Defects in Insulin-Receptor Internalization and Processing in Monocytes of Obese Subjects and Obese NIDDM Patients

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We investigated intracellular processing of the insulin-receptor complex in monocytes from 12 healthy control subjects, 11 obese nondiabetic subjects, and 13 obese patients with non-insulin-dependent diabetes mellitus (NIDDM) by measuring receptor internalization, recovery of cell-surface insulin binding after receptor internalization, and the release of intracellular intact insulin (insulin retroendocytosis). When monocytes from the three groups of subjects were exposed to 100 nM unlabeled insulin for 30 min at 37°C, the subsequent cell-surface ^{125}I-labeled insulin binding was reduced, but the total number of insulin receptors, measured by radioimmunoassay, was not changed. These findings indicate a redistribution of insulin receptors from the surface to the cell interior. Insulin-receptor internalization was significantly lower in monocytes of obese NIDDM patients (mean ± SE 17.8 ± 4.7%) than in obese subjects and healthy control subjects (33.5 ± 4.5%, $P < .05$, and 34.4 ± 3.7%, $P < .02$, respectively). Moreover, in downregulated cells, a complete recovery of the initial insulin binding was observed in control subjects but not in obese NIDDM patients or obese nondiabetic subjects. The release of internalized insulin was also reduced in obese NIDDM patients and obese subjects ($t_{1/2}$ = 49.0 ± 2.4 min, $P < .02$; 47.4 ± 5.7 min, $P < .05$; and 32.9 ± 3.8 in NIDDM patients, obese subjects, and control subjects, respectively). In the radioactivity released from monocytes of obese subjects and obese NIDDM patients, the percentage of intact insulin was higher ($P < .05$) than in control subjects, suggesting reduced intracellular insulin degradation in obese subjects and obese NIDDM patients. This study indicates that insulin-resistant obese subjects and obese NIDDM patients have multiple postbinding defects of the insulin-receptor intracellular processing. Among these defects, decreased insulin-receptor internalization is specifically associated with diabetic patients. *Diabetes* 38:1579–84, 1989

Glucose 1 mM = 18 mg/dl	Insulin 1 pM = 0.167 μU/ml

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Although it is well known that non-insulin-dependent diabetes mellitus (NIDDM) is characterized by tissue resistance to insulin action (1), the cellular mechanisms of this resistance are not well understood. Insulin binding to its receptor is often reduced in cells of NIDDM patients (2,3), but this defect is not believed to be the major cause of tissue resistance to insulin action; rather, insulin resistance has been attributed to a postbinding defect (4–6).

After insulin binding to its plasma membrane receptor, the insulin-receptor complex is internalized and processed by target cells (7–9). These events involve the recycling and degradation of both insulin and its receptor (10–14) or their translocation to other intracellular organelles (7–9). Past studies suggest that internalization (15–18) and intracellular processing (19,20) of the insulin-receptor complex may be related to the hormone action.

We and others have reported that human monocytes internalize (21,22) and degrade (23) insulin by a soluble neutral insulin-degrading enzyme in a fashion similar to typical insulin-target cells (24,25). We also reported that circulating monocytes from NIDDM patients have a decreased ability to internalize (21) and then degrade (23) insulin and that this defect is not associated with decreased intrinsic activity of the insulin-degrading enzyme (25). Decreased insulin internalization (22) and processing (26) in cells from NIDDM patients have also been reported by others.

To better understand the mechanism of these defects, in this study, we analyzed insulin-induced receptor internalization, recovery of cell-surface insulin binding after receptor internalization, and insulin retroendocytosis in monocytes from healthy subjects, obese subjects, and obese NIDDM

patients. The results indicate that multiple abnormalities of the insulin-receptor complex internalization and processing are present in obese subjects and more severely in obese patients with NIDDM.

RESEARCH DESIGN AND METHODS

Twelve healthy control subjects, 11 obese subjects, and 13 obese NIDDM patients were studied after giving informed consent. The characteristics of these subjects indicate that the three groups were matched for sex and age (Table 1). Diabetic patients were diagnosed according to the National Diabetes Data Group criteria (27) and had never been treated with insulin or antidiabetic agents. In the 2 wk preceding our study, all subjects were fed a weight-maintaining diet (20% protein, 50% carbohydrate, and 30% lipid) and allowed moderate physical activity. Monocytes were obtained from 150 ml of blood withdrawn after an overnight (12- to 13-h) fast.

Crystalline porcine insulin, Triton X-100, bacitracin, pronase, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (London). Highly purified porcine insulin (Novo, Copenhagen) was iodinated by the lactoperoxidase method (23). A14-^{125}I-labeled insulin (340–360 μCi/μg) was separated from the iodination mixture with reverse-phase high-performance liquid chromatography (RP-HPLC) (23).

Monocytes were prepared as previously described (28), and the percentage of monocytes was determined by the latex-bead method. Cell viability at the end of the experiments was always >95% judged by trypan blue exclusion.

Receptor internalization was initiated by adding 100 nM native insulin to 2 × 10^7 monocytes/ml in 50 mM HEPES buffer with 1% bovine serum albumin (BSA), pH 7.8, at 37°C (29). At the indicated times, cells were centrifuged at 4°C to remove unbound insulin and then acid washed with sodium acetate buffer (pH 4.5) at 4°C for 15 min to dissociate cell-surface–bound insulin (29). Labeled insulin binding to residual cell-surface receptors was then carried out at 4°C, a temperature that minimizes insulin internalization (21) and receptor recycling (11,29). In parallel experiments, similarly treated cells were first preincubated at 37°C, to allow the internalized receptors to return to the cell surface (11,29), and then insulin binding was studied at 4°C. Control experiments in which cells were not exposed to unlabeled insulin were carried through each step, and the results were used as control values.

Monocytes (2 × 10^7/ml) were incubated in binding buffer (50 mM HEPES with 1% BSA, pH 7.8; 21) with 66 pM ^{125}I-insulin in the absence or presence of 16.6 μM unlabeled insulin for 16 h at 4°C in a shaking bath (60–90 cpm). At the end of incubation, 160-μl aliquots were centrifuged at 8000 × *g* for 2 min in a Beckman microfuge. The pellets were washed once with 200 μl chilled buffer, and the tips were excised and counted in a γ-counter. Nonspecific binding was subtracted, and values were then normalized to 10^6 monocytes.

Cells were solubilized at 4°C for 60 min in 50 mM HEPES containing 1% Triton X-100, 1 mg/ml bacitracin, and 1 mM PMSF. Solubilized cells were then centrifuged at 8000 × *g* for 4 min in a Beckman microfuge at 4°C, and the supernatants were collected and used to measure total cellular insulin-receptor content.

To determine the intracellular binding activity, monocytes were first treated with 2.5 mg/ml pronase at 4°C for 120 min to destroy cell-surface receptors (10,30), washed four times, and solubilized, and then ^{125}I-insulin binding was carried out as previously described (21). ^{125}I-insulin binding to intact cells pretreated with pronase was reduced to <2% of initial binding, indicating the effectiveness of pronase treatment in destroying cell-surface insulin receptors. Intracellular insulin-binding activity in healthy control subjects (n = 4) was 20 ± 4% (mean ± SE) of total insulin-binding activity, measured in parallel experiments in solubilized monocytes that were not pretreated with pronase.

Many solubilized cells are necessary for an accurate estimation of the total number of insulin receptors by Scatchard analysis. To overcome this problem, we developed a direct radioimmunoassay of the insulin receptor. This assay is described in detail elsewhere (14). Briefly, it employs a specific antiserum for the human insulin receptor obtained in the rabbit (31) with a human placenta insulin receptor, highly purified by repeated affinity chromatography, as a standard. The same receptor preparation, labeled with ^{125}I by the Bolton-Hunter method at a specific activity of ~15 μCi/μg, is used as tracer. Bound versus free separation is obtained by precipitation with goat anti-rabbit γ-globulin. This assay can measure as little as 1 ng insulin receptor/ml of sample. Intra- and interassay coefficients of variation are 5 and 9%, respectively, and insulin up to 1 μM does not interfere. Cross-reactivity with the insulinlike growth factor I receptor is <10%.

Monocytes were incubated with 660 pM labeled insulin in HEPES buffer for 60 min at 37°C to achieve maximum insulin binding and internalization (21) and then acid washed to remove labeled insulin bound to surface receptors. Acid-washed cells were then resuspended in HEPES buffer at

TABLE 1
Clinical characteristics of study subjects

Subjects	*n* (M/F)	Age (yr)	Ideal body weight (%)	Blood glucose (mg/dl)	Immunoreactive insulin (μU/ml)	HbA_1 (%)
Control	8/4	53 ± 2	104 ± 2	82 ± 2	7 ± 1	
Obese	5/6	54 ± 3	154 ± 11*	84 ± 2	18 ± 3*	
Obese NIDDM	6/7	51 ± 2	149 ± 9*	170 ± 11*†	15 ± 2*	8.4 ± 0.3

Values are means ± SE. Blood glucose was determined by the glucose oxidase method with a Beckman glucose analyzer. Immunoreactive plasma insulin was determined with a commercial kit. Glycosylated hemoglobin (HbA_1) was determined by a chromatographic procedure (Boehringer Mannheim, Mannheim, FRG; normal range 4.7–7.0%). NIDDM, non-insulin-dependent diabetes mellitus.
*P < .01 vs. control subjects.
†P < .01 vs. obese subjects.

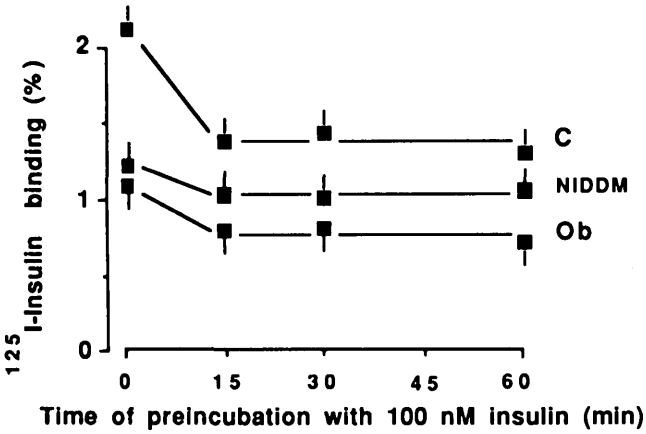

FIG. 1. Insulin-receptor internalization in human monocytes from healthy control subjects (C), obese nondiabetic subjects (Ob), and obese non-insulin-dependent diabetes mellitus patients (NIDDM). Cells were incubated at 37°C for indicated times with 100 nM insulin and acid washed (see RESEARCH DESIGN AND METHODS), and then ^{125}I-labeled insulin binding was carried out at 4°C for 16 h. Values are means ± SD of triplicate measurements in representative experiment performed with cells from each group of subjects.

37°C, and the amount of residual (intracellular) radioactivity was measured at different intervals by centrifuging cell aliquots in a Beckman microfuge and then washing and counting the cell pellets. The nature of the radioactivity released by the cells in the incubation medium was analyzed by trichloracetic acid (TCA) precipitability and RP-HPLC (23).

RESULTS

Insulin-receptor internalization. A typical study of insulin-receptor internalization in monocytes is shown in Fig. 1. In the monocytes from healthy control subjects, exposure to 100 nM unlabeled insulin at 37°C reduced the subsequent cell-surface insulin binding to ~65% of the control value. This effect was already observed after cells were exposed to insulin for 15 min, and no further significant change was observed thereafter for up to 60 min. When the temperature of preincubation with insulin was kept at 4°C, a reduction of <10% was observed (data not shown). Also, in monocytes from obese subjects and obese NIDDM patients, exposure to insulin produced a reduction of subsequent cell-surface insulin binding with a similar time course (Fig. 1). In contrast to cell-surface insulin binding, the total cell-receptor number measured by radioimmunoassay in three parallel experiments was not decreased after cell exposure to 100 nM unlabeled insulin (Table 2). Moreover, when cells exposed to insulin were treated with pronase to destroy cell-surface receptors and then solubilized, the residual (intracellular) insulin-binding activity was significantly higher in cells exposed to insulin (^{125}I-insulin binding 0.34 ± 0.05%) than in control cells (0.19 ± 0.03%, $n = 3$, $P < .05$). These findings indicate that in human monocytes exposed to insulin, a rapid and temperature-dependent redistribution of the insulin receptors occurs between the cell surface and the cell interior.

TABLE 2
Effect of exposure of monocytes to 100 nM unlabeled insulin on insulin-receptor radioimmunoassay and ^{125}I-labeled insulin binding

	Insulin-receptor radioimmunoassay (ng/ml)		^{125}I-insulin binding (%)	
Experiment	Untreated	Insulin treated	Untreated	Insulin treated
1	4.67	4.67	1.85	0.85
2	5.40	5.47	2.37	0.98
3	4.00	3.85	1.77	1.08
Mean ± SE	4.69 ± 0.21	4.66 ± 0.24	2.00 ± 0.18	0.97 ± 0.17*

Cells were preincubated or not with insulin at 37°C for 30 min and then acid washed (see RESEARCH DESIGN AND METHODS). ^{125}I-insulin binding to intact cells (4°C for 16 h) and radioimmunoassay were then performed.
*$P < .01$ vs. untreated cells.

In monocytes from obese NIDDM patients ($n = 13$), insulin-induced receptor internalization, measured by the reduction of ^{125}I-insulin binding after cells were exposed to 100 nM unlabeled insulin, was significantly lower than in monocytes from obese nondiabetic subjects ($n = 11$, $P < .05$) and control subjects ($n = 12$, $P < .02$; Fig. 2).

Recovery of cell-surface insulin binding after receptor internalization. Cells were first incubated with 100 nM unlabeled insulin for 30 min at 37°C, acid washed for 15 min at 4°C, and then rewarmed for 30 min at 37°C in the absence of insulin to allow the recovery of cell-surface insulin binding. ^{125}I-insulin binding was 66 ± 8% of the initial binding in downregulated monocytes from normal subjects ($n = 8$), and complete recovery of the initial binding (94 ± 5%) was obtained after rewarming. In contrast, downregulated monocytes from obese nondiabetic subjects ($n = 7$) and obese NIDDM patients ($n = 9$) had very little recovery of the initial binding after rewarming: in obese and diabetic patients, respectively, ^{125}I-insulin binding was 67 ± 5 and 88 ± 13% of the initial value after downregulation and 71 ± 8 and 91 ± 10% of the initial binding after rewarming.

Significant receptor internalization is an important prerequisite for reliable measurement of cell-surface insulin binding recovery. To exclude the possibility that the impaired insulin-binding recovery observed in NIDDM patients was the consequence of the inadequate sensitivity of our measurements in detecting very small variations in ^{125}I-insulin binding, we calculated the recovery of insulin binding in monocytes obtained from a selected group of five NIDDM patients with receptor internalization >20%. The insulin-binding recovery in the monocytes of the NIDDM patients was compared with the recovery obtained in monocytes from five control subjects and five obese nondiabetic subjects who had similar receptor internalization. The results confirm that, after receptor internalization, a significant impairment of cell-surface insulin-binding recovery is present in monocytes from obese subjects and obese NIDDM patients (Fig. 3).

Loss of intracellular radioactivity. We next examined the rate at which intracellular radioactivity was lost from monocytes previously incubated with 660 pM ^{125}I-insulin. Under our experimental conditions, monocytes from control subjects ($n = 8$) released nearly 80% of the intracellular radioactivity within 90 min (Fig. 4). At each time point studied, the rate of loss of intracellular radioactivity was reduced in the monocytes from obese subjects ($n = 7$) and obese NIDDM patients ($n = 9$; $P < .01$ by 2-way analysis of variance [ANOVA]; Fig. 4). As a consequence, the $t_{1/2}$ of the intra-

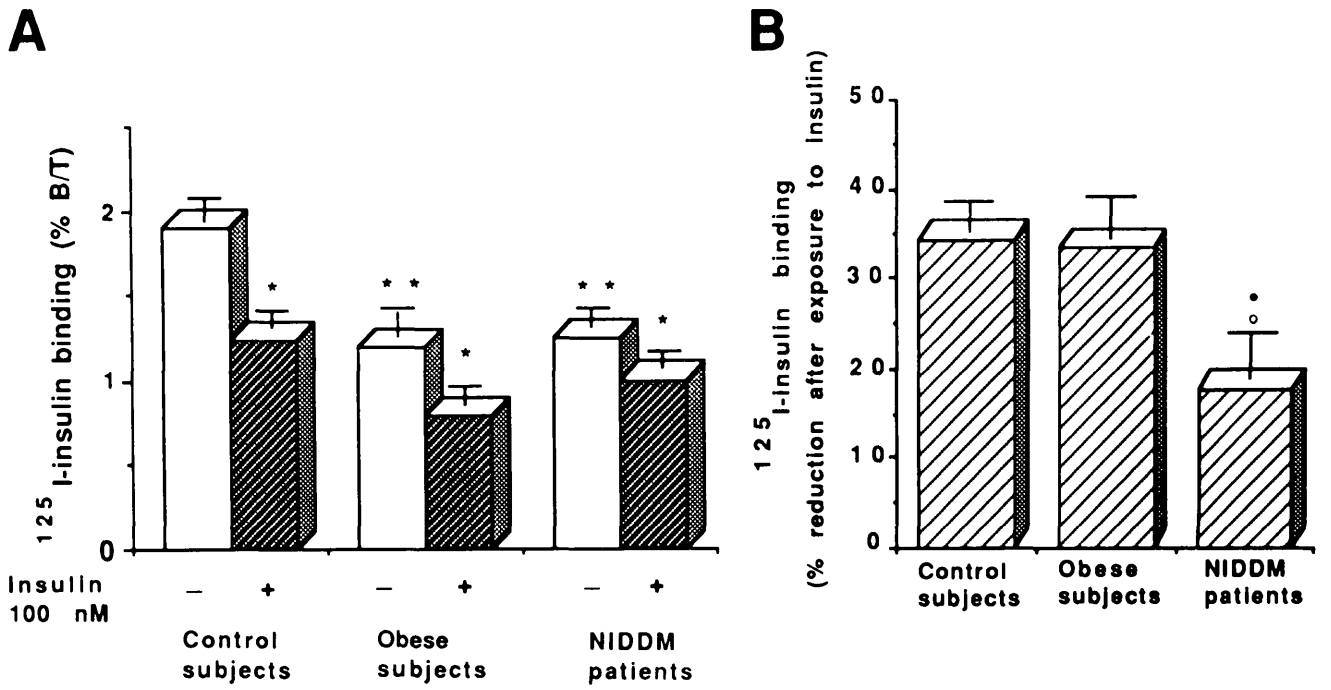

FIG. 2. Effect of preincubation with 100 nM unlabeled insulin on subsequent ^{125}I-labeled insulin binding to human monocytes of 12 healthy control subjects, 11 obese nondiabetic subjects, and 13 obese non-insulin-dependent diabetes mellitus (NIDDM) patients. *A*: human monocytes were preincubated with (+) or without (−) 100 nM insulin at 37°C for 30 min and then acid washed as described in RESEARCH DESIGN AND METHODS. ^{125}I-insulin binding was then performed at 4°C for 16 h. Data are normalized to 10^6 cells and expressed as means ± SE. $^{*}P < .01$ vs. untreated cells from same group of subjects; $^{**}P < .01$ vs. untreated cells of control subjects. *B*: data from *A* have been replotted. $^{\circ}P < .05$ vs. obese subjects; $^{\bullet}P < .02$ vs. control subjects.

cellular radioactivity release from monocytes of control subjects (32.9 ± 3.8 min) was significantly lower than that of monocytes from obese subjects or NIDDM patients (47.4 ± 5.7 min, $P < .05$, and 49.0 ± 2.4 min, $P < .02$, respectively).

The nature of the radioactive material released into the medium was examined by both TCA precipitability and RP-HPLC analysis. The RP-HPLC analysis indicated that during the first 10 min, intact A14-^{125}I-insulin was released (retroendocytosis) together with ^{125}I and six to seven labeled degradation products. No significant release of intact insulin was observed at later times (data not shown). The percentage of released radioactivity that was TCA precipitable was slightly but significantly higher in cells from obese subjects and obese NIDDM patients than in cells from control subjects ($P < .05$ by 2-way ANOVA; Fig. 5), indicating reduced insulin degradation in the monocytes from obese subjects and obese NIDDM patients (21).

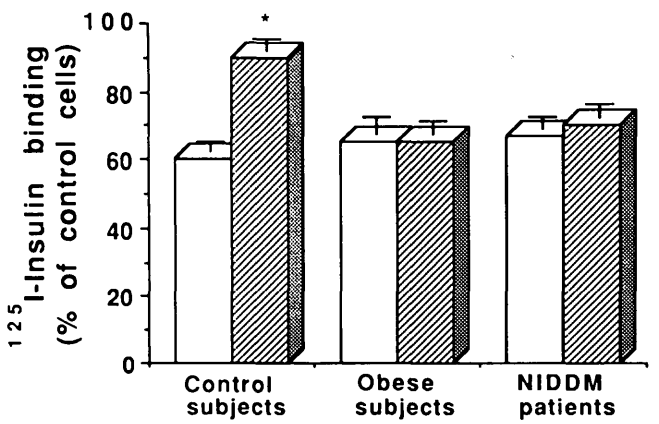

FIG. 3. Recovery of cell-surface insulin binding in downregulated monocytes from 5 healthy control subjects, 5 obese nondiabetic subjects, and 5 obese non-insulin-dependent diabetes mellitus (NIDDM) patients selected on basis of similar receptor internalization. ^{125}I-labeled insulin binding was performed after preincubation with unlabeled insulin (receptor internalization, *open bars*) and after unlabeled insulin removal (recovery of cell-surface insulin binding, *hatched bars*). Data are mean ± SE percentage of control cells (preincubated in absence of unlabeled insulin). $^{*}P < .01$ vs. receptor internalization in control subjects.

DISCUSSION

In a previous study, we observed a reduced proportion of intracellular insulin in monocytes from NIDDM patients incubated with ^{125}I-insulin compared with monocytes from control subjects (21). Similar results were also obtained by others in a preliminary study (22). The decreased proportion of intracellular insulin may be the result of reduced insulin-receptor internalization, increased release of internalized insulin, or both. To clarify this issue, we present evidence that insulin-induced receptor internalization is in fact decreased in monocytes from obese NIDDM patients. In these cells, the release of internalized insulin is delayed.

The reason for reduced insulin-receptor internalization in these patients is unknown. Recent observations indicate that receptor phosphorylation may be a prerequisite for receptor internalization (32–34) and that insulin-receptor phosphorylation is reduced in NIDDM patients (35–38). Therefore, impaired receptor phosphorylation may be a possible cause of the reduced receptor internalization in NIDDM patients. However, other receptor abnormalities, like an alteration in

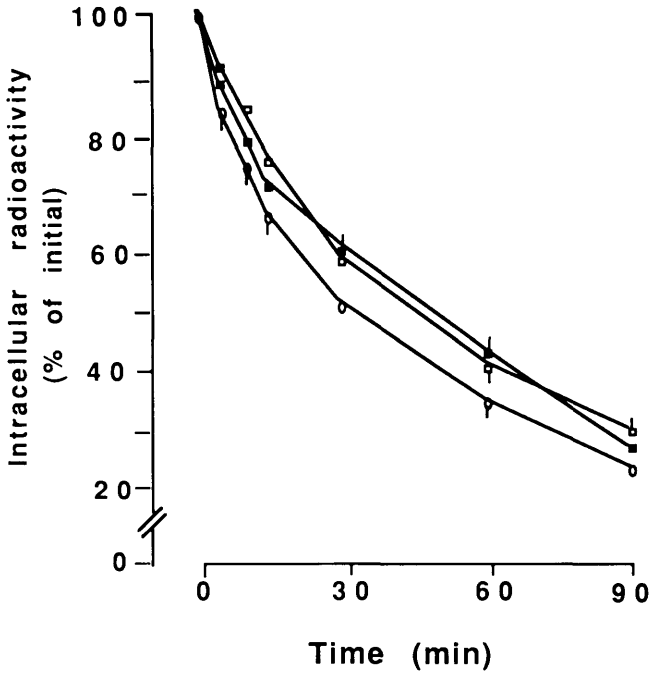

FIG. 4. Rate of loss of intracellular radioactivity from human monocytes from healthy control subjects (○), obese nondiabetic subjects (■), and obese non-insulin-dependent diabetes mellitus patients (□). Cells were incubated at 37°C for 60 min with 660 pM ^{125}I-labeled insulin, acid washed as described in RESEARCH DESIGN AND METHODS, and then resuspended at 37°C. Cell-associated radioactivity was then determined over next 90 min. Values are means, with SE given only when >2%.

glycosylation, may also be associated with reduced receptor internalization (39) and may possibly contribute to the reduced receptor internalization in the cells of NIDDM patients.

Impaired recovery of cell-surface insulin-binding activity was observed in monocytes from both obese NIDDM patients and obese nondiabetic subjects. After receptor internalization, recovery of insulin binding may be due to either receptor recycling or the insertion of new intracellular receptors into the plasma membrane (8,9). Therefore, the reduced insulin-binding recovery observed in monocytes from obese subjects and obese NIDDM patients may be due to an impairment of one or both of these mechanisms. In vitro long-term exposure to insulin leads to reduced insulin-receptor number, a consequence of both increased receptor degradation and decreased receptor biosynthesis (40). Because obese patients, with or without NIDDM, are hyperinsulinemic, they may have a decreased intracellular pool of insulin receptors, and consequently, this may contribute to the reduced recovery of cell-surface insulin-binding activity. However, this possibility is unlikely, because *1*) in other cell types, the recovery of surface insulin binding after short-term downregulation is entirely due to receptor recycling (10,11), and *2*) in human monocytes, the intracellular pool of insulin receptors represents only 20% of the total insulin-receptor number. Therefore, in these cells, the insertion of new intracellular receptors into the plasma membrane cannot be the major mechanism involved in the recovery of cell-surface insulin binding, which in normal subjects is ~30–40% of the initial value. Therefore, although other mechanisms cannot be excluded, receptor-recycling impairment is most likely the major cause of the lack of recovery observed in downregulated monocytes from obese subjects and obese NIDDM patients.

Finally, in monocytes from obese subjects and obese NIDDM patients, in addition to abnormal insulin-receptor internalization and processing, we also found abnormalities regarding insulin processing. We previously reported that monocytes from these patients degrade less insulin (23), and similar results were reported by others in adipocytes from obese NIDDM patients (26). We also found that the release of intracellular insulin is reduced in these patients

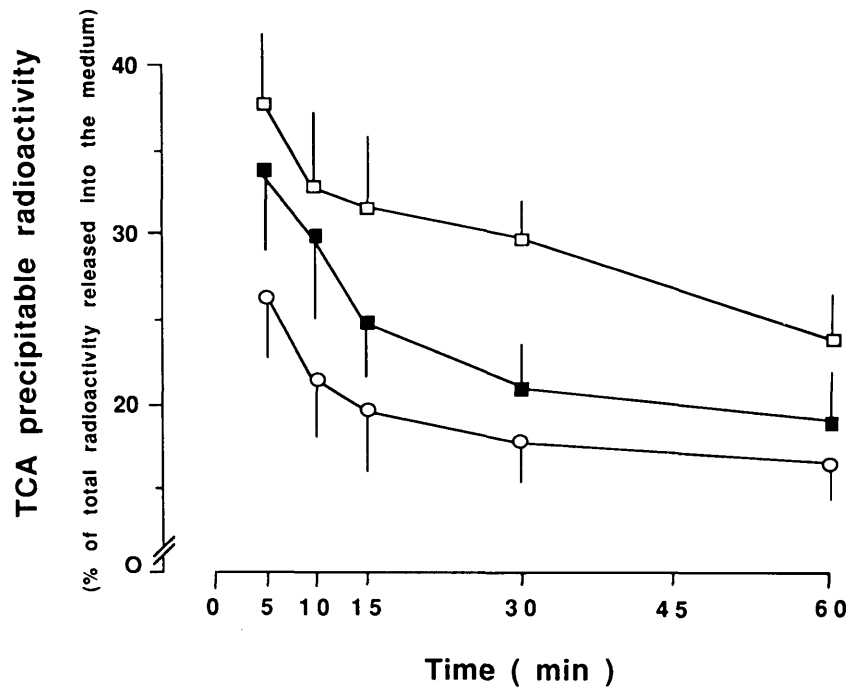

FIG. 5. Trichloracetic acid (TCA) precipitability of intracellular radioactivity released in medium. Monocytes from healthy control subjects (○), obese nondiabetic subjects (■), and obese non-insulin-dependent diabetes mellitus patients (□) were incubated at 37°C for 60 min with 660 pM ^{125}I-labeled insulin, acid washed to dissociate cell-surface–bound insulin, and then resuspended at 37°C to allow release of intracellular radioactivity. At indicated times, cells were centrifuged and supernatants utilized to determine TCA precipitability. Values are means ± SE.

and in obese subjects. Because the dissociation of insulin from its receptor is a prerequisite for receptor recycling (11) and insulin degradation and its subsequent release from the cell interior (12), an impaired dissociation of the insulin-receptor complex is one possible explanation for both alterations.

In adipocytes from obese insulin-resistant rats, we observed a reduction of both receptor recycling and release of internalized insulin (29). In addition, when these rats were made diabetic by a small dose of streptozocin, they also had reduced receptor internalization (41). Therefore, both the previous studies in rats and this study in humans suggest that a defect of insulin release and insulin-receptor recycling may be a generalized characteristic associated with obesity, whereas the defect in receptor internalization is a characteristic specifically associated with diabetes.

A relationship between the biological activity of insulin and the internalization and intracellular processing of the insulin-receptor complex is suggested by various in vitro evidence (14–20,42). We observed multiple abnormalities of insulin and its receptor internalization and processing in insulin-resistant states like obesity and NIDDM. Whatever their cause, these alterations may play a role in causing or aggravating the insulin resistance of these patients.

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