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**Research Article**

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# Studies on the Mechanism of Insulin Resistance in the Liver from Humans with Noninsulin-dependent Diabetes

## Insulin Action and Binding in Isolated Hepatocytes, Insulin Receptor Structure, and Kinase Activity

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### Abstract

We have developed a method to isolate insulin-responsive human hepatocytes from an intraoperative liver biopsy to study insulin action and resistance in man. Hepatocytes from obese patients with noninsulin-dependent diabetes were resistant to maximal insulin concentration, and those from obese controls to submaximal insulin concentration in comparison to nonobese controls. Insulin binding per cell number was similar in all groups. However, insulin binding per surface area was decreased in the two obese groups because their hepatocytes were larger. In addition, the pool of detergent-extractable receptor was further decreased in diabetics. Insulin receptors in all groups were unaltered as determined by affinity-labeling methods. However, insulin-stimulated insulin receptor kinase activity was decreased in diabetics. Thus, in obesity, decreased surface binding could explain resistance to submaximal insulin concentrations. In diabetes, diminished insulin-stimulated protein kinase activity and decreased intracellular pool of receptors could provide an explanation for postinsulin-binding defect(s) of insulin action in human liver.

### Introduction

Type II diabetes or noninsulin-dependent diabetes mellitus (NIDDM)<sup>1</sup> is a heterogeneous disorder characterized by defects in insulin secretion and insulin action (1–5). The overall insulin resistance in NIDDM, with or without obesity, has been well demonstrated with the use of several *in vivo* techniques (6–12).

The site and cellular mechanism of insulin resistance in NIDDM are not well established. The liver appears to play a fundamental role in the pathogenesis of fasting hyperglycemia because of the well-documented increased hepatic glucose production (8, 13, 14). The cause of the increased hepatic glucose production is not completely understood, but insulin resistance may play an important role by decreasing the ability of insulin

to suppress hepatic glucose production and by allowing an unopposed glucagon effect (15). The muscle plays the most important role in glucose disposal, and thus insulin resistance in muscle explains the postprandial hyperglycemia in NIDDM (16).

The cellular alterations responsible for insulin resistance are largely unknown. Using the euglycemic clamp technique to study insulin dose-response curves, both insulin-binding and postinsulin-binding defects in NIDDM were identified, and the post-binding defect seemed to be the predominant abnormality in patients with the higher degrees of fasting hyperglycemia (8). However, direct studies on the mechanism(s) of insulin resistance have only been performed in peripheral blood cells and adipocytes (7–9, 17, 18), which play a relatively minor role in overall glucose production and disposal (19, 20).

The goal of this study was to enhance our understanding of the mechanism(s) of insulin resistance in the liver of patients with NIDDM. To this end, we have developed a method to isolate insulin-responsive human hepatocytes from a 1–2 g liver biopsy. The liver tissue was obtained from a well-characterized population of morbidly obese patients with and without NIDDM, undergoing an elective gastric bypass (21, 22). Data on insulin action, insulin binding, and structure and function of the hepatic insulin receptors of these patients are presented here.

### Methods

**Materials.**  $\alpha$ -[<sup>14</sup>C]Aminoisobutyric acid (51.6 mCi/mmol), carrier-free Na <sup>125</sup>I, [*methoxyl*-<sup>3</sup>H]inulin (186.4 mCi/g), 3-0-[<sup>14</sup>C] methyl-D-glucose (58.0 mCi/mmol), L-[U-<sup>14</sup>C]leucine (300 mCi/mmol), and [<sup>14</sup>C]acetic acid, sodium salt (56.0 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Crude collagenase (type IV, lots 124F-6807, 25F-6806, 25F-6817, 44F-6812) and deoxyribonuclease were obtained from Sigma Chemical Co., St. Louis, MO; dispase was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN; and soybean trypsin inhibitor from Worthington Biomedical Corp., Freehold, NJ. Fraction V bovine serum albumin (BSA) was obtained from Armour Pharmaceutical, Co., Kankakee, IL, and it was further purified by charcoal treatment (23). Crystalline porcine insulin was kindly provided by Dr. Ronald Chance of Eli Lilly & Co., Indianapolis, IN. All other chemicals were reagent grade.

**Human subjects.** 11 morbidly obese patients with NIDDM and an equal number of morbidly obese patients without diabetes were studied. The clinical and biochemical data of these patients are presented in Table I. These patients were admitted to the hospital to undergo gastric bypass for the treatment of morbid obesity. We also studied a nonobese nondiabetic group composed of four subjects who had uncomplicated gallstone disease and were admitted for elective cholecystectomy, and five brain-dead organ donors. The biochemical data on the liver of these two clinically different subgroups of nonobese subjects were identical. Thus, they have been combined and referred to as the nonobese comparison group. The value of the organ donor's liver should be underscored because we have the entire liver from the donors. This tissue has been fundamental

Portions of this work have been presented at the 45th Annual Meeting of the American Diabetes Association, Baltimore, MD (1985) and the Joint Conference of the American Diabetes Association for the Study of Obesity, Toronto, Ontario, Canada (1985).

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1. *Abbreviations used in this paper:* AIB,  $\alpha$ -aminoisobutyric acid; IVGTT, intravenous glucose tolerance test; KRB, Krebs-Ringer bicarbonate (buffer); NIDDM, noninsulin-dependent diabetes mellitus; PAGE, polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

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Table I. Clinical and Biochemical Data in Obese Patients with and without NIDDM

	Obese NIDDM	Obese control
Number and sex	7 females 4 males	9 females 2 males
Age (yr)	44±3	37±4
Weight (kg)	137±6	129±9
Percent body fat	49±1	48±1
Height (cm)	170±3	166±3
Fasting plasma glucose (mg/dl)	181±18	86±1*
Fasting plasma insulin (μU/ml)	39±5	35±5
Peak plasma insulin during IVGTT (μU/ml)	63±14	153±24*
K <sub>a</sub> rate (%/min glucose disappearance)	0.56±0.07	1.40±0.08*
Duration of obesity (yr)		
Preadolescence	6	7
Postadolescence	5	4
Duration of diabetes (yr)	5±2	
Treatment of diabetes	Insulin	3
(number of patients)	Sulfonylureas	2
	None	6

\*  $P < 0.05$ .

for the development of several of the methodologies reported here. The characteristics of the nonobese comparison group were as follows: age, 34±4 y; sex, five male, four female; weight, 67±3 kg; height, 169±5 cm; fasting plasma glucose, 108±7. None of the subjects studied had any diseases or had taken any medications known to alter carbohydrate metabolism, with the exception of the obesity and NIDDM themselves, and the administration of 1 g of methylprednisolone intravenously in four of the organ donors immediately before the organ procurement.

All subjects had maintained constant body weight during the months preceding admission. Records of food intake of the morbidly obese patients demonstrate that ~15% of the calories were derived from proteins, 39% from fat, and 49% from carbohydrates, which is the typical distribution of calories for this population of patients in North Carolina (24). Because of the effect that the caloric intake and its distribution might have on this study, the last eight morbidly obese patients studied were admitted into the hospital 4 d before surgery. During this period, they remained active at approximately their prehospital exercise level. They received a weight maintaining diet providing 50% of the calories as carbohydrates, 30% as fat (polyunsaturated/saturated fat ratio of 0.4 and cholesterol content of 600 mg), and 20% as protein. This diet was basically the same as the one that the patients were taking as outpatients. Furthermore, fasting plasma glucose and insulin and their responses to an intravenous glucose (25 g) tolerance test (IVGTT) were identical in the same subjects studied on the ad libitum diet and the control diet. Also, the biochemical data on the liver from the same group of patients on the ad libitum diet and the control diet were similar.

Every morbidly obese patient without known diabetes mellitus had a 75-g oral glucose tolerance test as an outpatient. Their carbohydrate intake was >150 g daily for the preceding 3 d before the test. The criteria of the National Diabetes Data Group (25) were used to classify these patients as nondiabetics. Oral glucose tolerance tests were not performed in the morbidly obese patients with diabetes with the exception of one who was not known to have NIDDM, but had a fasting plasma glucose of 282 mg/dl and a peak glucose of 429 mg/dl during the test. The morbidly obese subjects with or without NIDDM had a 25-g IVGTT as

outpatients to determine the rate of glucose disappearance ( $K_a$  rate) of plasma glucose and insulin (26). Eight of these subjects had the IVGTT repeated the third day of their hospitalization, while they were on a controlled diet as described above. Insulin or oral hypoglycemic agents were discontinued before admission into the hospital. Rapidly acting insulin was used only if the preprandial plasma glucose was over 300 mg/dl.

The subjects underwent surgery after an overnight fast. General anesthesia was induced with a short-acting barbiturate, and maintained by phentanyl and nitrous oxide-oxygen mixture. Only saline was given intravenously before the liver biopsy. After opening the abdomen, a 1–2-g liver biopsy specimen was obtained from the left lobe of the liver. Informed written consent was obtained from all patients after they were informed about the nature and potential risks of the study.

*Preparation of freshly isolated human hepatocytes.* After biopsy, the liver specimen (1–2 g) was immediately immersed in calcium-free ice cold Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, supplemented with 5 mM glutamate, 11 mM glucose, and 5 mM sodium pyruvate previously gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> (buffer A). The tissue was brought into the laboratory within 5 min. It was then blotted dry between filter papers to remove superficially adherent blood to facilitate the slicing procedure. Tissue was held between two plastic petri dish covers and cut manually into ~0.5-mm-thick slices with a heavy-duty blade. These slices were then incubated twice in buffer A for 10 min at 37°C under 95% O<sub>2</sub>/5% CO<sub>2</sub> atmosphere. This incubation was repeated twice with the same buffer containing 5 mM EGTA. The liver slices were then transferred to 20 ml of Hanks' balanced salt solution without Mg<sup>++</sup>, 5 mM CaCl<sub>2</sub>, containing 1 mg/ml collagenase type IV, 75 μg/ml deoxyribonuclease, 22 μg/ml dispase, and 50 μg/ml soybean trypsin inhibitor (buffer B). They were incubated at 37°C, 95% O<sub>2</sub>/5% CO<sub>2</sub> in 125-ml Erlenmeyer flasks in a circular water bath at 200 rpm. After the first 15 min, the supernatant containing isolated hepatocytes was decanted, the cells were separated by centrifugation and incubated in buffer A containing 2.4 mM CaCl<sub>2</sub> and 3% BSA at 37°C, 95% O<sub>2</sub>/5% CO<sub>2</sub>. Fresh buffer B was added to the undigested tissue and the same operation was repeated four times at 10-min intervals. The remaining undigested tissue was filtered through a nylon mesh using buffer A. The isolated hepatocytes were pooled and washed three times with buffer A containing 2.4 mM CaCl<sub>2</sub> and 1% BSA with 2 min of centrifugation between the washing at 800 rpm. Hepatocytes were then suspended to a final concentration of 0.5–1 × 10<sup>6</sup> cells/ml in the same buffer containing 3% BSA, and after 30 min of preincubation at 37°C, 95% O<sub>2</sub>/5% CO<sub>2</sub>, the cells were centrifuged and resuspended at 0.5–1 × 10<sup>6</sup> cells/ml to determine biological responses and insulin binding.

*Cell counting, sizing, and indicators of viability of freshly isolated human hepatocytes.* Freshly isolated hepatocytes were counted in a hemocytometer. The intracellular water space was estimated using 3-O-[<sup>14</sup>C]methyl-D-glucose and adjusting for the trapping of extracellular water by the [methoxyl-<sup>3</sup>H]inulin space measurement as reported (27). The cell surface area was calculated assuming that each cell was a sphere by measuring the cytoplasmic area using the Videoplan image analysis System (Zeiss, Oberkochen, Federal Republic of Germany) on cytocentrifuge (Shandon Southern Instruments, Inc., Sewickley, PA) prepared material. Measurements were performed on Papanicolaou-stained smears. From each slide 100 cells were randomly selected. Manually using a cursor, the cytoplasm of these cells was outlined as displayed on a videomonitor and the data were stored on a floppy disk and the mean±standard deviation of the cell surface area was computed (28). Cell viability was assessed by its morphologic integrity, exclusion of trypan blue, linearity of [<sup>14</sup>C]leucine and [<sup>14</sup>C]acetate incorporation into proteins and lipids, respectively, and the ability of the cells to maintain normal ATP concentration during the incubation procedure (29).

*Bioassay of insulin action in freshly isolated human hepatocytes.* The ability of insulin to stimulate the uptake of α-aminoisobutyric acid (AIB), a nonmetabolizable analogue of alanine, was used as a bioassay of insulin action. Freshly isolated human hepatocytes were preincubated for 2 h in the absence and presence of insulin (1 × 10<sup>-9</sup> M and 1 × 10<sup>-7</sup> M) at 37°C (30). After preincubation, [<sup>14</sup>C]AIB (0.1 mM, 1.4 mCi/mmol) was

added to the incubation mixture. Because AIB uptake was linear for at least 20 min, the reaction was terminated at 10 min to obtain the initial rate of uptake as previously reported for rat hepatocytes (30).

**Insulin binding in freshly isolated human hepatocytes.** Insulin was iodinated with chloramine T according to the method of Cuatrecasas (31). Freshly isolated hepatocytes were incubated at 4°C for 16 h in the presence of labeled insulin ( $1 \times 10^{-10}$  M) and increasing concentrations of unlabeled insulin. Insulin binding was expressed as specific cell-associated  $^{125}\text{I}$ -insulin binding after subtractions of nonspecific binding which was determined in the presence of excess  $1 \times 10^{-6}$  M unlabeled insulin. The nonspecific insulin binding was consistently <15% of the total binding.

**Insulin binding to human liver plasma membranes.** The liver tissue was homogenized in 50 ml of a 1 mM  $\text{NaHCO}_3$  buffer, containing 0.5 mM  $\text{CaCl}_2$ , pH 7.5, as described by Ray (32), and the plasma membranes were isolated by the aqueous two-phase polymer method of Lesko et al. (33). The membranes were suspended in 0.25 M Sucrose, 50 mM Tris buffer, pH 7.4, and kept at  $-70^\circ\text{C}$  until they were used. Insulin binding activity was measured as described by Arner et al. (34) with minor modifications. Approximately 20  $\mu\text{g}$  of membrane protein was incubated in 350  $\mu\text{l}$  of Krebs-Ringer phosphate buffer, pH 7.4, containing 3% BSA. Membranes were incubated with  $^{125}\text{I}$ -insulin ( $1 \times 10^{-10}$  M) and increasing concentrations of unlabeled insulin at 4°C for 16 h. The membrane bound  $^{125}\text{I}$ -insulin was separated from free  $^{125}\text{I}$ -insulin by the centrifugation method of Rodbell et al. (35).

**Partially purified insulin receptors from human liver.** The liver specimen was immediately compressed between precooled copper planchets, dropped into liquid nitrogen, and stored at  $-70^\circ\text{C}$ . The frozen liver was then used to prepare a liver microsomal fraction as described by Blackshear et al. (36). Insulin receptors were solubilized for 60 min at room temperature with 50 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 2  $\mu\text{M}$  leupeptin, 2  $\mu\text{M}$  pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml bacitracin, and 1,000 U/ml aprotinin. The insoluble material was separated from solubilized material by centrifugation at 100,000 g for 90 min at 4°C. The soluble extract was diluted 1:2 with 50 mM Tris-HCl buffer containing various protease inhibitors and equilibrated at 4°C with 2 ml of wheat germ agglutinin (WGA) sepharose. The unabsorbed material was extensively washed with 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM  $\text{MgCl}_2$ , 0.1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The glycoproteins were then eluted from the WGA sepharose column by the above buffer containing 0.3 M *N*-acetyl glucosamine. The protein concentration was measured by the method of Miller (37) after the removal of interfering substances (38). This partially purified insulin receptor preparation was used to measure, under identical conditions,  $^{125}\text{I}$ -insulin binding and tyrosine specific protein kinase activity, and to affinity label the insulin receptor with  $^{125}\text{I}$ -insulin as described below.

**$^{125}\text{I}$ -insulin binding in partially purified insulin receptors from human liver.** 50- $\mu\text{l}$  aliquots of partially purified solubilized receptors (10  $\mu\text{g}$  of protein) were incubated in a final volume of 250  $\mu\text{l}$  in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 and 10 mM  $\text{MgCl}_2$  in the presence of  $^{125}\text{I}$ -Insulin ( $1 \times 10^{-10}$  M) and increasing concentrations of unlabeled insulin for 16 h at 4°C. The  $^{125}\text{I}$ -insulin bound to receptor was precipitated by adding bovine gamma-globulin and 12.5% polyethylene glycol at 4°C as previously described (30).

**Tyrosine-specific protein kinase activity of human liver insulin receptors.** The tyrosine-specific protein kinase activity of the solubilized insulin receptor was measured by the method of Grunberger et al. (39), with minor modifications. After preincubation of the insulin receptor with different concentrations of insulin for 16 h at 4°C, as in the insulin binding assay, [ $^{32}\text{P}$ ]ATP (100  $\mu\text{M}$ ) was added in the presence of 2.5 mg/ml exogenous substrate, Glu<sup>80</sup>-Tyr<sup>20</sup>, 10 mM  $\text{MgCl}_2$ , and 0.5 mM  $\text{MnCl}_2$ . After 20 min at room temperature, the reaction was stopped with 10% trichloroacetic acid (TCA) containing 10 mM pyrophosphate and 3 mg/ml BSA. The insulin receptor kinase activity was expressed as picomoles of  $^{32}\text{P}$  incorporated per milligram of Glu<sup>80</sup>-Tyr<sup>20</sup> per minute per milligram of solubilized insulin receptor.

**Affinity labeling of the insulin receptor with  $^{125}\text{I}$ -insulin.** After optimal

$^{125}\text{I}$ -insulin binding in the absence and presence of unlabeled insulin ( $1 \times 10^{-6}$  M) to purified insulin receptors as described in the preceding section, the  $^{125}\text{I}$ -insulin receptor complex was covalently cross-linked with 0.5 mM disuccinimidyl suberate for 10 min at 4°C by the method of Pilch and Czech (40). The reaction was stopped by adding 50 mM Tris-HCl (pH 7.0) containing 1% sodium dodecyl sulfate, 0.005% bromophenol blue, and 2% glycerol in the presence of 50 mM dithiothreitol and boiling for 3 min. Polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (41) in a 7.5% acrylamide resolving gel and a 4.5% acrylamide stacking gel. The gels were fixed, stained with 0.25% Coomassie blue, destained, dried, and autoradiographed for 24 h at  $-70^\circ\text{C}$  with Kodak X-OMAT film using lightening plus screen.

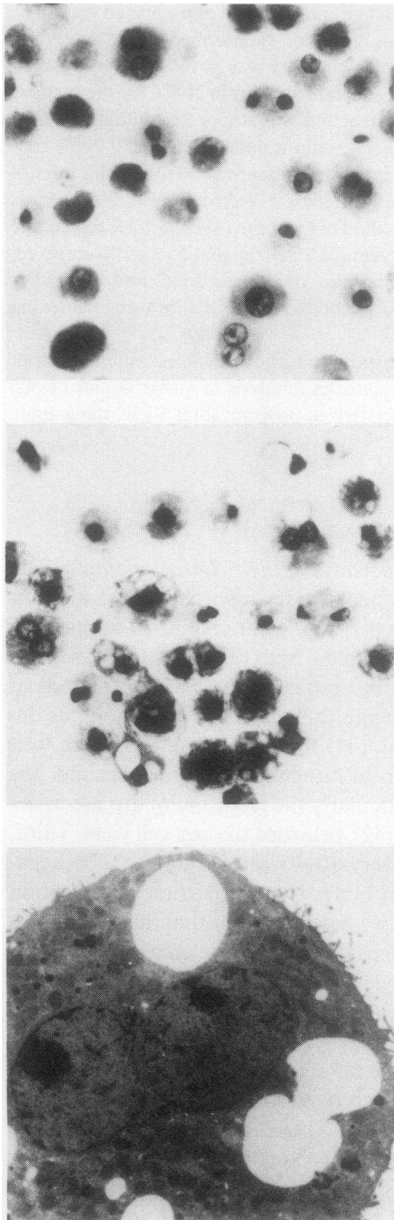
**Statistical analysis.** Descriptive statistics (mean  $\pm$  standard error of the mean) and comparative statistics (analysis of variance and *t* tests) were performed using a computerized program (Stat View, the graphic statistics for the Macintosh).

## Results

**Isolation of human hepatocytes and their characterization.** The method to isolate human hepatocytes reported here is a modification of other methods (42–45). After several attempts, four points become clear, which are the basis of the method described here. First, the four 10-min preincubation washing periods at 37°C of the liver slices before the enzymatic treatment, as described by Gebhardt et al. (43) for rat hepatocytes, were fundamental for the isolation of human hepatocytes. Second, the enzymatic treatment, particularly the inclusion of dispase as described by Maekubo et al. (42), provided the best cell yield. Third, the sequential removal every 10–15 min of the isolated hepatocytes from the digestion buffer is an important modification we have introduced since we have observed that the continuous exposure of the isolated hepatocytes with the enzymes results in a sharp decrease in cell yield. Finally, although we were well aware of the importance in selecting a collagenase preparation for isolation of rat hepatocytes, we learned that, for the isolation of human hepatocytes, it is the single most important factor to consider. Human liver specimens from organ donors were divided into four equal portions. Then, hepatocytes were isolated in an identical manner as described in Methods, except that four different lots of collagenase type IV (Sigma Chemical Co.) were used. Lot nos. 124F6807 and 25F6817 resulted in a cell yield which was consistently  $<2.5 \times 10^6$  cells per gram of liver, whereas lot nos. 25F6806 and 44F6812 gave a cell yield  $>8 \times 10^6$  cells per gram of liver. The collagenase preparation (4177 CLSII W2H209, Worthington Biochemical Corp.), which we have used to isolate a high yield of insulin responsive rat hepatocytes for the last 3 yr, gave the lowest cell yield of all the collagenase preparations we have tested. Lot no. 44F6812 was used for the execution of the experiments described herein.

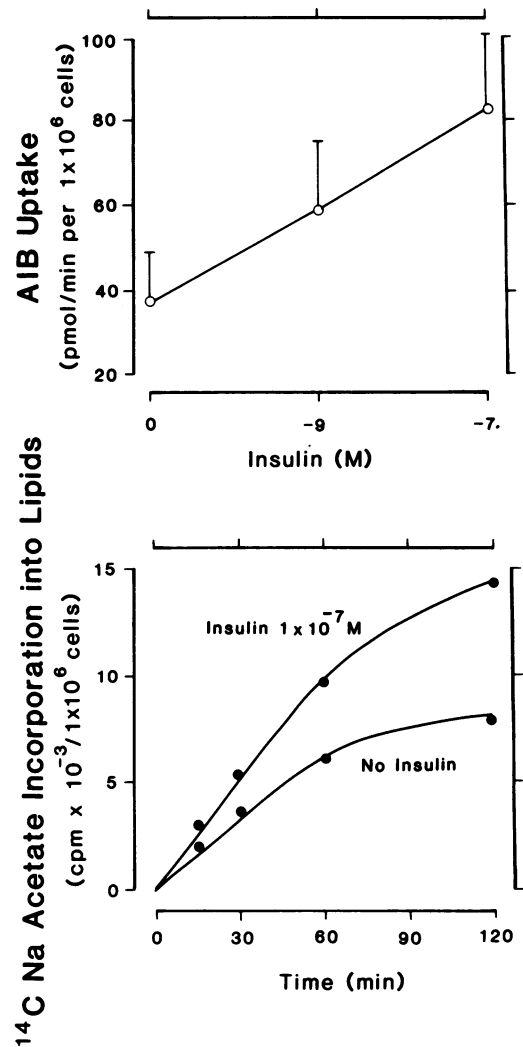
Human hepatocytes isolated in this manner are morphologically intact as shown in Fig. 1. Over 90% of the cells exclude trypan blue. They incorporate [ $^{14}\text{C}$ ]leucine into proteins linearly for at least 2 h, and they maintain initial ATP concentrations ( $\sim 1 \times 10^{-2}$  M) for at least 2 h of incubation. The ATP concentration of the isolated human hepatocytes is identical to that reported for isolated rat hepatocytes (46). Furthermore, these isolated human hepatocytes are responsive to insulin with regard to its ability to stimulate AIB uptake and [ $^{14}\text{C}$ ]acetate incorporation into total lipids above basal as demonstrated in Fig. 2.

Freshly isolated hepatocytes from the patients with morbid obesity were morphologically intact and viable, in spite of sig-



**Figure 1.** Morphology of freshly isolated human hepatocytes. Human hepatocytes were isolated as described in Methods. The cells were processed for light and electron microscopy. The upper panel is a light micrograph of hepatocytes from a nonobese subject, the middle panel from a morbidly obese patient, and the lower panel is an electron micrograph from a morbidly obese patient.

nificant histologic differences with regard to fat infiltration or the degree of fibrosis of the liver specimens. Furthermore, the cell yield per gram of tissue was similar whether the patients had obesity and diabetes ( $10 \pm 3 \times 10^6$  cells per gram liver,  $n = 6$ ), obesity alone ( $11 \pm 1 \times 10^6$  cells per gram liver,  $n = 4$ ), or were nonobese ( $14 \pm 2 \times 10^6$  cells per gram liver,  $n = 3$ ). The morphology of the isolated hepatocytes correlates well with that of the intact liver with regard to the degree of fatty infiltration (Fig. 2). The intracellular water space was also similar in the three groups of patients. However, the volume of hepatocytes of the obese subjects calculated using the Videoplan image analysis system ( $3.1 \pm 0.9 \mu\text{l}/1 \times 10^6$  cells in obese NIDDM;  $2.4 \pm 0.7 \mu\text{l}/$



**Figure 2.** Indicators of viability of freshly isolated human hepatocytes. AIB uptake (*upper panel*). Freshly isolated human hepatocytes ( $0.5\text{--}1 \times 10^6$  cells/ml) were preincubated in the presence and absence of insulin for 2 h in KRB buffer supplemented with 3% BSA at  $37^\circ\text{C}$ , 95%  $\text{O}_2/5\%$   $\text{CO}_2$ . Then AIB, 0.1 mM (1.4 mCi/mmol) was added to the incubation mixture. Because the reaction was linear for at least 20 min, the reaction was terminated at 10 min to obtain initial rates of uptake. The figure shows the dose-response curve of insulin-stimulated AIB uptake (mean  $\pm$  SEM) from three nonobese subjects. [ $^{14}\text{C}$ ]Na acetate incorporation into lipids (*lower panel*). Human hepatocytes from one nonobese subject were incubated in the presence and absence of insulin  $1 \times 10^{-7}$  M and [ $^{14}\text{C}$ ]acetic acid (sodium salt, 5 mM, 0.01 mCi/mmol). At different times, total lipids were extracted into a mixture of chloroform, water, and methanol.

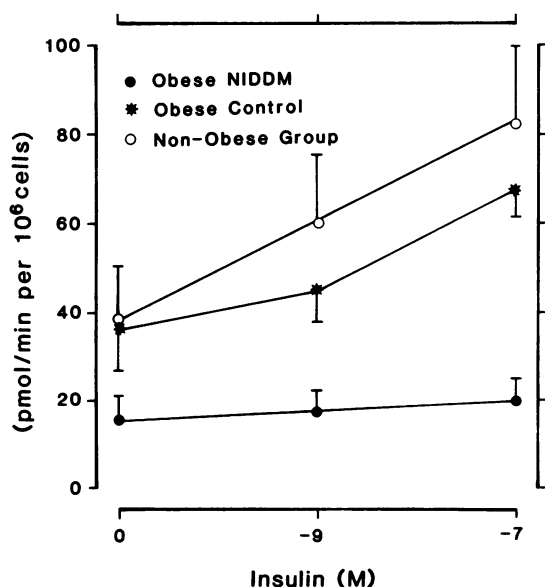
$1 \times 10^6$  cells in obese controls) was about twice of that of the nonobese subjects ( $1.3 \pm 0.4 \mu\text{l}/1 \times 10^6$  cells). The surface areas of the hepatocytes were  $1,033 \pm 325$ ;  $854 \pm 260$ ; and  $586 \pm 195 \mu\text{m}^2$  for the obese NIDDM, obese control, and the nonobese groups, respectively (mean  $\pm$  standard deviation from 200 cells from each group).

It is clear that the cells from the obese patients with or without NIDDM, even though the cells have the same water space as those from the nonobese subjects, are larger because of fat infiltration, as can be seen by simple inspection in Fig. 1.

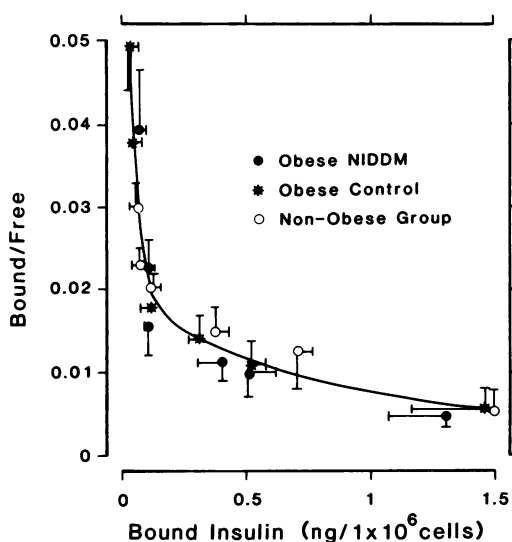
*Insulin action in isolated hepatocytes.* The ability of insulin

to stimulate AIB uptake was used as a bioassay of insulin action (Fig. 3). The basal rate of AIB uptake was increased in the hepatocytes from nondiabetic subjects when compared with the obese subjects with NIDDM. However, this difference was not statistically significant. Basal AIB uptake in freshly isolated hepatocytes from humans was similar to that previously reported (30) in freshly isolated rat hepatocytes ( $37 \pm 13$  pmol/ $10^6$  cells/min in humans;  $46 \pm 6$  pmol/ $10^6$  cells/min in rats). The insulin-stimulated AIB uptake was determined using submaximal ( $1 \times 10^{-9}$  M) and maximal ( $1 \times 10^{-7}$  M) concentrations of insulin. Insulin at  $1 \times 10^{-9}$  M and  $1 \times 10^{-7}$  M stimulated AIB uptake  $43 \pm 17\%$  and  $118 \pm 18\%$  above basal ( $P < 0.05$ – $0.005$ ), respectively, in human hepatocytes from nonobese subjects. Human hepatocytes from morbidly obese subjects were also responsive to insulin, but this response was significant ( $P < 0.005$ ) only with maximal concentrations of insulin. Because of the limited amount of material for these studies, these data only suggest a shift to the right in the dose-response curve in the patients with morbid obesity. Hepatocytes from patients with obesity and NIDDM were unresponsive to submaximal or maximal concentrations of insulin. Thus far, this data demonstrates that the human liver from NIDDM studied in vitro is resistant to insulin with regard to AIB uptake. The next part of this study was designed to define further the mechanism of this insulin resistance.

**Insulin binding.** Fig. 4 demonstrates Scatchard plots of insulin binding in freshly isolated hepatocytes performed at  $4^\circ\text{C}$ . These plots show that the apparent affinity of the insulin receptor for insulin and the number of binding sites are the same in the three groups of patients when the data are expressed per  $1 \times 10^6$  cells. The concentration of native insulin displacing 50% of tracer  $^{125}\text{I}$ -insulin ( $1 \times 10^{-10}$  M) was  $1.8 \times 10^{-9}$  M,  $0.8 \times 10^{-9}$  M, and



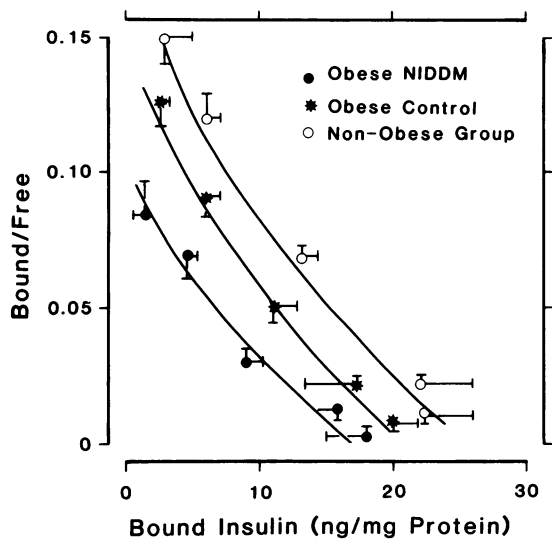
**Figure 3.** Insulin action in freshly isolated human hepatocytes. Freshly isolated human hepatocytes ( $0.5$ – $1 \times 10^6$  cells/ml) were preincubated at  $37^\circ\text{C}$ , under  $95\% \text{O}_2/5\% \text{CO}_2$  atmosphere in the presence and absence of different concentrations of insulin for 2 h in KRB buffer supplemented with 3% BSA. Then, AIB ( $0.1$  mM,  $1.4$  mCi/mmol) was added and 10 min later the reaction was stopped. The figure shows the dose-response curve of insulin-stimulated AIB uptake (mean $\pm$ SEM) from five obese patients with NIDDM ( $\bullet$ ), three obese controls ( $*$ ), and three nonobese subjects ( $\circ$ ).



**Figure 4.** Insulin binding to freshly isolated human hepatocytes. Freshly isolated human hepatocytes ( $0.5$ – $1 \times 10^6$  cells/ml) were incubated for 16 h at  $4^\circ\text{C}$  with a tracer amount of  $^{125}\text{I}$ -insulin ( $1 \times 10^{-10}$  M) and increasing concentrations of unlabeled insulin ( $0$ – $10^{-6}$  M) in KRB buffer supplemented with 3% BSA. The cells were separated from the medium by centrifugation through dibutylphthalate oil. Nonspecific  $^{125}\text{I}$ -insulin binding, defined as the residual radioactivity present in the presence of  $1 \times 10^{-6}$  M insulin, was subtracted from total bound radioactivity to determine specific  $^{125}\text{I}$ -insulin binding. The figure shows the Scatchard plots of the insulin binding data (mean $\pm$ SEM) from four obese patients with NIDDM ( $\bullet$ ), three obese controls ( $*$ ), and four nonobese subjects ( $\circ$ ).

$1.3 \times 10^{-9}$  M for the obese NIDDM, obese, and nonobese subjects, respectively, further suggesting that the binding affinity for insulin is the same in the three groups. However, since the volume of hepatocytes from nonobese subjects was smaller than in the obese patients,  $^{125}\text{I}$ -insulin binding is higher in the nonobese controls when expressed as per unit of cell surface area.

The data in isolated hepatocytes represent cell surface insulin binding because at  $4^\circ\text{C}$  insulin internalization and degradation are negligible. Thus, we next studied the total pool of detergent extractable insulin receptors from a portion of the liver specimen prior to cell isolation. Fig. 5 shows the Scatchard plots of insulin binding in detergent solubilized receptor extracts. The concentration of native insulin displacing 50% of tracer  $^{125}\text{I}$ -insulin ( $1 \times 10^{-10}$  M) was  $0.8 \times 10^{-9}$  M,  $0.8 \times 10^{-9}$  M, and  $1.2 \times 10^{-9}$  M for the obese NIDDM, obese control, and nonobese groups, respectively, suggesting that the binding affinity for insulin is the same in the three groups as shown by the parallelism of the Scatchard plots. Analysis of variance of these plots demonstrates significant differences ( $P < 0.01$ – $0.005$ ) between the three groups at low concentrations of insulin ( $1 \times 10^{-10}$  M to  $1 \times 10^{-9}$  M, the first three points in the Scatchard plots), but no differences at the insulin concentrations  $> 5 \times 10^{-9}$  M. At low concentrations of insulin, the bound/free ratio and the bound insulin was lower ( $P < 0.005$ ) in the NIDDM patients when compared with the nonobese subjects. The obese subjects were in between the two groups, but were more similar to the nonobese subjects than to the diabetics. The only difference ( $P < 0.05$ ) between the obese and nonobese subjects was in the bound insulin at  $3 \times 10^{-10}$  M insulin concentration (second point in the Scatchard plots). When compared with the diabetics, the obese controls were dif-

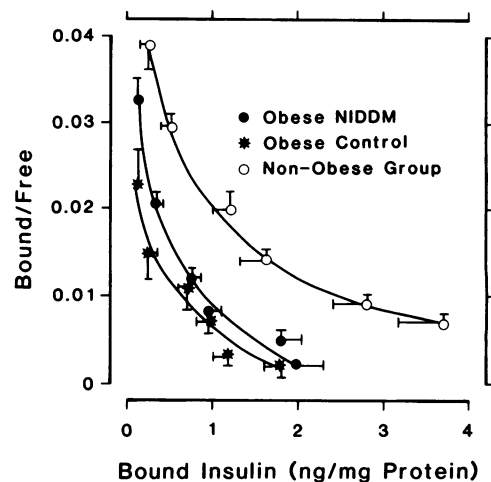


**Figure 5.** Insulin binding to solubilized WGA-purified insulin receptors from human liver. WGA-purified insulin receptors ( $10 \mu\text{g}$  of protein) were incubated with  $^{125}\text{I}$ -insulin ( $1 \times 10^{-10} \text{ M}$ ) and increasing concentrations of unlabeled insulin ( $0\text{--}10^{-6} \text{ M}$ ) in a total volume of  $250 \mu\text{l}$  of  $50 \text{ mM}$  Tris buffer containing  $0.1\%$  Triton X-100. After incubation for  $16 \text{ h}$  at  $4^\circ\text{C}$ , the receptor and receptor-bound insulin were precipitated with polyethylene glycol ( $12.5\%$  final concentration) using bovine  $\gamma$ -globulin as carrier protein. The figure shows the Scatchard plots of the specific insulin-binding data (mean  $\pm$  SEM) from seven obese patients with NIDDM ( $\bullet$ ), seven obese controls ( $*$ ), and seven nonobese subjects ( $\circ$ ). Analysis of variance of these plots demonstrates significant differences ( $P < 0.01\text{--}0.005$ ) between the three groups at low concentrations of insulin ( $1 \times 10^{-10} \text{ M}$  to  $1 \times 10^{-9} \text{ M}$ , the first three points in the Scatchard plots). The obese control was in between the other two groups. When compared to the obese NIDDM it was different at  $1 \times 10^{-10} \text{ M}$  and  $1 \times 10^{-9} \text{ M}$  insulin ( $P < 0.05$ , first and second point in the Scatchard plots). The only difference between obese and nonobese control was at  $3 \times 10^{-10} \text{ M}$  insulin ( $P < 0.05$ , second point in the Scatchard plots).

ferent at  $1 \times 10^{-10} \text{ M}$  (bound and bound/free,  $P < 0.05$ ) and  $1 \times 10^{-9} \text{ M}$  insulin concentrations (bound/free,  $P < 0.025$ ).

These data in detergent-solubilized receptor extracts are different from what could be predicted from the insulin-binding data in freshly isolated hepatocytes corrected for cell surface area. Insulin binding in nonobese controls should have been greater than in the obese subjects with or without diabetes, without any differences between these two latter groups. Thus, we raised the questions: (a) whether the data from the isolated hepatocytes were not representative of the *in vivo* data owing to redistribution of the insulin receptors during the 2-h isolation procedure; and also, (b) whether the differences between the obese patients with and without diabetes on the detergent-extractable insulin binding were due to varying degrees of solubilization rates owing to differences in membrane composition and thus not representative of *in vivo* data.

In an attempt to answer these questions, we first performed insulin binding in partially purified liver plasma membrane isolated from liver biopsies prior to cell isolation. Fig. 6 shows these data, which are essentially as predicted from the insulin binding in isolated hepatocytes. At every insulin concentration, insulin binding was higher in the nonobese control ( $P < 0.05\text{--}0.005$ ) when compared with the obese with and without NIDDM. There was no difference between these two latter groups except that at



**Figure 6.** Insulin binding to liver plasma membranes from human liver. Liver plasma membranes ( $20 \mu\text{g}/\text{protein}$ ) were incubated in Krebs-Ringer phosphate buffer containing  $3\%$  BSA in the presence of  $^{125}\text{I}$ -insulin ( $1 \times 10^{-10} \text{ M}$ ) and increasing concentrations of insulin ( $0\text{--}10^{-6} \text{ M}$ ) for  $16 \text{ h}$  at  $4^\circ\text{C}$ . Then the membranes were separated from the medium by centrifugation. The figure shows the Scatchard plots of the specific insulin binding data (mean  $\pm$  SEM) from five obese patients with NIDDM ( $\bullet$ ), five obese controls ( $*$ ), and five nonobese subjects ( $\circ$ ).

$1 \times 10^{-10} \text{ M}$  and  $5 \times 10^{-9} \text{ M}$  insulin concentrations (the first and fifth points in the Scatchard plots) insulin binding was increased in the obese NIDDM when compared with the obese controls.

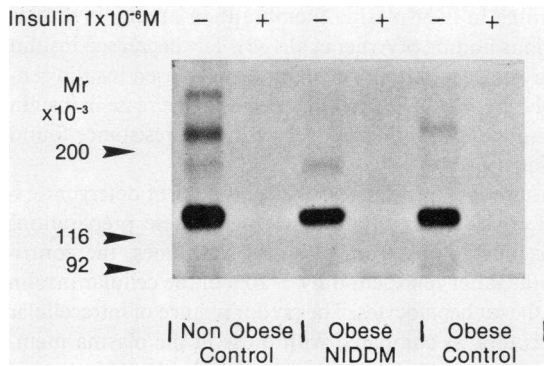
Finally, when a given amount of liver plasma membrane with equal insulin binding activity from obese subjects with or without NIDDM and nonobese subjects was solubilized and the insulin receptors were partially purified on a WGA-Sepharose column, the insulin-binding activity recovered in the three groups was the same, demonstrating that the rate of solubilization of detergent-extractable insulin receptor was unaffected by possible differences in chemical composition.

Fig. 7 is an autoradiogram of the  $\alpha$ -subunit of the insulin receptor cross-linked with  $^{125}\text{I}$ -insulin using  $0.5 \text{ mM}$  disuccinamidyl suberate under reducing conditions and subjected to SDS-PAGE as described in the Methods section. There is no difference in the characteristics of this band in patients from different groups. As can be seen in Fig. 7, the changes in insulin binding described in Fig. 5 are reflected in affinity labeling in that with identical protein concentrations the nonobese group that has the highest insulin binding also demonstrates the greatest radioactivity associated with the  $\alpha$ -subunit of the insulin receptor. The different pattern of high molecular weight bands of low intensity in the groups was not a consistent finding and it is probably due to a varying degree of reduction by dithiothreitol in the different receptor preparations.

**Insulin receptor kinase.** The ability of insulin to stimulate the phosphorylation of the artificial exogenous substrate Glu $^{80}$ -Tyr $^{20}$ , was used to assess the protein kinase activity of the insulin receptors. These experiments were performed in parallel and under identical conditions as those performed in Fig. 5 for  $^{125}\text{I}$ -insulin binding. Thus, the data can be expressed as per milligram of protein and also by bound insulin.

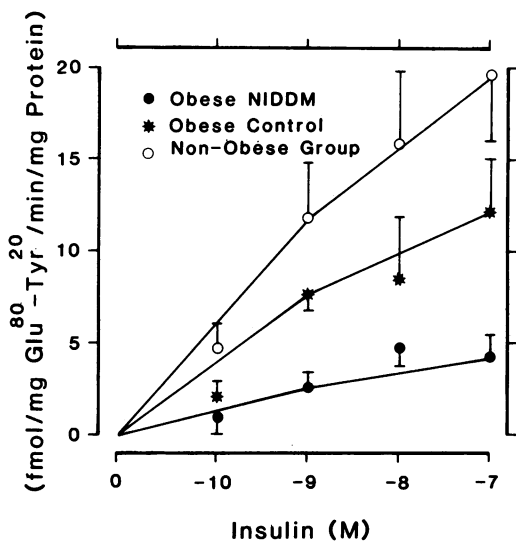
Fig. 8 demonstrates the ability of different concentrations of





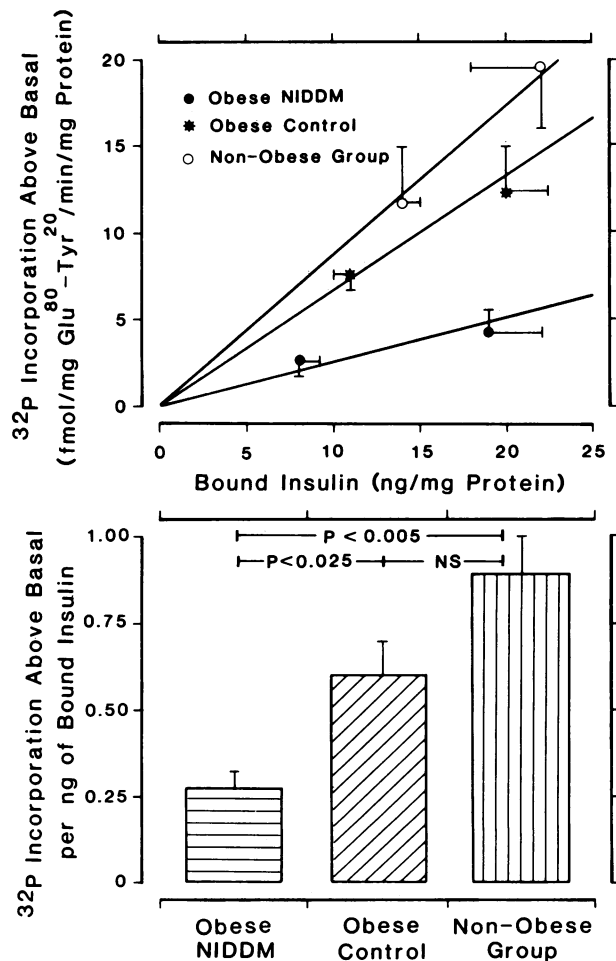
**Figure 7.** Autoradiography of SDS-PAGE of solubilized insulin receptors.  $^{125}\text{I}$ -insulin ( $1 \times 10^{-9}$  M) binding was performed in solubilized WGA-purified insulin receptor as described in the legend to Fig. 5, in the presence and absence of unlabeled insulin. The  $^{125}\text{I}$ -insulin receptor complex was cross-linked with 0.5 mM disuccinimidyl suberate, reduced with 50 mM dithiothreitol, and subjected to SDS electrophoresis in a 7.5% acrylamide resolving gel. The figure shows the  $\alpha$ -subunit of insulin receptor from a nonobese, obese NIDDM, and obese control.

insulin to stimulate the insulin receptor kinase above basal. Basal kinase activity was  $11.7 \pm 1.8$ ,  $13.78 \pm 1.0$ , and  $13.6 \pm 1.4$  fmol of  $^{32}\text{P}$  incorporated per milligram of Glu $^{80}$ -Tyr $^{20}$ /min/mg of protein in the obese NIDDM, obese controls, and nonobese comparison groups, respectively. The data in Fig. 8 clearly show that, whereas insulin stimulates the insulin receptor kinase in the two groups



**Figure 8.** Dose-response curve of insulin-stimulated insulin receptor kinase activity in human liver. WGA-purified insulin receptors from liver ( $10 \mu\text{g}$  of protein) were incubated with different concentrations of insulin under identical conditions to that of  $^{125}\text{I}$ -insulin binding described in Fig. 5. Then,  $^{32}\text{P}$ ATP ( $100 \mu\text{M}$ ) was added in the presence of Glu $^{80}$ -Tyr $^{20}$  ( $2.5 \text{ mg/ml}$ ),  $10 \text{ mM MgCl}_2$ , and  $0.5 \text{ mM MnCl}_2$ . After 20 min at room temperature, the reaction was stopped with 10% TCA containing  $10 \text{ mM}$  pyrophosphate and 3% BSA. The figure shows the data (mean  $\pm$  SEM) from three or seven obese patients with NIDDM ( $\bullet$ ), three or seven obese controls ( $\ast$ ), and three or seven nonobese subjects. The lower number of observations were at  $1 \times 10^{-10}$  M and  $1 \times 10^{-8}$  M insulin.

of patients without diabetes, it had a blunted effect on the liver insulin receptor from subjects with NIDDM. Statistical analysis was not performed on the data generated with  $1 \times 10^{-10}$  M and  $1 \times 10^{-8}$  M insulin because liver from only three patients was used in each group. However, at  $1 \times 10^{-9}$  M and  $1 \times 10^{-7}$  M insulin ( $n = 7$ ), this analysis demonstrates that there was no significant difference between the two nondiabetic groups, and that these two groups separately were significantly different from the NIDDM group ( $P < 0.025$  at both insulin concentrations for the obese controls;  $P < 0.025$  and  $P < 0.005$  for  $1 \times 10^{-9}$  M and  $1 \times 10^{-7}$  M, respectively, for the nonobese group). Although these data are expressed as per milligram of protein, the three- to fivefold higher stimulation of the insulin receptor kinase in the nondiabetics ( $12 \pm 3$  in obese;  $20 \pm 4$  in nonobese), when compared with the NIDDM patients ( $4 \pm 1$ ), cannot be explained by the relatively small differences in insulin binding. The upper panel of Fig. 9 demonstrates the insulin-stimulated protein kinase activity of insulin receptors plotted against the amount of bound insulin. It can be seen that for a given amount of insulin bound, the receptors from the NIDDM patients are less responsive to insulin than those from the nondiabetic groups. The lower panel



**Figure 9.** Insulin-stimulated insulin receptor kinase activity in human liver. (Upper panel): The insulin receptor kinase data from Fig. 8 is plotted against the bound insulin from Fig. 5. (Lower panel): The same data are normalized by nanograms of insulin specifically bound to the insulin receptor.



of the same figure shows that each nanogram of bound insulin stimulates the insulin receptor kinase more than double ( $P < 0.025$ ) and more than triple ( $P < 0.005$ ) in the obese controls and nonobese group than the stimulation in the NIDDM subjects.

## Discussion

We have developed a method to isolate  $\sim 20 \times 10^6$  human hepatocytes from an intraoperative liver biopsy. These cells appear to be morphologically and biochemically intact. Furthermore, they respond to submaximal and maximal concentrations of insulin. The long range goal of our laboratory is now to understand better the insulin action and resistance in human liver at the cellular and subcellular level. This technical advance can be of maximal value only if it can be applied to a well-characterized human population undergoing elective surgery.

Our institution performs approximately 100 gastric bypass procedures yearly for the treatment of morbid obesity. The patients undergoing surgery comprise an interesting population of whom 27% have NIDDM, 20% have impaired glucose tolerance, and the remainder have normal glucose tolerance tests as defined by the National Diabetes Data Group (25).

Although 80% of patients with NIDDM are obese, we recognize that we are studying only those who are extremely obese. Even though *in vivo* studies have not demonstrated fundamental differences between obese and nonobese patients with NIDDM (6, 47), we do not wish to imply that the data presented here in morbidly obese patients with NIDDM can be extrapolated to the entire population with NIDDM. However, this is the only large population with NIDDM, noncritically ill and relatively homogeneous, from whom liver tissue can be obtained without any significant additional risk at the same time that a therapeutic gastric bypass is performed.

The initial step in this study was to see whether isolated hepatocytes from patients with NIDDM were resistant to insulin, and then to see whether any changes in insulin receptor structure or function could explain the insulin resistance.

Human hepatocytes from obese patients with NIDDM are unresponsive to maximal insulin concentration with regard to AIB uptake, and those from obese subjects without NIDDM appear to have a diminished response to submaximal insulin concentration but they respond normally to maximal insulin concentration. Our data, however, do not imply that the human liver is universally resistant to insulin. It is important to note that in a given metabolic state one or more tissues may be resistant to insulin, but others may be responsive. Likewise, one tissue may be resistant to one hormone action but not to another.

Insulin binding in freshly isolated hepatocytes from obese patients with and without NIDDM is similar to the nonobese group when it is related to number of cells. However, if insulin binding is expressed per unit of cell surface area, it is decreased in both obese groups. This fact is also reflected by insulin-binding studies using liver plasma membranes. However, in plasma membrane preparation, insulin binding at tracer insulin concentrations ( $1 \times 10^{-10}$  M) is increased in NIDDM compared to weight-matched nondiabetics. It is possible that these subtle changes in insulin binding are affected by the cell isolation procedure or that the preparation of liver plasma membrane exposes some additional insulin-binding sites that are not normally available at the cell surface. Our demonstration of decreased

insulin binding in liver plasma membrane in obesity is similar to the previous finding of Arner et al. (34). The decreased insulin binding may at least partially explain the decreased insulin sensitivity in obesity. However, a similar degree of decrease in insulin binding cannot explain the most severe insulin resistance found in obese diabetics.

The insulin binding data on the total pool of detergent extracts is of considerable interest. Although these preparations contain insulin binding from plasma membranes, the contributions of the latter represent only  $\sim 20\%$  of the cellular insulin binding in the rat hepatocytes. The predominance of intracellular insulin receptors, as compared with those in the plasma membranes, has been previously reported (27, 48–51). This pool of receptors is significantly decreased in obese patients with NIDDM and only slightly decreased in obese controls. Thus, in obesity alone, the decrease in insulin receptor appears to be mainly at the plasma membrane, whereas in NIDDM there is also a decrease in the intracellular pool of insulin receptors. The mechanism of these alterations is largely unknown. However, our ability to have a viable cell preparation should permit future studies of insulin receptor synthesis, degradation, and recycling.

Because the structural characteristics of the hepatic insulin receptor in the three groups of patients we have studied were identical, at least as determined by the affinity-labeling method of Pilch and Czech (40), and we believe that the changes in insulin binding observed in NIDDM cannot, by themselves, explain the insulin resistance, we studied the insulin receptor kinase activity—one of the newly recognized functions of the insulin receptor (52).

The interaction of insulin with its receptor in the plasma membrane is known to trigger both the phosphorylation and dephosphorylation of several cellular proteins, which may be a basic regulatory mechanism in hormone action (53–55). Recently, it has been shown that insulin binding induces phosphorylation of the  $\beta$ -subunit of the insulin receptor and that the insulin receptor itself is a tyrosine-specific protein kinase with the ability to phosphorylate exogenous substrates and presumably, also, endogenous substrates (52, 56–60). Thus, it has been hypothesized that the phosphorylation and protein kinase activity of the insulin receptor might mediate insulin action. Indeed, several lines of evidence (61–64), but not all (36, 65–68), support this hypothesis.

Our study suggests that diminished insulin protein kinase activity of the insulin receptor provides a possible mechanism for the postinsulin-binding defect of insulin action in the hepatocytes from patients with NIDDM. Insulin-stimulated protein kinase activity of insulin receptors is clearly diminished in NIDDM, whether the data are expressed as per milligram of protein or as per nanogram of insulin specifically bound to the receptor. Because of the limited amount of human liver available, receptor autophosphorylation could not be studied. Thus, our studies do not imply that autophosphorylation of the insulin receptor is also defective in NIDDM in that the dissociation between autophosphorylation and the ability to phosphorylate exogenous substrates has already been reported (64). Furthermore, because the biological significance of these experiments performed in a free-cell system is not completely clear at this time, and there are important differences in these processes if they are examined in an intact cell system (69), our data are only supportive of the hypothesis that the insulin resistance in NIDDM is at least partially due to a defect in the insulin receptor kinase.

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