Enhanced escape of non-esterified fatty acids from tissue uptake: its role in impaired insulin-induced lowering of total rate of appearance in obesity and Type II diabetes mellitus

S. C. Riemens, W. J. Sluiter, R. P. F. Dullaart

Department of Endocrinology, University Hospital Groningen, The Netherlands

Abstract

Aims/hypothesis. To estimate non-esterified fatty acids kinetics in patients with Type II (non-insulin-dependent) diabetes mellitus and obese subjects in the postabsorptive state and during hyperinsulinaemia using non-equilibrium tracer conditions.

Methods. We evaluated the effect of hyperinsulinaemia [euglycaemic clamp with insulin infused at $30 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (3–4 h) and $150 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (3 h)] on non-esterified fatty acid kinetics, traced with [^{14}C]-palmitate using non-equilibrium tracer conditions in non-obese and obese healthy subjects and Type II diabetic patients (10 per group). Michaelis-Menten kinetics were applied for total non-esterified fatty acid disposal, which was assumed to be composed of total arterial plasma non-esterified fatty acid rate of appearance (equalling the rate of disappearance) and tissue uptake of non-esterified fatty acids derived from intravascular triglyceride hydrolysis. A model was developed to calculate the rate of escape of non-esterified fatty acids from tissue uptake and the net rate of tissue lipolysis.

Results. Total arterial plasma non-esterified fatty acid rate of appearance was lower in non-obese healthy subjects than in the other groups at low insulin infu-

sion ($p < 0.05$) and in obese Type II diabetic patients at high insulin infusion ($p < 0.05$). Plasma triglycerides were also lowest in non-obese healthy subjects during hyperinsulinaemia ($p < 0.05$ from other groups). The rate of escape from tissue uptake decreased during hyperinsulinaemia ($p < 0.05$ for each group) but remained higher in obese Type II diabetic patients ($p < 0.05$ from non-obese healthy subjects). In contrast, net rate of tissue lipolysis was not different between the groups at baseline and its decline during hyperinsulinaemia ($p < 0.05$ for each group) was similar in all groups.

Conclusion/interpretation. This study challenges the view that the antilipolytic effect of insulin is impaired in Type II diabetes and obesity. We suggest that a high plasma triglyceride concentration causes a higher escape of non-esterified fatty acids from tissue uptake, leading to an impaired suppression of total arterial plasma rate of appearance during a low degree of hyperinsulinaemia in obese subjects and Type II diabetic patients and during a high degree of hyperinsulinaemia in obese Type II diabetic patients. [Diabetologia (2000) 43: 416–426]

Keywords Non-esterified fatty acids, insulin, Type II diabetes mellitus, obesity, lipolysis, triglycerides.

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Corresponding author: R. P. F. Dullaart, MD, PhD, Department of Endocrinology, State University Hospital Groningen, PO Box 30.001, 9700 RB Groningen, The Netherlands

Abbreviations: TBD, Total body NEFA disposal; ESC, rate of escape of NEFA from tissue uptake; Ext, extraction coefficient; RL, net rate of tissue lipolysis; TR_a , total arterial plasma

NEFA rate of appearance; LPL, lipoprotein lipase; HL, hepatic lipase; apo, apolipoprotein; V_d , distribution volume; k , fractional removal constant; MCR, metabolic clearance rate; V , rate of disposal; V_m , maximum transport capacity; S , actual NEFA concentration at the site of transport; K_m , NEFA concentration at the site of transport at half maximum disposal rate; A , reaction rate constant of intravascular triglyceride hydrolysis; TU, tissue uptake; B , V_m/V_d ; PF, plasma flow.

Non-esterified fatty acids (NEFA) are important substrates for hepatic triglyceride synthesis [1]. Furthermore, NEFA could compete with glucose for substrate oxidation as originally postulated [2] or, as shown more recently, impair glucose uptake in muscle by inhibition of glucose transport or phosphorylation [3]. Increased plasma NEFA (and glycerol) concentrations could also impair suppression of hepatic glucose production by insulin [4, 5]. Increased NEFA availability is, therefore, thought to play an important part in the pathogenesis of hypertriglyceridaemia and insulin resistance of glucose metabolism as observed in Type II (non-insulin-dependent) diabetes mellitus and obesity [1, 6, 7].

Insulin efficiently lowers plasma NEFA, which is predominantly due to its antilipolytic action by inhibiting hormone-sensitive lipase in adipose tissue [1]. Several [8–10], but not all [11, 12] *in vitro* studies have shown that the antilipolytic action of insulin is unimpaired in adipose tissue from patients with Type II diabetes and obese subjects. Using prolonged infusion of radiolabelled palmitate to trace plasma NEFA and applying equilibrium tracer conditions, it has been documented that the effect of insulin to maximally suppress the rate of appearance of plasma NEFA is impaired in Type II diabetes [13, 14]. In obesity, the postabsorptive rate of appearance of plasma NEFA could be increased as a consequence of the enlarged fat mass, whereas maximum responsiveness to insulin is unaltered [13, 15, 16]. In these studies [13–16], the rate of appearance of NEFA was considered to reflect influx of NEFA derived from adipose tissue lipolysis and hence a blunted suppression of NEFA influx by insulin was taken as evidence in support of impaired antilipolysis by insulin *in vivo*. This assumption may, however, need some modification. Firstly, NEFA derived from lipoprotein lipase (LPL)-mediated intravascular triglyceride hydrolysis is now recognised to escape in part from extraction by peripheral, e.g. adipose, tissues both in the postabsorptive and in the postprandial state [17–19], thus contributing to NEFA influx. Secondly, plasma NEFA are quickly re-esterified to triglyceride by the liver [20–22]. Together this will lead to underestimation of the rate of appearance of NEFA with prolonged radiolabel infusion in the basal state, because of recirculation of label derived from intravascular hydrolysis of radiolabelled triglyceride [22]. We have recently developed and validated a method to measure NEFA kinetics during non-equilibrium tracer conditions with short-term [^{14}C]-palmitate infusion, thereby avoiding the effect of label recirculation on the measurement of NEFA rate of appearance [22]. In the present study, this method was used to document the effect of insulin on NEFA kinetic variables in non-obese and obese healthy subjects and patients with Type II diabetes. Furthermore, a model is provided to estimate the contributions of the net rate of

lipolysis and of the rate of appearance of NEFA that escape from tissue uptake after intravascular release to total arterial plasma rate of appearance during postabsorptive and hyperinsulinaemic circumstances.

Subjects and methods

Subjects. The protocol was approved by the local medical ethics committee. Written informed consent was obtained from all participants. Smokers were excluded. Type II diabetes was diagnosed according to National Diabetes Group Criteria [23] and age at onset was over 40 years in the patients with Type II diabetes. None of the diabetic patients had suffered from ketoacidotic periods or was treated with insulin. In the non-diabetic subjects, diabetes was excluded by a 75-g oral glucose tolerance test with a fasting venous blood glucose less than 6.7 mmol/l and 2-h blood glucose concentration less than 7.8 mmol/l as cut-off values. Excluded were subjects with a fasting plasma cholesterol higher than 8.0 mmol/l or triglyceride concentration above 4.5 mmol/l or both, hypertension (systolic blood pressure > 160 mmHg or diastolic blood pressure > 95 mmHg or both), macroproteinuria (> 500 mg/day) and microalbuminuria (overnight urinary albumin excretion rate > 20 µg/min), clinically manifest cardiovascular disease and a family history of hyperlipidaemia or premature cardiovascular disease. The thyreotropin concentration, liver function tests and serum creatinine concentration were within the normal range. Alcohol intake was three or less consumptions a day. No medication other than sulphonylurea and metformin was allowed. Body mass index (BMI) was calculated as weight (kg) divided by height (m^2). Obesity was defined as BMI exceeding 27 kg/ m^2 . To assess body composition bioelectrical impedance analysis (BIA) was used with a tetrapolar BIA-101 analyser (RJL Systems, Detroit, Mich., USA). After application of an alternating electrical current of 800 µA at 50 kHz, resistance and reactance were assessed [24]. Fat-free mass was estimated by using the equation supplied by the manufacturer. Body fat was calculated by subtracting fat-free mass from body weight.

The participants were ten non-obese healthy subjects, ten obese healthy subjects, ten non-obese patients with Type II diabetes and ten obese patients with Type II diabetes. They were individually matched for age (within 5 years). Patients with Type II diabetes were matched for diabetes duration (within 5 years). Most of the subjects also participated in another study that aimed to evaluate the effect of hyperinsulinaemia on plasma lipid transfer proteins [25].

Procedure. The subjects adhered to their habitual diet and the Type II diabetic patients continued their blood glucose lowering drugs until the study day. They refrained from drinking alcohol on the day before the study and were kept fasting after 20.00 hours. On the study day they remained supine from 8.00 hours onwards. One hand vein was cannulated and the canula was kept patent with a NaCl drip (154 mmol/l, 30 ml/h). This hand was placed in a thermoregulated box with an ambient temperature of 55 °C to obtain arteriatised venous blood. This procedure is a suitable alternative to arterial blood sampling for NEFA tracer studies [26]. Two forearm veins of the contralateral arm were cannulated for infusion of dextrose, insulin and [$1\text{-}^{14}\text{C}$]-palmitate. Non-esterified fatty acid kinetic variables were obtained by infusion of [$1\text{-}^{14}\text{C}$]-palmitate as a tracer using non-equilibrium tracer conditions as described recently [22]. In brief, [$1\text{-}^{14}\text{C}$]-palmitate (approximately 0.2 µCi/min, equivalent to 7.5 kBq/min) was infused for 10 min at baseline after 1 h of supine rest and at the end of the two insulin in-

fusion steps (see below). During each of the three tracer infusion periods blood samples were taken at the start of the infusion and at 2-min intervals thereafter for 20 min. In this manner NEFA kinetic variables were calculated at the upstroke (0–10 min) and downstroke (10–20 min) of the plasma palmitate specific activity curve and the result of the upstroke and downstroke measurements were averaged. By this method the radiation dose is limited to 0.16 mSv per measurement. After the baseline period, a two-step hyperinsulinaemic euglycaemic clamp was carried out with insulin infused at a rate of $30 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ for 3 h (4 h in Type II diabetic patients to gradually lower blood glucose to target concentrations), followed by an infusion rate of $150 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ for 3 h. An insulin bolus of 5 mU/kg was given directly before the insulin infusions. Blood glucose was kept at approximately 0.6 mmol/l below fasting concentrations in non-diabetic subjects. Target blood glucose concentration was 4.4 mmol/l in Type II diabetic patients. Blood glucose was measured at intervals of 5–10 min. Blood glucose concentration was allowed to decline initially and then maintained at these targets by a variable dextrose infusion (20% w/w) to which KCl was added to prevent hypokalaemia. The mean glucose infusion rate (in $\text{mmol} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) during the last hour of each insulin step was used to estimate exogenous glucose disposal. Additional blood samples for measurement of plasma triglycerides and insulin were taken at baseline and at the end of each insulin step. Plasma was obtained 20 min after intravenous injection of 50 U/kg of heparin for lipoprotein lipase (LPL) and hepatic lipase (HL) measurement 1–2 weeks before the study. This was done to avoid on effect of heparin treatment on triglyceride and NEFA metabolism during the experiments.

Laboratory methods. The [$1\text{-}^{14}\text{C}$]-palmitate [specific activity, 1.85–2.29 GBq/mmol (CFA23, Amersham, UK)] was prepared for intravenous infusion as described [22]. Radiochemical purity was well over 95%. Blood was collected into EDTA (1.5 mg/ml) containing tubes and was placed immediately on ice. Plasma was obtained within 30 min by centrifugation at 3000 rpm for 15 min at 4°C. Samples were frozen immediately at –70°C until analysis. Plasma NEFA were measured using a kit from Wako Chemicals (Neuss, Germany; catalogue no. 994–75409). Radioactivity was counted after extraction of NEFA with a mixture of chloroform and heptane (CHM, 1:1, v/v) to which methanol was added (98:2, v/v) [22]. Co-extraction of triglyceride was prevented by addition of 1.0 ml of 35 mmol/l phosphate buffer (pH 6.4) to 0.2 ml of the samples before extraction with CHM. No internal standard was used to monitor recovery of NEFA, which is virtually complete. A 5-ml aliquot of duplicate samples of the organic phases was combined in a counting vial, dried at 37°C under nitrogen stream and counted after addition of scintillation fluid, with a counting error below 1%.

Lipoprotein lipase and HL activities were assayed as described [27]. Lipids were measured in plasma and in the high density lipoprotein (HDL)-containing supernatant fraction after removal of apolipoprotein (apo) B-containing lipoproteins with polyethylene glycol-6000 [28]. Plasma cholesterol and triglycerides were measured using enzymatic methods. Apo B was assayed by immunoturbidimetry (Boehringer Mannheim, Mannheim, Germany, catalogue no. 726494). Blood glucose was measured with a Yellow Springs Glucose Analyser (model 23A, Yellow Springs, Yellow Springs, Ohio, USA). Glycated haemoglobin (HbA_{1c}) was measured by high performance liquid chromatography (Bio-Rad, Veenendaal, The Netherlands, reference range 4.6–6.1%). Plasma-free insulin was assayed by radioimmunoassay (Novo Nordisk Immunochemical Department, Copenhagen, Denmark).

Calculations of NEFA kinetic variables. The following NEFA kinetic variables were calculated: plasma NEFA concentration, total rate of appearance (TR_a , in $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$), distribution volume (V_d , in l/m^2), fractional removal constant (k , in % per min) and metabolic clearance rate (MCR, in $\text{ml} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$), where $\text{MCR} = k \cdot V_d$. Because of the extremely short half life of plasma NEFA [22, 29], it is preferable to interpret V_d in terms of a volume flow, making k an extraction coefficient. The mathematics used to calculate these NEFA kinetics variables have been described elsewhere [22]. The within-day coefficients of variation (CVs) of plasma NEFA, TR_a , V_d , k and MCR using this method are 5.1%, 5.8%, 6.8%, 12.6% and 8.2%, respectively.

The amount of intravascularly released NEFA from triglyceride hydrolysis that escapes from tissue uptake was estimated assuming that at physiological concentrations NEFA disposal into tissues is a facilitated process that follows Michaelis-Menten kinetics [30–33]. The rate of whole-body total NEFA dis-

posal (TBD), is thus equivalent to $V = \frac{V_m \cdot [S]}{[S] + K_m}$. This means

that the rate of disposal (V) is governed by the maximum transport capacity (V_m), the actual NEFA concentration at the site of transport (S) and that at the site of transport at half maximum disposal rate (K_m). The actual NEFA concentration at the site of transport is composed of the measured arterial plasma NEFA concentration and the NEFA released intravascularly from triglyceride hydrolysis by LPL: $S = \text{plasma NEFA} + A \cdot \text{LPL} \cdot \text{plasma triglyceride}$, where A represents the reaction rate constant of intravascular triglyceride hydrolysis. Thus TBD has two components because of the two sources of NEFA that make up S :

1. the rate of disposal of arterial plasma NEFA, amounting to $V_m \cdot \text{plasma NEFA}/([S] + K_m)$ and
2. the rate of disposal of intravascularly released NEFA (tissue uptake, TU), amounting to $V_m \cdot A \cdot \text{LPL} \cdot \text{plasma triglyceride}/([S] + K_m)$.

In this study, arterial plasma NEFA disposal was measured as TR_a , assuming that this rate of appearance equals the rate of disappearance during all kinetic experiments [13–16]. This results in equation 1: $TR_a = \text{MCR} \cdot \text{plasma NEFA} = V_m \cdot \text{plasma NEFA}/([S] + K_m)$. Rearrangement of equation 1 results in equation 2: $1/\text{MCR} = ([S] + K_m)/V_m = (\text{plasma NEFA} + A \cdot \text{LPL} \cdot \text{plasma triglyceride} + K_m)/V_m$. The V_m is dependent on the plasma flow (PF) through tissues capable of NEFA uptake (PF). Plasma flow can be expressed as a fraction of V_d . Thus, V_m was expressed in relation to V_d , giving equation 3: $1/\text{MCR} = (\text{plasma NEFA} + A \cdot \text{LPL} \cdot \text{plasma triglyceride} + K_m)/(B \cdot V_d)$, where B represents V_m/V_d . Using multiple regression analysis with $1/\text{MCR}$ as a dependent variable and plasma NEFA/ V_d and the product of LPL and plasma triglyceride/ V_d as independent variables the mean values of B , K_m and A can be disclosed. Further, we evaluated whether obesity, the diabetic state and the plasma insulin concentration statistically influenced the values of B , K_m and A in this multiple regression analysis. It should be noted that $B \cdot V_d/K_m$ is the theoretical maximum MCR at $S = 0$. In this situation, extraction is 100% and $B \cdot V_d/K_m$ equals PF through tissues that are capable of NEFA uptake.

With the use of the calculated mean A of the whole group, S can be approximated for each subject during the three kinetic experiments as $S = \text{plasma NEFA} + A \cdot \text{LPL} \cdot \text{plasma triglyceride}$. Using the group means of A , B and K_m the concomitant rate of intravascular release of NEFA is approximated as $A \cdot \text{LPL} \cdot \text{plasma triglyceride} \cdot B \cdot V_d/K_m$ of which only a part is taken up directly by tissue: $\text{TU} = A \cdot \text{LPL} \cdot \text{plasma triglyceride}$

Table 1. Clinical characteristics, blood glucose, plasma insulin and glucose infusion rates in non-obese and obese healthy subjects and patients with Type II diabetes

BMI groups <i>n</i>	Healthy subjects		Patients with Type II diabetes	
	< 27 kg/m ² 10	> 27 kg/m ² 10	< 27 kg/m ² 10	> 27 kg/m ² 10
Age (years)	51 (44–60)	55 (53–58)	55 (44–60)	56 (53–60)
Diabetes duration (years)	–	–	3 (1–16)	4 (3–8)
HbA _{1c} (%)	5.3 (5.0–5.7)	5.5 (5.4–5.8) ^b	7.0 (6.4–8.2) ^a	7.0 (6.4–7.9) ^a
BMI (kg/m ²)	23.7 (21.6–25.5)	28.9 (28.3–29.7) ^a	25.3 (23.3–25.7) ^b	29.8 (28.1–36.8) ^a
Fat mass (kg)	12 (9–19)	24 (19–28) ^a	15 (14–17) ^b	22 (17–28) ^a
Blood glucose (mmol/l)				
baseline	5.1 (4.5–5.3)	5.4 (5.0–5.7)	8.0 (7.0–9.0) ^a	8.2 (7.0–9.9) ^a
1 ⁰ step	4.5 (4.4–5.0) ^c	4.7 (4.1–4.9) ^c	4.4 (4.3–4.6) ^c	4.5 (4.3–5.1) ^c
2 ⁰ step	4.8 (4.2–5.0) ^c	5.0 (4.6–5.1) ^c	4.4 (4.4–4.5) ^c	4.6 (4.5–4.8) ^c
Plasma insulin (mU/l)				
baseline	10 (9–11)	15 (10–20)	10 (6–15)	15 (11–19)
1 ⁰ step	28 (25–30) ^c	35 (29–54) ^c	31 (28–47) ^c	36 (33–49) ^c
2 ⁰ step	139 (121–166) ^c	214 (157–278) ^{a,c}	166 (131–187) ^c	194 (175–229) ^{a,c}
Glucose infusion rate (mmol · min ⁻¹ · m ⁻²)				
1 ⁰ step	1.47 (1.20–2.27)	0.97 (0.62–1.15) ^{a,b}	0.79 (0.33–1.05) ^a	0.22 (0.16–0.44) ^a
2 ⁰ step	3.34 (2.49–3.84) ^d	2.74 (2.54–3.12) ^{b,d}	2.27 (1.37–2.60) ^{a,d}	1.42 (1.10–1.96) ^{a,d}

Data in medians (interquartile ranges). Plasma insulin concentrations at baseline and at the end of each insulin infusion step, blood glucose and glucose infusion rates at the last hour of each insulin infusion step are given. ^a $p < 0.05$ from non-obese

healthy subjects; ^b $p < 0.05$ from obese patients with Type II diabetes; ^c $p < 0.05$ from baseline; ^d $p < 0.05$ from first insulin infusion step

ide · $B \cdot V_d / ([S] + K_m)$. As a consequence, the concomitant coefficient of extraction (Ext) amounts to $K_m / ([S] + K_m)$. The part of the NEFA that originates from intravascular triglyceride hydrolysis, which escapes from direct uptake by tissue (ESC) will appear in the circulation and will approximate $ESC = (1 - Ext) \cdot A \cdot LPL \cdot \text{plasma triglyceride} \cdot B \cdot V_d$. If this part ESC is subtracted from the total rate of appearance TR_a , an estimate of the net rate of tissue lipolysis (RL) is obtained. Finally, as both TR_a and TU are known, TBD can be calculated as $TBD = TR_a + TU$.

Statistical analysis. Because of non-parametric distribution of variables, data are given in medians (interquartile ranges) unless stated otherwise. Between group comparisons of variables was carried out by Kruskal-Wallis analysis of variance. Within group changes in variables were evaluated by Friedman's two-way analysis of variance. Duncan's method was applied to correct for multiple comparisons. Multiple regression analysis was used to evaluate the independent relations between variables. A two-sided p value less than 0.05 was taken as significant.

Results

Age was similar in the groups and diabetes duration was not different between non-obese and obese patients with Type II diabetes (Table 1). Haemoglobin A_{1c} was higher in the diabetic than in the non-diabetic groups. Of the non-obese diabetic patients, two were treated with diet alone, four with diet plus sulphonylurea and four received sulphonylurea in combination with metformin. In addition to diet, four of the obese diabetic patients were treated with sulpho-

nylurea, five received sulphonylurea in combination with metformin and one patient was medically treated with metformin. Body mass index and fat mass were similarly higher in the obese healthy and obese subjects with Type II diabetes than in the non-obese groups (Table 1). Blood glucose was higher in diabetic patients at baseline but during the clamp no between-group differences in blood glucose were present (Table 1). In the combined subjects, the CV of blood glucose during high-dose insulin infusion was $6.4 \pm 3.4\%$. Plasma insulin was not statistically significantly different between the groups at baseline. During the second insulin infusion step plasma insulin was higher in obese healthy subjects and in obese patients with Type II diabetes than in lean healthy subjects. (Table 1). Glucose infusion rate was highest in non-obese healthy subjects and lowest in obese Type II diabetic patients (Table 1).

Plasma total cholesterol was not different between the groups but HDL cholesterol was lowest in obese Type II diabetic patients and highest in non-obese healthy subjects (Table 2). Plasma apo-B was higher in the diabetic groups than in non-obese healthy subjects. Lipoprotein lipase activity in post-heparin plasma was not significantly different between the groups but HL activity was higher in obese healthy and obese Type II diabetic subjects than in non-obese healthy subjects (Table 2). Baseline plasma triglyceride was lowest in non-obese healthy subjects and highest in obese Type II diabetic patients (Table 2). During the second insulin infusion step plasma triglyceride decreased but its change was not statistically

Table 2. Baseline plasma lipid variables, lipoprotein lipase (LPL) and hepatic lipase (HL) activities in post-heparin plasma as well as plasma triglycerides at baseline and during hyperinsulinaemia in non-obese and obese healthy subjects and patients with Type II diabetes

BMI groups <i>n</i>	Healthy subjects		Patients with Type II diabetes mellitus	
	< 27 kg/m ²	> 27 kg/m ²	< 27 kg/m ²	> 27 kg/m ²
	10	10	10	10
Plasma total cholesterol (mmol/l)	4.73 (4.40–5.08)	4.89 (4.52–5.83)	5.10 (4.64–5.62)	4.83 (4.50–5.69)
HDL cholesterol (mmol/l)	1.31 (1.12–1.48)	1.18 (0.92–1.28) ^b	1.12 (0.98–1.27) ^b	0.87 (0.73–1.01) ^a
Apolipoprotein B (g/l)	0.75 (0.70–0.83)	0.93 (0.70–1.19)	0.97 (0.78–1.18) ^a	1.09 (0.96–1.15) ^a
LPL activity (U/l)	127 (115–172)	103 (98–150)	144 (137–172)	95 (72–143)
HL activity (U/l)	383 (234–429)	518 (372–699) ^a	361 (262–504)	620 (306–707) ^a
Plasma triglycerides (mmol/l)				
baseline	0.90 (0.81–0.99)	1.01 (0.78–1.64) ^b	1.27 (0.89–1.69) ^b	2.00 (1.58–2.95) ^a
1 ⁰ step	0.61 (0.54–0.81) ^c	0.98 (0.67–1.60) ^{a,b}	0.98 (0.67–1.45) ^{a,b}	1.81 (1.36–2.97) ^a
2 ⁰ step	0.51 (0.44–0.58) ^c	0.89 (0.65–1.54) ^{a,c}	0.87 (0.56–1.21) ^{a,c}	1.74 (1.09–2.80) ^a

Data in medians (interquartile ranges). ^a $p < 0.05$ from non-obese healthy subjects; ^b $p < 0.05$ from obese patients with Type II diabetes; ^c $p < 0.05$ from baseline

Table 3. Non-esterified fatty acid (NEFA) kinetic variables at baseline and during hyperinsulinaemia in non-obese and obese healthy subjects and patients with Type II diabetes

BMI groups <i>n</i>	Healthy subjects		Patients with Type II diabetes	
	< 27 kg/m ²	> 27 kg/m ²	< 27 kg/m ²	> 27 kg/m ²
	10	10	10	10
Plasma NEFA (μmol/l)				
baseline	469 (453–537)	485 (364–571) ^f	636 (489–863)	791 (608–871) ^e
1 ⁰ step	31 (17–47) ^g	73 (34–97) ^{f,g}	54 (41–260) ^{e,g}	160 (144–170) ^{e,g}
2 ⁰ step	15 (12–25) ^g	28 (18–39) ^{f,g}	34 (7–65) ^g	62 (37–85) ^{e,g}
V_d (l/m ²) ^a				
baseline	2.1 (1.7–2.3)	1.8 (1.5–2.0)	1.9 (1.8–2.2)	1.8 (1.5–2.1)
1 ⁰ step	2.3 (2.1–2.4)	1.7 (1.6–2.2)	2.0 (1.8–2.4)	2.0 (1.6–2.1)
2 ⁰ step	2.3 (2.2–3.2)	2.5 (1.8–3.1)	2.4 (1.7–3.0)	2.3 (2.1–2.6) ^g
k (%/min) ^b				
baseline	27 (24–31)	26 (23–34)	26 (23–29)	24 (18–43)
1 ⁰ step	36 (30–40)	42 (32–47)	34 (30–50)	31 (23–36)
2 ⁰ step	38 (32–53) ^g	51 (45–63) ^{f,g}	39 (34–47) ^g	31 (29–36)
MCR (ml · min ⁻¹ · m ⁻²) ^c				
baseline	463 (420–585)	507 (389–618)	486 (475–717)	455 (389–640)
1 ⁰ step	762 (648–873) ^g	701 (612–993) ^{f,g}	788 (539–1044) ^{f,g}	580 (499–655) ^e
2 ⁰ step	974 (844–1076) ^g	992 (841–2340) ^{f,g}	1032 (896–1210) ^{f,g}	748 (616–993) ^g
TR_a (μmol · min ⁻¹ · m ⁻²) ^d				
baseline	219 (186–326)	253 (198–297)	305 (242–447)	357 (218–633)
1 ⁰ step	29 (15–39) ^g	53 (39–58) ^{e,g}	74 (39–227) ^{e,g}	86 (72–113) ^{e,g}
2 ⁰ step	12 (10–26) ^g	39 (29–55) ^g	31 (11–71) ^g	40 (37–58) ^{e,g}

Data in medians (interquartile ranges). ^a V_d , distribution volume; ^b k , fractional removal constant; ^c MCR, metabolic clearance rate; ^d TR_a , total rate of appearance; ^e $p < 0.05$ from non-obese healthy subjects; ^f $p < 0.05$ from obese patients with Type II diabetes; ^g $p < 0.05$ from baseline

significant in obese Type II diabetic patients. At this high rate of insulin infusion plasma triglyceride was lower in non-obese healthy subjects than in the other groups.

Baseline plasma NEFA were lower in non-obese healthy subjects than in obese Type II diabetic patients (Table 3). At the first insulin infusion step plasma NEFA were lower in non-obese healthy subjects than in both diabetic groups, whereas after the second insulin infusion step plasma NEFA were lower in non-obese healthy than in obese patients with

Type II diabetes. The distribution volume (V_d) was not different between the groups at baseline and during hyperinsulinaemia (Table 3). In the separate groups, the changes in V_d during hyperinsulinaemia did not reach statistical significance in most situations. In the combined groups, the increase in V_d was significant during each insulin infusion step ($p < 0.05$ from baseline, data not shown). The fractional disappearance rate (k) was similar in the groups at baseline and rose in all groups during hyperinsulinaemia (Table 3). As a result, the metabolic clearance rate,

Fig. 1. Lineweaver-Burk plots showing the relation between $1/S$ (S denotes the apparent NEFA concentration at the site of transport) and $1/TBD$ (TBD denotes the whole body total NEFA disposal) at baseline (line A), during insulin infused at $30 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (line B) and during insulin infused at $150 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (line C). The values of K_m and V_m were obtained by multiple regression analysis (see text). The intercepts between the regression lines and the x-axis represent $-1/K_m$, where $K_m = 850 \text{ } \mu\text{mol/l}$. The intercepts between the regression lines and the y-axis represent $1/V_m$, where $V_m = 829, 889$ and $1065 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$ for lines A, B and C, respectively. $\square$, $\blacksquare$, $\circ$, $\bullet$ represent median (interquartile range) values in non-obese and obese healthy subjects and in non-obese and obese patients with Type II diabetes, respectively

$\text{MCR} (= k \cdot V_d)$, which was not different between the groups at baseline, increased in all groups during hyperinsulinaemia (Table 3). At baseline, TR_a tended to be higher in obese Type II diabetic patients than in non-obese healthy subjects ($p < 0.10$, Table 3). The TR_a decreased greatly during hyperinsulinaemia in all groups. At the first insulin step TR_a was significantly lower in non-obese healthy subjects than in the other groups. At the second insulin infusion step TR_a remained lower in non-obese healthy than in obese subjects with Type II diabetes (Table 3).

Based on equation 3, multiple regression analysis with $1/\text{MCR}$ as a dependent variable resulted in the following formula: $1/\text{MCR} = (1.97 + 2.32 \cdot 10^{-3} \cdot \text{plasma NEFA} + 2.10 \cdot 10^{-3} \cdot \text{LPL} \cdot \text{plasma triglyceride})/V_d$ (120 data sets, multiple $r = 0.899$ with p values for the three coefficients of < 0.01 , < 0.001 and 0.041 , respectively). Applying the coefficients of this formula, K_m was calculated to be $(1.97/2.32) \cdot 10^3 \text{ } \mu\text{mol/l} = 850 \text{ } \mu\text{mol/l}$ and A was calculated to be $(2.10/$

$2.32) \cdot 10^{-3} \text{ l/U} = 0.904 \text{ l/U}$ in the 3 kinetic experiments. The V_m was calculated as $(1/2.32) \cdot 10^3 V_d$, resulting in values of 829, 889 and $1065 \text{ } \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$ in the basal situation and at low-dose and high-dose insulin infusion, respectively, using the averaged V_d values of the 40 participants of 1.92, 2.06 and $2.47 \text{ l} \cdot \text{min}^{-1} \cdot \text{m}^{-2}$ at the three kinetic experiments. Multiple regression analysis did not show significant contributions of the diabetic state or obesity to the values of V_m , K_m and A ($p > 0.10$ for all). There was also no significant contribution of the plasma insulin concentration on any of these variables ($p > 0.10$ for all). In a separate model, LPL was substituted by HL. No influence of HL activity on $1/\text{MCR}$ was found ($p = 0.89$).

Using the calculated variables K_m and A and V_m we approximated, for each subject, the actual NEFA concentration at the site of transport (S), the extraction coefficient (Ext), the rate of disposal of intravascularly released NEFA (TU), the rate of escape of intravascularly released NEFA (ESC), the net rate of tissue lipolysis (RL) and rate of whole-body total NEFA disposal (TBD). Figure 1 shows the Lineweaver-Burk plot of the linear relation between $1/S$ and $1/\text{TBD}$ for the three kinetic experiments using the variables from the multiple regression analysis. Also depicted are the median (interquartile range) values of $1/S$ and $1/\text{TBD}$ from each group during the experiments. Figure 2 shows the relations between plasma insulin and S , Ext , ESC and RL for each group. The S declined in all groups with increasing plasma insulin (Fig. 2). At baseline, S was higher in obese patients with Type II diabetes than in non-obese and obese healthy subjects. At both insulin infusion steps, S was lower in the non-obese healthy subjects than in the other groups. Changes and differences in TBD paralleled those in S (data not shown). The Ext rose in all groups during hyperinsulinaemia (Fig. 2). At baseline, Ext was lower in obese patients with Type II diabetes than in non-obese and obese healthy subjects. At the low and high rate of insulin infusion, Ext was higher in non-obese healthy subjects than in the other groups. The ESC decreased in each group with increasing plasma insulin (Fig. 2). At baseline and at the first insulin infusion step, ESC was higher in both groups with Type II diabetes than in non-obese healthy subjects. At the second insulin infusion step, ESC was higher in obese Type II diabetic patients than in non-obese healthy subjects. Thus, as suggested by Figure 2, both obesity and the diabetic state seemed to result in similar alterations in S , Ext and ESC in relation to the prevailing plasma insulin concentration. In obese Type II diabetic patients, these abnormalities seemed to be aggravated. The RL declined in all groups during hyperinsulinaemia (Fig. 2). In contrast to the three former variables, RL was, however, not different between the groups in any of the kinetic experiments.

Fig. 2A–D. Relations between plasma insulin and S, the apparent NEFA concentration at the site of transport (**A**), Ext, the extraction coefficient (**B**), ESC, the rate of escape of intravascularly released NEFA (**C**) and RL, the net rate of tissue lipolysis (**D**). □, ■, ○, ● represent median (interquartile range) values in non-obese and obese healthy subjects and in non-obese and obese patients with Type II diabetes, respectively. ^a $p < 0.05$ from non-obese healthy subjects, ^b $p < 0.05$ from baseline

Multiple regression analysis was used to evaluate the influence of the glucose infusion rate, the diabetic state, obesity and the two levels of hyperinsulinaemia on the actual values of TBD and RL. In this analysis, the diabetic state, obesity and the two levels of hyperinsulinaemia were considered as categorical variables. The TBD was found to be negatively related to glucose infusion rate ($p = 0.014$) and was increased by both the diabetic state ($p = 0.009$) and obesity ($p = 0.014$, multiple $r = 0.542$). In this model, the degree of hyperinsulinaemia did not contribute independently ($p = 0.98$) to the actual TBD. The RL was only inversely related to the degree of hyperinsulinaemia ($p = 0.011$) without a significant influence of the glucose infusion rate ($p = 0.786$), the diabetic state ($p = 0.135$) and obesity ($p = 0.93$).

Discussion

As expected [13, 14, 16, 34, 35], this study showed that the plasma NEFA concentration is increased in the fasting state and is less suppressible by insulin in obese Type II diabetic patients compared with non-obese healthy subjects. Using non-equilibrium tracer conditions, we found impaired suppression of the arterial plasma NEFA rate of appearance, TR_a , in obese subjects and patients with Type II diabetes at low-dose insulin infusion and also in obese patients with Type II diabetes at high-dose insulin infusion. This again seems to agree with previous data obtained by prolonged tracer infusion [13–16].

Total arterial plasma NEFA rate, as assessed in the present study, is composed of both the rate of appearance of NEFA derived from tissue stores and of NEFA appearing from intravascularly hydrolysed triglyceride that escape from tissue uptake [20–22]. The rate of appearance of NEFA, measured by prolonged tracer infusion, is, in contrast, considered to represent only their release from tissue stores [13–16, 36, 37]. This assumption holds true in those situations where the specific radioactivity of the fatty acids in circulating triglyceride equals that of circulating NEFA. Such conditions are met if the circulating pool of esterified fatty acids is completely formed by re-esterification of circulating NEFA. In the basal

state this assumption is likely to be correct [38] but during hyperinsulinaemia and abundant supply of glucose, *de novo* synthesis of fatty acids contributes to a relevant extent to hepatic triglyceride production [38]. Hence, under such circumstances, the rate of appearance of NEFA, assessed by prolonged tracer infusion, is bound to measure intravascularly released NEFA that escape from tissue uptake together with NEFA released from tissue stores. Therefore, impaired insulin-induced suppression of the rate of appearance of NEFA so measured does not necessarily imply an impaired antilipolytic action of insulin, but could also be attributable to an enhanced escape from direct uptake of intravascularly released NEFA.

Unlike measurement of NEFA kinetics during equilibrium tracer conditions, the non-equilibrium method enables measurement of factors that constitute MCR, i.e. the distribution volume, V_d and the fractional removal constant, k . In the basal state, V_d was approximately 1.9 l/m². Regarding V_d in terms of a volume flow and assuming a haematocrit of 0.4, V_d would represent a blood flow of about 3.2 l · min⁻¹ · m⁻², a value similar to the cardiac output. When the data from the groups were combined, V_d rose with increasing plasma insulin, in keeping with the positive inotropic effect of insulin and the insulin-induced increase in perfusion of tissues like muscle and adipose tissue as shown in some studies [17, 39]. Using actual values of V_d , we were able to approximate both components of TR_a , i.e. the net rate of tissue lipolysis, RL and the rate of intravascular escape from tissue uptake, ESC by application of a model of whole-body total NEFA disposal, TBD. The model presented here is based on several assumptions. Firstly, as shown *in vitro*, transmembrane NEFA transport was considered to be a facilitated diffusion process [30–33] that follows Michaelis-Menten kinetics. Indeed, several protein factors have been identified that facilitate long-chain fatty acid transport across cell membranes [33, 40, 41]. It can be argued that the low unbound NEFA concentrations that are likely to be present under our experimental conditions make simple diffusion of NEFA across cell membranes of minor importance in NEFA transmembrane transport [30, 33]. It should be noted, however, that the physiology of NEFA transmembrane transport is still under debate [33, 41, 42]. With the assumption that transmembrane NEFA transport follows Michaelis-Menten kinetics, TBD would then follow Michaelis-Menten kinetics as well, in analogy with such kinetics of whole-body glucose disposal [43, 44]. In our model, the action of lipases on intravascular triglyceride metabolism and, hence, on NEFA kinetics was crucial. No effect of HL activity on NEFA kinetics could be shown and this lipase was, therefore, not considered further. We assumed that the rate of intravascular triglyceride hydrolysis is proportional to LPL activity, the arterial plasma

triglyceride concentration and the plasma flow (PF) in tissues capable of hydrolysing triglyceride. Lipoprotein lipase activity was measured in post-heparin plasma shortly before the experiments because the biological effect of the low LPL activity in native pre-heparin plasma is unclear. Because there was no independent contribution of the plasma insulin concentration to the reaction rate constant of intravascular triglyceride hydrolysis (A), the total amount of active LPL (corresponding to $A \cdot \text{LPL}$) was considered to remain unaltered during the experiments, as suggested previously [17]. In healthy subjects, LPL activity has been shown to increase in adipose tissue [45, 46] and to decrease in skeletal muscle [46] in response to insulin. In another study, the increase in native plasma LPL activity after insulin was not different from that after saline infusion [47]. Furthermore, we assumed that plasma triglycerides were completely hydrolysed by LPL [18, 48] and that the changes in the composition of triglyceride-rich lipoproteins occurring after insulin infusion [49, 50] were not so prominent that the use of the plasma triglyceride concentration as the substrate concentration for LPL action became inappropriate. Finally, it was assumed that intravascular triglyceride hydrolysis was proportional to tissue plasma flow, as witnessed by the virtually unchanged arterial-venous plasma triglyceride difference in adipose tissue, despite a large increase in blood flow, as evoked by adrenalin [48].

Applying multiple regression analysis, it was shown that the model sufficiently described the NEFA concentration at the site of transport at half maximum disposal rate, K_m , and the maximum transport capacity, V_m (expressed per body surface area). These characteristics of the NEFA transport system were unaffected by the presence of Type II diabetes and obesity. The maximum transport capacity (V_m) rose during hyperinsulinaemia in proportion with the increase in V_d . Moreover, the distribution of blood supply to the various organs involved in NEFA metabolism seemed to remain similar during hyperinsulinaemia because both V_m ($B \cdot V_d$) and PF ($B \cdot V_d / K_m$) kept a constant relation to V_d . This was expected because changes in cardiac output and tissue perfusion, particularly muscle, are highly correlated under hyperinsulinaemic conditions [39]. As insulin infusion evoked a rise in V_d , the rate of intravascular triglyceride hydrolysis ($A \cdot \text{LPL} \cdot B \cdot V_d$) increased during hyperinsulinaemia despite a lack of change in the reaction rate constant of intravascular triglyceride hydrolysis, A .

Using the model-derived constants, the coefficient of extraction of NEFA across tissues, Ext, as well as the two components of TR_a , RL and ESC, could be approximated for the individual subjects under the three experimental conditions. The Ext increased during hyperinsulinaemia, but did not reach 1.0 in any group. Accordingly, retention of NEFA in adi-

pose tissue is incomplete after a high fat meal combined with a hyperinsulinaemic clamp [17, 18]. The methodology used in our study assesses only that part of tissue lipolysis that results in actual NEFA release. Under the present experimental conditions, with oral glucose lowering agents being withheld only during the experiments and evaluating adequately controlled Type II diabetic patients, RL (expressed per body surface area) was not different between the groups in the basal state and after hyperinsulinaemia. This proves that in this situation, abnormalities in the antilipolytic response to insulin (judged by RL) do not contribute to an important extent to the impaired suppression of TR_a in Type II diabetic patients and in obese subjects at a low rate of insulin infusion and in obese Type II diabetic patients at a high rate of insulin infusion. Instead, our study not only confirmed a considerable contribution of ESC to TR_a in non-diabetic subjects in the postabsorptive state [17, 22] but also showed an enhanced ESC at baseline and at a low rate of insulin infusion in both groups with Type II diabetes and also at a high rate of insulin infusion in obese Type II diabetic patients. We suggest that this enhanced escape is a consequence of the increased NEFA concentration at the site of transport, S, due to enhanced intravascular triglyceride hydrolysis. This increased release of NEFA is based solely on the higher plasma triglyceride concentration associated with Type II diabetes and obesity because LPL activity and PF were similar in all groups. An increased NEFA concentration at the site of transport will lead to a higher TBD. Consequently, TBD was positively related to the diabetic state and obesity during hyperinsulinaemia.

It is plausible that these abnormalities in NEFA kinetics in Type II diabetes and obesity result from altered regulation of triglyceride-rich lipoprotein secretion in response to insulin. The insulin-induced decrease in hepatic apo B secretion has been shown to be impaired in Type II diabetes [51], although this has not always been observed [52]. In obesity, the decrease in apo B secretion and in plasma triglyceride after insulin is impaired as well [49]. In that report, the lowering of hepatic VLDL triglyceride secretion was unaltered but the insulin dose was approximately threefold lower than that in our study [49]. Because the plasma NEFA concentration is an important determinant of hepatic triglyceride synthesis [1, 53], we postulate that an impaired suppression of hepatic triglyceride secretion by insulin in Type II diabetes and obesity will lead to an enhanced TR_a and, hence, to a futile NEFA cycle.

The negative relation between NEFA and glucose metabolism has been widely reported [2–7, 13–16, 54, 55]. Our study showed a negative interrelation between the glucose infusion rate during hyperinsulinaemia and TBD, although the absence of hepatic glucose production measurement does not allow us to

explain the precise relation between NEFA kinetics and peripheral insulin sensitivity of glucose metabolism. No independent effect of hyperinsulinaemia on actual TBD was found. Others have shown that the decrease in NEFA oxidation during hyperinsulinaemia [56] is a consequence of lowered NEFA concentrations rather than a direct effect of insulin [14]. Of note, the present experiments showed that RL was unrelated to glucose infusion rate during hyperinsulinaemia, which suggests with the aforementioned restrictions that there was no direct interrelation between net rate of tissue lipolysis and insulin sensitivity of glucose metabolism.

This study suggests that the enhanced TR_a during hyperinsulinaemia in adequately controlled patients with Type II diabetes and obese subjects does not result from a defect in suppressibility of tissue lipolysis but from escape of NEFA uptake after intravascular triglyceride hydrolysis. This, in turn, is likely to be attributable to higher plasma triglyceride concentrations which could be related to impaired lowering of triglyceride secretion by insulin. Such a defect could represent one of the main components of the complex abnormalities in overall lipid handling in these conditions, which bare an increased risk of cardiovascular disease.

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