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Diabetogenic Impact of Long-Chain ω-3 Fatty Acids on Pancreatic β-Cell Function and the Regulation of Endogenous Glucose Production

MARK J. HOLNESS, GEMMA K. GREENWOOD, NICHOLAS D. SMITH, AND MARY C. SUGDEN

Department of Diabetes and Metabolic Medicine, Barts and the London, Queen Mary's School of Medicine and Dentistry, University of London, London E1 4NS, United Kingdom

In healthy individuals, peripheral insulin resistance evoked by dietary saturated lipid can be accompanied by increased insulin secretion such that glucose tolerance is maintained. Substitution of long-chain ω-3 fatty acids for a small percentage of dietary saturated fat prevents insulin resistance in response to high-saturated fat feeding. We substituted a small amount (7%) of dietary lipid with long-chain ω-3 fatty acids during 4 wk of high-saturated fat feeding to investigate the relationship between amelioration of insulin resistance and glucose-stimulated insulin secretion (GSIS). We demonstrate that, despite dietary delivery of saturated fat throughout, this manipulation prevents high-saturated fat feeding-induced insulin resistance with respect to peripheral glucose disposal and reverses insulin hypersecretion in response to glucose *in vivo*. Effects of long-chain ω-3 fatty acid enrichment to lower GSIS were also observed in perifused islets suggesting a direct effect on islet function. However, long-chain ω-3 fatty acid enrichment led to hepatic insulin resistance with respect to suppression of glucose output and impaired glucose tolerance *in vivo*. Our data demonstrate that the insulin response to glucose is suppressed to a greater extent than whole-body insulin sensitivity is enhanced by enrichment of a high-saturated fat diet with long-chain ω-3 fatty acids. Additionally, reduced GSIS despite glucose intolerance suggests that either long-chain ω-3 fatty acids directly impair the β-cell response to saturated fat such that insulin secretion cannot be augmented to normalize glucose tolerance or β-cell compensatory hypersecretion represents a response to insulin resistance at the level of peripheral glucose disposal but not endogenous glucose production. (*Endocrinology* 144: 3958–3968, 2003)

INSULIN RESISTANCE IS a fundamental defect of the insulin resistance syndrome that is characterized by hyperglycemia, hyperinsulinemia, and dyslipidemia, and can predict the development of type 2 diabetes (1). Increased dietary fat is an important contributor to the development of insulin resistance (2–4), which reflects a diminished response to insulin in its target tissues, in particular muscle and liver, which accumulate excess lipid (reviewed in Refs. 5 and 6). There is accumulating evidence that increased plasma fatty acids decrease muscle glucose uptake and reduce insulin-stimulated insulin receptor substrate (IRS)-1 tyrosine phosphorylation (7), and IRS-1-associated phosphatidylinositol 3-kinase activity (7, 8). Infusion of lipid (mainly 18:2 fatty acids) in rats leads to accumulation of 18:2 fatty acyl-coenzyme A (CoA) and transient accumulation of diacylglycerol in muscle, increased IRS-1 Ser^{307} phosphorylation, decreased IRS-1 tyrosine phosphorylation, and IRS-1-associated phosphatidylinositol 3-kinase activity (9). Finally, fatty acid-induced muscle insulin resistance may reflect increased fatty acid utilization at the expense of glucose utilization (Randle's glucose-fatty acid cycle) (10). Increased plasma fatty acids increase hepatic gluconeogenesis; however, this does not necessarily increase hepatic glucose production due to compensatory reduction in glycogenolysis, defined as hepatic autoregulation, possibly in part because of fatty acid-induced increases in insulin secretion (reviewed in Ref. 11). Breakdown of autoregulation may occur if glycogenolysis is limited by glycogen depletion and may not further decrease to provide hepatic autoregulation of basal hepatic glucose production (reviewed in Ref. 11). IRS-2 may be an important component signaling insulin action in the liver. Insulin receptor deficient hepatocytes exhibit selective reduction of IRS-2, but not IRS-1, phsophorylation, impaired IRS-2 activation and impaired insulin action (12). IRS-2-deficient mice develop type 2 diabetes, together with insulin resistance, defective insulin signaling in liver, but not muscle (13) and decreased suppression of endogenous glucose production (14).

In healthy, nondiabetic individuals, a regulated negative feedback loop allows compensation for physiological changes in insulin sensitivity by inverse changes in pancreatic β-cell insulin secretion in relation to the degree of glucose tolerance (15, 16). However, β-cell compensation for insulin resistance may also be geared to a requirement to normalize the blood or tissue lipid profile, and a glucocentric emphasis may not be entirely justified. Indeed, liver steatosis is closely associated with the insulin resistance syndrome and obesity (17) and is characteristic of patients with type 2 diabetes (18). Irrespective of the stimulus to secretion, β-cell compensation

Abbreviations: ACL, ATP-citrate lyase; AIR, acute insulin response; CoA, coenzyme A; DI, disposition index; EGP, endogenous glucose production; ΔG, the incremental blood glucose values integrated over the 30-min period after the injection of glucose; GSIS, glucose-stimulated insulin secretion; HIFAT, high-saturated fat diet; ω3-HIFAT, long-chain ω-3 fatty acid enriched high-saturated fat diet; ΔI, the incremental plasma insulin values integrated over the 30-min period after the injection of glucose; IR, insulin resistance; IRS, insulin receptor substrate; ISI_{clamp}, insulin sensitivity index at euglycaemia; k, rate of glucose disappearance; LOFAT, control rats; NEFA, nonesterified fatty acids; PPAR, peroxisome proliferator-activated receptor; *R*d, glucose disposal rates.

may be inadequate during the development and progression of type 2 diabetes, such that it is insufficient to maintain blood glucose and blood and tissue lipid levels within the normal physiological range. Because IRS-2 null mice fail to increase β-cell mass in response to the development of insulin resistance, IRS-2 may play an important role in the control of β-cell mass during β-cell compensation (13).

In a rodent model (high-saturated fat-fed Wistar rats), peripheral insulin resistance evoked by increased dietary provision of saturated fat for a 4-wk period is accompanied by increased insulin secretion, and glucose tolerance is maintained (3, 19, 20). Hence, as suggested from the finding that very long-term (10 month) high-saturated fat feeding of Wistar rats leads to severe insulin resistance but not diabetes (21), this model effectively reproduces the compensatory response to dietary-induced insulin resistance seen in healthy rather than diabetes-prone individuals. Enhanced insulin secretion in healthy high-saturated fat-fed Wistar rats is consistent with the concept that insulin resistance begets hyperinsulinemia, with undefined cross-talk between the periphery and the islet that signals insulin insensitivity. A potential involvement of adipokines in mediating intertissue communication has been suggested by correlations between hepatic steatosis and plasma leptin (positive) and adiponectin (negative) levels in high-saturated fat diet (HIFAT) rats treated with peroxisome proliferator-activated receptor (PPAR) agonists (22). An alternative possibility is that insulin resistance and hyperinsulinemia during high-saturated fat feeding arise simultaneously as a result of an, as yet undefined, primary event. In support of the latter, the effect of high-saturated fat feeding to enhance glucose-stimulated insulin secretion (GSIS) *in vivo* is retained in perifused islets (20). This result appears to eliminate acute influences of circulating factors, including systemic lipid and adipokine delivery to the islet, and implicates a stable change in islet function.

Epidemiological evidence suggests that insulin resistance in association with hyperinsulinemia is linked to the ingestion of saturated, rather than unsaturated, fat (23–25). However, gross modification of the type of fatty acids included in the diet (saturated *vs.* unsaturated) is unnecessary to prevent the adverse effect of dietary saturated fat on insulin action. Thus, the substitution of long-chain ω-3 fatty acids from fish oil for only a small percentage (6–7%) of saturated fat in the diet prevents the development of insulin resistance in response to high-saturated fat feeding (26, 27). The mechanism underlying this prevention remains conjectural, but may include changes in fatty acid composition in membrane phospholipids that influence membrane stability and fluidity or insulin signaling (28–31). The impact of such a subtle change in dietary fatty acid compensation on insulin secretion in relation to changes in insulin sensitivity and glucose tolerance has not been evaluated.

In the present study, we investigated the relationship between amelioration of insulin resistance and changes in insulin secretion during 4 wk of high-saturated fat feeding through substitution of a small amount (7%) of dietary lipid with long-chain ω-3 fatty acids from fish oil. Studies of insulin action were conducted in intact conscious rats using the euglycemic-hyperinsulinemic clamp technique. Changes in insulin action were examined in relation to systemic and hepatic triglyceride levels, hepatic glycogen levels and plasma leptin concentrations. GSIS was studied *in vivo* after iv glucose challenge and perifusions of isolated islets were used to identify persistent effects of modulation of dietary lipid composition on the islet itself.

Materials and Methods

Materials

General laboratory reagents were purchased from Roche Diagnostics (Lewes, East Sussex, UK) or from Sigma (Poole, Dorset, UK). Kits for determination of insulin and glucose concentrations were purchased from Mercodia (Uppsala, Sweden) and Roche Diagnostics, respectively. Wako kits for measurement of plasma nonesterified fatty acids (NEFA) and triglyceride concentrations were purchased from α Labs (Eastleigh, Hampshire, UK). Plasma leptin was measured by RIA using kits from Linco Research (St. Louis, MO).

Animals

All studies were conducted in adherence to the regulations of the United Kingdom Animal Scientific Procedures Act (1986). Female albino Wistar rats (200–250 g) were purchased from Charles River (Margate, Kent, UK). Rats were maintained at a temperature of 22 ± 2 C and subjected to a 12-h light, 12-h dark cycle. We did not assess the stage of the estrous cycle that the rats were in at the time of experimentation. Control rats (LOFAT) were given free access to standard, low-fat/high-carbohydrate rodent diet purchased from Special Diet Services (Witham, Essex, UK) (52% carbohydrate, 15% protein, 3% lipid, and 30% nondigestible residue, by weight). HIFAT rats were given free access to a semisynthetic diet high in saturated fat (see Refs. 3 and 32), henceforth referred to as HIFAT. The HIFAT contained 34% carbohydrate, 19% protein, and 22% lipid (lard as the major source of lipid, together with corn oil [1.9 g/100 g diet] to prevent essential-fatty acid deficiency) by weight (3). The lipid component of the high-saturated fat diet comprised 16% saturated fatty acids (mainly stearic), 16% monounsaturated fatty acids (mainly oleic), and 7% polyunsaturated fatty acids (mainly linoleic) by energy. The second experimental high-fat diet (long-chain ω-3 fatty acid enriched high-saturated fat diet, ω3-HIFAT) was also lard/corn oil based, but approximately 7% of the dietary saturated fatty acids were replaced with long-chain ω-3 fatty acids from marine oil (Marine TG 18/30, kindly provided by Dr. D. Horrobin, Scotia Pharmaceuticals, Guildford, Surrey, UK). By gas liquid chromatography analysis, 49% of the long-chain ω-3 fatty acid was eicosapentaenoic acid (20:5) and 33% was docosahexaenoic acid (22:6). The two high-fat diets were prepared at 3-d intervals using components supplied by Special Diet Services, with the exception of the saturated fat component (lard), which was purchased locally. Rats were maintained on the high-fat diets for 4 wk. In all experiments, rats were allowed access *ad libitum* to water.

Euglycemic-hyperinsulinemic clamps

For euglycemic-hyperinsulinemic clamp studies, each rat was fitted with two chronic indwelling cannulas under Hypnorm [fentanyl citrate (0.315 mg/ml)/fluanisone (10 mg/ml); 1 ml/kg body weight, ip injection] and Diazepam (5 mg/ml; 1 ml/kg body weight, ip injection). One cannula was placed in the right jugular vein, and the other cannula was placed in the left jugular vein (for infusion and sampling, respectively). Normal food intake was resumed within 2–3 d and studies were conducted at 5–7 d after surgery. On the day of the experiment, food was withdrawn at the end of the dark (feeding) phase and euglycemic-hyperinsulinemic clamps were performed in conscious, unstressed, freely moving rats in the postabsorptive state at 1400 h (*i.e.* at 6 h after food withdrawal). Further details of the procedures are given in Refs. 33 and 34. In brief, after a 90-min equilibration period, a primed continuous iv infusion of insulin was given at a fixed rate (either 2.1 or 4.2 mU/kg body weight·min) for 2 h. The higher insulin dose was selected on the basis of previous studies demonstrating that in rats maintained on standard (high carbohydrate/low fat) diet, it produces steady-state plasma insulin concentrations in the physiological range observed after

ingesting a carbohydrate-rich meal (35). A variable rate of glucose infusion was initiated at 1 min after the start of insulin infusion. Blood (0.1 ml) was sampled from the right jugular vein at 5- to 10-min intervals to monitor the establishment of steady-state conditions. Blood withdrawn during the euglycemic clamps was replaced with an equal volume of saline. Steady state was reached after 60–90 min. Coefficients of variance of blood glucose concentrations during hyperinsulinemic clamp were less than 12% in all studies. Whole-body body glucose kinetics were estimated in awake, unstressed, freely moving rats in the basal (postabsorptive) state and during euglycemic-hyperinsulinemia by use of primed (0.5 μCi) continuous (0.2 μCi/min per rat) iv infusion of [3-^{3}H] glucose. Blood samples (0.15 ml) were obtained at 60, 75, and 90 min after the initiation of [3-^{3}H] glucose infusion in the basal state and at 90, 105, and 120 min after the initiation of infusion of [3-^{3}H] glucose and insulin in the hyperinsulinemic state. Endogenous glucose production (EGP) and whole-body glucose disposal rates (*R*d) were calculated as described in Refs. 33 and 34.

Intravenous glucose challenge

Glucose was administered as an iv bolus (0.5 g glucose/kg body weight; 150 μl/100 g body weight) to conscious, unrestrained rats (see Ref. 3). Glucose was injected via a chronic indwelling jugular cannula and blood samples (100 μl) were withdrawn at intervals from the indwelling cannula, which was flushed with saline after the injection of glucose to remove residual glucose. Samples of whole blood (50 μl) were deproteinized with $ZnSO_4$/$Ba(OH)_2$, centrifuged (10,000 $\times$ *g*) at 4 C, and the supernatant retained for subsequent assay of blood glucose. The remaining blood sample was immediately centrifuged (10,000 $\times$ *g*) at 4 C, and plasma was stored at −20 C until assayed for insulin. The calculated acute insulin response (AIR) was calculated as the mean of suprabasal 2- and 5-min plasma insulin values. Insulin and glucose responses during the glucose tolerance test were used for calculation of the incremental plasma insulin values integrated over the 30-min period after the injection of glucose (ΔI) and the corresponding incremental integrated plasma glucose values (ΔG). The insulin resistance (IR) index was calculated as the product of the areas under the glucose and insulin curves after glucose challenge. The rate of glucose disappearance (k) was calculated from the slope of the regression line obtained with log-transformed glucose values from 2–15 min after glucose administration.

Islet isolation and perifusion

Rats were anesthetized by injection of sodium pentobarbital (60 mg/ml in 0.9% NaCl; 1 ml/kg body weight ip) and, once locomotor activity had ceased, pancreases excised and islets were isolated by collagenase digestion (36). Free islets were collected under a dissecting microscope with a 20 μl pipette into HEPES-buffered Hanks' balanced salts solution containing 5% BSA. Insulin release from freshly isolated islets was measured in a perifusion system as described by Hughes *et al.* in 1992 (37). In this system, 50 islets were housed in small chambers on Millicell culture inserts. Islets were perifused in basal medium [Krebs-Ringer containing 20 mM HEPES (pH 7.4), 5 mg/ml BSA, and 2 mM glucose] for 60 min at a flow rate of 1 ml/min at 37 C before collection of fractions. Glucose concentrations were then modified as indicated. Fractions (2 ml) were collected at 2-min intervals and stored at −20 C before assay for insulin.

Analytical methods

Plasma glucose concentrations were determined by a glucose oxidase method (Roche Diagnostics). Plasma immunoreactive insulin concentrations were measured by ELISA, using rat insulin as a standard (Mercodia). Plasma leptin concentrations were determined by RIA using a commercial kit, using rat leptin standards (rat leptin RIA kit, Linco Research). Plasma NEFA and triglyceride concentrations were determined spectrophotometrically using commercial kits. Hepatic glycogen concentrations were measured in freeze-clamped liver samples as described in Ref. 38. Hepatic triglyceride concentrations were estimated using hydrolysis of triglyceride with subsequent enzymatic assay of glycerol using a commercial kit, as described in Ref. 39.

Statistical analysis

Results are presented as the mean ± SE (SEM), with the numbers of rats or islet preparations in *parentheses*. Statistical analysis was performed by ANOVA followed by Fisher's *post hoc* tests for individual comparisons or Student's *t* test as appropriate (Statview, Abacus Concepts, Inc., Berkeley, CA). A *P* value of less than 0.05 was considered to be statistically significant.

Results

Phenotypic comparison of LOFAT, HIFAT, and ω3-HIFAT groups

Although caloric intake was 41% and 47% higher in the HIFAT and ω3-HIFAT groups, respectively, compared with the LOFAT group, the increased caloric intake was not associated with increased body weight gain during the 4-wk period of high-fat feeding (Table 1). There were no obvious differences in physical activity or adiposity between the dietary groups (results not shown). However, postabsorptive insulin levels were significantly higher (by 56%; $P < 0.05$) in the HIFAT compared with the LOFAT group, an effect that was reversed in the ω3-HIFAT group (Table 1). The postabsorptive glucose level was not significantly different between the HIFAT group and LOFAT groups but was significantly higher in the ω3-HIFAT group compared with the HIFAT group. Hence, in the postabsorptive state, the basal insulin:glucose ratio, an index of the insulin response to basal glycemia, was slightly, but not statistically significantly, elevated in the HIFAT group compared with the LOFAT group, but this effect was completely reversed in the ω3-HIFAT group by a combination of lowered insulin and elevated glucose, such that the basal insulin:glucose ratio was signif-

TABLE 1. Phenotypic comparison of LOFAT, HIFAT, and ω3-HIFAT groups

	LOFAT	HIFAT	ω3-HIFAT
Food intake (kcal/d)	55.4 ± 8.6 (9)	78.0 ± 7.7 (9)	81.3 ± 4.3^a (9)
Initial body weight (g)	237 ± 3 (9)	238 ± 6 (9)	228 ± 3 (9)
Final body weight (g)	264 ± 5 (9)	274 ± 6 (9)	272 ± 11 (9)
Plasma insulin (μU/ml)	16 ± 2 (8)	25 ± 4^a (9)	14 ± 2^b (7)
Blood glucose (mM)	4.1 ± 0.3 (8)	4.0 ± 0.1 (9)	4.3 ± 0.1^b (7)
Insulin:glucose ratio (μU/mmol)	4.4 ± 0.8 (8)	6.0 ± 0.9 (9)	3.2 ± 0.5^b (7)
Plasma triglyceride (mM)	0.42 ± 0.08 (7)	0.79 ± 0.16^a (8)	0.34 ± 0.07^b (7)
Plasma NEFA (mM)	0.41 ± 0.05 (7)	0.45 ± 0.07 (7)	0.44 ± 0.04 (6)
Plasma leptin (ng/ml)	5.9 ± 0.7 (8)	4.6 ± 1.4 (8)	5.7 ± 1.5 (7)
Hepatic triglyceride (μmol/g wet wt)	14.1 ± 1.7 (11)	38.4 ± 2.7^c (12)	30.6 ± 1.7^{b,c} (10)

Plasma insulin, leptin, NEFA, triglyceride, and blood glucose concentrations were determined using commercial kits. Data are means ± SEM, with the numbers of rats in *parentheses*. Statistically significant differences from LOFAT rats are indicated by: ^{a}, $P < 0.05$; ^{c}, $P < 0.01$. Statistically significant differences between ω-3 HIFAT *vs.* HIFAT groups are indicated by: ^{b} $P < 0.05$.

icantly lower in the ω3-HIFAT group compared with the HIFAT group (by 47%; $P < 0.05$).

Hepatic steatosis in relation to systemic lipid and leptin concentrations

Fasting plasma triglyceride levels were higher (by 88%; $P < 0.05$) in the HIFAT group compared with the LOFAT group (Table 1), but this effect was abolished by provision of long-chain ω-3-enriched HIFAT diet. Plasma NEFA concentrations were unaffected by feeding either the HIFAT or ω3-HIFAT diets (Table 1). HIFAT feeding led to a 2.7-fold increase in liver triglyceride content compared with the LOFAT group; this increase was moderately attenuated (by 20%; $P < 0.05$) by long-chain ω-3 enrichment of the HIFAT diet. Although liver triglyceride content was marginally lower in the ω3-HIFAT group compared with the HIFAT group, the liver triglyceride concentration in the ω3-HIFAT group was still 2.2-fold higher than that of the LOFAT group. Plasma leptin levels in the fed state were unaffected by high-saturated fat feeding (LOFAT, 4.7 ± 1.2 ng/ml [n = 9]; HIFAT, 4.0 ± 0.8 ng/ml [n = 6]), but were significantly increased [(4-fold; $P < 0.001$) by ω3-HIFAT, 15.8 ± 2.0 ng/ml (n = 6)]. In contrast, plasma leptin levels in the ω3-HIFAT group did not differ from those of the LOFAT or HIFAT groups in the postabsorptive state (Table 1). Hence, there was no clear association of plasma leptin levels with hepatic triglyceride concentrations in these study groups.

Hepatic glucoregulation in response to increased dietary lipid

Analysis of hepatic glycogen concentrations in LOFAT, HIFAT, and ω3-HIFAT groups with free access to diet revealed significantly lowered hepatic glycogen storage in the HIFAT group (by 30%; $P < 0.001$) (Fig. 1). Hepatic glycogen concentrations were further significantly lowered by enrichment of the HIFAT diet with long-chain ω-3 fatty acids (by 26%; $P < 0.05$) such that glycogen levels were 48% lower than corresponding values in LOFAT rats ($P < 0.001$) (Fig. 1). Nevertheless, hepatic glycogen concentrations in all three groups were appropriate for the prevailing plasma insulin concentrations in the absorptive state (Fig. 1).

Using [3-^{3}H] glucose, we measured EGP, which reflects glucose production by the liver and kidney, in the postabsorptive state in the three dietary groups. EGP was lower in the HIFAT group compared with the LOFAT group (Fig. 2), suggesting that the relative hyperinsulinemia observed in the HIFAT group in the postabsorptive state (Table 1) is able to restrain EGP. In marked contrast, EGP was markedly increased by supplementation of the HIFAT with long-chain ω-3 fatty acids (Fig. 2), which may contribute to the postabsorptive hyperglycemia observed in this group (Table 1). We went on to analyze to what extent modulation of dietary lipid content influenced the effect of insulin to suppress EGP. A fixed rate of insulin infusion raised steady-state plasma insulin concentrations to 117 ± 17 μU/ml (n = 6) in the CON rats and to 105 ± 7 μU/ml (n = 6) in the HIFAT rats. During hyperinsulinemia, glucose was infused to maintain blood glucose concentrations and steady-state glucose concentrations were 4.2 ± 0.2 mM (n = 6) and 4.0 ± 0.2 mM (n = 6), respectively, in the LOFAT and HIFAT groups. Under these

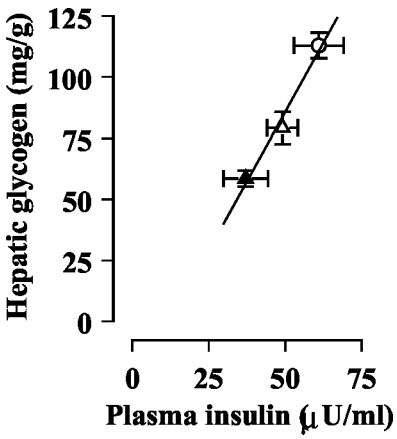

FIG. 1. Correlation between plasma insulin and hepatic glycogen concentrations in the fed state in control rats fed LOFAT (*open circle*), HIFAT (*open triangle*) or ω3-HIFAT (*closed triangle*) diets. Plasma immunoreactive insulin concentrations were measured by ELISA, using rat insulin as a standard. Hepatic glycogen concentrations were measured in freeze-clamped liver samples. Results are means ± SEM for 12 LOFAT, nine HIFAT, and six ω3-HIFAT rats. The correlation yielded an r value of 0.99.

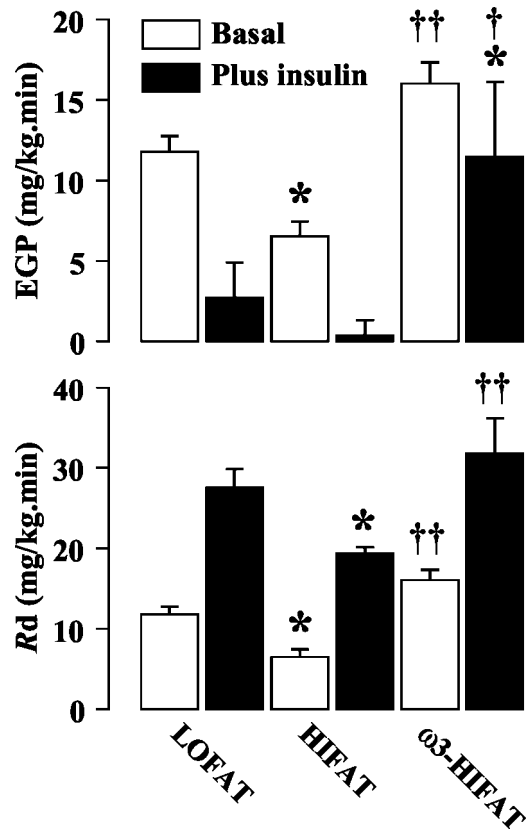

FIG. 2. Rates of EGP (*top panel*) and *R*d (*bottom panel*) in the basal state and after 2 h euglycemic-hyperinsulinemia in LOFAT, HIFAT, and ω3-HIFAT rats. Further details are provided in the legend to Fig. 1. Results are presented as means ± SEM for six LOFAT, six HIFAT, and five ω3-HIFAT rats. Statistically significant differences from LOFAT rats are indicated by: *, $P < 0.05$. Statistically significant effects of long-chain ω-3 fatty acid enrichment of the HIFAT are indicated by: †, $P < 0.05$; ††, $P < 0.01$.

conditions, EGP was markedly suppressed in both the LOFAT (by 75%; $P < 0.01$) and HIFAT (by 94%; $P < 0.001$) groups (Fig. 2). Because steady-state insulin concentrations did not differ significantly between the HIFAT and LOFAT groups and the rate of insulin infusion was identical, insulin clearance rates were not significantly different between the two groups [LOFAT, 11.1 ± 1.7 ml/min (n = 6); HIFAT, 11.0 ± 0.5 ml/min (n = 6)]. The same fixed rate of insulin infusion raised steady-state plasma insulin concentrations to only 58 ± 4 μU/ml (n = 5) in the ω3-HIFAT group, indicating a significantly augmented rate of insulin clearance in the ω3-HIFAT group [18.1 ± 1.9 ml/ml (n = 5)] relative to either LOFAT or HIFAT groups ($P < 0.01$ for both comparisons). During hyperinsulinemia, steady-state glucose concentrations in the ω3-HIFAT group were maintained at approximately the level found in the basal state in this group (4.0 ± 0.4 mM, n = 5). Under these conditions, EGP was resistant to suppression by insulin in the ω3-HIFAT group and, although a trend toward a lower EGP was evident during insulin stimulation (a 28% decline in EGP, which did not achieve statistical significance), EGP remained significantly higher than rates observed in the LOFAT and HIFAT groups ($P < 0.05$ for both comparisons) (Fig. 2). Hence, at a steady-state insulin concentration of approximately 58 μU/ml, which is substantially higher than that found in the postabsorptive state, there is a marked failure in insulin's ability to suppress EGP in the long-chain ω-3 fatty acid-supplemented high-fat-fed group. To determine whether the impairment in insulin's ability to suppress EGP in the ω3-HIFAT group reflected the lower steady-state plasma insulin level achieved during insulin infusion, we undertook further studies in the HIFAT group using a lower rate of insulin infusion to achieve steady-state plasma insulin concentrations comparable to those observed in the ω3-HIFAT group. Infusion of insulin at a rate of 2.1 mU/kg body weight per min for 2 h resulted in steady-state insulin concentrations of 35 ± 2 μU/ml (n = 4) in the HIFAT rats, together with complete suppression of EGP [−0.5 ± 1.2 mg/kg·min (n = 4); $P < 0.01$]. Thus, the impaired action of insulin to suppress EGP in the ω3-HIFAT group does not reflect the lower circulating insulin concentrations attained during the insulin infusion studies.

Glucose disposal during hyperinsulinemic-euglycemic clamp and after iv glucose challenge

We analyzed insulin-stimulated glucose disposal *in vivo* in LOFAT, HIFAT, and ω3-HIFAT groups at steady-state euglycemia. Insulin infusion in the LOFAT group resulted in a 2.3-fold increase in *R*d (Fig. 2). Despite complete suppression of EGP, high-saturated fat (HIFAT) feeding significantly impaired insulin-mediated *R*d (by 30%; $P < 0.05$), such that the increment in *R*d elicited by insulin was 18% lower in the HIFAT group compared with the LOFAT group (Fig. 2). Previous studies have shown that peripheral (but not hepatic) insulin resistance is ameliorated on changing the composition of a high-fat diet from one containing predominantly linoleate (18:2) + oleate (18:1) (linoleate > oleate) to one containing predominantly stearate (18:0) + oleate (oleate > stearate) (40). Holding both the period of high-saturated fat feeding (4 wk) and the total lipid content constant, the replacement of 7% of the dietary saturated fat with long-chain ω-3 fatty acids markedly and significantly increased (by 63%; $P < 0.01$) insulin-stimulated *R*d compared with the HIFAT group (Fig. 2). The magnitude of the increase in peripheral insulin sensitivity evoked by the inclusion of long-chain ω-3 fatty acids in the high-saturated fat diet was such that *R*d values of ω3-HIFAT group did not differ significantly from those of the LOFAT group (Fig. 2). Indeed, the increment in *R*d elicited by insulin in the ω3-HIFAT group was 22% higher than that observed in the HIFAT group, and was identical to that observed in the LOFAT group (Fig. 2). As a consequence, even though steady-state insulin values at euglycemia were considerably lower, *R*d values of the ω3-HIFAT group tended to exceed those of the LOFAT group (Fig. 2). Our data indicate that long-chain ω-3 fatty acid supplementation of a HIFAT prevents the adverse effect of high-saturated fat on whole-body insulin action through improved peripheral glucose disposal; however, this insulin-sensitizing action is partially offset by impaired suppression of EGP.

To evaluate possible relationships between altered hepatic and peripheral insulin sensitivity and glucose tolerance, we proceeded to analyze whole-body glucose clearance rates *in vivo* following an iv glucose challenge, which elevates insulin levels through endogenous insulin secretion. Administration of iv glucose (500 mg/kg) elevated blood glucose levels to approximately 10 mM in all three groups. Overall glucose tolerance, as indicated by a nonsignificant 30% increase in ΔG, was slightly impaired by high-saturated fat feeding (HIFAT group) (Fig. 3). Nevertheless, k values for rates of glucose disappearance over the first 15 min after glucose challenge did not differ significantly between the LOFAT and HIFAT groups (Fig. 3). In contrast, overall glucose tolerance was significantly impaired by enrichment of the high-saturated fat diet with dietary long-chain ω-3 fatty acids: ΔG was significantly increased, whereas k was significantly lowered (Fig. 3). These data imply that, despite enhanced peripheral insulin sensitivity, insulin secretion is inadequate to maintain glucose tolerance in the ω3-HIFAT group.

Effects of long-chain ω-3 fatty acid supplementation on glucose-stimulated insulin secretion in vivo in relation to overall glucose tolerance

AIR and ΔI values after iv administration of glucose (500 mg/kg) in LOFAT, HIFAT, and ω3-HIFAT rats are shown in Fig. 4. We confirmed previous findings of an effect of high-saturated fat feeding for 4 wk to elicit enhanced insulin secretion after iv glucose challenge (3, 19). HIFAT rats exhibited a 1.8-fold increase in AIR ($P < 0.01$), together with a 1.8-fold increase in ΔI (the total suprabasal 30-min area under insulin curve) ($P < 0.05$). The IR index, the product of the areas under the glucose and insulin curves after glucose challenge, significantly increased (by 2.2-fold; $P < 0.01$) after high-saturated fat feeding (Fig. 4), confirming the development of insulin resistance shown using the euglycemic-hyperinsulinemic clamp (see Fig. 2). Because glucose tolerance is little affected by HIFAT feeding, it can be concluded that there is adequate β-cell compensation for the impairment in glucose disposal introduced by high-fat feeding. To

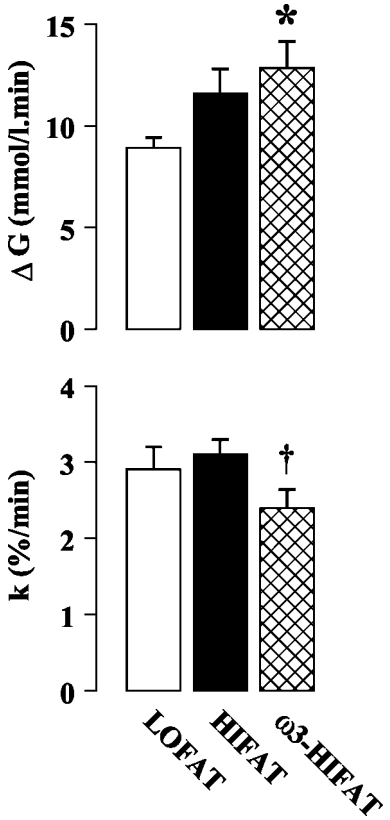

FIG. 3. Long-chain ω-3 fatty acid enrichment of the HIFAT impairs glucose clearance after iv glucose challenge in high-fat-fed rats. Glucose was administered as an iv bolus (0.5-g glucose/kg body weight) to LOFAT, HIFAT, or ω3-HIFAT rats. Studies were undertaken in conscious, unrestrained rats in the postabsorptive state. Blood samples were withdrawn at intervals for measurement of blood glucose using a commercial kit. Glucose responses during the glucose tolerance test were used for calculation of the ΔG and are shown in the *top panel*. Rates of glucose disappearance (k), calculated from the slopes of the regression lines obtained with log-transformed glucose values from 2–15 min after glucose administration and expressed as percentage per minute, are shown in the *bottom panel*. Further details are provided in the legend to Fig. 1. Results are means ± SEM for eight LOFAT rats, nine HIFAT rats, or seven ω3-HIFAT rats. Statistically significant differences from LOFAT rats are indicated by: *, $P < 0.05$. Statistically significant effects of long-chain ω-3 fatty acid enrichment of the HIFAT are indicated by: †, $P < 0.05$.

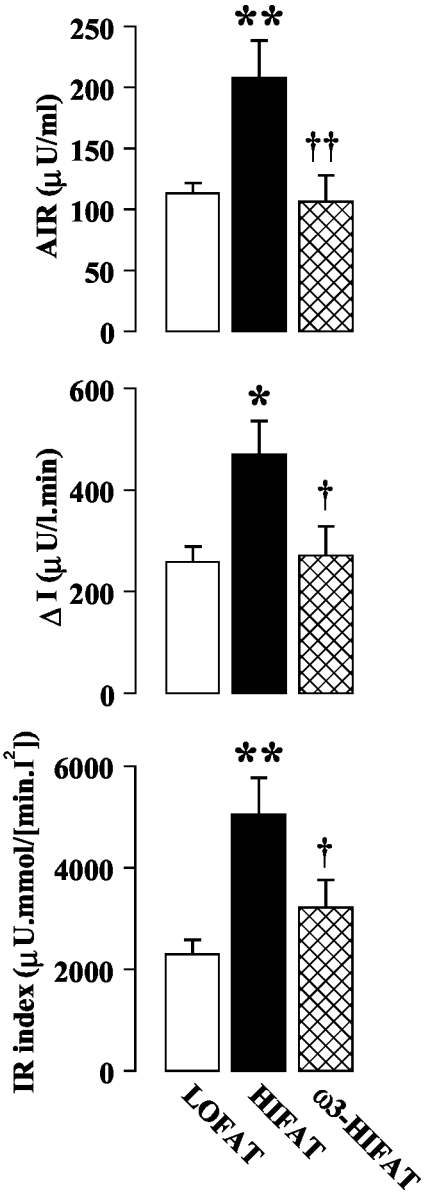

FIG. 4. Long-chain ω-3 fatty acid enrichment of the HIFAT impairs glucose-stimulated insulin secretion after iv glucose challenge in high-fat-fed rats. Blood samples were withdrawn from LOFAT, HIFAT, and ω3-HIFAT rats at intervals after an iv glucose bolus for measurement of plasma insulin and blood glucose using commercial kits. AIRs, calculated as the mean of suprabasal 2- and 5-min plasma insulin values, are shown in the *top panel*. Insulin responses during the glucose tolerance test were used for calculation of the ΔI and are shown in the *middle panel*. The IR index, calculated as the product of the areas under the glucose and insulin curves after glucose challenge in the postabsorptive state are shown in the *bottom panel*. Results are means ± SEM for eight LOFAT rats, nine HIFAT rats, or seven ω3-HIFAT rats. Statistically significant differences from LOFAT rats are indicated by: * $P < 0.05$; ** $P < 0.01$. Statistically significant effects of long-chain ω-3 fatty acid enrichment of the high-saturated fat diet are indicated by: †, $P < 0.05$; ††, $P < 0.01$.

further explore the relationship between insulin secretion and action in the LOFAT and HIFAT groups, we calculated the disposition index (DI), the product of insulin secretion measured during iv glucose tolerance tests (AIR) and insulin sensitivity index measured at euglucaemia during the euglycemic-hyperinsulinemic clamps (ISI_{clamp}). There are many different ways to calculate SI_{clamp} (see *e.g.* Ref. 41); however, we chose to define SI_{clamp} as GIR (the steady-state glucose infusion rate required to maintain euglycemia during insulin infusion) divided by the steady-state insulin concentration during insulin infusion. This allowed an evaluation of insulin's ability to stimulate glucose disposal and suppress endogenous glucose production at eugluycaemia. Calculated DI values at steady-state insulin concentrations in the high-physiological range (~100 μU/ml) are therefore 23.8 mg·ml/μU·min·kg and 35.7 mg·ml/μU·min·kg for LOFAT and HIFAT rats, respectively. These data demonstrate that the enhanced ability of the β-cells of HIFAT rats to secrete insulin in response to glucose actually exceeds that required to compensate for impaired insulin action, as assessed by the euglycemic-hyperinsulinemic clamp. However, because k after glucose challenge is not significantly

increased in the HIFAT group, it is implied that β-cell compensation also takes into account factors in addition to insulin resistance that adversely influence glucose clearance, which may include impaired glucose effectiveness [*e.g.* due to operation of the glucose-fatty acid cycle (10)]. The relative response of insulin to a rapid increase in glycemia elicited by iv glucose challenge was greatly attenuated in the ω3-HIFAT group compared with the HIFAT group (Fig. 4). This effect was reflected by a substantial decrease in AIR (49%; $P < 0.01$) accompanied by a significant decline in ΔI (42%), such that the effects of high-saturated fat feeding to increase AIR and ΔI were both completely reversed (Fig. 4). Because of lowered glucose-stimulated insulin levels, the IR index was significantly lower (by 36%; $P < 0.05$) in the ω3-HIFAT group compared with that found after high-saturated fat feeding, even though glucose tolerance was adversely affected, implying that the IR index parallels insulin's action on peripheral glucose disposal, rather than insulin action at the level of endogenous glucose production. At the insulin infusion rate of 4.2 mU/kg body weight·min, the calculated DI for the ω3-HIFAT group (37.2 mg.ml/μU·min·kg) was similar to that obtained for the HIFAT group and greater than that obtained for the LOFAT group at the higher insulin infusion rate. Comparison of DI values for HIFAT and ω3-HIFAT groups at similar steady-state insulin concentration during the euglycemic-hyperinsulinemic clamps (insulin infusion rate of 2.1 mU/kg body weight·min) revealed an effect of enrichment of a high-saturated fat diet by long-chain ω-3 fatty acids to lower DI (HIFAT, 54.4 mg.ml/μU·min·kg; ω3-HIFAT, 37.2 mg·.ml/μU·min·kg).

Long-chain ω-3 fatty acid supplementation of a HIFAT modulates insulin secretion by isolated perifused islets

Differences in GSIS *in vivo* introduced by lowering the degree of saturation of the fatty acid component of a high-fat diet are associated with impaired GSIS from the perfused pancreas *ex vivo* (40). To examine whether effects of varying dietary fatty acid composition on insulin secretion were observed *ex vivo*, we tested whether the persistent effect of high-saturated fat feeding on insulin release by perifused islets was modified by long-chain ω-3 fatty acid supplementation. These additional experiments also eliminate any acute influences that *in vivo* variables, including altered islet lipid delivery and hepatic insulin clearance, might exert on insulin responses. Overall patterns of insulin release were measured during stepwise glucose perifusion designed to generate a rise in perifusate glucose concentrations to 8 mM for 16 min. After this, the perifusate glucose concentration was increased to 16 mM for a further 16 min, then lowered to basal levels over a total 2-h perifusion period. Basal rates of insulin release by perifused islets isolated from HIFAT rats were significantly higher (by 47%; $P < 0.05$) than rates of insulin release by LOFAT perifused islets (Fig. 5). Although raising the perifusate glucose concentration to 8 mM did not significantly increase rates of insulin release by perifused islets from HIFAT rats, insulin release rates were significantly higher than corresponding rates from perifused LOFAT islets. Raising the perifusate glucose concentration to 16 mM resulted in more rapid increases in and higher rates of insulin release compared with LOFAT islets. Peak rates of insulin release by perifused islets from HIFAT rats were 58% higher ($P < 0.01$) than corresponding peak rates of insulin release by perifused LOFAT islets (Fig. 5). Once perifusate glucose

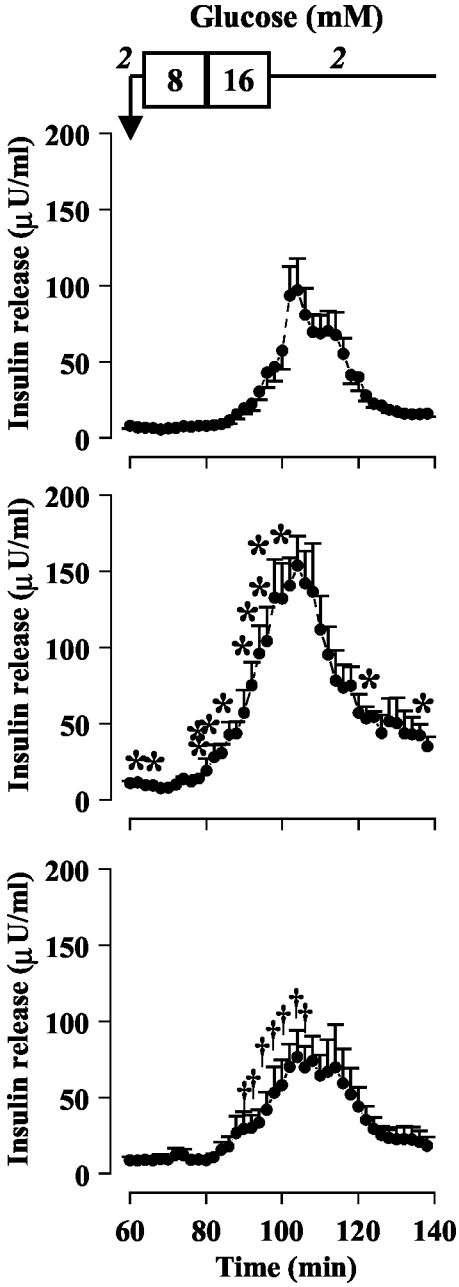

FIG. 5. Long-chain ω-3 fatty acid enrichment of the HIFAT modulates insulin secretion by isolated perifused islets. Islets isolated from LOFAT (*top panel*), HIFAT (*middle panel*), or ω3-HIFAT (*bottom panel*) rats were perifused in basal medium containing 2 mM glucose for 60 min at a flow rate of 1 ml/min at 37 C before collection of fractions. Perifusate glucose concentrations were then modified as indicated. Fractions (2 ml) were collected at 2-min intervals and stored at −20 C before assay for insulin using commercial kits. Results are means ± SEM for 10 LOFAT rats, five HIFAT rats, or five ω3-HIFAT rats. Statistically significant effects of high-saturated fat feeding are indicated by: *, $P < 0.05$. Statistically significant effects of long-chain ω-3 fatty acid enrichment of the high-saturated fat diet are indicated by †, $P < 0.05$.

concentrations returned to basal, rates of insulin release by perifused islets from HIFAT rats remained approximately 2-fold higher than corresponding LOFAT values (Fig. 5). The replacement of 7% of the dietary saturated fat with long-chain ω-3 fatty acids significantly decreased insulin release from perifused islets under conditions of glucose stimulation. When the glucose concentration was raised from 8–16 mM, rates of insulin release by ω3-HIFAT islets were significantly lower (by 55%; $P < 0.05$) than those of HIFAT islets and did not differ significantly from corresponding rates of insulin release by LOFAT islets (Fig. 5). These data suggest that long-chain ω-3 fatty acid enrichment of a HIFAT prevents the effects of high-saturated fat feeding to enhance insulin secretion by perifused islets *ex vivo*.

Discussion

We demonstrate here that, despite dietary delivery of saturated fat throughout, substitution of only 7% of total dietary lipid with long-chain ω-3 fatty acids prevents the effect of high-saturated fat feeding for 4 wk to elicit insulin resistance with respect to peripheral glucose disposal *in vivo* and insulin hypersecretion in response to glucose *in vivo* and *ex vivo*. However, these effects were associated with insulin resistance with respect to suppression of endogenous glucose production and impaired glucose tolerance *in vivo*. Reduced hyperinsulinemia despite glucose intolerance suggests two alternative scenarios. Either long-chain ω-3 fatty acids directly impair the β-cell response to saturated fat such that insulin secretion cannot be augmented (*i.e.* long-chain ω-3 fatty acids prevent a direct effect of saturated fat on the β-cell to amplify GSIS) and/or β-cell compensatory hypersecretion recognizes the sensitivity of peripheral glucose disposal, but not of endogenous glucose production, to insulin. Irrespective of the mechanism, these data imply that the insulin response to glucose is suppressed to a greater extent than whole-body insulin sensitivity is enhanced by enrichment of a HIFAT by long-chain ω-3 fatty acids.

Our results are compatible with the observation that enhanced endogenous insulin secretion after glucose challenge is adequate to compensate for the degree of insulin resistance elicited by high-saturated fat feeding and thereby to suppress EGP in the HIFAT group and, together with stimulation of *R*d, maintain glucose tolerance. The data also support the concept of a hyperbolic insulin sensitivity-secretion relationship (42). In contrast to HIFAT rats, insulin infusion was unable to suppress EGP completely in the ω3-HIFAT group, although rates of glucose disposal were comparable to those observed in the LOFAT group. The impact of substitution of 7% of total dietary lipid in the HIFAT diet with long-chain ω-3 fatty acids to impair suppression of EGP by insulin in the ω3-HIFAT group did not reflect the lower steady-state plasma insulin concentrations observed during insulin infusion because EGP was completely suppressed in HIFAT rats infused with insulin at a lower rate that resulted in steady-state insulin concentrations comparable to those in the ω3-HIFAT group. The pattern of change of plasma insulin concentrations after glucose challenge was similar in the ω3-HIFAT and LOFAT groups, suggesting that increased insulin clearance in the ω3-HIFAT group was not significant over the timescale of these experiments (results not shown). From this, it follows that, although the AIR to iv glucose in the ω3-HIFAT group would be predicted to stimulate glucose disposal at least to rates comparable to those found in the LOFAT group, there is insufficient compensatory insulin secretion to suppress EGP in the ω3-HIFAT group. It appears likely that impaired glucose tolerance in the ω3-HIFAT group compared with HIFAT and LOFAT groups arises because of an impaired response of EGP to elevated insulin, rather than impaired stimulation of peripheral glucose disposal.

Although the tracer dilution method used in the present study measures total endogenous glucose production by both liver and kidney, greater than 75% of EGP originates in the liver (43). Consequently, the impact of substitution of 7% of total dietary lipid in the HIFAT with long-chain ω-3 fatty acids to impair suppression of EGP by insulin is likely to reflect, at least in part, hepatic insulin resistance. In obese individuals with type 2 diabetes, insulin-stimulated glucose uptake is decreased by 30–40% compared with nondiabetic controls (44), possibly as a consequence of tissue lipid oversupply relative to oxidation (45). Positive correlations have been reported between increased intracellular triglyceride content and insulin resistance in both muscle and liver (46, 47). Increased delivery of fatty acids via liver-specific overexpression of lipoprotein lipase increases hepatic triglyceride concentration from approximately 20 μmol/g to approximately 30 μmol/g in transgenic mice, an effect associated with greatly impaired suppression of endogenous glucose production by insulin (47). It was suggested that a direct and causative relationship existed between the accumulation of intracellular fatty acid-derived metabolites and control of hepatic metabolism by insulin (47). However, this degree of hepatic triglyceride accumulation did not affect glucose production in the basal state (47). In the present experiments, hepatic triglyceride accumulation was such that it would be predicted that hepatic insulin resistance would be evident in both HIFAT and ω3-HIFAT groups. However, the HIFAT used in the present study elicits only a relatively moderate suppression of *R*d, with complete suppression of EGP during hyperinsulinemia at euglycemia.

Other possible mechanisms present themselves. An attractive possibility is that long-chain ω-3 fatty acids modify hepatic IRS-2 expression and/or activation by phosphorylation. Thus, as observed in the ω3-HIFAT group, mice specifically deficient in IRS-2 develop insulin resistance, defective insulin signaling in liver, but not muscle (13), and decreased suppression of endogenous glucose production (14). Insulin receptor deficient hepatocytes exhibit selective reduction of IRS-2, but not IRS-1, phosphorylation, impaired IRS-2 activation, and impaired insulin action (12).

Measurement of insulin release from pancreas of fasted rats perfused with 12.5 mM glucose and 0.5 mM of a range of individual fatty acids of varying chain length and degree of saturation revealed that the fold stimulation of insulin secretion was greater for saturated [*e.g.* palmitate (16:0) or stearate (18:0)] *vs.* unsaturated [*e.g.* linoleate (C18:2), oleate (C18:1), or palmitoleate (C16:1)] fatty acids and was greater for longer chain [*e.g.* palmitate (16:0) or stearate (18:0)] *vs.* shorter chain [*e.g.* octanoate (C8:0)] fatty acids. The lipid

component of high-fat diet used in the present study comprised mainly stearate, oleate, and linoleate. Despite the presence of unsaturated C18 fatty acids, which only modestly affect stimulation of insulin secretion in perfused pancreas, the high-fat diet markedly enhanced insulin secretion after iv glucose challenge. Conversely, supplementation of 7% of the lipid component of the high-fat diet with long-chain ω-3 fatty acids from marine oil, of which 49% was eicosapentaenoic acid (20:5) and 33% was docosahexaenoic acid (22:6), greatly attenuated the enhancement of GSIS elicited by the high-fat diet, indicating that the influence of the degree of unsaturation to lower GSIS exceeded the influence of increasing chain length to enhance GSIS. Glucose regulation of insulin release is mediated by metabolic signals that are generated secondary to increased glucose metabolism. Oxidative metabolism generates triggers for GSIS via the closure of ATP-sensitive potassium channels, plasma membrane depolarization, opening of voltage-gated calcium channels, and a resultant increase in cytosolic Ca^{2+}, which triggers exocytosis (48). A second, as yet less well-defined, pathway occurs independently of ATP-sensitive potassium channels (49, 50) (reviewed in Ref. 48). This may involve increased influx of glucose carbon into the tricarboxylic acid cycle leading to the increased production of intermediates (*e.g.* malate, citrate, glutamate) that then leave the mitochondria and, through poorly understood mechanisms, stimulate insulin release. Cataplerosis via citrate and ATP-citrate lyase, shown to be important for GSIS through the adverse effect of specific inhibition of ATP-citrate lyase (51), allows the production of cytoplasmic acetyl-CoA, which acts as a precursor for the synthesis of malonyl-CoA (and thence other acyl-CoAs). Malonyl-CoA prevents the mitochondrial oxidation of long-chain acyl-CoA, allowing the accumulation of undefined lipid signaling molecules. PPARα activation reverses the enhancement of GSIS induced by high-saturated fat feeding (20). We have therefore postulated that compensatory insulin hyperresponsiveness during high-fat feeding may reflect increased exogenous provision of a precursor of a lipid-intermediate that is normally synthesized endogenously (20). However, contrasting with the current study, treatment of high-fat-fed rats with the specific PPARα ligand WY14,643 for 24 h *in vivo* reverses insulin hypersecretion *in vivo* without impairing glucose tolerance (20), suggesting that an improved insulin action diminishes the requirement for compensatory insulin secretion.

Others have shown that peripheral insulin action is improved and hepatic steatosis is greatly lessened by treatment with PPARα and PPARγ agonists, although improved hepatic insulin action does not achieve significance unless a dual PPARα/γ agonist is used (22). Saturated fatty acids, including palmitate and stearate, do not activate PPARα at physiological concentrations (52). Although polyunsaturated fatty acids can activate PPARα in liver (*e.g.* Refs. 53 and 54) and a high-unsaturated fat (safflower oil based) diet increases PPARα expression in liver (55), long-chain ω-3 fatty acid enrichment of the high-saturated fat diet did not markedly attenuate hepatic steatosis in the present experiments. Hence, its action to impair insulin action, selective for the liver, appears to counter any potentially beneficial affect of PPARα activation on hepatic glucose homeostasis.

Our present studies show that, even under conditions of a sustained increase in saturated fatty acid delivery to the islet, the dietary provision of small quantities of long-chain ω-3 fatty acids prevents the effect of high-saturated fat feeding to augment GSIS *in vivo*. Effects of long-chain ω-3 fatty acid enrichment were also observed *in vitro* (perifused islets), suggesting that indirect actions mediated through altered insulin clearance cannot entirely explain differences in insulin levels before and after glucose challenge between the HIFAT and ω3-HIFAT groups. Prolonged increased exposure to fatty acids can precipitate β-cell failure through a lipotoxic effect on the β-cell (56, 57). However, this scenario would not be consistent with the enhanced GSIS observed with perifused islets from the HIFAT group in the present experiments. Dobbins *et al.* (40) found that a soy oil-based diet showed insulin secretion rates both *in vivo* and *in vitro* that were lower than those found in control rats maintained on low-fat/high-carbohydrate diet. These authors suggested that the polyunsaturated fatty acids found in soy oil (predominantly linoleic acid) might elicit a diminished insulin response. However, in the present study, long-chain ω-3 fatty acid enrichment of the HIFAT did not impair GSIS to such an extent that it was lower than that found in the LOFAT group. Hence, although a direct diabetogenic action of long-chain ω-3 fatty acids on the islet itself cannot be excluded, it seems more likely that long-chain ω-3 fatty acids target the same pathway through which saturated fatty acids amplify GSIS. This might be achieved, for example, via inhibition of islet endogenous lipid synthesis or by increasing clearance of a lipid molecule derived from saturated fat that amplifies GSIS.

Finally, the tissue-selective effects of long-chain ω-3 fatty acid supplementation on insulin action, with impaired suppression of EGP but augmented insulin-stimulated *R*d, imply that the β-cell response to high-saturated fat reflects the development of insulin resistance at the level of peripheral glucose disposal, but not, of endogenous glucose production. The primary site of insulin resistance in the high-saturated fat-fed rat is skeletal muscle (2). In the model used here, insulin action in adipose tissue is not impaired, as evidenced by unaltered NEFA and leptin levels (3, 58). It seems reasonable to suggest that peripheral insulin resistance and augmented insulin secretion during high-saturated fat feeding arise simultaneously through a common action on skeletal muscle and the pancreatic β-cell that is countered by small quantities of dietary long-chain ω 3-fatty acids.

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Address all correspondence and requests for reprints to: Professor M. C. Sugden, Department of Diabetes and Metabolic Medicine, Medical Sciences Building, Queen Mary, University of London, Mile End Road, London E1 4NS, United Kingdom. E-mail: m.c.sugden@qmul.ac.uk.

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