Effect of Free Fatty Acids on Insulin Receptor Binding and Tyrosine Kinase Activity in Hepatocytes Isolated From Lean and Obese Rats

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We demonstrated previously that high physiological concentrations of free fatty acids (FFA) rapidly decrease insulin binding, degradation, and action in isolated rat hepatocytes. In this study, hepatocytes from lean and obese Sprague-Dawley rats (Alab, Stockholm) were preincubated with or without 0.4 mM oleic acid, and the effect on insulin binding and tyrosine kinase activity was measured. In the absence of exogenous FFA, insulin binding was reduced in hepatocytes from obese compared with lean rats (mean ± SE reduction 44 ± 7%, $n = 8$, $P < 0.01$). Furthermore, the inhibitory effect of oleic acid added to hepatocytes from lean rats ($n = 8$; 40 ± 9%, $P < 0.01$) was not seen in cells from obese rats. Treating obese rats with Etomoxir, a carnitine palmitoyl transferase I inhibitor, increased insulin binding to isolated hepatocytes by 41 ± 13% ($n = 5$, $P < 0.05$). There was no difference in total binding to partially purified insulin receptors from solubilized hepatocytes from lean and obese rats, whether cells were or were not preincubated with oleic acid. Tyrosine kinase activity of partially purified receptors from basal or insulin-stimulated cells was not affected by either obesity, treatment with Etomoxir, or preincubating the cells with oleic acid. Thus, both obesity and elevated ambient FFA levels are associated with impaired insulin cell surface binding to isolated hepatocytes, possibly through an effect of lipid oxidation on the internalization/recycling of the insulin-receptor complex without any perturbation of the receptor tyrosine kinase activity. The data suggest that the reduced insulin binding to hepatocytes from obese rats is due to elevated ambient FFA levels. *Diabetes* 41:294–98, 1992

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Obesity is associated with insulin resistance and hyperinsulinemia (1–3). However, not only the amount but also the distribution of body fat is important because individuals with an abdominal fat distribution are more insulin resistant than equally obese individuals with a gluteo-femoral distribution (4–7). The reason for this is unclear but may be linked to differences in regional fat cell metabolism. Thus, both intraabdominal (8) and subcutaneous abdominal (9) fat cells are more responsive to lipolytic hormones than fat cells from other locations. Furthermore, the antilipolytic effect of insulin seems to be blunted in visceral fat (10). The relevance of these in vitro observations to the in vivo situation has been verified with microdialysis (11). These observations led to the hypothesis that elevated free fatty acid (FFA) levels and/or FFA turnover in subjects with abdominal obesity (12) may be of importance for the development of insulin resistance.

Obese subjects with an abdominal fat distribution have a reduced hepatic insulin uptake (13,14). A possible cellular mechanism for this was recently demonstrated in two independent studies on isolated hepatocytes where high physiological FFA concentrations markedly reduced insulin binding, degradation, and action (15,16). Moreover, the inhibitory effect of FFA is energy dependent and does not alter the total cellular number of insulin receptors or their binding characteristics, indicating that the receptor internalization/recycling is influenced (15). In this study, the effect of FFA on insulin binding to hepatocytes from obese rats was compared with that in cells from lean rats. The influence of FFA on basal and insulin-stimulated tyrosine kinase activity of partially purified insulin receptors from lean and obese rats was also investigated. To further elucidate the role of FFA on insulin binding and action in obesity, rats were treated with Etomoxir, a lipid oxidation inhibitor.

RESEARCH DESIGN AND METHODS

Bovine serum albumin (BSA, fraction V), collagenase (type IV), dibutylphthalate oil, EDTA, HEPES, oleic acid, Glu^{80},Tyr^{20}, benzamidine, pepstatin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma (St. Louis, MO). Sepharose (4B CNBr activated) was obtained from Pharmacia (Uppsala, Sweden), wheat-germ agglutinin (WGA) from Biokemiska Separationscentralen (Uppsala, Sweden). A14-Tyr-[^{125}I]monoiodopork insulin and regular insulin were from Novo (Copenhagen), and Etomoxir was a kind gift from Dr. H. Wolf, BYK Gulden (Konstanz, Germany).

Male Sprague-Dawley rats were used for all experiments. The rats were kept at a constant temperature and had free access to laboratory chow (22.5% protein, 72.5% carbohydrate, 5% fat, and sufficient vitamins and minerals) throughout the experiments. The rats were divided into two groups by mean ± SE body weight: lean (n = 8; 200 ± 5 g, 20 days old) and obese (n = 8, 410 ± 5 g, 40 days old). All studies were performed on rats in the fed state. In a separate set of experiments, obese rats (n = 6; 400 ± 5 g, 40 days old) were treated with Etomoxir (50 mg/kg i.p.), a carnitine palmitoyl transferase I inhibitor, for 3 days before isolation of hepatocytes.

Preparation of isolated hepatocytes. Hepatocytes were isolated according to the method of Seglen et al. (17), with modifications (15). Briefly, after induction of general anesthesia, the liver was perfused through a portal cannula with calcium-free Krebs Ringer–bicarbonate-HEPES buffer (KRbH) containing 5.5 mM glucose and 2 mM EDTA (pH 7.4) followed by KRbH containing 5.5 mM glucose and 0.05% collagenase for 5 min. The cells were washed extensively in KRbH with 3% BSA, and the hepatocytes were separated from the nonhepatocytes by centrifugation at 50 × g for 1 min. All subsequent experiments were carried out at 37°C in KRbH containing 5.5 mM glucose and 3% BSA or with 2.3% BSA where 0.7% BSA-FFA complex was added to give the same BSA concentration in all samples. Over 95% of the cells excluded trypan blue before and after incubation with or without oleic acid. The size of the hepatocytes was the same in nonobese and obese rats. Incubating the cells with oleic acid did not elicit any changes in cell size (data not shown).

Preparation of albumin-bound FFA. Oleic acid (0.2 M in ethanol) was diluted 1:25 into KRbH (pH 7.4) containing 20% "fatty acid–poor" BSA at 60°C. The fatty acid–albumin molar ratio was kept at <3 to ensure that the fatty acids were bound to albumin. Control experiments revealed that the BSA used contained <0.1 mM FFA at a BSA concentration of 3%. The FFA content of the different albumin preparations was measured with an enzymatic colorimetric method (Wako, Neuss, Germany).

Insulin binding to intact cells. Insulin binding was carried out essentially as described by Gammeltoft et al. (18), with modifications (15). A14-Tyr-[^{125}I]monoiodoinsulin (final concn 200 pM), and increasing concentrations of unlabeled insulin were added to the isolated hepatocytes (cell conc ~10^5 cells/ml). The incubations were performed for 45 min at 37°C in a final vol of 1 ml. The bound insulin was separated from the unbound by centrifuging the cells through a layer of dibutylphtalate oil in Eppendorf microfuge tubes. The tips of the tubes were cut, and the radioactivity was measured. Insulin binding was expressed as specific cell-associated radioactivity after subtraction of nonspecific binding, which averaged 10–15%.

Solubilization and partial purification of insulin receptors. Isolated hepatocytes at a concn of 5 × 10^6 cells/50 ml were preincubated in KRbH containing BSA in the presence or absence of 0.7 nM insulin and 0.4 mM oleic acid. After preincubating the cells at 37°C for 45 min in a shaking water bath (gas phase 95% O_2/5% CO_2), the cells were centrifuged at 200 × g, and the pellet was resuspended in a 1:1 vol of ice-cold solubilizing solution (final concn 1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10 mM $MgSO_4$, 2 μM pepstatin, 300 U/L aprotinin, 1 mM PMSF, 2 μM pepstatin, 10 mM bensamidine, pH 7.4). After solubilizing, the cellular extract was centrifuged at 7000 × g for 15 min at 4°C. The pellet was discarded, and the nonsoluble material of the supernatant was separated by centrifugation at 70 000 × g for 60 min. The clear supernatant was recycled twice over columns with 0.75–1.00 ml wheat-germ agarose at 4°C with an average flow rate of 0.5 ml/min. The receptors were eluated from the WGA columns in fractions of 2 ml × 1 ml with 0.3 M N-acetyl-D-glucosamine.

Insulin binding to solubilized receptors. Aliquots of the receptor preparations were incubated in duplicates with ^{125}I-labeled insulin (final concn 0.1 nM) at 4°C for 20 h in the absence or presence of increasing concentrations of unlabeled insulin. The bound hormone was separated from free hormone by precipitation with polyethylene glycol as described previously (15).

Insulin receptor tyrosine kinase activity. After preincubation of 50 μl WGA eluate in the presence or absence of 0.7 μM insulin and 2 mM $MnCl_2$ for 15 min at 20°C, ATP at a concn of 15 μM was added. After 15 min of incubation, [^{32}P]ATP (~10^6 cpm) was added in the presence of 2.5 mg/ml Glu^{80},Tyr^{20} as exogenous substrate and unlabeled ATP (final concn 15 μM). After another 15 min at 20°C, a 40-μl sample was spotted on a Whatman 3-M filter paper wetted with 10% trichloroacetic acid containing 10 mM $Na_4P_2O_7$. The filter paper was washed four times in ethanol and acetone, and the radioactivity was counted in a scintillation counter. The radioactivity obtained in samples without Glu^{80},Tyr^{20} was subtracted as background.

Statistics. Statistical significance was evaluated with Student's t test for paired or unpaired data as appropriate.

RESULTS

Effect of obesity and oleic acid on insulin binding. Figure 1*A* shows the effect of 0.4 mM oleic acid on specific insulin binding to isolated hepatocytes from lean rats. As reported previously, oleic acid at this concentration reduced the cell-associated insulin binding by ~40%. The apparent K_d was 1.09 ± 0.27 (n = 8) and 1.09 ± 0.27 nM in control cells and cells exposed to oleic

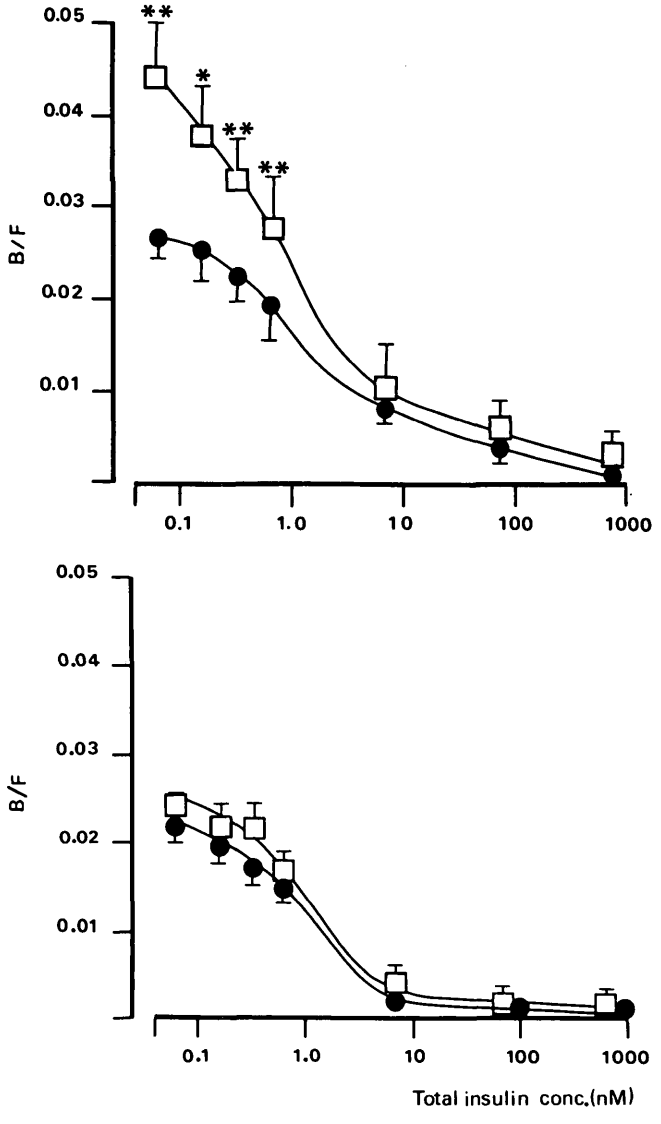

FIG. 1. *A*: effect of oleic acid on specific ^{125}I-labeled insulin binding to isolated hepatocytes from lean rats. *B*: effect of oleic acid on specific ^{125}I-insulin binding to isolated hepatocytes from obese rats. Hepatocytes (10^5/ml) were incubated for 45 min at 37°C with 200 pM ^{125}I-insulin and increasing concentrations unlabeled insulin in the absence (□) or presence (●) of 0.4 mM oleic acid. Data are means ± SE of 8 experiments. *P < 0.05, **P < 0.01.

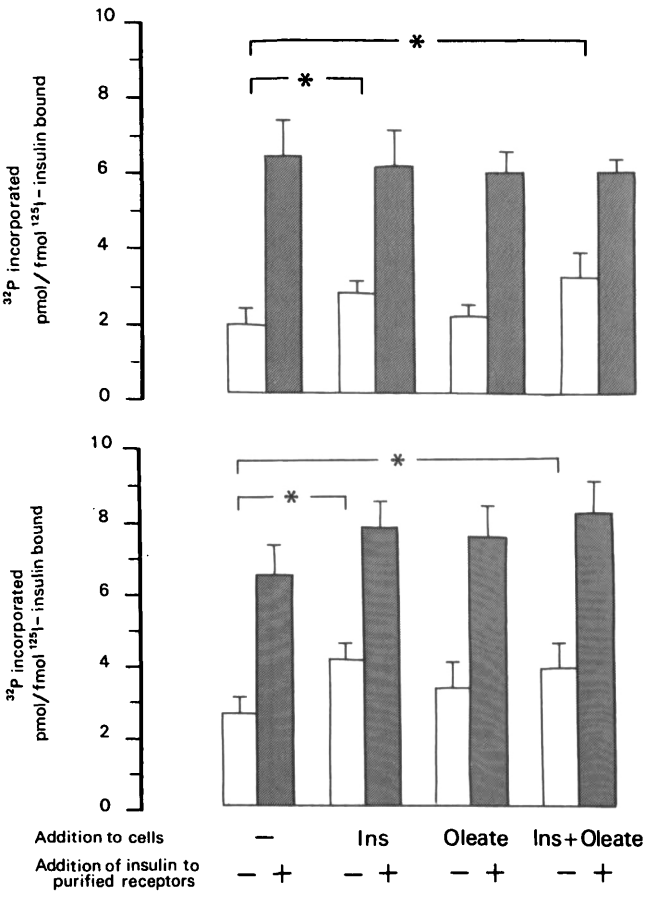

FIG. 2. *A*: basal and insulin-stimulated tyrosine kinase activity of solubilized and partially purified insulin receptors from hepatocytes of lean rats. *B*: basal and insulin-stimulated tyrosine kinase activity of insulin receptors from hepatocytes from obese rats. Cells were preincubated for 45 min with and without 0.4 mM oleic acid, 0.7 nM insulin, or oleic acid plus insulin. Wheat-germ agglutinin–purified insulin receptors were incubated for 15 min at 20°C with 2 mM $MnCl_2$ in the presence or absence of 1 μM insulin as indicated. Incorporation of [^{32}P]ATP to Glu^{80},Tyr^{20} was assayed as described in METHODS. Data are means ± SE of 6 experiments. *P < 0.05.

acid, respectively, indicating that receptor affinity was not affected by FFA. This was further validated by analyzing the data according to Scatchard (data not shown).

Figure 1*B* shows insulin binding to isolated hepatocytes from obese rats in the presence or absence of 0.4 mM oleic acid. These experiments, performed in parallel to those in Fig. 1*A*, demonstrate that insulin binding is decreased ~40% in hepatocytes from obese compared with lean rats. Moreover, the addition of 0.4 mM oleic acid to cells from obese rats did not further decrease insulin binding. The apparent K_d was similar in control cells and cells exposed to oleic acid (n = 8; 1.03 ± 0.14 and 1.01 ± 0.11 nM, respectively), indicating a reduction in the number of binding sites for insulin.

There was no difference in the yield of partially purified insulin receptors from solubilized hepatocytes preincubated in the absence or presence of oleic acid, insulin, or oleic acid plus insulin for 45 min (data not shown). Insulin binding to purified insulin receptors from obese and nonobese rats was also comparable. Furthermore, oleic acid had no direct effect on the ability of insulin to bind to partially purified receptors (data not shown).

Effect of obesity and oleic acid on insulin receptor tyrosine kinase activity. Insulin receptor tyrosine kinase activity was significantly increased when intact cells were preincubated with insulin or insulin and 0.4 mM oleic acid for 45 min before isolation of the insulin receptors (Fig. 2*A*). When insulin was added to partially purified receptors, no differences in maximally insulin-stimulated tyrosine kinase activity were seen between any of the four groups studied (Fig. 2*A*).

Tyrosine kinase activity was increased if intact cells from obese rats were preincubated with 0.7 nM insulin (Fig. 2*B*). This increase was not inhibited by the addition of oleic acid. The maximally insulin-stimulated tyrosine kinase activity after addition of insulin to the partially purified receptors was similar in all four groups (Fig. 2*B*). Basal, unstimulated tyrosine kinase activity was also

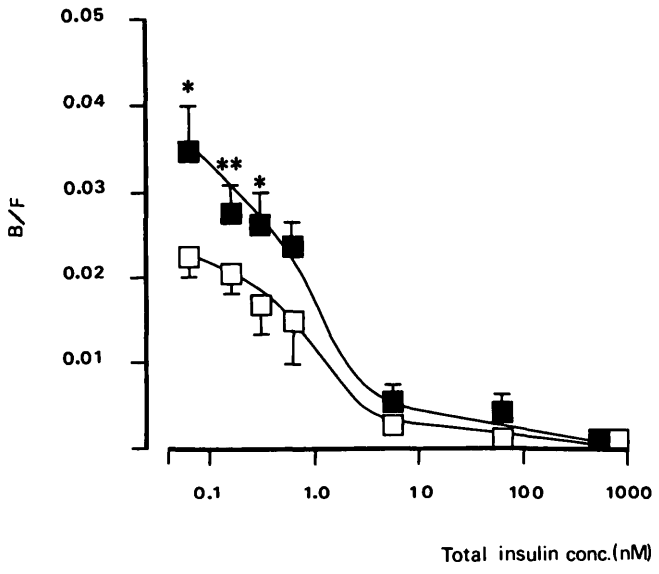

FIG. 3. Specific insulin binding to isolated hepatocytes from Etomoxir-treated (■) and untreated (□) obese rats. Hepatocytes (10^6/ml) were incubated for 45 min at 37°C with 200 pM ^{125}I-labeled insulin and increasing concentrations of unlabeled insulin. Data are means ± SE of 5 experiments. *$P < 0.05$, **$P < 0.01$.

similar in receptors from obese and lean rats. Preincubating hepatocytes with oleic acid for 45 min had no effect on the insulin receptor tyrosine kinase activity in cells from either group, and oleic acid did not change the insulin response (Fig. 2*B*).

Effect of treating obese rats with Etomoxir. Specific insulin binding to hepatocytes from Etomoxir-treated obese rats was increased 41 ± 13% ($n = 5$; $P < 0.05$) compared with cells from untreated obese rats (Fig. 3). The apparent K_d of the insulin binding sites in hepatocytes from Etomoxir-treated rats was similar ($n = 6$; 1.02 ± 0.06 nM) to that seen in the other groups as reported above. Etomoxir treatment of the rats did not influence the yield of partially purified insulin receptors or their binding capacity. Furthermore, both unstimulated and maximally insulin-stimulated tyrosine kinase activity of partially purified receptors from Etomoxir-treated rats were similar to the activities in the other groups (data not shown).

DISCUSSION

This study shows that preincubating hepatocytes from obese male rats with FFA did not further decrease insulin binding, which was reduced ~40% compared with cells from lean rats. A similar reduction in the obese state has been demonstrated in hepatocytes from obese Sprague-Dawley (19) and *fa/fa* (20) rats in isolated hepatocytes and purified liver plasma membranes from the *ob/ob* mouse (21) and to liver membranes from obese *fa/fa* rats (22) and obese human subjects (23,24).

In our investigation, it was also shown that total insulin binding to partially purified receptors from preincubated or nonpreincubated solubilized cells was similar in lean and obese rats, showing that neither obesity nor elevated FFA levels influence the total number of cellular binding sites or their binding characteristics. This is in accordance with recent studies demonstrating an unchanged insulin binding to partially purified receptors from solubilized hepatocytes of 2- and 4-mo-old male Wistar rats (25) and nonobese and obese human subjects (24). Moreover, our data show for the first time that preincubating hepatocytes with FFA does not change basal or insulin-stimulated tyrosine kinase activity in WGA-purified insulin receptors from lean and obese rats. Similar tyrosine kinase activity has been demonstrated previously in lean and obese animals (25) and humans (24). Thus, this study demonstrates that both obesity and high ambient FFA levels influence the relative distribution of the insulin receptors between the cell surface and the intracellular compartment without changing the total cellular number of insulin receptors or their basal and insulin-stimulated tyrosine kinase activity.

We previously demonstrated that high ambient physiological FFA concentrations induce an ~40% decrease in the available insulin binding sites in isolated hepatocytes from lean rats (15). This reduction seems to be due to an altered rate of receptor internalization/recycling without affecting the degradation of the insulin receptor complex (15). The mechanisms for the inhibitory effect of FFA on insulin binding to isolated rat hepatocytes has been further investigated elsewhere (16). These authors found a decrease in the amount of insulin internalized when the hepatocytes were preincubated with palmitate, whereas the dissociation and/or release of cell-associated internalized insulin was not influenced by the FFA exposure. Recently, we demonstrated that the inhibitory effect of FFA on both insulin action and binding to hepatocytes from lean rats was prevented by treating the rats with Etomoxir, indicating that the FFA effect is linked to their metabolism and oxidation (26). Also, the hepatic triglyceride concentrations are negatively correlated to insulin clearance as measured in the in situ perfused liver in obese rats (27).

To investigate the specificity of the FFA effect on the insulin-receptor interaction, ^{125}I-labeled cyanopindolol binding to the β-receptors of isolated hepatocytes was investigated in the presence and absence of 0.4 mM oleic acid. No effect of oleic acid on ligand binding to the β-receptor was seen (data not shown). The specificity of the FFA effect on the insulin receptor interaction is also indicated by the finding that insulin binding was not further decreased by FFA exposure in vitro of hepatocytes from obese rats.

Note that these findings may reflect an effect of aging rather than obesity per se because it is well known that the tissue sensitivity to insulin decreases with age (28). Previous studies have shown that aging is associated with a reduced insulin binding and action in human adipocytes (29). The effect of aging is also additional to that of obesity (30). However, the reduced insulin binding to hepatocytes from old obese rats is normalized after fasting and weight reduction, suggesting that obesity rather than age is responsible (19,20). This finding that Etomoxir increased the number of binding sites at the cell surface further supports the concept that the reduction in insulin binding to hepatocytes from old obese rats is a

consequence of obesity and high FFA levels rather than age per se.

Another explanation for the Etomoxir effect in the obese rats could be a reduction in plasma insulin levels, followed by an upregulation of the number of hepatic insulin receptors. However, this is not supported by studies in insulin-sensitive and insulin-resistant animals, where systemic Etomoxir treatment did not influence the insulin concentrations in vivo (31,32). The novel finding of the effect of Etomoxir in the obese state supports the concept that elevated FFA levels play an important role for the reduced insulin binding (and action) in obesity. Moreover, a reduced number of binding sites for insulin leads to a decreased receptor-mediated insulin clearance by the liver. In the in situ perfused rat liver, high physiological FFA concentrations also inhibit hepatic insulin clearance (33). Reduced insulin uptake by the liver has been reported in human subjects with abdominal obesity and glucose intolerance (13,14). Thus, one consequence of the impaired insulin binding by elevated FFA levels may be to reduce the clearance of insulin and to contribute to the peripheral hyperinsulinemia in insulin-resistant obese subjects. However, direct studies in the pertinent situation are needed to elucidate this possibility.

Taken together, our results show that obesity and high ambient FFA levels influence the internalization/recycling of the hepatic insulin receptors leading to a reduced cell surface binding without affecting the ability to activate the receptor tyrosine kinase. It is likely that the reduced insulin binding to hepatocytes from obese rats and the associated insulin resistance is due to the elevated ambient FFA levels.

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