THE DEFECT IN INSULIN RECEPTORS IN OBESE-HYPERGLYCEMIC MICE: A PROBABLE ACCOMPANIMENT OF MORE GENERALIZED ALTERATIONS IN MEMBRANE GLYCOPROTEINS

Kwen-Jen Chang, Donna Huang, and Pedro Cuatrecasas

Departments of Pharmacology and Experimental Therapeutics, and Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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SUMMARY. Although the liver plasma membranes of obese-hyperglycemic (ob/ob and db/db) mice bind less insulin than the membranes from thin litter mates, nearly equally depressed binding is observed for the general plasma membrane markers, concanavalin A and wheat germ agglutinin. Glucagon binding is virtually unchanged, and adenylate cyclase activity from obese mice is more sensitive to glucagon. Isolated kidney cells from obese mice have reduced insulin binding, but the decrease in WGA and con A binding is even more profound. Cultured (48 to 72 hours) spleen lymphocytes also have grossly reduced WGA and con A binding. The apparent change in insulin binding in obese animals may be but a minor part of generalized alterations in membrane glycoproteins. Factors complicating comparisons of hormone binding in different metabolic states are considered.

Recent studies have demonstrated decreased insulin binding to liver (1, 2) and fat cell (3) membranes and lymphocytes (4) from obese-hyperglycemic mice (ob/ob). This syndrome has been proposed (1-4) as a model for defective insulin-receptor interactions in the insulin-resistant state of obesity.

In adult rats, large fat cells have the same number (and affinity) of insulin receptors as small cells from young rats (5). Despite the apparent metabolic resistance to insulin, glucose transport in these large cells responds quite normally to insulin (6); thus, the aberration appears to be distal to insulin binding and sugar transport. In circulating lymphocytes from obese human subjects, the number of insulin binding sites per cell was originally reported to be unchanged (7), although more recently (8) it has been described as being reduced. Large adipocytes from obese subjects have similar numbers and affinities of insulin binding sites as small adipocytes from normal-weight subjects (9).

METHODS. C57BL/6J mice were obtained from Jackson Laboratory, Bar Harbor, Maine, and fed ad libitum on purina rat chow. ^{125}I-Labeled insulin (10), glucagon (11), WGA and con A (12) have been described. Binding was determined in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.1% bovine serum albumin. Binding was measured by centrifugation (5 min) in a Beckman Microfuge using a layer of oil consisting of a mixture of one part dinonylphathalate and 2 parts of dibutylphathalate. All data are the mean of duplicate or triplicate replications, and are corrected for nonspecific binding, measured in the presence of 1 μM of native hormone, 0.1 M N-acetyl-D-glucosamine (for WGA) or 0.1 M α-methyl-D-mannopyranoside (for con A). Binding was linearly dependent upon membrane protein concentration. Protein was determined (13) after heating for 40 min at 100° in 1 N NaOH.

Homogenates were fractionated by differential centrifugation in isotonic (0.25 M) sucrose containing 5 mM Tris·HCl, pH 7.4 (14). Plasma membranes were obtained by centrifugation (40,000 x g, 30 min) of the postmitochondrial supernatant. Kidney minces were washed once in Hanks' balanced salt solution and incubated with 1 mg/ml of collagenase and 0.05% trypsin in a Ca^{2+}- and Mg^{2+}-free Hanks' solution for 30 min at 37° with vigorous shaking. Undissociated tissue was removed by filtration (nylon screen). The cell suspension was washed three times with sterile Hanks' solution and cultured for 3 days in Eagles' minimum essential medium with 2 mM L-glutamine, 10% fetal calf serum and 50 units/ml of penicillin-streptomycin. Kidney cells grew in suspended aggregates while fibroblasts adhered to the substratum. Only the floating kidney cells were used in binding assays; viability was confirmed by ^{3}H-thymidine incorporation into DNA.

RESULTS. Binding studies on liver fractions. Livers from young (one month old) obese (ob/ob) mice are grossly abnormal in appearance. They are heavily infiltrated with fat, and they weigh 2- to 3-times more than those of the thin litter mates, despite a difference of only 50% in total body weight. Adipose tissue (epididymal) weighs 8-times more in these obese mice. On fractionation of liver homogenates, 65% of the insulin binding activity is recovered in the plasma membrane fraction of obese mice and thin litter mates (Table 1). About 25%

TABLE 1. Insulin Binding to Fractions of Liver Homogenates from ob/ob Mice and Thin Litter Mates. Ten mice (1 month old) were used. The nuclear and mitochondrial fractions were obtained at 600 and 12,000 x g (14). The plasma membrane was obtained at 40,000 x g (30 min) and the microsomes at 100,000 x g (60 min). Binding was measured at 60 min (24°) with 5.4×10^5 cpm ^{125}I-insulin (1.8 Ci per μmole).

Fraction	Thin Litter Mates			ob/ob mice		
	protein (mg)	insulin bound (cpm/mg)	(cpm/liver)	protein (mg)	insulin bound (cpm/mg)	(cpm/liver)
Nuclear	99	160,000	1.6×10^6	198	58,000	1.1×10^6
Mitochondrial	180	25,000	4.5×10^5	480	10,000	4.6×10^5
Plasma memb.	104	412,000	4.1×10^6	128	175,000	2.2×10^6
Microsomal memb.	30	22,000	6.6×10^4	30	30,000	9.0×10^4

of the activity is retained in the nuclear fraction. The insulin binding activity (per mg of protein) in the obese mice membranes is about 40% that of the controls. Total binding per liver (or per animal) is about 1/2 that of the thin litter mates, despite a 2.5-fold difference in the weight of the livers. The 40,000 x g "membrane" pellet, which contains most of the insulin binding activity, was used for all further studies. Glucagon binding per mg of membrane protein is not decreased in the young obese mice (Table 2); total gluca-

TABLE 2. Binding of Insulin, Glucagon, WGA and Con A to Liver Plasma Membranes of ob/ob Mice. Mice were about one month old. Percent binding activity, relative to that found in thin litter mates, is given in parenthesis.

Membrane marker	^{125}I-Labeled ligand bound			
	Thin litter mates		ob/ob mice	
	(cpm/mg)	(cpm/liver)	(cpm/mg)	(cpm/liver)
^{125}I-Insulin				
1.5 x 10^{-9} M (540,000 cpm)	412,000	4 x 10^6	175,000 (42%)	2.2 x 10^6 (55%)
^{125}I-Glucagon				
8.5 x 10^{-10} M (256,000 cpm)	208,000	2.2 x 10^6	208,000 (100%)	2.6 x 10^6 (118%)
^{125}I-WGA				
0.8 μg/ml (15,600 cpm)	14,000		6,700 (48%)	
8 μg/ml (160,000 cpm)	125,000		80,000 (64%)	
80 μg/ml (1.6 x 10^6 cpm)	800,000	8.3 x 10^6	440,000 (55%)	5.6 x 10^6 (67%)
^{125}I-Con A				
0.5 μg/ml (48,000 cpm)	202,000		150,000 (74%)	
50 μg/ml (320,000 cpm)	1.3 x 10^6	13.6 x 10^6	766,000 (58%)	9.8 x 10^6 (72%)

gon binding per liver is increased. However, in obese mouse membranes the binding of the general plasma membrane markers (12, 14), con A and WGA, is, like insulin binding, substantially decreased (Table 2). If the binding of hormones is expressed in relation to WGA binding rather than to protein, the fall in insulin binding is very minor (if significant) while glucagon binding is actually increased by nearly two-fold.

Membranes from relatively old (4 months, 65 g) db/db obese mice bind much less (30%) insulin, but there is a similarly striking reduction of WGA and con A binding, as well as of glucagon binding (Table 3). The apparent differences in glucagon binding between ob/ob and db/db mice may be related to age effects.

Adenylate cyclase. The basal and NaF- and isoproterenol-stimulated adenylate cyclase of liver membranes of obese mice are somewhat decreased if expressed on the basis of protein (Table 4). However, the enzyme is more sensitive to glucagon, and the total glucagon-stimulated activity is greater. The hormone-stimulated activities are much higher than those reported previously (1).

Binding to cultured kidney cells and cultured lymphocytes. Insulin binding to isolated kidney cells (cultured for 48 hours) from young (1 month, 30 g) obese (ob/ob) mice is nearly the same as in their thin (19 g) litter mates (Table 5). The binding of WGA and con A is reduced much more markedly than is insulin binding. In kidney cells from older obese (db/db) mice the fall in in-

TABLE 3. Comparison of Insulin, Glucagon, WGA and Con A Binding Activities of Liver Membranes of db/db Mice and Their Thin Litter Mates. db/db Mice (65 g, 4 months old) and their same aged thin litter mates (32 g) were studied. The specific activities of the radioligands were 1.6 Ci/μmoles, 1.6 Ci/μmole, 0.14 μCi/μg and 0.14 μCi/μg for ^{125}I-labeled insulin, glucagon, WGA, and Con A, respectively.

Membrane marker	^{125}I-Labeled ligand bound (cpm/mg)	
	Thin litter mates	db/db mice
^{125}I-Insulin (1.5 nM)	48,000	15,000 (31%)
^{125}I-Glucagon (0.8 nM)	32,000	8,500 (27%)
^{125}I-WGA (80 μg/ml)	241,000	92,400 (38%)
^{125}I-Con A (20 μg/ml)	1,500,000	632,000 (40%)

TABLE 4. Adenylate Cyclase Activity in Liver Plasma Membranes of ob/ob and Thin Litter Mates. Mice were one month old. The protein concentrations were 1.4 and 1.85 mg/ml for thin litter mates and ob/ob mice, respectively, where production of cyclic AMP is the same. Fold stimulation and percent (relative to control mice) adenylate cyclase activity (15-17) are in parenthesis.

Condition	Adenylate cyclase activity[1]			
	Thin litter mates		ob/ob mice	
	pmoles/min/mg	pmoles/min per liver	pmoles/min/mg	pmoles/min/liver
Basal	44 ± 2	496 ± 24	29 ± 1.5 (66%)	432 ± 21 (90%)
NaF (10 mM)	280 ± 7 (6.3X)	3298 ± 82	217 ± 10 (7.4X) (77%)	3208 ± 160 (97%)
Glucagon (5 μM)	446 ± 18 (10X)	4992 ± 190	385 ± 9 (13X) (86%)	5702 ± 120 (117%)
Isoproterenol (20 μM)	133 ± (3X)	1493 ± 150	86 ± 5 (3X) (65%)	1274 ± 75 (85%)

[1]Mean value ± standard deviation of triplicate replications.

sulin binding is more apparent, but again the decrease is even more striking for WGA and con A. Spleen lymphocytes (purified on nylon wool columns) from old db/db obese mice bind 50% as much con A and WGA as control cells after 72 hours of culture (18) in the absence or presence of mitogenic doses of con A (not shown).

Effect of starvation. Temporary food restriction in the obese-hyperglycemic syndrome results in near-normalization of body weight, blood glucose and

TABLE 5. Binding of Insulin, WGA and Con A to Isolated Cultured Kidney Cells of Obese Mice. Young ob/ob (one month old, 30 g) and old db/db (60 g) mice were used. The binding relative to control cells is in parenthesis.

^{125}I-Labeled membrane marker	^{125}I-Labeled ligand bound (cpm/10^6 cells)	
	Thin litter mates	ob/ob mice
Young ob/ob mice		
Insulin (522,000 cpm)	4,600	4,300 (92%)
WGA (107,000 cpm)	122,000	92,500 (76%)
Con A (144,000 cpm)	60,000	45,200 (75%)
Old db/db mice		
Insulin (160,000 cpm)	6,700	4,500 (72%)
WGA (161,000 cpm)	293,000	124,000 (43%)
Con A (184,000 cpm)	130,000	64,000 (50%)

insulin concentrations, and it restores the impaired insulin sensitivity (19). In thin mice, starvation (3 days) causes a proportional reduction in the total binding activity and a slight increase in the specific binding activity of insulin, glucagon and WGA (Table 6). In obese mice there is a two-fold increase in total, and a 3-fold increase in specific, insulin binding. Glucagon binding in the obese mouse decreases by 40% (total) and 20% (specific). Although in obese mice the specific activity of WGA binding increases, the restoration is less marked than for insulin binding.

Fat cells. Attempts to prepare (with collagenase) isolated fat cells from the epidydimal fat pads of obese-hyperglycemic mice were unsuccessful. Although the material appeared to contain a good number of very large fat cells by flotation and by phase contrast microscopy, after staining with methylene blue virtually no intracellular nuclei were seen in the large globular structures. Many free nuclear clumps were detected, some adhering to the large globules. Very few intact fat cells were obtained, even with the mildest digestions. Thus, these extraordinarily fragile cells release large fat globules which can be mistaken for isolated fat cells. It was therefore not possible to study insulin binding in fat cells or fat cell membranes.

DISCUSSION. Although insulin binding appears to be decreased in various tissues of obese-hyperglycemic mice, the relationship of this "fall" to other changes in the plasma membrane complicate interpretations of possible "changes" in receptor concentrations. Attempts to relate the fall in binding to insulin re-

TABLE 6. Effect of Starvation of Obese Mice on the Binding of Insulin, Glucagon and WGA to Liver Plasma Membranes. Three month old ob/ob mice and their thin litter mates were starved for 3 days. Binding was determined in the presence of 4.5×10^5 cpm ^{125}I-labeled insulin (1.6 Ci.μmole), 4×10^5 cpm ^{125}I-labeled glucagon (1.6 Ci/μmole) and 1.5×10^6 cpm ^{125}I-labeled WGA (0.15 μCi/μg).

Variable studied	Thin litter mates		ob/ob mice	
	Control	Starved	Control	Starved
Animal, average weight (g)	23	17	46	35
Average liver weight (g)	1.3	0.7	2.8	1.3
Plasma membrane per liver (mg)	28	12	40	26
Insulin binding, cpm/mg	79,000	100,000	22,000	58,000
total	2.2×10^6	1.2×10^6	0.86×10^6	1.53×10^6
Glucagon binding, cpm/mg	210,000	240,000	166,000	148,000
total	5.9×10^6	2.9×10^6	6.6×10^6	3.9×10^6
WGA binding, cpm/mg	2×10^6	2.5×10^6	1.3×10^6	1.6×10^6
total	56×10^6	30×10^6	53×10^6	42×10^6

sistance, hyperinsulinemia, or any other metabolic abnormalities must be especially tempered with caution. This "defect" in insulin binding cannot be viewed as being specific since similar "defects" are observed in the binding of the general membrane markers (14), con A and WGA. These lectins bind to numerous glycoproteins in membranes, and in quantities which far exceed insulin binding; for example, in fat cells the binding of these lectins is 10^4-times greater than is the binding of insulin (12). If the present methods of comparing bind- are valid, it is perhaps more likely that a generalized defect in glycoprotein biochemistry may exist in the obese state. Since insulin receptors appear to be glycoproteins (20), the fall in insulin binding may be simply a reflection of a more generalized alteration. Indeed, the facts that in kidney cells the fall in lectin binding is more profound than that of insulin binding, and that on starvation the recovery of insulin binding is more dramatic than the recovery of WGA binding, suggest that if a generalized glycoprotein defect actually exists, it may be of more fundamental importance. Viewed in this light, the observation that glucagon binding is not "decreased" in obesity might be more significant metabolically.

It is difficult to analyze hormone binding in such abnormal states as the obese-hyperglycemic syndrome, especially using tissues as complex as liver. The subcellular and chemical composition of such a tissue, which in these two states differs even visually, is surely grossly different and complicated.

Large cell volumes will result in disporportionate intracellular compared to plasma "membranes", and the proteins and lipids in the membranes may differ. Fatty deposits will yield interfering fat globule "membranes" of different protein and receptor composition. Binding of membrane markers in grossly different metabolic states is further complicated by heterogeneity of the cell populations. Changes in the cell composition can cause changes in total binding without actual changes in binding per cell, especially if (as is commonly the case) the receptor is present in only a few cells. This is probably the case in human circulating cells where a very few contaminating cells (probably macrophages) rather than resting lymphocytes bind significant quantities of insulin (18, 21). Thus, it is most difficult to tell whether differences in hormone binding in crude "lymphocytes" from different metabolic states (4, 8, 22) reflect differences in cell composition or in binding per cell.

It has been suggested (7, 8, 23) that the apparent decrease in insulin receptors in certain insulin-resistant states may result from chronic elevations in serum insulin, perhaps by a direct regulatory effect of insulin on peripheral tissues (23). In the obese-hyperglycemic mouse, insulin resistance occurs before hyperglycemia and hyperinsulinemia (19), suggesting mechanisms other than hyperinsulinemia. In addition, large adipocytes from obese, hyperinsulinemic subjects do not display defects in insulin binding (9) and large, insulin "resistant" fat cells from rats bind insulin and respond (glucose transport) to insulin normally (5, 6). No compelling evidence exists to implicate abnormal receptor function in obese or other insulin "resistant" states. Indeed numerous studies in large, "resistant" fat cells from animals (23-25) and humans (26-30) show alterations in intracellular lipid and carbohydrate metabolism, and recent studies in rat (5, 6) or human (9) large fat cells suggest defects distal to initial binding. In guinea pig fat cells, which are resistant in glucose transport and oxidation but not antilipolysis, an abnormality possibly related to glucose transport exists in a plasma membrane phosphorylation system (31). It is likely that insulin "resistance" represents a wide spectrum of complex metabolic expressions of multiple etiologies.

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