In Vivo Regulation of Plasma Free Fatty Acids in Insulin Resistance

S. Mook, C.J.M. Halkes, S. Bilecen, and M. Castro Cabezas

Elevated plasma free fatty acid (FFA) concentrations as seen in obesity, insulin resistance, and type 2 diabetes are partly caused by impaired inhibition of intracellular lipolysis in adipose tissue, and this is considered to be part of the insulin resistance syndrome (IRS). Based on predicted insulin resistance at the level of intracellular lipolysis, patients with the IRS would loose weight by disinhibited lipolysis. Since this is not the case in clinical practice, impaired stimulation of intracellular lipolysis must also play a role. We studied acute plasma FFA changes, representing stimulation and inhibition of intracellular adipose tissue lipolysis, in obese patients with IRS and in healthy controls. Thirteen insulin-resistant (IR) subjects (7 men and 6 women) and 10 controls (6 men and 4 women) underwent a mental stress test (20 minutes) preceded by 60 minutes of rest. After mental stress, an oral glucose tolerance test (OGTT) was performed. Baseline FFA levels were higher in IR patients compared to controls (0.59 ± 0.06 and 0.31 ± 0.06 mmol/L, respectively; $P = .004$). During the 20 minutes of mental stress, FFAs increased significantly in IR subjects from 0.55 ± 0.07 to 0.67 ± 0.07 mmol/L ($P < .001$) and from 0.21 ± 0.04 to 0.36 ± 0.07 mmol/L in controls ($P = .001$). Although the absolute change of plasma FFA was not different, the relative increase was lower in IR subjects (28% ± 7%) compared to controls (89 ± 24%; $P = .02$). Despite the more pronounced mean maximal insulin concentration during the OGTT in IR subjects compared to controls (600.0 ± 126.6 pmol/L and 208.1 ± 30.0 pmol/L, respectively), the relative decrease of FFAs was lower in IR subjects (11% ± 5% *v* 36% ± 11% in controls after 30 minutes; $P = .04$). In conclusion, our study shows impaired acute responses of plasma FFAs upon stimulation by mental stress and inhibition by endogenous insulin in insulin resistance in vivo. The presence of both defects helps to understand weight maintenance in insulin resistance.

FREE FATTY ACIDS (FFAs) play a central role in the metabolic disturbances seen in the insulin resistance syndrome (IRS).[1-4] In the postabsorptive state, the FFA release from adipocytes depends on the interaction between hormones and intracellular processes that have been partially elucidated.[5-10] Hormone-mediated regulation of in vivo lipolysis occurs by means of reversible phosphorylation and translocation of hormone-sensitive lipase (HSL) and perilipine.[5-7] In humans, catecholamines are the major activators of in vivo lipolysis, leading to phosphorylation of HSL through the induction of the cyclic adenosine monophosphate (cAMP)-dependent protein kinase, resulting in an elevation of plasma FFA levels.[5,10] Insulin is the most potent antilipolytic hormone by activation of a phosphodiesterase, which results in dephosphorylation and deactivation of HSL, thereby lowering plasma FFA levels.[5,6]

HSL is an intracellular enzyme and is not present in extracellular fluids; therefore, most of our knowledge on adipocyte lipolysis is based on in vitro and ex vivo studies using subcutaneous adipocytes obtained by biopsies. A novel technique using microdialysis that allows measurement of interstitial levels of glycerol in adipose tissue has increased our understanding of in vivo lipolysis.[11-14] In theory, a defective intracellular lipolytic cascade can lead to accumulation of TGs in the adipocytes and may cause obesity and therefore insulin resistance.

Further insight into in vivo regulation of FFA handling by adipocytes has been obtained from studies performing hyperinsulinemic clamps. In these experiments, intracellular lipolysis was inhibited by supraphysiological concentrations of insulin. It has been reported that the blunted FFA suppression observed in obese, insulin-resistant (IR) patients represents resistance of intracellular inhibition of lipolysis to the action of insulin in IRS.[15-18] In theory, insulin resistance of intracellular lipolysis may impair cellular FFA uptake and cause a higher FFA flow out of the adipocytes, which could explain in part the elevated FFA levels frequently seen in insulin resistance.

In a recent in vivo study we showed that stimulation of intracellular lipolysis by endogenous catecholamines leads to a plasma FFA increase within 10 minutes in healthy volunteers and that inhibition by insulin under physiological conditions can not be overruled by endogenous catecholamines.[19] In a different study using the same methodology, we showed that stimulation and inhibition of in vivo intracellular lipolysis was not disturbed in patients with familial combined hyperlipidemia using lipid-lowering drugs.[20] Using this test, the acute regulation of FFAs can be studied under physiological conditions.[20]

The purpose of the present study was to compare the acute effects of inhibition and stimulation of in vivo lipolysis under physiological conditions in obese subjects with insulin resistance and in insulin-sensitive volunteers.

MATERIALS AND METHODS

Subjects

The study protocol was approved by the Human Investigations Review Committee of the University Medical Centre Utrecht. Thirteen IR subjects were recruited from the Endocrinology Outpatient Clinic of the University Medical Centre Utrecht and by advertisement. The major inclusion criterion was the presence of the metabolic syndrome according to National Cholesterol Education Program (NCEP) guidelines.[20] Excluded were patients with liver, renal, or thyroid failure; manifest cardiac failure; or a daily alcohol intake of 3 units or more. Patients with overt diabetes on the basis of fasting glucose (≥7.0

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mmol/L) or using oral hypoglycemic drugs, were excluded. Ten healthy normolipidemic volunteers were recruited by advertisement. All were healthy volunteers, who had a negative family history for diabetes mellitus type 2 and a body mass index less then 30 kg/m^2. None of the volunteers were using drugs known to affect lipid metabolism and all had normal fasting plasma glucose concentrations. On the morning of inclusion, weight, height, blood pressure, and waist-to-hip ratio were measured, and fasting venous blood samples were taken for the determination of lipids, lipoproteins, glucose, FFA, and insulin concentrations.

Mental Stress Tests

All subjects underwent a mental stress test as described.[18,19] The participants visited the metabolic ward of our laboratory, after a 12-hour overnight fast, where an intravenous cannula was placed in a brachial vein. The cannula was kept open by a continuous 0.9% saline infusion. All peripheral blood samples were obtained from the cannula in sodium EDTA (2 mg/mL), placed on ice, and centrifuged immediately for 15 minutes at 3,000 rpm at 4°C. An inhibitor of lipoprotein lipase (tetrahydrolipstatin, Roche, Basel, Switzerland) was added to the plasma immediately after centrifugation in order to block in vitro lipolysis.[21] During the first 60 minutes of the test the subjects remained supine, in a room without disturbing stimuli. Over the next 20 minutes, the participants were subjected to 2 types of mental stress test consisting of letters and figures as described in detail elsewhere.[18,19,22] After the mental stress period, the subjects remained supine for 40 minutes. Peripheral blood samples were obtained before the mental stress period ($t = -60$ to 0 minutes) at 10-minutes intervals. During the 20 minutes of mental stress ($t = 0$ to 20 minutes), blood samples were taken at 5-minute intervals and after the stress period ($t = 20$ to 60 minutes) at 10-minute intervals.

Oral Glucose Tolerance Test

Immediately after the mental stress test, an oral glucose tolerance test (OGTT) was performed. The subjects ingested a solution of 300 mL containing 75 g glucose. Blood samples were collected at baseline and at 30 minutes and 60 minutes for determination of FFA and insulin concentrations.

Analytical Methods

Plasma samples were stored at −20°C immediately after centrifugation. FFA concentrations were measured in duplicate in plasma samples by enzymatic colorimetric method (Wako Chemicals, Neuss, Germany). Insulin concentrations were measured by commercial enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden). Plasma glucose was measured by glucose oxidase dry chemistry (Vitros GLU slides) and colorimetry. Cholesterol and TGs were determined using a Vitros 250 analyzer (Johnson & Johnson, Rochester, NY). Plasma apolipoprotein (apo) B was measured by nephelometry using apo B monoclonal antibodies (Behring Diagnostics NV, OSAN 14/15, Marburg, Germany). Intra-assay and interassay coefficients of variation for the analytical assays described above are all below 10% in our laboratory. For estimation of insulin sensitivity, the homeostasis model assessment was calculated: HOMA = glucose · insulin/22.5.[23]

Statistics

All values are expressed as the mean ± SEM. Differences between groups were tested by analysis of variance (ANOVA). Normality was tested with the Kolmogorov-Smirnov test, and if non-normality occurred, as in the case of plasma TGs, plasma insulin, and HOMA, calculations were performed after logarithmic transformation. For statistical analysis of changes in FFA concentrations, repeated-measures ANOVA was used, with the least significant difference (LSD) test used for post hoc analysis. For estimation of total amount of FFA released by mental stress, the area under the FFA curve (AUC) and the FFA AUC corrected for the FFA concentration at time 0 (dAUC) were calculated for the stress period. For statistical analysis of differences between FFA changes in IR subjects compared to controls, the nonparametric Mann-Whitney test was used.

Statistical significance was reached when $P < .05$ (2-tailed). Calculations were performed using SPSS/PC+ 10.0 (SPSS Inc, Chicago, IL).

Table 1. Anthropometric and Fasting Biochemical Characteristics of 13 Insulin-Resistant Subjects and 10 Healthy Volunteers

	IR Subjects (n = 13)	Controls (n = 10)	*P* Value
Gender	7 M/6 F	6 M/4 F	
Age (yr)	49 ± 3	34 ± 4	.004
BMI (kg/m^2)	31.0 ± 1.0	23.5 ± 0.7	<.001
WHR	0.99 ± 0.02	0.80 ± 0.01	<.001
BP diastolic (mm Hg)	89 ± 3	76 ± 3	.003
BP systolic (mm Hg)	138 ± 4	124 ± 4	.026
TG (mmol/L)	2.27 ± 0.27	1.01 ± 0.13	.001
Total cholesterol (mmol/L)	5.8 ± 0.3	4.9 ± 0.3	NS
Apo B (g/L)	1.10 ± 0.07	0.97 ± 0.05	NS
FFA (mmol/L)	0.59 ± 0.06	0.31 ± 0.06	.003
Insulin (pmol/L)	99.6 ± 16.2	33.6 ± 3.6	<.001
Glucose (mmol/L)	5.8 ± 0.2	4.8 ± 0.1	<.001
HOMA	4.2 ± 0.6	1.2 ± 0.1	<.001

NOTE. Data are mean ± SEM.

Abbreviations: BMI, body mass index; WHR, waist-to-hip ratio; BP, blood pressure; TG, triglyceride; Apo, apolipoprotein; HOMA, homeostasis model assessment.

RESULTS

General Characteristics

Thirteen patients with insulin resistance and 10 healthy controls participated in this study. General characteristics of all participants are given in Table 1. The IR group consisted of 7 men and 6 women. The control group (6 men and 4 women) consisted of healthy subjects with normal insulin sensitivity and normal fasting plasma lipids. IR patients were older and had an increased body mass index, waist-to-hip ratio, and blood pressure compared to controls. Fasting plasma TG, insulin, and glucose concentrations were higher in IR patients. Plasma cholesterol and apo B concentrations were not different. Among the IR subjects, 3 were using lipid-lowering drugs (1 fibrate, 1 statin, and 1 combination of fibrate and statin) and 5 were treated with antihypertensive drugs (3 with angiotensin-converting enzyme [ACE] inhibitors and 2 with a combination of ACE inhibitors and diuretics).

FFA Concentrations

During the first 60 minutes, the FFA concentrations decreased significantly in controls (from 0.31 ± 0.06 mmol/L at $t = -60$ minutes to a minimum of 0.21 ± 0.04 mmol/L at $t = -20$ minutes; $P = .04$) but did not change significantly in IR patients (from 0.59 ± 0.06 mmol/L at $t = -60$ minutes to 0.50 ± 0.04 mmol/L at $t = -20$ minutes; $P = .09$). At the beginning of stress, at $t = 0$ minutes, FFA concentrations were significantly higher in IR subjects compared to controls (0.55 ± 0.07 and 0.21 ± 0.04 mmol/L, respectively; $P = .004$) and remained elevated during the whole mental stress test (Fig 1).

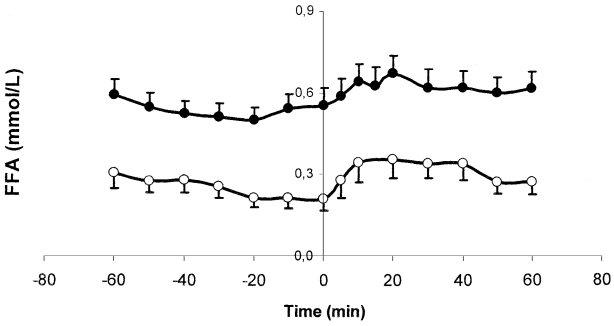

Fig 1. Mean changes in FFA concentrations during the mental stress test (mental stress period from t = 0 to t = 20) in 13 IR subjects (●) and 10 healthy controls (○). Data are means ± SEM.

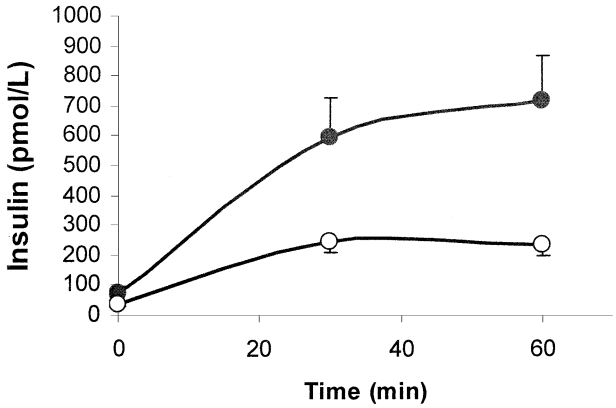

Fig 3. Mean absolute change in insulin concentration during OGTT in 13 IR subjects (●) and 10 healthy controls (○). Data are means ± SEM.

Mental Stress-Induced Activation of Lipolysis

During mental stress, FFA levels increased in IR subjects (to 0.67 ± 0.07 mmol/L; $P = .005$) and in controls (to 0.36 ± 0.07 mmol/L; $P = .009$). The absolute FFA increase was 0.12 ± 0.03 mmol/L in IR subjects and 0.15 ± 0.04 mmol/L in controls (not significant) (Fig 1). The maximum relative increase of FFA was significantly lower in IR subjects (28% ± 7%) compared to controls (89% ± 24%; $P = .02$) (Fig 2). The FFA AUC from t = 0 to t = 20 minutes in IR subjects (12.4 ± 1.3 mmol · h/L) was significantly higher compared to controls (5.9 ± 1.4 mmol · h/L; $P = .004$). The FFA dAUC was not significantly different in IR subjects and controls (1.31 ± 0.36 mmol · h/L and 1.89 ± 0.60 mmol · h/L, respectively). After the mental stress period, FFA concentrations returned towards basal FFA concentrations in both groups.

Insulin Concentrations During OGTT

Insulin concentrations increased after ingestion of glucose, in IR subjects from 73.9 ± 21.0 pmol/L to a maximum of 717.3 ± 151.5 pmol/L ($P = .001$) after 1 hour and in controls from 34.5 ± 5.3 pmol/L to 248.8 ± 36.5 pmol/L ($P < .001$), 30 minutes after glucose ingestion (Fig 3).

Insulin-Mediated Inhibition of Lipolysis During OGTT

FFA concentrations decreased significantly both in IR subjects (from 0.58 ± 0.06 mmol/L at t = 0 minutes to 0.27 ± 0.06 mmol/L at t = 60 minutes; $P < .001$) and in controls (from 0.40 ± 0.06 mmol/L at t = 0 minutes to 0.10 ± 0.01 mmol/L at t = 60 minutes; $P = .001$) (Fig 4). The initial decrease of FFA at 30 minutes after glucose was significantly lower in the IR subjects (11% ± 5%) compared to controls (36% ± 11%; $P = .04$). The relative decrease of FFA after 60 minutes was also lower in IR subjects (54% ± 6%) compared to controls (73% ± 4%; $P = .02$).

DISCUSSION

Obesity, insulin resistance, and type 2 diabetes are disorders in which fasting plasma FFA concentrations are usually elevated.[2-4,24] High plasma FFA can induce both an atherogenic lipid profile by very-low-density lipoprotein overproduction, and alterations in glucose metabolism.[24-26] In the present study, in vivo

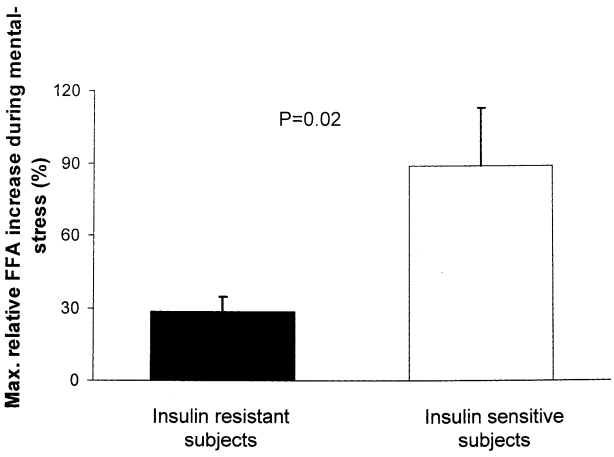

Fig 2. Maximum mean relative increase of plasma FFA during 20 minutes of mental stress in 13 IR subjects (■) and 11 healthy controls (□). Data are means ± SEM.

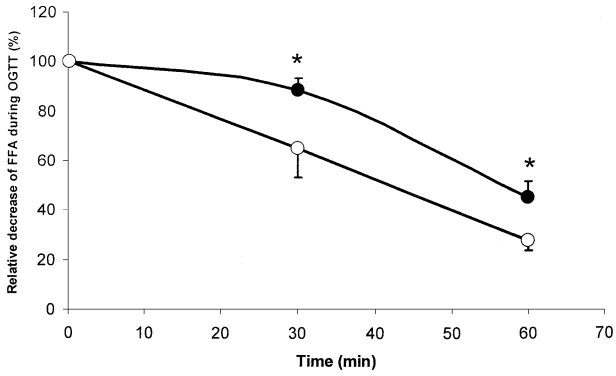

Fig 4. Relative decrease of FFA during OGTT in 13 IR subjects (●) and 10 healthy controls (○). Data are means ± SEM. *$P < .05$ for decrease *v* baseline compared to controls.

FFA metabolism in IR patients was investigated under physiological conditions. The results of this study are in line with the general consensus that insulin resistance is accompanied by impaired stimulation of intracellular lipolysis in combination with a blunted insulin-mediated suppression of FFAs. Since HSL is one of the key enzymes of FFA metabolism in the postabsorptive state, and is regulated by both catecholamines and insulin, it is likely that FFA changes reflect changes of phosphorylation and translocation of HSL and possibly also of perilipin.

The absolute increase of plasma FFA during mental stress was 20% lower (not significant) in IR subjects compared to controls, but the difference in relative increase did reach statistical significance. In our opinion, these data suggest impaired stimulation of intracellular lipolysis during mental stress. However, there were more fatty acids circulating in the blood in the IR subjects than in the controls already in baseline conditions. This may be interpreted as a result of higher intracellular lipolysis in IR subjects, impaired cellular uptake, or a combination of both. The fact remains that upon stimulation by mental stress, the acute rise (within 20 minutes) of plasma FFAs was lower in IR subjects, suggesting less release form intracellular depots, most likely by defective intrallular lipolysis. It should be underlined that plasma FFA concentrations do not reflect exactly the FFA fluxes and that variables such as plasma volume and adipose tissue mass should be taken into account in future studies.

Arner and coworkers have shown an impaired catecholamine-induced intracellular lipolysis in adipocyte biopsies from IR and obese subjects,[27-30] which is in agreement with our in vivo findings. An impaired inducibility of intracellular lipolysis by catecholamines can be caused by defects at several levels in the lipolytic cascade.[7-9,27,31-33] The design of our in vivo study does not allow to draw final conclusions on the localization of the molecular defect in IR subjects.

Despite the higher insulin levels in IR subjects after the OGTT, the FFA suppression was blunted in IR subjects. Since insulin is a strong inhibitor of intracellular lipolysis, even a small increase of insulin may result in suppression of plasma FFAs.[17,34] This impaired FFA decrease, in spite of high plasma insulin levels, suggests insulin resistance at the sites of inhibition of intracellular lipolysis, in agreement with other studies.[14-17,35]

The antilipolytic action of insulin is partly mediated by means of phosphodiesterase activation, resulting in an inactive, dephosphorylated isoform of HSL.[5,6] Insulin resistance at the level of HSL could result in a large amount of the active, phosphorylated isoform of HSL at the basal state and therefore in an elevated fasting FFA concentration. Assuming that the majority of the HSL is in the active, phosphorylated isoform, increase of catecholamines by mental stress may only activate a small amount of HSL protein that is still in the inactive form, thus explaining the lower relative increase of FFAs during mental stress in IR subjects observed in our study. This concept is supported by the combination of impaired stimulation and impaired suppression of in vivo lipolysis. On the other hand, the elevated insulin concentrations seen in IR subjects could also contribute to the blunted FFA increase during mental stress through the antilipolytic action of insulin, but since several studies including our study have shown a resistance to insulin-mediated FFA suppression, this does not seem to be the case in IR subjects.[14-17]

In conclusion, this study demonstrates that IR subjects have impaired mental stress-induced increase of plasma FFA concentrations, suggesting a defective stimulatory pathway of acute intracellular lipolysis at the level of the adipose tissue, in combination with a blunted insulin-mediated suppression of plasma FFAs. The combination of both defects helps to understand why patients with insulin resistance maintain their body weight despite impaired inhibition of intracellular lipolysis.

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