Free–Fatty Acid Inhibition of Insulin Binding, Degradation, and Action in Isolated Rat Hepatocytes

JAN SVEDBERG, PER BJÖRNTORP, ULF SMITH, AND PETER LÖNNROTH

The effect of free fatty acids (FFAs) on insulin binding and action was investigated in isolated rat hepatocytes. Oleic acid (0.4 mM) added to the cells rapidly (within 45 min) reduced insulin binding and degradation (each by 45%; $P < 0.001$, $n = 7$) without changing the apparent receptor affinity. The effect was concentration dependent; a half-maximal inhibitory effect occurred at 0.150 ± 0.050 mM (mean ± SE). Oleic acid exerted no effect on insulin binding in energy-depleted (KCN-treated) cells. Oleic, palmitic, stearic, palmitoleic, and eicosapentaenoic acids were equally effective in reducing insulin binding. FFA did not change insulin binding to partially purified insulin receptors, thus excluding a direct effect on the insulin receptor. Furthermore, binding to partially purified receptors from solubilized cells pretreated with 0.2 mM oleic acid was not changed, indicating the effect of FFA in intact cells is on the rate of receptor internalization and/or recycling. Concomitant with the effect on insulin binding, oleic acid elicited a concentration-dependent reduction in nonstimulated cellular [^{14}C]aminoisobutyric acid uptake (AIB; 29 ± 8%, $P < 0.05$) and decreased the maximal effect of insulin (39 ± 7%, $P < 0.05$). Thus, in a concentration-dependent manner, different fatty acids can reduce the number of binding sites for insulin and the degradation of insulin by isolated liver cells. Basal and insulin-stimulated AIB transport was reduced, suggesting the presence of postbinding perturbations. These data suggest that FFA exerts an important modulating effect on insulin action in the liver. *Diabetes* 39:570–74, 1990

Obesity is generally associated with insulin resistance and hyperinsulinemia (1–3). The insulin resistance is present in peripheral tissues and liver (3). It has become evident that obese subjects with the male type of fat distribution (abdominal obesity) are more prone to develop insulin resistance (4–6), hypertension, cardiovascular disease (7–9), and cancer (9) than obese subjects with the gluteofemoral type of obesity.

Large fat cells are more responsive to lipolytic stimulation than small adipocytes both in the absence and presence of insulin (10). In addition, regardless of size, abdominal adipocytes are more lipolytically active than fat cells in the gluteofemoral region (11).

Intra-abdominal adipose tissue, which is drained by the portal vein, is particularly responsive to lipolytic stimuli (12). These observations have led to the hypothesis that elevated free–fatty acid (FFA) levels in obese subjects with an abdominal fat distribution may be at least partly responsible for the insulin resistance (13). In support of this, it has been demonstrated that acutely elevated FFA levels can induce insulin resistance both in peripheral tissues and liver (14,15).

Studies have shown that severely obese and glucose-intolerant subjects with an abdominal fat distribution have a decreased clearance of insulin by the liver (16,17). Insulin clearance is mediated through the binding of the hormone to its receptor (18). The number of insulin receptors in liver cells is reduced in glucose-intolerant obese subjects (19). Because we have found that high FFA levels in the portal blood are correlated with a decreased clearance of insulin in rat liver (20), we hypothesize that FFA exerts an effect on insulin binding to liver cells. To explore this possibility, we investigated the effect of different fatty acids on insulin binding, degradation, and action in isolated rat hepatocytes. The data show that FFA indeed reduces insulin binding and degradation and is capable of exerting postbinding perturbations of insulin action.

From the Department of Medicine I and II, the Wallenberg Laboratory, Sahlgren's Hospital, Gothenburg, Sweden.

Address correspondence and reprint requests to Dr. Jan Svedberg, The Wallenberg Laboratory, Dept. of Medicine I, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden.

Received for publication 6 April 1989 and accepted in revised form 4 January 1990.

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RESEARCH DESIGN AND METHODS

Bovine serum albumin (BSA; fraction V), collagenase (type IV), dibutyl phthalate oil, EDTA, HEPES, oleic acid, palmitic acid, palmitoleic acid, stearic acid, and eicosapentaenoic acid were obtained from Sigma (St. Louis, MO); A14-Tyr-[^{125}I]monoiodo–pork insulin was from Novo (Copenhagen); and [1-^{14}C]aminoisobutyric acid (AIB; 50 mCi/mmol) was from Du Pont-NEN (Boston, MA). Sprague-Dawley rats (150–200 g) were obtained from Alab (Stockholm).

Preparation of isolated hepatocytes. Hepatocytes were isolated from fed male rats according to the method originally described by Seglen (21). The rodents were anesthetized with phenobarbital, and the liver was perfused through a portal cannula. The perfusion was started with a Ca^{2+}-free Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 5.5 mM glucose and 2 mM EDTA, pH 7.4. The perfusate was changed after 5 min to KRBH containing 5.5 mM glucose and 0.05% collagenase for another 5 min. Throughout the experiment, the mediums were gassed vigorously with 95% O_2/5% CO_2 directly into the perfusion reservoir, which was kept at 37°C.

The liver capsule was torn apart with two forceps, and the liver cells were suspended in KRBH containing 3% BSA. The suspension was filtered through a nylon mesh (100 μm) and centrifuged at 50 × *g* for 1 min to separate the hepatocytes from the nonhepatocytes and stromal elements. The supernatant was discarded, and the hepatocytes were resuspended in the buffer at 37°C. The washing procedure was repeated twice. To eliminate as much of the effect of proteases as possible, the cells were washed in a shaking (120-rpm) water bath at 37°C for 10 min. All subsequent experiments were carried out at 37°C in KRBH containing 5.5 mM glucose and 3% BSA and gassed with 95% O_2/5% CO_2.

Freshly isolated hepatocytes were counted in a Buerkner chamber to obtain the cell number. Control experiments have shown that trypan blue was excluded by >90% of the cells.

Solubilization and partial purification of insulin receptors. After preparation and washing of the hepatocytes, the cells were centrifuged at 200 × *g*, and the pellet was resuspended in a volume of 1:1 in ice-cold buffer (1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10 mM $MgSO_4$, 2.1 μM leupeptin, 240 U/L aprotinin, 29 μM phenylmethylsulfonyl fluoride, 1.5 μM pepstatin, final concn, pH 7.4). The cells were frozen at −70°C for 1 h, thawed on ice, and subsequently disrupted by homogenization in a glass homogenizer with a Teflon pestle (Janke & Kunkel, Staufen, FRG). After solubilization, the cellular extract was centrifuged at 7000 × *g* for 15 min at 4°C. The supernatant was discarded, and the nonsoluble material was separated by centrifugation at 70,000 rpm for 60 min. The clear supernatant was recycled twice over 0.75–1.00 ml of wheat-germ agarose at 4°C with an average flow rate of 0.5 ml/min. The receptors were eluted from the column in fractions of 2 × 1 ml with 0.3 M *N*-acetyl-D-glucosamine. The fractions were combined, aliquoted, and frozen at −70°C before the binding experiments were performed.

Insulin binding and degradation in intact cells. Insulin binding was carried out essentially as described by Gammeltoft et al. (22). A14-Tyr-[^{125}I]monoiodoinsulin (final concn 200 pM) and increasing concentrations of unlabeled insulin were added together to isolated hepatocytes (cell concn ~10^5 cells/ml) and incubated at 37°C in a final volume of 1 ml. After 45 min of incubation, 300-μl samples were drawn and added to Eppendorf microfuge tubes containing 100 μl dibutyl phthalate oil. The cell-associated ^{125}I-labeled insulin was then separated from the unbound insulin by centrifugation. The tip of the tube was cut and the radioactivity that was associated with the cells measured. Essentially no unbound ^{125}I-insulin passed through the oil under the conditions used. Insulin binding was expressed as specific cell-associated radioactivity after subtraction of nonspecific binding. Nonspecific binding was expressed as radioactivity remaining in the presence of 7 μM unlabeled insulin and averaged 10–15%.

To assess the degradation of insulin, 100 μl of cell-free medium was added to 1 ml of 10% trichloroacetic acid (TCA). The tubes were centrifuged at 50 × *g* for 10 min, the pellet was washed once, and the radioactivity was counted. Insulin degradation was expressed as the percentage of nonprecipitable radioactivity.

Insulin binding to solubilized receptors. Aliquots of the receptor preparations were incubated in duplicate with ^{125}I-insulin (final concn 0.1 nM) at 4°C for 20 h in the absence and presence of increasing concentrations of unlabeled insulin in a total volume of 100 μl. Receptor-bound hormone was separated from free hormone by precipitation with 1% immunoglobulin G and 30% polyethylene glycol. The pellet was washed twice, and the radioactivity was counted. Nonspecific binding, defined as the radioactivity remaining in the presence of 1 μM unlabeled insulin, was subtracted from total binding.

[^{14}C]AIB uptake. [^{14}C]AIB uptake was studied with some modifications of the method described by Fehlman et al. (23). Isolated hepatocytes were preincubated for 30 min in the absence and presence of insulin (40 pM to 1.4 nM) at 37°C. After the preincubation period, [^{14}C]AIB (0.68 μM) was added to the vials. The reaction was terminated after 45 min by centrifuging the cells through dibutyl phthalate oil.

Preparation of albumin-bound FFA. Fatty acids (0.2 M in ethanol) were diluted 1:25 into KRBH, pH 7.4, containing 20% BSA at 60°C. The mixture was gently agitated to dissolve the fatty acids, and the pH was adjusted to 7.4. 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 solution was diluted into KRBH to the concentrations needed in each experiment. The FFA content of the different albumin preparations was measured with an enzymatic colorimetric method (Wako, Neuss, FRG).

Statistics. Statistical significance was evaluated with Student's *t* test for paired or unpaired data as appropriate. Linear regression analyses were performed according to the least-squares method. Data are reported as means ± SE.

RESULTS

Effect of FFA on insulin binding and degradation. Figure 1 shows the effect of 0.4 mM oleic acid on specific insulin binding to hepatocytes. At this concentration, oleic acid reduced the cell-associated insulin binding by 45%. The ap-

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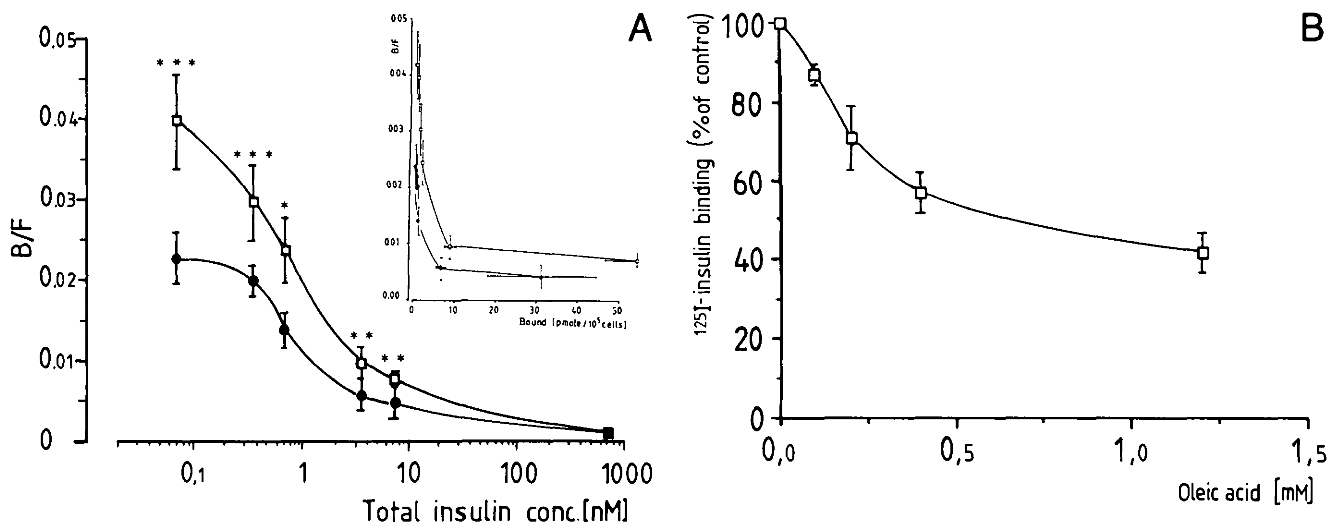

FIG. 1. *A*: specific insulin binding to isolated rat hepatocytes. Hepatocytes (10^5/ml) were incubated for 45 min at 37°C with ^{125}I-labeled insulin (200 pM) and increasing concentrations of unlabeled insulin (0–10 μM) in absence (□) or presence (●) of oleic acid (0.4 mM). Data are means ± SE of 7 experiments. *Inset* shows analysis of data according to Scatchard. B/F, ratio of bound to free insulin. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. *B*: influence of different concentrations of oleic acid on specific ^{125}I-insulin binding at tracer concentrations. Data are expressed as percentage of binding in absence of oleic acid. Data are means ± SE of 3 experiments.

parent K_d was 1.20 ± 0.35 nM (n = 7) and 1.20 ± 0.07 nM in control cells and cells exposed to oleic acid, respectively, indicating that receptor affinity was not affected by FFA. This was further demonstrated when the data were calculated according to Scatchard (24; Fig. 1*A*, *inset*). Thus, the effect of oleic acid was to reduce the number of binding sites for insulin. Figure 1*B* shows the effect of different concentrations of oleic acid on insulin binding. The maximal inhibitory effect of 58 ± 4% was exerted at 1.2 mM and half-maximal effect at 0.150 ± 0.050 mM.

There was no difference in the binding to partially purified insulin receptors from solubilized hepatocytes preincubated in the absence or presence of 0.2 mM oleic acid for 45 min (100 ± 17 vs. 96 ± 8%, respectively; 100% = 0.18 fmol ^{125}I-insulin/μg protein). Furthermore, 0.2 mM oleic acid had no direct effect on the ability of insulin to bind to partially purified insulin receptors.

The importance of ATP for the effect of oleic acid was investigated in cells exposed to 2 mM KCN for 5 min before the binding assay. This procedure prevents receptor internalization and further processing of the hormone-receptor complex (25,26).

Figure 2 shows that insulin binding is decreased by 55 ± 6% in energy-depleted cells without any shift in receptor affinity. Furthermore, 0.4 mM oleic acid did not further reduce cell-associated insulin in these cells.

The effect of oleic acid was also compared to equimolar concentrations of palmitic, stearic, palmitoleic, and eicosapentaenoic acid (Fig. 3). Comparable effects on insulin binding were seen with all the fatty acids studied.

Oleic acid at a concentration of 0.4 mM reduced the ^{125}I-insulin degradation, as estimated by measuring the precipitability in 10% TCA, from 6.0 ± 0.4 to 3.2 ± 0.2% (n = 10, $P < 0.01$). Furthermore, there was a significant linear correlation between the decrease in insulin binding and the decrease in degradation by different concentrations of oleic acid (Fig. 4).

Control experiments have shown that oleic acid had no effect on insulin degradation in a cell-free system or in medium in which liver cells had been preincubated for 45 min before the assay (data not shown).

Effect of oleic acid on [^{14}C]AIB uptake. [^{14}C]AIB uptake was 1.9 ± 0.2 nM/10^5 cells (Fig. 5). At high concentrations of insulin (0.7 nM), an ~50% increase of [^{14}C]AIB uptake was found. The half-maximal effect of insulin was seen at 70 ± 19 pM. In the presence of 0.4 mM oleic acid, basal [^{14}C]AIB uptake was significantly decreased by 32% ($P <$ 0.05).

The maximal insulin effect was also reduced by ~40%. Hence, a proper estimation of the ED_{50} for insulin could not be achieved. The concentration dependency of the inhibitory effect of oleic acid on AIB transport was similar to that on

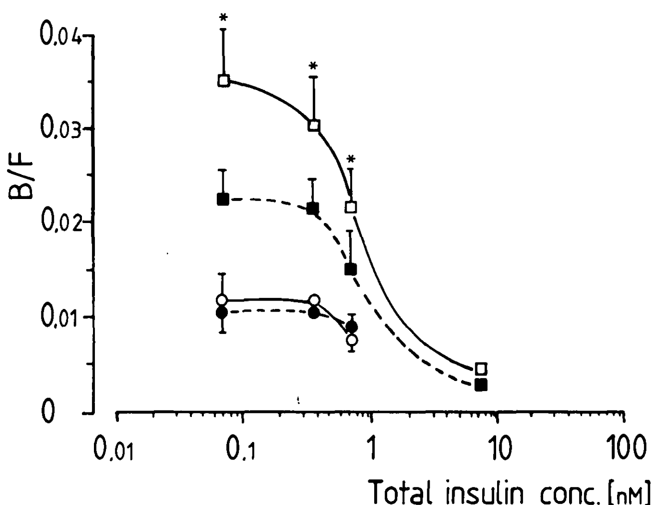

FIG. 2. Specific insulin binding to normal (*squares*) and energy-depleted (*circles*) cells in absence (*open symbols*) or presence (*closed symbols*) of 0.4 mM oleic acid. Data are means ± SE of 4 experiments. *$P < 0.05$.

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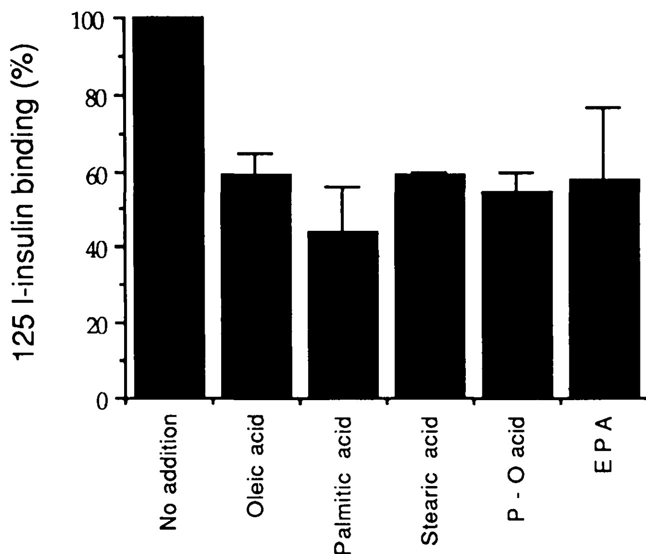

FIG. 3. Influence of different fatty acids at 0.4 mM on specific ^{125}I-labeled insulin binding to rat hepatocytes. Data are means ± SE of 5 experiments. P-O, palmitoleic acid; EPA, eicosapentaenoic acid.

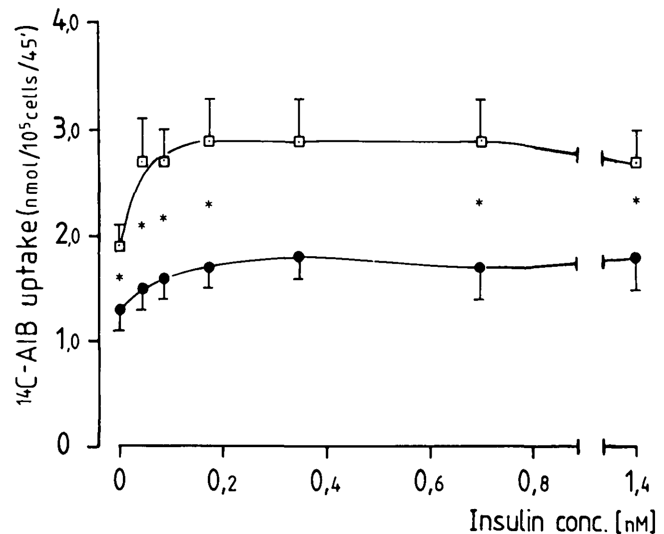

FIG. 5. Basal and insulin-stimulated [^{14}C]aminoisobutyric acid (AIB) uptake. Rat hepatocytes were preincubated at 37°C in absence and presence of different concentrations of insulin and in absence (□) and presence (●) of 0.4 mM oleic acid for 30 min. [^{14}C]AIB was added, and cellular uptake was measured after 45 min. Data are means ± SE of 7 experiments. $P < 0.05$.

insulin binding, i.e., half-maximal effect at 0.15 ± 0.05 mM (n = 2; data not shown).

DISCUSSION

This study shows that different fatty acids can rapidly (within 45 min) decrease the number of binding sites for insulin in isolated hepatocytes. The prevention of this effect by a respiratory inhibitor like KCN indicates that it is mediated through an energy-requiring process. Previous studies on cultured 3T3 cells (27) and reconstituted receptors (28) have shown the importance of the ambient FFA level in the composition of the cell membrane and the binding characteristics of the insulin receptor. However, the effect of FFA on hepatocytes in our study seems to be regulated through an entirely different mechanism in that it is rapid, energy requiring, and comprises all types of FFA tested. It seems reasonable to assume that such an acute effect of FFA is not due to changes in the composition of the cell membrane.

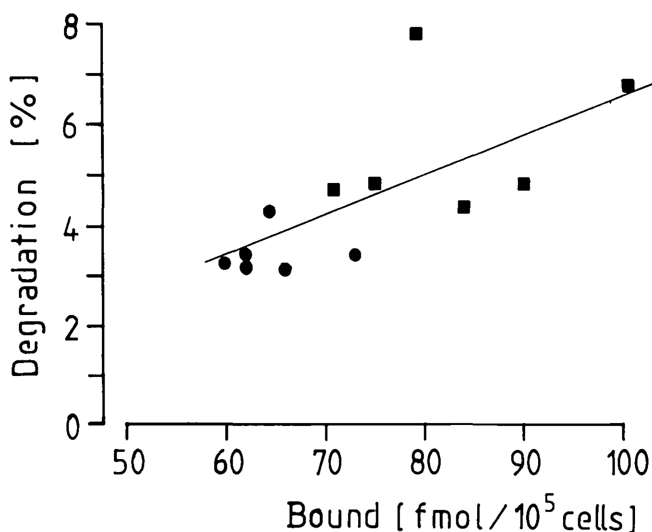

FIG. 4. Correlation between binding and degradation of ^{125}I-labeled insulin (200 pM) by isolated rat hepatocytes in absence (■) or presence (●) of 0.4 mM oleic acid. Data are from 6 experiments. $y = 0.794x + 0.137$, $r = 0.669$, $P < 0.02$.

The observation that FFA added to intact cells did not lead to a reduction in binding to partially purified receptors from solubilized cells suggests that FFA alters the processing of the hormone-receptor complex. This conclusion is further supported by the finding that FFA did not directly interfere with insulin binding to solubilized receptors. However, our data do not indicate whether FFA alters the rate of receptor internalization or recycling, although the degradation of insulin receptors is clearly not affected by FFA under the current conditions.

As noted previously, the technique for preparation of hepatocytes seems to be crucial for the kinetics of insulin binding and for the insulin effect (22). In this study, the isolated cells were extensively washed before the incubations, which were performed at a low cell concentration. This treatment results in minimum cellular leakage of proteases and, consequently, reduces nonspecific insulin binding and insulin degradation in the incubation medium (22). In our study, the cellular sensitivity for the effect of insulin on amino acid uptake was assayed at a concentration of ~10^5 cells/ml. At this low cell concentration, the half-maximal effect was seen at ~70 pM, whereas ED_{50} for the insulin effect was nearly 10 times higher in incubation systems with higher cell concentrations (29–31). Treatment of isolated hepatocytes with KCN leads to rapid ATP depletion and, consequently, inhibition of internalization and further processing of the hormone-receptor complex (25,26).

It is reasonable to assume that the effect of FFA on insulin binding also affects the rate of receptor-mediated insulin degradation. In accordance with this, the concentration of oleic acid inhibiting insulin binding by ~45% also decreased the insulin degradation by ~45%. Furthermore, the reduction in insulin binding and degradation was closely correlated. Further evidence for the direct effect of FFA on receptor-

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mediated degradation was provided by the observation that FFA did not change insulin degradation in a cell-free system.

Insulin degradation was assayed by TCA precipitability, which underestimated insulin degradation (18). However, the amount of degraded insulin detected by this method is probably proportional to that estimated by more sensitive methods like radioreceptor assays or radioimmunoassays. Thus, the results obtained indicate that physiological levels of FFA decrease insulin binding and receptor-mediated insulin degradation in liver cells.

The predicted consequence of reduced insulin-receptor binding would be a rightward shift of the dose-response curve for insulin (32). In this study, increased levels of oleic acid effectively reduced basal amino acid uptake and the maximal insulin effect, indicating the existence of postreceptor perturbations. The marked postreceptor perturbation precludes a correct estimation of ED_{50} for insulin.

The physiological importance of the effect of FFA on insulin binding, degradation, and action is not yet clear. The fact that the maximal inhibitory effect of FFA was seen at physiological concentrations may indicate that the regulation is important during the normal switch between anabolic and catabolic conditions. The physiological importance of elevated portal FFA is supported by the observation that obese human subjects have fewer binding sites for insulin in the hepatocytes (19). This may lead to a decrease in hepatic insulin clearance, which would be in agreement with the observation that insulin uptake by the liver is decreased in obese rats (33) and glucose-intolerant human subjects with abdominal obesity (17,34).

The concept is further supported by the fact that portal FFA levels correlate negatively with hepatic insulin clearance in experimentally obese rats (20). Moreover, preliminary data show that oleic acid decreases insulin uptake by the perfused rat liver in a concentration-dependent fashion (35).

In summary, physiological levels of different fatty acids effectively decrease insulin binding and degradation in isolated hepatocytes. Furthermore, both basal and insulin-stimulated AIB uptake are decreased, indicating the presence of postbinding perturbations. These findings are important in understanding the decreased hepatic insulin uptake and clearance and the reduced insulin effect seen in several clinical states accompanied by increased FFA levels.

ACKNOWLEDGMENTS

This study was supported by grants from the Swedish Medical Research Council (project B89-19x-251-27A-3506).

The expert technical assistance of Barbro Carlander, Irene Leonardsson, and Birgitta Svalstedt is gratefully acknowledged.

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