.01$). Based upon these, IDD vs. YC (insulin 40 and 400 ng/ml), $p < 0.05$; IDD vs. YC (insulin 4000 ng/ml), $p < 0.01$; IDD vs. NIDD (insulin 4000 ng/ml), $p < 0.01$.

^b Least significant differences were calculated to be 0.05 ($p = 0.05$) and 0.06 ($p = 0.01$). Thus, all of these ratios are significantly different from control ($p \leq 0.01$)

nificantly smaller stimulatory effect of insulin in IDD than YC at 40, 400 and 4000 ng/ml insulin. Additionally, at 4000 ng/ml, insulin-stimulated leucine incorporation into protein was less in IDD than NIDD. Differences in basal leucine incorporation into protein were not significant by the Newman-Keuls comparison procedure.

Glucose uptake was also significantly enhanced at all insulin concentrations for combined donor types (Table 3). No significant differences in the effect of insulin on glucose uptake were found between donor types.

Discussion

Although less responsive to insulin than the adipocyte, the cultured human fibroblast has been shown to be responsive to physiological concentrations of insulin [9, 10]. This has been shown for glucose uptake, uridine incorporation into RNA and α -amino-isobutyrate uptake. Glucose oxidation to CO₂ [11] and leucine incorporation into protein [9] were less sensitive parameters of insulin action in fibroblasts.

In monocytes or in isolated adipocytes, insulin binding and insulin action appear to be related to the physiological status of the donor. Thus, when plasma insulin was high, decreased binding of insulin to freshly isolated monocytes was observed, due to changes in both receptor affinity and concentration [19–21]. Adipose cells from obese subjects were less responsive to insulin [22]. In contrast, in investiga-

Table 3. Basal uptake of glucose, and mean insulin ratios for insulin-stimulated uptake

Donor type	Basal uptake (mean cpm $\pm$ SEM)	Mean insulin ratios Insulin concentration (ng/ml)			
		4.0	40	400	4000
YC	89 $\pm$ 15	1.14	1.40	1.52	1.76
OC	84 $\pm$ 12	1.16	1.41	1.67	1.91
IDD	116 $\pm$ 23	1.15	1.32	1.54	1.69
NIDD	73 $\pm$ 4	1.19	1.28	1.49	1.57
Mean insulin ratios for combined donor types		1.19 ^a	1.28 ^a	1.49 ^a	1.57 ^a

^a Least significant differences were calculated to be 0.09 ($p = 0.05$) and 0.11 ($p = 0.01$). Thus, all of these ratios are significantly different from control ($p \leq 0.01$)

tions of insulin action in fibroblast cultures, where cells are many generations removed from the donor and cells may be tested under identical culture conditions, any changes observed are more likely to reflect intrinsic cellular differences and not the *in vivo* insulin levels. Thus, changes may be related to the genetic status of the donor.

Previously demonstrated abnormalities in plating efficiency, growth rate, population density at confluence, replicative lifespan and protein synthesis in fibroblasts from patients with diabetes mellitus suggest that intrinsic cellular abnormalities are present [1–7]. It was, therefore, reasoned that if differences in insulin action were demonstrable in fibroblasts from patients with diabetes, this would represent further evidence for intrinsic cellular changes in diabetes.

In YC and NIDD, insulin-stimulated uridine incorporation into RNA was greater than in OC. These observations, however, are likely secondary to the two-fold increase in basal uridine incorporation into RNA in OC. Why a difference exists in basal incorporation of uridine into RNA in OC is unclear, but could be attributable to enhanced RNA synthesis, alterations in uridine, UMP, UDP or UTP pool sizes, or increased RNA turnover, none of which were measured in the present study. Since the fibroblasts were rendered mitotically quiescent at the time of study, it seems highly unlikely that these changes are due to differences in growth rates between IDD and YC or NIDD and OC during the actual labelling interval.

The effect of insulin on leucine incorporation into protein was significantly less in IDD than in YC at all 3 insulin concentrations utilized. Here the lesser insulin response in IDD vs. YC may not be entirely explained by the apparently lower basal incorporation of leucine into protein in YC since although both OC and NIDD had basal incorporations which

appear higher than in YC, their responses to insulin were similar to those of YC. The other significant difference, IDD vs. NIDD, occurred only at insulin 4000 ng/ml. Alterations in total protein and collagen synthesis have already been defined in fibroblasts from diabetic donors in a prior study in which basal protein and collagen synthesis rates per cell were greater in IDD than NIDD cells [6]. The addition of hydrocortisone caused a decrease in synthesis by IDD cells and an increase by NIDD cells [6]. The differences in insulin-stimulated leucine incorporation into protein observed in this study may be secondary to alterations in amino acid pool size, protein degradation or protein synthesis.

The present demonstration of alterations of insulin-stimulated leucine incorporation into protein in IDD provides additional evidence for intrinsic cellular abnormalities in protein metabolism in diabetes mellitus. Most of the data in this investigation, however, would indicate no differences in insulin-stimulated response of fibroblasts between diabetic and control donors. Whether any of the cellular alterations described in fibroblasts of diabetic donors represent changes inherited with the disorder, or are properties evolving from an *in vivo* selection process within the abnormal diabetic milieu which allows fibroblasts with these *in vitro* properties to survive is an important distinction that remains to be resolved.

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