Free Fatty Acids Impair Hepatic Insulin Extraction in Vivo

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Hyperinsulinemia is a common finding in obesity and results from insulin hypersecretion and impaired hepatic insulin extraction. In vitro studies have shown that free fatty acids (FFAs), which are often elevated in obesity, can impair insulin binding and degradation in isolated rat hepatocytes. To investigate whether FFAs impair hepatic insulin extraction (E_H) in vivo, either saline (SAL) or 10% Intralipid (0.03 ml · kg^{-1} · min^{-1}) plus heparin (0.44 U · kg^{-1} · min^{-1}) (IH) was infused into normal dogs to elevate FFA levels. Insulin was infused intraportally at 18 pmol · kg^{-1} · min^{-1} for 150 min (period A, high insulin dose), and then at 2.4 pmol · kg^{-1} · min^{-1} for another 150 min (period B, low insulin dose). After the low portal insulin dose, additional insulin was infused peripherally at 8.4 pmol · kg^{-1} · min^{-1} for 120 min (period C) to assess the clearance of insulin from the peripheral plasma. In 16 paired experiments, FFA levels were 1,085 ± 167, 1,491 ± 240, 1,159 ± 221 μmol/l (IH) and 221 ± 44, 329 ± 72, 176 ± 44 μmol/l (SAL) in periods A, B, and C, respectively. Peripheral insulin levels were greater with IH (P < 0.001) than with SAL in all periods (1,620 ± 114, 126 ± 12, 1,050 ± 72 pmol/l for IH vs. 1,344 ± 168, 96 ± 4.2, 882 ± 60 pmol/l for SAL). Glucose clearance was impaired by IH in all periods (P < 0.05), whereas glucose production was slightly increased by IH during period B. Peripheral insulin clearance (CI) and E_H were calculated from the insulin infusion rate and insulin concentration data in each period by taking into account the nonlinearity of insulin kinetics. CI was lower (P < 0.01) with IH (9.6 ± 0.6, 12.0 ± 0.9, 10.2 ± 0.6 ml · kg^{-1} · min^{-1}) than with SAL (11.2 ± 1, 13.6 ± 0.7, 11.9 ± 0.9 ml · kg^{-1} · min^{-1}) in periods A, B, and C. E_H was also lower (P < 0.05) with IH (25 ± 4, 40 ± 5, 32 ± 5%) than with SAL (30 ± 2.8, 47 ± 3, 38 ± 3%). We conclude that FFAs can impair hepatic insulin extraction in vivo at high and low insulin levels, an effect that may contribute to the peripheral hyperinsulinemia of obesity. *Diabetes* 48:766–774, 1999

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ANOVA, analysis of variance; CI, peripheral insulin clearance; CPT-1, carnitine palmitoyl transferase 1; EC_{50}, concentration for half-maximal uptake; E_H, hepatic insulin extraction; FFA, free fatty acid; ID, internal diameter; IH, Intralipid plus heparin; LPL, lipoprotein lipase; PKC, protein kinase C; SAL, saline.

Hyperinsulinemia is a common feature of obesity and insulin-resistant states and is implicated in the pathogenesis of cardiovascular disease (1). In obesity, the main cause of hyperinsulinemia is insulin hypersecretion (2), an event that is either secondary or primary to insulin resistance, or both. It has been clearly demonstrated that in addition to insulin hypersecretion, obese humans, as well as animal models of obesity, have a reduced hepatic extraction of insulin (2–7). Because ~50% of insulin produced by the pancreas is removed by the liver during the first portal passage before reaching the systemic circulation, the liver is a main site of insulin extraction. Therefore, a decline in hepatic insulin extraction would have a pronounced effect on the resulting peripheral levels of insulin.

One factor that may account for the impaired hepatic insulin extraction in obesity is insulin hypersecretion, which may exceed the hepatic insulin extraction capacity. Another factor that may influence the hepatic insulin extraction capacity is the presence of elevated circulating and hepatic free fatty acid (FFA) levels. High FFA levels commonly occur in obesity (8,9) and in insulin-resistant states (10). Data from in vitro studies have shown that FFAs can impair insulin binding and degradation in rat hepatocytes (11–14). In situ studies have demonstrated an impairing effect of FFAs on hepatic insulin clearance in the perfused rat liver (15). However, the in vivo effect of FFAs on hepatic insulin extraction has not been fully investigated. The objective of this study was therefore to investigate the effect of FFAs on hepatic insulin extraction in vivo. The dog model was used to control the portal insulin levels by direct intraportal infusion of insulin, a technique impossible to perform in humans. FFAs can stimulate the β-cell of the pancreas to secrete insulin, and the resultant high portal insulin concentrations may differentially affect hepatic insulin extraction. Therefore, we controlled the level of hyperinsulinemia by inhibiting endogenous secretion of insulin with high doses of somatostatin, which are well tolerated in dogs, and replaced insulin at high or low dose rates via the portal vein. Plasma FFA levels were experimentally raised by an infusion of Intralipid and heparin, and hepatic insulin extraction was derived using a combination of portal and peripheral insulin infusions.

RESEARCH DESIGN AND METHODS

Experimental animals and surgical preparation. This study was carried out on eight overnight-fasted normal male dogs. Mongrel dogs weighing 20–35 kg and at least 1 year old underwent vessel cannulation, performed under general anesthesia induced with sodium thiopental and maintained with halothane and nitrous oxide along with assisted ventilation. A Silastic cannula (0.04-inch

internal diameter [ID]; Baxter Healthcare, McGraw Park, IL) was inserted into the portal vein via a branch of the splenic vein and advanced until the tip was ~1.0 cm beyond the point of confluence of the splenic vein with the portal vein—that is, ~5 cm from the branching point of the portal vein into its left and right bifurcations to the liver. The portal cannula served for infusion. We did not have sampling catheters in the portal vein, because portal sampling catheters require more extensive surgery (16) and have a high failure rate, which makes it difficult to carry out paired experiments in the same dog. Three additional Silastic cannulas, which served for peripheral infusion (one 0.04-inch ID and two 0.03-inch ID), were inserted into a jugular vein and advanced into the superior vena cava. In addition, a Silastic cannula (0.04-inch ID) was advanced into the aortic arch through a carotid artery. The arterial cannula served for sampling, and the jugular and portal cannulas served for infusions. The cannulas were tunneled subcutaneously and exteriorized at the back of the neck. They were filled with 1,000 U/ml heparin (Hepalean; Organon Teknika, Ontario, Canada) and flushed with saline regularly (every 4 days) to maintain patency. Heparin activates lipoprotein lipase, which results in release of FFAs and glycerol. When using heparin as an anticoagulant in the above concentration, we do observe an increase in basal FFAs versus those in noncatheterized dogs, despite our efforts to avoid systemic heparinization during catheter flushing. When using citrate as an alternative anticoagulant, the failure rate of the catheters is close to 100%. When using less heparin, the failure rate of the catheters increases substantially to the point of making it difficult to perform repeated experiments in the same dog.

The dogs received 15 g/kg food per day, mixed with 500 g of canned beef chunks. The food was supplemented with folic acid and iron to assist erythrocyte production and prevent anemia due to the blood sampling during the experiments. Only healthy dogs were selected, with a hematocrit >35%. The experiments were performed after an 18-h overnight fast. All procedures were in accordance with the Canadian Council of Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto.

Experimental design. Sixteen paired experiments were conducted in eight normal, fasted, and conscious dogs. After a basal resting period of 30 min (–30 to 0 min), at time 0, either saline (SAL) (0.03 ml · kg^{-1} · min^{-1}) or 10% Intralipid (0.03 ml · kg^{-1} · min^{-1}) (a triglyceride emulsion) plus heparin (0.44 U · kg^{-1} · min^{-1}) (IH) was given to elevate circulating FFA levels. Heparin releases lipoprotein lipase (LPL) from the surface of the peripheral endothelium of capillaries, and LPL

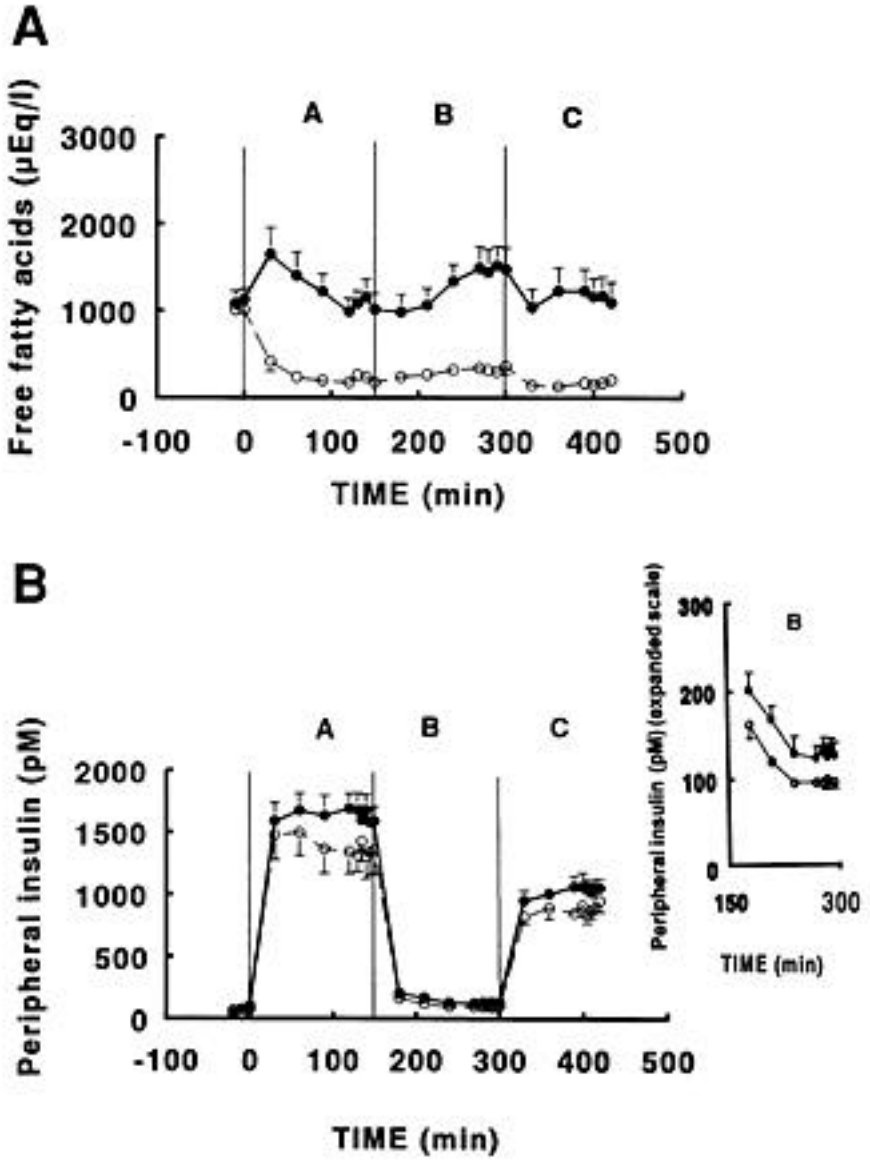

FIG. 1. Plasma free fatty acids (*A*) and peripheral insulin levels (*B*) in periods A, B, and C. Measurements were taken before (basal) and during infusion of 10% Intralipid at 0.03 ml · kg^{-1} · min^{-1} plus heparin at 0.44 U · kg^{-1} · min^{-1} (n = 8) (●) or SAL infusion (n = 8) (○) in period A (high-rate portal insulin infusion, 3 mU · kg^{-1} · min^{-1}), period B (low-rate portal insulin infusion, 0.4 mU · kg^{-1} · min^{-1}), and period C (low-rate portal insulin infusion, 0.4 mU · kg^{-1} · min^{-1}, plus peripheral insulin infusion, 1.4 mU · kg^{-1} · min^{-1}). A glucose clamp was maintained by an exogenous glucose infusion. Values are presented as means ± SE from eight experiments in each treatment. The plasma FFA levels were either basal or higher than basal (P < 0.001, period B) throughout the experiment. The peripheral insulin levels were significantly different between treatments (P < 0.001).

catalyzes the breakdown of the triglyceride emulsion to FFAs and glycerol. IH infusion is a standard nontoxic method to elevate FFA levels in vivo. We did not directly infuse FFAs because high-rate FFA infusion, as required to elevate FFA levels during high-rate insulin infusion, may be toxic and cause hemolysis, hemoglobinuria, and thrombus formation (17) unless FFAs are bound to albumin in a very high molar ratio (which might not be suitable for in vivo infusions). Simultaneously with IH or SAL, a high-dose intraportal insulin infusion (18 pmol · kg^{-1} · min^{-1}) was given to achieve high levels of insulin (period A). These levels were comparable to those observed in massively obese human subjects after an oral glucose load (18). After 150 min, the intraportal insulin infusion rate was lowered (2.4 pmol · kg^{-1} · min^{-1}) to obtain basal insulin levels (period B). After another 150 min, an additional insulin infusion (8.4 pmol · kg^{-1} · min^{-1}) was given peripherally to assess the effect of FFAs on the clearance of insulin from the peripheral plasma (period C). All insulin infusions were prepared in saline containing ~4% of the dog's own plasma. Somatostatin (0.8 µg · kg^{-1} · min^{-1}) was given throughout the experiment to inhibit endogenous insulin secretion, and glucagon was replaced intraportally at a rate resulting in basal levels (0.65 ng · kg^{-1} · min^{-1}). A variable rate of glucose (25% dextrose; Abbott Laboratories, Montreal, Quebec, Canada) was infused to prevent insulin-induced decline in plasma glucose and was adjusted according to plasma glucose concentrations determined every 5 min on a glucose analyzer (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). At time 0, a primed infusion of [3-^{3}H]glucose (79 µCi + 0.79 µCi/min) (New England Nuclear, Boston, MA) was started (period A). The [3-^{3}H]glucose was purified by high-performance liquid chromatography (HPLC) to remove contaminants, which have been shown to induce errors in the determinations of glucose production. The tracer rate was lowered to 0.52 µCi/min during period B and increased back to 0.79 µCi/min during period C in an attempt to minimize changes in plasma glucose specific activity, which have been shown to affect the calculation of glucose turnover in nonsteady states.

Arterial samples were taken every 10 min for 30 min during the basal period, and then every 5 min throughout the experiment. Blood samples were obtained for FFAs, insulin, glucagon, C-peptide levels, triglycerides, glycerol, lactate, β-hydroxybutyrate, and [3-^{3}H]glucose specific activity.

The blood samples for insulin, glucagon, and C-peptide were collected in EDTA (Sigma, St. Louis, MO) and 2,000 kallikrein IU Trasylol (Bayer, Etobicoke, Ontario, Canada). The samples for FFAs, triglycerides, and glycerol were collected in EDTA and Trasylol and 5 µl (74.7 ng/ml blood) tetra-hydrolipostatin (Hoffman La Roche, Mississauga, Ontario, Canada). Tetra-hydrolipostatin, an inhibitor of all lipases including lipoprotein lipase (19,20), was added to the tubes to prevent the in vitro hydrolysis of triglycerides to FFAs and glycerol. The blood samples for [3-^{3}H]glucose were collected in tubes containing 2.5 mg sodium fluoride (Fisher, Fairlawn, New Jersey) and dried heparin (50 USP units per 1.0-ml sample). Blood samples for β-hydroxybutyrate and lactate were collected in tubes containing an equal volume of 10% perchloric acid (BDH, Toronto, Ontario, Canada). Within an hour after collection, the samples were centrifuged at 800*g* at 4°C, and the supernatant was stored at –20°C for later analysis.

Laboratory methods. Plasma glucose concentrations were measured by the glucose oxidase method on a Glucose Analyzer II during the experiment. Plasma FFA levels were determined using the fluorometric method of Miles et al. (21). The radioimmunoassay for plasma insulin levels was determined using a kit from Pharmacia AB (Uppsala, Sweden). The interassay coefficient of variation determined on reference plasma was <7%. The range of linearity was 18–1,440 pmol/l. To avoid bias, samples from both periods A and C were diluted 1:1. The insulin recovery of the assay was between 97 and 99% on undiluted and diluted samples. Heparin has been known to interfere with insulin radioimmunoassays; it does not interfere with the Pharmacia kit, according to the manufacturer. We verified this ourselves by collecting blood samples at different insulin concentrations in two separate tubes, one with and one without heparin, and found that the results did not differ from each other. For the determination of the [3-^{3}H]glucose specific activity, 200 µl plasma was deproteinized in equal volumes of 5% zinc sulfate and 0.3N barium hydroxide that had been titrated. One milliliter H_2O, 0.4 ml $Ba(OH)_2$, and 0.4 ml $ZnSO_4$ were added. The samples were deproteinized, and 1 ml of the supernatant was evaporated to eliminate tritiated water. Water and liquid scintillation solution (Ready Safe; Beckman, Fullerton, CA) were added, and the radioactivity of the [3-^{3}H]glucose was measured in a β-scintillation counter (Camberra Packard, Meriden, CT). Aliquots of the infused glucose tracer and the labeled glucose infusate were diluted with pre–tracer equilibrium plasma and assayed together with the plasma samples. The plasma glucagon assay was performed using a kit from Diagnostics Products (Los Angeles, CA). Plasma C-peptide levels were determined in Dr. K. Polonsky's laboratory (Chicago) by a non-equilibrium double-antibody radioimmunoassay procedure for dog C-peptide (22). The detection limit of the assay is 0.06 pmol/ml. Plasma triglycerides and glycerol were determined using enzymatic colorimetric assay (kits 877557 and 148270; Boehringer Mannheim Diagnostic Laboratories, Mannheim, Germany). Plasma lactate and β-hydroxybutyrate were determined using the enzymatic fluorometric continuous-flow assay (23) with a fluoromicrophotometer (Aminco, Silver Spring, MD).

Calculations

Hepatic insulin extraction. Fractional hepatic insulin extraction (E_H) (dimensionless or percentage) is the rate of hepatic insulin extraction divided by the hepatic insulin load. The rate of hepatic insulin extraction

TABLE 1
Estimated hepatic insulin, glucagon, glucose, lactate, triglyceride, glycerol, and β-hydroxybutyrate levels with IH or SAL infusions during periods A, B, and C

	Basal period	Period A	Period B	Period C
Estimated hepatic insulin (pmol/l)				
SAL	104 ± 11	2,346 ± 186	240 ± 8.4	1,002 ± 66
IH	125 ± 22	2,598 ± 114	270 ± 13	1,158 ± 66
Glucagon (pg/ml)				
SAL	59.6 ± 3.6	62.8 ± 4.0	58.6 ± 3.0	57.7 ± 4.0
IH	64.5 ± 5.1	63.5 ± 3.0	64.5 ± 8.0	60.1 ± 5.0
Glucose levels (mmol/l)				
SAL	5.4 ± 0.14	5.2 ± 0.13	5.3 ± 0.14	5.4 ± 0.14
IH	5.6 ± 0.14	5.7 ± 0.12	5.9 ± 0.07	5.6 ± 0.14
Lactate (µmol/l)				
SAL	375 ± 52	831 ± 97	475 ± 34	721 ± 91
IH	378 ± 38	746 ± 74	408 ± 39	573 ± 56
Triglyceride (mmol/l)				
SAL	3.2 ± 0.3	1.9 ± 0.2	1.9 ± 0.3	1.8 ± 0.3
IH	3.5 ± 0.4	4.1 ± 0.5	4.3 ± 0.6	4.6 ± 0.7
Glycerol (mmol/l)				
SAL	0.23 ± 0.03	0.26 ± 0.07	0.24 ± 0.07	0.26 ± 0.08
IH	0.36 ± 0.1	0.66 ± 0.16	0.68 ± 0.14	0.61 ± 0.12
β-Hydroxybutyrate (µmol/l)				
SAL	25.4 ± 4.4	6.3 ± 0.5	5.8 ± 0.4	5.0 ± 0.1
IH	19.4 ± 2.7	9.3 ± 1.3	15.0 ± 4	9.3 ± 1.6

Data are means ± SE. Period A, high-rate portal insulin infusion; period B, low-rate portal insulin infusion; period C, low-rate portal insulin infusion plus peripheral insulin infusion.

can be determined during the portal insulin infusion periods from the difference between the portal insulin infusion rate (R_{PO}) (in picomoles per kilogram per minute) and the rate of systemic insulin appearance. The rate of systemic insulin appearance is calculated as the product of arterial insulin concentration (I_A) (in picomoles per liter) and peripheral insulin clearance (Cl) (in milliliters per kilogram per minute). Thus, for periods A and B, E_H is calculated as

$$E_H = \frac{R_{PO} - I_A Cl}{R_{PO}} \quad (1)$$

Assuming linearity of the insulin system, peripheral insulin clearance is the same in all periods and can therefore be calculated during the peripheral insulin infusion (period C) as $Cl = R_{PE}/\Delta I_A$, where R_{PE} (picomoles per kilogram per minute) is the fixed rate of peripheral insulin infusion and ΔI_A (picomoles per liter) is the rise in insulin levels induced by the peripheral insulin infusion (R_{PE}) in period C. Insulin clearance may not be independent of insulin concentration, however, and thus insulin clearance calculated during the peripheral insulin infusion period (period C) may not be the same as during the portal infusion periods. To overcome this problem, we used a model that accounts for the nonlinearity of insulin kinetics. In this model, we have expressed peripheral insulin clearance (Cl) as a sum of a hepatic component (Cl_H) and an extrahepatic component (Cl_{EH}) such that

$$Cl = Cl_H + Cl_{EH} \quad (2)$$

Because $Cl_H = F_H E_H$ where F_H (milliliters per kilogram per minute) is hepatic plasma flow and E_H is fractional hepatic insulin extraction, then

$$Cl = F_H E_H + Cl_{EH} \quad (3)$$

Equation 3 is valid in general conditions, provided that insulin extraction in the lungs is negligible (24). Peripheral insulin clearance is also the ratio between systemic insulin appearance and arterial insulin concentration. When insulin is infused both peripherally and portally at rates R_{PE} and R_{PO}, respectively, the systemic insulin appearance is $R_{PE} + (1 - E_H)R_{PO}$, where $(1 - E_H)R_{PO}$ is the amount of insulin that, after portal insulin infusion, is not extracted by the liver. Thus

$$Cl = \frac{R_{PE} + (1 - E_H)R_{PO}}{I_A} \quad (4)$$

By combining Eqs. 4 and 3, we obtain

$$\frac{R_{PE} + (1 - E_H)R_{PO}}{I_A} = F_H E_H + Cl_{EH} \quad (5)$$

Solving for E_H, we obtain

$$E_H = \frac{R_{PE} + R_{PO} - Cl_{EH} I_A}{F_H I_A + R_{PO}} \quad (6)$$

Equation 6 expresses the relationship between fractional hepatic insulin extraction E_H, and the measured variables R_{PE}, R_{PO}, and I_A.

To calculate E_H using Eq. 6, we assumed a constant value for F_H of 20 ml/min of plasma per kg body wt (25,26). We also assumed that the extrahepatic insulin clearance, Cl_{EH}, is constant throughout the experiment.

Hepatic insulin uptake (U_H) was assumed to follow Michaelis-Menten kinetics. Hepatic insulin uptake is the product of hepatic plasma flow (F_H), fractional hepatic insulin extraction (E_H), and hepatic insulin concentration (I_H),

$$U_H = F_H E_H I_H = \frac{U_{max} I_H}{EC_{50} + I_H} \quad (7)$$

where U_{max} (maximal uptake) (picomoles per kilogram per minute) and EC_{50} (insulin concentration for half-maximal uptake) (in picomoles per liter) are the Michaelis-Menten parameters.

Solving for E_H,

$$E_H = \frac{U_{max}}{F_H (EC_{50} + I_H)} = \frac{U_{max}}{EC_{50} F_H (1 + I_H/EC_{50})} \quad (8)$$

By defining E_O, the extrapolation of fractional hepatic insulin extraction at zero insulin concentration (dimensionless, or %), as

$$E_O = U_{max}/F_H EC_{50} \quad (9)$$

Eq. 8 can be rearranged as

$$E_H = \frac{E_O}{1 + I_H/EC_{50}} \quad (10)$$

Note that as hepatic insulin concentration increases, Eq. 7 predicts that hepatic insulin uptake increases toward a maximal value U_{max}, and Eq. 10 predicts that fractional hepatic insulin extraction decreases progressively from its maximum value, E_O.

Using a modification of the Fick's principle as in Ader and Bergman (27), hepatic sinusoidal insulin concentration (I_H, where I_H = systemic insulin + the insulin added by the portal infusion) is calculated as

$$I_H = I_A + \frac{R_{PO}}{F_H} \quad (11)$$

By inserting this value into Eq. 10 and by substituting E_H from Eq. 10 with Eq. 6, the following equation is obtained:

$$\frac{E_O}{1 + (I_A + R_{PO}/F_H)/EC_{50}} = \frac{R_{PE} + R_{PO} - Cl_{EH} I_A}{F_H I_A + R_{PO}} \quad (12)$$

Equation 12 relates the unknown parameters E_O, EC_{50}, and Cl_{EH} to the measured variables R_{PE}, R_{PO}, and I_A. If the three experimental periods are considered, Eq. 12 yields a system of three equations in three unknowns, and can thus be solved for E_O, EC_{50}, and Cl_{EH}. To solve the system of nonlinear equations, Eq. 12 was first rearranged to express I_A as a function of known parameters and the unknowns E_O, EC_{50}, and Cl_{EH}. The values of E_O, EC_{50}, and Cl_{EH} were then obtained by least-squares fit of the measured insulin concentration I_A in periods A, B, and C of each experiment.

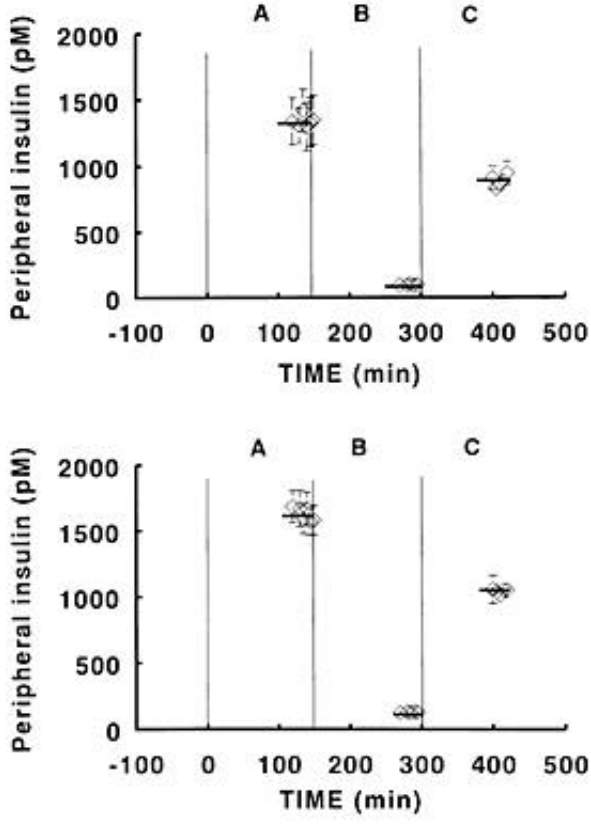

FIG. 2. Average model fit to the steady-state insulin concentrations in the SAL or IH infusion groups. The residual model error (root mean square of the differences between the measured and the model-predicted arterial insulin concentrations) was not different from the standard deviations of the arterial insulin concentrations.

Once E_O, EC_{50}, and CI_{EH} are obtained, fractional hepatic insulin extraction can be calculated using Eq. 10 for any value of hepatic insulin concentration. Peripheral insulin clearance is then obtained from Eq. 3. If this value of peripheral insulin clearance is used in Eq. 1, the same value of fractional hepatic insulin extraction as that calculated from Eq. 10 is obtained. Thus, the model can be regarded as a method for estimating the appropriate value of insulin clearance to be used with Eq. 1.

The reported values of fractional hepatic insulin extraction in periods A, B, and C are relative to the hepatic insulin concentration calculated in each period using Eq. 10. In the SAL and IH experiments, hepatic insulin concentrations in the corresponding periods are not identical. To compare fractional hepatic insulin extraction in SAL and IH at the same hepatic insulin concentration in IH, in Eq. 10 the hepatic insulin concentration observed in SAL was used for both the SAL and IH experiments.

Glucose turnover. Despite our efforts to modify tracer infusion rates, plasma glucose specific activity was not constant. Therefore, we calculated glucose turnover with a previously published two-compartment model (28) using the canine model parameters reported by Dobbins et al. (29). Glucose clearance was calculated as the ratio between the model-determined glucose disappearance rate and arterial glucose concentration. Tracer-determined glucose production was calculated as the difference between tracer-determined glucose turnover and the exogenous glucose infusion rate.

Statistical analysis. Data are expressed as mean ± SE. All results refer to $n = 8$ in both IH and SAL groups unless otherwise specified. Two-way analysis of variance (ANOVA) was carried out for differences between experimental groups. Data were also analyzed within each group for differences between the experimental periods. For the latter calculations, two-way ANOVA followed by Tukey's t test was used. Correlations were assessed with linear regression analysis. Calculations were performed using SAS software (SAS Statistical Analysis System, Cary, NC).

RESULTS

Hormones and metabolites. In the SAL protocol, FFA levels (Fig. 1) declined from basal ($P < 0.001$) at the onset of the insulin infusion. In the IH protocol, FFA levels remained at basal levels or higher than basal (period B, $P < 0.001$) throughout the experiment. With IH, FFA levels were six to nine times higher ($P < 0.001$) than with SAL in all periods.

Basal peripheral insulin levels (Fig. 1) in the SAL treatment were 60 ± 6.0 pmol/l and increased to 1,344 ± 168, 96 ± 4.2, and 882 ± 60 pmol/l in periods A, B, and C, respectively. In the IH protocol, basal insulin levels were 72 ± 12 pmol/l and rose to 1,620 ± 114, 126 ± 12, and 1,050 ± 72 pmol/l in periods A, B, and C, respectively. Insulin levels were higher ($P < 0.001$) with IH versus SAL in all three periods.

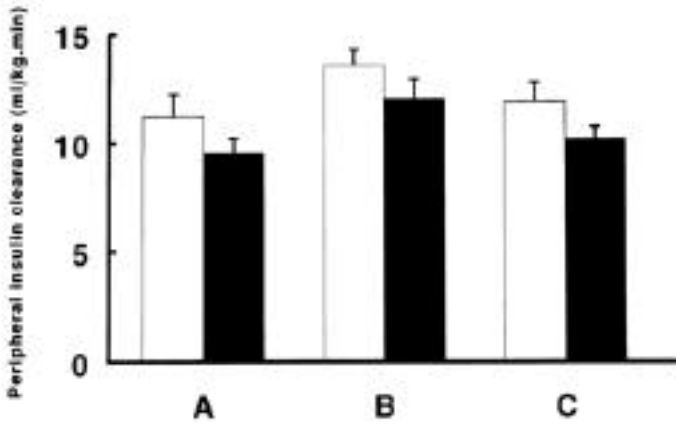

FIG. 3. Peripheral insulin clearance with SAL ($n = 8$) (□) and IH ($n = 8$) (■) in periods A, B, and C. Calculations were performed using a method that takes into account the nonlinearity of insulin kinetics (see METHODS). Peripheral insulin clearance was significantly different between treatments using ANOVA ($P < 0.01$).

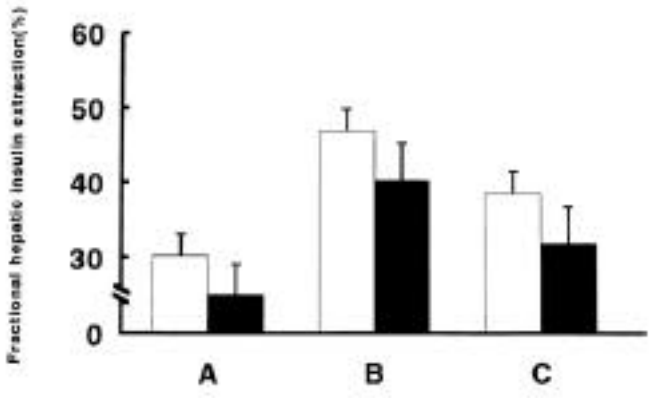

FIG. 4. Fractional hepatic insulin extraction (percentage) with SAL ($n = 8$) (□) and IH ($n = 8$) (■) in periods A, B, and C. The fractional hepatic insulin extraction was calculated using a method that takes into account the nonlinearity of insulin kinetics (see METHODS) at the hepatic insulin levels found in SAL. The fractional hepatic insulin extraction was significantly different between treatments using ANOVA ($P < 0.05$).

Plasma C-peptide levels as measured in four dogs were suppressed to below the detection limit of the dog C-peptide assay (0.06 pmol/ml) in both IH and SAL at all times and during all insulin infusion periods, indicating that endogenous insulin secretion was effectively inhibited.

Estimated hepatic insulin levels (Table 1) were higher with IH versus SAL in all three periods ($P < 0.001$). Plasma glucagon levels (Table 1) remained constant, and there were no significant differences from basal in SAL or IH or between treatments in all periods, indicating an effective basal replacement of glucagon. Plasma glucose levels (Table 1) were maintained at euglycemia throughout the experiment with the variable glucose infusion (GINF). Lactate levels (Table 1) were higher than basal with SAL in periods A and C ($P < 0.05$). The levels of lactate were also higher than basal with IH in periods A and C ($P < 0.05$). There were no significant differences between SAL and IH.

Triglyceride levels (Table 1) rose from basal with IH; however, the rise was only significant in period C ($P < 0.05$). With SAL, the levels of triglycerides were lower than basal in all three periods. As expected, the triglyceride levels were higher with IH than with SAL in all three periods ($P < 0.001$). Glycerol levels (Table 1) rose from basal with IH infusion. The rise was significant only in periods A and B ($P < 0.05$). The levels of glycerol did not change from basal with SAL in spite of an anticipated fall due to insulin's suppressive action on lipolysis. As expected, the levels of glycerol were significantly higher with IH compared with SAL ($P < 0.01$). With SAL (Table 1), β-hydroxybutyrate levels fell from basal ($P < 0.05$). With IH, the levels were not significantly different from basal. The β-hydroxybutyrate levels were suppressed significantly less with IH than SAL in all three periods ($P < 0.01$).

Model fit. The average model fit to the steady-state insulin concentrations in the SAL and IH groups is shown in Fig. 2. The residual model error (root mean square of the differences between the measured and the model-predicted arterial insulin concentrations) was not different from the standard deviation of the arterial insulin concentrations. By pooling all 16 cases, the residual model errors of periods A, B, and

TABLE 2
Glucose infusion rate, glucose clearance rate, and glucose production with IH or SAL infusions during periods A, B, and C

	Period A	Period B	Period C
Glucose infusion rate (μmol · kg^{-1} · min^{-1})			
SAL	85.0 ± 7.8	16.7 ± 2.8	83.4 ± 11.7
IH	76.2 ± 7.8	10.6 ± 2.8	71.7 ± 10.0
Glucose clearance (ml · kg^{-1} · min^{-1})			
SAL	16.0 ± 0.9	4.3 ± 0.5	16.4 ± 1.3
IH	12.7 ± 1.4	2.8 ± 0.6	11.6 ± 1.7
Glucose production (μmol · kg^{-1} · min^{-1})			
SAL	1.5 ± 2.8	4.5 ± 1.1	–0.2 ± 2.2
IH	–1.7 ± 2.8	8.2 ± 1.7	5.6 ± 4.5

Data are means ± SE. Period A, high-rate portal insulin infusion; period B, low-rate portal insulin infusion; period C, low-rate portal insulin infusion plus peripheral insulin infusion.

C were 110 ± 9, 13 ± 1, and 98 ± 15 pmol/l, respectively, while the standard deviations of the arterial insulin concentrations were 102 ± 8, 12 ± 2, and 91 ± 14 pmol/l. Thus the model adequately predicts the arterial insulin concentrations in the three steady-state periods.

In the SAL group, the parameters of Eq. 12 were: E_O = 50 ± 3%, EC_{50} = 4,254 ± 841 pmol/l, and Cl_{EH} = 4.6 ± 1.0 ml · kg^{-1} · min^{-1}. In the IH group, E_O = 45 ± 5% and Cl_{EH} = 4.2 ± 1.0 ml · kg^{-1} · min^{-1}. In one dog, the estimated EC_{50} value was very high because insulin clearance did not decrease with increasing insulin concentrations, presumably because it was already very low at low insulin concentrations in period B. In the other seven dogs, EC_{50} = 4,626 ± 1,832 pmol/l.

Peripheral insulin clearance and hepatic insulin extraction. The peripheral insulin clearance, calculated by taking into account the nonlinearity of insulin kinetics, is presented in Fig. 3. In both IH and SAL groups, peripheral insulin clearance decreased with increasing insulin concentrations (P < 0.01 for differences between periods) and was lower with IH (P < 0.01) than with SAL (11.2 ± 1, 13.6 ± 0.7, 11.9 ± 0.9 ml · kg^{-1} · min^{-1} (SAL) vs. 9.6 ± 0.6, 12.0 ± 0.9, 10.2 ± 0.6 (IH) ml · kg^{-1} · min^{-1}, in periods A, B, and C, respectively).

Peripheral insulin clearance has a hepatic and an extrahepatic component (see METHODS). The values for the extrahepatic clearance, Cl_{EH}, which are reported in the preceding section, were not significantly different between treatments.

With both SAL and IH treatment, the hepatic insulin fractional extraction, expressed as a percentage (Fig. 4), was less with increasing hepatic sinusoidal insulin levels (P < 0.05 for differences between periods). The fractional hepatic insulin extraction, calculated using the hepatic insulin levels found in SAL, was lower (P < 0.05) with IH (25 ± 4, 40 ± 5, 32 ± 5%) vs. SAL (30 ± 3, 47 ± 3, 38 ± 3%) in periods A, B, and C, respectively.

Significant differences between treatments in both peripheral insulin clearance (calculated in period C) and fractional hepatic insulin extraction were also found assuming linearity of insulin kinetics (peripheral insulin clearance: 9.7 ± 0.6 ml · kg^{-1} · min^{-1} [IH] vs. 11.5 ± 0.8 ml · kg^{-1} · min^{-1} [SAL], P < 0.001, data not shown; fractional hepatic insulin extraction: 17.1 ± 5% [IH] vs. 21.2 ± 3% [SAL] in period A and 48.2 ± 6% [IH] vs. 53.8 ± 3% [SAL] in period B; P < 0.05; data not shown).

Glucose turnover. The glucose infusion rate (Table 2) was significantly lower with IH than with SAL (P < 0.05) in both periods A and B. The difference between treatments just failed to reach statistical significance in period C (P = 0.052). Glucose clearance (Table 3) was less in all three periods with IH compared with SAL (P < 0.05). Glucose production (Table 4) was greater with IH versus SAL in period B (P < 0.05) and was completely suppressed in periods A and C with both IH and SAL. There was a trend for glucose production to be correlated with FFAs (P = 0.056). There was also a trend toward an inverse correlation between fractional hepatic insulin extraction and glucose production in period B (P = 0.051).

DISCUSSION

This study is the first to demonstrate that FFAs impair hepatic insulin extraction in vivo, at low and at high portal insulin concentrations. Evidence that FFAs may affect processes related to hepatic insulin extraction first emerged from in vitro studies. Studies in isolated rat hepatocytes showed that low physiologic concentrations of FFAs decreased insulin binding and degradation in proportion to a decreased receptor number (11–14). In the in situ perfused rat liver, physiologic FFA concentrations caused a decline in hepatic insulin extraction (15). The in vivo effect of FFAs on hepatic insulin extraction, however, is not entirely clear. Ideally, the problem should be addressed in humans, but portal insulin infusion cannot be performed in humans and portal insulin delivery can be obtained only by stimulating insulin secretion. Isolation of the FFA effect is difficult, since FFAs acutely stimulate insulin secretion and the resultant elevation in hepatic sinusoidal insulin may lead to lower fractional hepatic insulin extraction due to nonlinear insulin kinetics. In addition, insulin per se might downregulate receptor numbers, thus impairing the hepatic insulin extraction process (7). However, indirect human data show an association between peripheral insulin concentrations (30,31) or peripheral insulin clearance (30) and hepatic triglyceride content (measured by computed tomography scan [30] or directly [31]). The latter is proportional to the main provider of portal FFA, namely visceral adipose tissue mass (32). Some studies in humans in which FFA levels were elevated with an Intralipid plus heparin infusion did not show an impairing effect of FFAs on peripheral insulin clearance (33–35), which includes a hepatic and an extrahepatic component. FFAs may have a detectable effect on hepatic insulin extraction only in young

subjects with high extraction and very low hepatic triglyceride content, since the inhibitory effect of FFAs on insulin clearance was attenuated in the triglyceride-rich liver of fat, old rats with an already reduced insulin clearance (15). Alternatively, since the majority of the in vivo human studies were performed at lower insulin levels than in ours, a small inhibitory effect of FFAs might have been masked. Furthermore, since insulin was infused peripherally in these studies, the impact of the liver on the resultant peripheral insulin levels was less than with physiologic portal insulin delivery. In agreement with our findings, Hennes et al. (36) showed in humans that elevated FFAs decreased whole-body insulin clearance (which includes both first-pass hepatic and peripheral insulin clearance). We have obtained similar findings in humans (A. Carpentier, S.D. Mittelman, B. Lamarche, R.N. Bergman, A. G., G.F.L., unpublished observations). In all of these studies showing an FFA effect, insulin was delivered portally by stimulating insulin secretion with hyperglycemic clamps. In the present study, hepatic insulin extraction was calculated using a method that takes into account the nonlinearity of insulin kinetics. This was a necessary choice, as it would be contradictory to assume constancy of peripheral insulin clearance (which includes both a hepatic and an extrahepatic component) in the presence of changes of fractional hepatic insulin extraction, as we found using the linear method. Studies in humans and animals agree that the peripheral insulin clearance is constant up to insulin levels of 600 pmol/l (37–42); however, our hepatic insulin levels were >600 pmol/l in both periods A and C. Because CI decreases with increasing insulin levels, CI, which was calculated in period C, was likely overestimated in A and underestimated in B when using the linear method. Hence, the hepatic insulin extraction derived using the linear method was underestimated in A and overestimated in B, as evidenced from the difference with the data obtained using the nonlinear method.

The main assumptions of the nonlinear method are Michaelis-Menten kinetics of hepatic insulin extraction and linearity of extrahepatic insulin extraction. Michaelis-Menten kinetics are frequently used to describe receptor-mediated events such as hepatic insulin extraction. Renal insulin extraction, which comprises most of the extrahepatic insulin extraction, is linearly dependent on insulin concentrations up to 900 pmol/l (43–47). This value corresponds to the peripheral insulin levels found in period C in the present study. While the majority of studies have been able to show linearity beyond this level, there is at least one study that did not (48). If the results during period A, where peripheral insulin concentrations were >900 pmol/l, are biased by our assumption of constant extrahepatic clearance, a similar bias would have occurred in both the IH and the SAL groups, and therefore our main conclusions would likely not be altered. Incidentally, a significant difference in the hepatic insulin extraction was found between treatments both by taking into account the nonlinearity of hepatic insulin extraction and by assuming linearity of insulin kinetics (hepatic and extrahepatic extraction).

It could be argued that the effect of FFAs on impairing hepatic insulin extraction was not large. This may have been in part because the dogs had high basal FFA levels due to the chronic heparinization of the catheters. The high basal exposure to FFAs may have increased the hepatic triglyceride content and attenuated the effect of the Intralipid infusion.

The mechanism of the FFA effect on hepatic insulin extraction may depend on cellular metabolism, since in vitro studies suggest that the FFA inhibition of insulin binding does not occur at 4°C or in hepatocytes pretreated with potassium cyanide (KCN) (11,12). Fatty acid oxidation may partly mediate the effect of FFAs on insulin binding. Consistent with this hypothesis, in our study β-hydroxybutyrate levels (index of hepatic FFA oxidation) were higher with IH compared with SAL. Both the pretreatment of rats with etomoxir (an inhibitor of carnitine palmitoyl transferase 1 [CPT-1]) (49) and the addition of methyl palmoxirate (another CPT-1 inhibitor) to rat hepatocytes (14) either completely or partially prevented the FFA-induced decline in insulin binding. FFAs or products of FFA oxidation may decrease the number of surface receptors by increasing the rate of insulin receptor internalization, decreasing the rate of receptor recycling, or both. In addition, FFAs may activate protein kinase C (PKC) (50), which can increase [^{125}I]insulin and insulin receptor internalization (51,52). This would not necessarily lead to an increased hepatic insulin extraction, if unoccupied receptors were preferentially internalized. Our preliminary studies on isolated rat hepatocytes indicate that the FFA-induced reduction in insulin binding may be linked to PKC activation (S. Chen, S.R. Wiesenthal, L. Lam, V. Tchipashvili, H. Yoshii, I.G. Fantus, A. Giacca, unpublished observations).

In the present study, glucose clearance was significantly less in all three periods with IH than with SAL in accordance with Randle's cycle (53). In humans, the inhibition of glucose clearance by FFAs may be time-dependent, as it has usually only been observed in studies lasting for longer than 2–3 h (54). In contrast, we observed that IH impairs glucose clearance after 1.5 h of Intralipid infusion. The rapid effect of IH on glucose clearance may be species-specific, since Sindelar et al. (55) and Rebrin et al. (56) also showed a rapid effect of IH on impairing glucose clearance in dogs. In the present study, glucose production was completely suppressed by the high insulin levels (periods A and C), independent of FFAs. At low insulin levels (period B), glucose production was greater with IH, consistent with a stimulatory effect of FFAs on gluconeogenesis as previously shown (34,57). These results are in accordance with our previous studies in dogs (58) and humans (59), which show that glucose production is suppressed by both direct hepatic and indirect peripheral effects. The indirect peripheral effect of insulin on glucose production is mainly mediated by the FFA inhibition due to the peripheral antilipolytic effect of insulin (25,55,56,59).

A trend toward an inverse correlation between hepatic insulin extraction and glucose production was found in period B. Thus, hepatic insulin resistance may be associated with decreased insulin extraction, an association that might merely reflect two independent effects of FFAs. It is also possible that decreased binding could affect both hepatic insulin extraction and action. However, FFAs have known effects on insulin action that are postreceptor and therefore independent of insulin binding. Conceivably, the same postreceptor mechanisms that induce hepatic or peripheral insulin resistance may also induce changes in insulin binding. The association between decreased extraction and action (increased glucose production) has been interpreted in the past as indicating that insulin needs to be internalized to be active; however, this theory has subsequently been discounted (60).

The IH infusion resulted in greater triglyceride levels than with SAL, as expected. An effect of circulating triglycerides on hepatic insulin extraction cannot be excluded; however, this effect may be mediated indirectly by the provision of intrahepatic FFAs. The IH infusion resulted in elevations of glycerol from the breakdown of the triglyceride in the Intralipid and from free glycerol in the Intralipid. While the relationship between glycerol and hepatic insulin extraction has not been investigated, an increase in glycerol phosphate turnover may cause a rise in diacylglycerol, which could stimulate PKC (52), and thus, insulin receptor internalization. To simultaneously control for the effect of triglyceride, glycerol, and heparin, we are now carrying out studies with direct infusion of FFAs. In these studies, we are also correlating the FFA effect on extraction with the prestudy hepatic triglyceride content.

In conclusion, we found that an elevation of circulating FFAs from an Intralipid plus heparin infusion decreased peripheral insulin clearance and hepatic insulin extraction. This effect may be a compensatory mechanism to overcome the insulin resistance associated with elevated FFAs. However, the resulting peripheral hyperinsulinemia may promote atherogenesis. Although the effect of FFAs on insulin clearance was not large in this study, any degree of hyperinsulinemia generated by this effect, acting over a prolonged period of time, might be of clinical relevance because of its potential in promoting cardiovascular disease.

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