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Morphometric documentation of abnormal intramyocellular fat storage and reduced glycogen in obese patients with Type II diabetes

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Abstract

Aims/hypothesis. Insulin resistance of skeletal muscle has been associated with increased lipid availability. This study aimed to estimate volume fractions of intramyocellular triglyceride droplets and glycogen granules in skeletal muscle using electron microscopy and furthermore, relate these findings to insulin sensitivity and the level of circulating lipids.
Methods. We compared 11 obese patients with Type II (non-insulin-dependent) diabetes mellitus and 11 obese normoglycaemic subjects matched for age and sex. Glucose metabolism was determined using the euglycaemic hyperinsulinaemic clamp technique ($40\ mU \cdot m^{-2} \cdot min^{-1}$) coupled with indirect calorimetry and tritiated glucose. On the second day, using an automatic procedure, a fasting muscle biopsy was carried out and processed for electron microscopy. Volume fractions of intramyocellular structures were estimated by pointcounting on photographic pictures in a blinded manner.
Results. Insulin-stimulated total glucose disposal rate was lower in the Type II diabetic subjects compared with the obese normoglycaemic subjects (4.96 ± 049 vs $10.35 \pm 0.89\ mg \cdot min^{-1} \cdot kg\ ffm^{-1}$, $p < 0.001$) as was glucose storage (2.03 ± 0.50 vs 6.59 ± 0.83, $p < 0.001$). The electron microscopy study revealed that the diabetic subjects had higher intramyocellular amounts of triglyceride (1.43 ± 0.21 vs $0.39 \pm 0.07\,\%$, $p < 0.001$) and lower amounts of glycogen (3.53 ± 0.33 vs $6.94 \pm 0.54\,\%$, $p < 0.001$). Mitochondrial volume was identical indicating equal aerobic capacity. The fractional intramyocellular lipid volume was found to be positively associated with fasting NEFA ($r = 0.63$, $p = < 0.05$ and $r = 0.79$, $p = < 0.05$) and triglyceride ($r = 0.74$, $p = 0.01$ and $r = 0.62$, $p < 0.05$) in the obese diabetic and normoglycaemic cohorts respectively. Intramyocellular lipid content was negatively correlated to insulin sensitivity ($r = –0.71$, $p < 0.02$) in the obese diabetic group whereas no significant association was found in the obese normoglycaemic group.
Conclusion/interpretation. This study shows that fat accumulates intramyocellulary while glycogen stores are simultaneously reduced in obese subjects with Type II (non-insulin-dependent) diabetes mellitus. Quantitatively, a major component of the excessive lipid accumulation could be secondary in origin, related to the diabetic state in itself, although a contribution from the altered insulin action cascade of obesity and diabetes cannot be excluded. In both groups significant positive relations were found between circulating and intramyocellular lipid. [Diabetologia (2001) 44: 824–833]

Keywords Type II diabetes, insulin sensitivity, skeletal muscle, intramyocellular, electron microscopy, triglyceride, glycogen

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Abbreviations: FFM, Fat free mass; NMR, nuclear magnetic resonance; cpm, counts per minute; Rd, total glucose disposal; GS, glucose storage; Si, insulin sensitivity index

Over the last decade, the role of excessive lipid availability in the pathogenesis of Type II (non-insulin-dependent) diabetes mellitus has been the subject of increasing attention [1–4]. It has recently been suggested that the disease could be better characterized in terms of defects in fat metabolism with secondary changes in carbohydrate metabolism and in beta-cell

secretory capacity [5]. An inverse relation exists between glucose and fat as metabolic fuels, with inappropriate increases (by infusion of lipid emulsion) in the supply of fatty acids, leading to an impairment of glucose utilization and a reduction in insulin sensitivity, whereas insulin sensitivity improves if the NEFA metabolism is modified pharmacologically [6–12].

Skeletal muscle is generally believed to be important for the removal of NEFA from the circulation [2, 13], and it usually contains substantial amounts of stored triglyceride that provide an efficient energy source for muscles. Obese subjects with Type II diabetes have an increased rate of NEFA turnover per unit of skeletal muscle mass, in addition to being insulin resistant [2, 14]. Although it is difficult to disentangle cause and effect, an excess accumulation of triglyceride in skeletal muscle could play a primary pathophysiological role in the causation of the reduced insulin sensitivity. Thus, in a group of obese Pima Indians with normal glucose tolerance investigators found muscle triglyceride to be closely related to the degree of insulin resistance [15]. Other investigators using a semiquantitative histological method were able to demonstrate a negative association between glycogen synthase activity and intracellular lipid in a population of normoglycaemic obese women [16]. Recently, the nuclear magnetic resonance (NMR) spectroscopy technique has been used to determine muscle lipid content and showed that intramyocellular lipid concentrations was inversely correlated to insulin sensitivity in a group of normal weight non-diabetic subjects and in relatives of subjects with Type II diabetes [17–20]. The NMR studies have been conducted on net cross-sections of muscle, whereas the current study uses quantitative electron microscopy to investigate anatomically the intramyocellular space.

This study, therefore, used electron microscopy EM to estimate the intramyocellular volume of triglyceride in skeletal muscle of obese Type II diabetic subjects and matched obese normoglycaemic subjects. We related these findings to insulin sensitivity as determined by the euglycaemic hyperinsulinaemic clamp method. The EM technique offers the additional advantage of a simultaneous quantification of the intramyocellular glycogen content and the assessment of mitochondria, as well as the localisation and morphology intramyocellulary of these organelles and substrates.

Subjects and methods

Subjects. A total of 11 subjects with Type II diabetes and 11 obese subjects with no family history of diabetes were recruited for the study. All were Caucasians and followed a sedentary lifestyle. The obese subjects were matched for age, sex and body mass index and all had a normal glucose tolerance, as assessed by an oral glucose tolerance test. Of the subjects with Type II diabetes, two were on sulfonylurea therapy, five were on metformin therapy and four were on diet therapy alone. Altogether five of the diabetic subjects and two of the obese normoglycaemic subjects were being treated for hypertension. All anti-diabetic medication had been discontinued 3 weeks before the start of the metabolic studies. During this period mean fasting plasma glucose concentrations increased from 9.8 ± 1.2 to 12.3 ± 1.3 mmol/l, subjects were asymptomatic and weight did not change. None of the participants suffered from a major disease other than diabetes and hypertension as assessed by clinical investigation and standard laboratory examinations. Subjects were instructed not to change their diet and amount of physical activity during the study and to avoid excessive physical exercise at least 3 days before all metabolic investigations. Informed written consent was obtained from all subjects after the purpose and potential risks of the study were explained to them. The study was approved by the regional ethical committee and was in accordance with the Helsinki declaration.

Study protocol. All studies began at 0800 hours after a 10- to 12-h overnight fast. The design of the study in which subjects were examined three times is illustrated graphically on Figure 1.

Euglycaemic hyperinsulinaemic clamp. The diabetic subjects were admitted to the clinical research centre the evening before the start of the study in order to induce euglycaemic steady state (about 5.2 mmol/l) by a variable infusion of insulin using an antecubital vein catheter. Blood samples for plasma glucose analyses were drawn from a catheter inserted into a dorsal wrist vein of the opposite arm. The obese normoglycaemic subjects were admitted to the centre at 0800 hours at which time intravenous catheters were inserted. During the study the subjects remained in a supine position with the hand designated for collection of blood samples placed and maintained in a heated Plexiglas box for arterialization of venous blood [21]. After a 30-min relaxation period, at time zero, a surface adjusted priming dose of 3-^{3}H glucose was given (14 μCi/m^2) (DuPont-New England Nuclear, Boston, Mass., USA), followed by a continuous infusion of 3-^{3}H glucose (0.14 $\mu Ci \cdot min^{-1} \cdot m^{2(-1)}$), which was continued throughout the study periods. After a 120-min basal tracer equilibration period, insulin (Actrapid, Novo-Nordisk, Bagsvaerd, Denmark) was infused at a rate of 40 $mU \cdot min^{-2} \cdot min^{-1}$ for 180 min and euglycaemia maintained using a variable infusion of 18 % glucose. To keep plasma specific activity constant at the basal level during the clamp, 3-^{3}H glucose was added to the glucose infusate (HOTGINF) as previously described [22]. Steady-state periods were defined as the last 30 min during basal and insulin stimulated measurements, respectively.

Glucose and lipid oxidation. Indirect calorimetry was done using a computerized flow through canopy gas analyser system (Deltatrac, Datex, Helsinki, Finland) as previously described [10]. After an equilibration period of 10 min, the average gas exchanges recorded over the two 30-min steady state periods were used to calculate rates of glucose oxidation, lipid oxidation and energy expenditure [23]. The protein oxidation rate was estimated from urinary urea nitrogen excretion (1 g of nitrogen = 6.25 g of protein) and corrected for changes in pool size [24].

Calculations. Rates of total glucose appearance (Ra) and glucose disposal (Rd), were calculated from the plasma concentrations of tritiated glucose and plasma glucose using Steele's non-steady-state equations [25]. In these calculations, the dis-

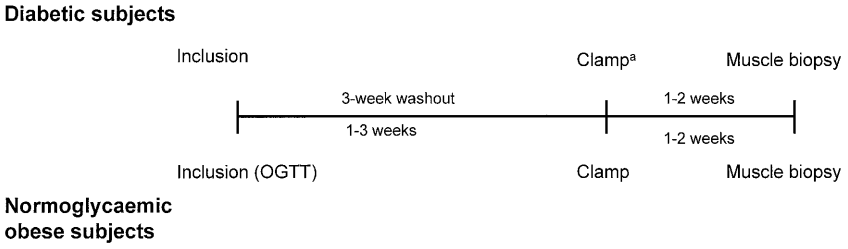

Fig. 1. Illustration of study design. [a]Overnight normalization of plasma glucose

tribution volume of glucose was taken as 200 ml/kg body weight and the pool fraction as 0.65. Hepatic glucose output was calculated as the difference between Ra and the glucose infusion rate (GINF). During the basal equilibration period plasma glucose specific activity (cpm · mg^{-1} glucose) was 788.3 ± 41.9 at 20 min and 776.4 ± 45.9 after 120 min in the diabetic group and 806.0 ± 28.1 and 834.3 ± in the obese normoglycaemic group confirming steady state. Specific activities at the end of the second pre-defined steady state period were 897.3 ± 59.4 and 824.3 ± 36.9 in the two groups respectively. The in vivo glycolytic flux rates were calculated from the rates of generation of plasma ^3H_2O from 3-^{3}H glucose as previously described [26, 27]. Briefly, this measurement is based on the observation that essentially all of tritium in the C-3 position is lost to water at the triose-isomerase reaction during the glycolytic process. Thus, the glycolytic flux rate can be calculated by multiplying the slope of the regression line obtained from the plot between time and ^3H_2O content (cpm · ml^{-1} · min^{-1}) with total body water (ml) estimated from the bioimpedance measurement and divided by plasma specific activity (cpm/mg). In these calculations, plasma water was assumed to be 93 % of the total plasma volume [27]. Linearity of the accumulation of plasma tritiated water radioactivity was obtained from –100 to 0 min during basal measurements ($r = 0.94 \pm 0.01$ and $r = 0.94 \pm 0.02$) and from 40 to 180 min during insulin infusion ($r = 0.96 \pm 0.01$ and $r = 0.97 \pm 0.01$) in the obese diabetic and obese normoglycaemic group, respectively.

Rates of glucose storage (GS) were calculated as the difference between Rd and glycolytic flux. Non-oxidative glucose disposal as the difference between Rd and glucose oxidation as determined by indirect calorimetry. The insulin sensitivity indices of Rd and GS which mainly represent skeletal muscle glucose processing were expressed as the glucose metabolic parameter per increment in plasma insulin induced by the hyperinsulinaemic clamp compared with baseline values, standardized to the plateau clamp glucose concentration, as described in a study from our laboratory [26, 28, 29]. Body composition was estimated with the bioimpedance method using the formula of Lukaski [30]. All data of glucose metabolism are given as mg · min^{-1} · kg ffm^{-1}.

Muscle biopsy. As the induction of euglycaemia by an overnight insulin infusion could influence muscle substrate volumes at least theoretically, we deliberately chose (in both groups) to obtain the muscle biopsies on a second day in order to reflect habitual fasting conditions [31]. Thus, a percutaneous muscle biopsy (about 20–30 mg) was taken under local anaesthesia (2 % lidocaine without epinephrine) from musculus vastus lateralis of the leg 20 cm above the knee using an automatic procedure (Pro-mag Automatic Biopsy System, Manan Medical Products, Northbrook, Ill., USA). Biopsies were immediately fixed in buffered 2 % glutaraldehyde. The tissue was cut in smaller pieces, postfixed in osmium tetroxid block stained with uranyl acetate and imbedded in Epon. Semithin sections were cut out from two blocks and technically well preserved areas were selected by the technician for ultrathin sections. Ultrathin sections were grid stained with lead citrate and analysed in a Philips EM208 electron microscope. For each subject 12 pictures were taken randomly for morphometric analysis. The morphometric analysis was performed on blinded material by one of the authors. Initially, counts were done on 12 pictures in 4 subjects. It was found, however, that from 5 pictures onwards, pictures gave rise to variation in estimates not surmounting 10 % which is in accordance with previous stereological studies [32]. Consequently, 5 pictures were chosen for the rest of the subjects. Volume fractions of lipid, glycogen and mitochondria were estimated according to standard stereological principles by counting the points in a test system which hit the structure and dividing them by the total number of points hitting muscle fibers [32, 33]. The final magnification was 14 700 and the density of points was 2240 per micrograph.

Analytical procedures. Bedside plasma glucose (for adjustment of the variable glucose infusion during the clamp) was measured using a glucose oxidase method (Glucose Analyzer II, Beckmann Instruments, Fullerton, Calif, USA). Tritiated glucose activity was measured as previously described [22]. Plasma glucose was measured using a glucose dehydrogenase Method (Merck Diagnostica, Darmstadt, Germany) on an Axon-Technicon (Bayer-Diagnostics, München, Germany). Plasma insulin was analysed by a two-site, time-resolved immunofluorometric assay (DELFIA) [34]. Non-esterified fatty acids were measured by an enzymatic colorimetric method (Wako Chemicals GmbH, Neuss, Germany) as was triglyceride (Boehringer Mannheim – Diagnostica, Mannheim, Germany). Serum urea nitrogen was measured using an enzymatic method on an Technicon Axon (Technicon Instruments, Tarrytown, N. Y., USA). Urinary urea nitrogen was measured using an enzymatic method on a Cobas Mira S (Roche, Basle, Switzerland).

Statistical analysis. Differences in means between the groups or within groups were tested using unpaired or paired *t*-tests respectively (two-tailed). In the absence of normality, variables were logarithmically transformed to achieve normal distribution before statistical calculation. For correlation analysis Spearman's rho was employed. All analyses were performed with the SPSS computer programme, version 7.5 for Windows (SPSS, Chicago, Ill., USA). Data are presented as means ± SE or means (95 %-Confidence intervals) unless otherwise stated. A p value of less than 0.05 was considered significant.

Table 1. Clinical characteristics of the study subjects

Cohort	Type II diabetic subjects	Normoglycaemic obese subjects	*p*-value
Number (f/m)	11 (1/10)	11 (2/9)	NS
Age (years)	52.3 ± 1.7	53.6 ± 3.0	NS
Duration of diabetes (years)	3.3 ± 0.8		
BMI (kg/m^2)	31.8 ± 1.6	31.4 ± 1.4	NS
Weight (kg)	92.7 ± 3.9	95.7 ± 4.2	NS
FFM (kg)	57.0 ± 2.3	51.9 ± 2.4	NS
FM (kg)	35.7 ± 4.9	43.8 ± 2.8	NS
Fasting plasma glucose (mmol/l)	12.3 ± 1.2	5.6 ± 0.1	< 0.001
Fasting plasma NEFA (mmol/l)	0.65 ± 0.07	0.36 ± 0.03	< 0.005
Fasting plasma insulin (pmol/l)	62.7 (32.5–121.1)[a]	46.8 (34.3–64.1)[a]	NS
Fasting plasma triglyceride (mmol/l)	3.0 (1.7–5.3)[a]	1.1 (1.1–1.5)[a]	< 0.005
Fasting serum cholesterol (mmol/l)	6.3 (5.4–7.3)[a]	6.1 (5.7–6.6)[a]	NS
Fasting serum HDL-cholesterol (mmol/l)	1.1 (0.8–1.1)[a]	1.4 (1.1–1.6)[a]	< 0.01

[a] Logarithmically transformed

Results

Clinical and fasting laboratory characteristics of the subjects are summarized in Table 1. Plasma glucose concentrations at the clamp study were similar in the diabetic and obese normoglycaemic subjects during both the basal (5.8 ± 0.2 vs 5.9 ± 0.1 mmol/l, NS) and insulin stimulated (5.4 ± 0.2 vs 5.5 ± 0.1 mmol/l, NS) steady state period. Because of the overnight normalization of plasma glucose, basal plasma insulin increased in the diabetic subjects (182.3 ± 28.9 vs 43.1 ± 5.6 pmol/l, $p = 0.001$) but was more or less the same during insulin stimulation (384.0 ± 24.1 vs 369.1 ± 20.7 pmol/l, p = NS). As noted for plasma glucose, overnight insulin infusion resulted in identical basal NEFA concentrations (0.43 ± 0.08 vs 0.44 ± 0.06 mmol/l, p = NS) whereas clamp procedures revealed a reduced suppression of NEFA in the diabetic group (0.22 ± 0.04 vs 0.01 ± 0.0 mmol/l, $p < 0.001$).

During insulin stimulation, GINF, Rd, glucose oxidation, non-oxidative glucose metabolism, glycolytic flux and GS were significantly lower in the diabetic subjects. In contrast, lipid oxidation was significantly higher. All steady state rates of glucose and lipid metabolism are presented in Table 2. Resistance to the action of insulin was noted in the Type II diabetic subjects for Si_{RD} and Si_{GS} when these clamp metabolic data were expressed as rates of change per unit change in insulin, that is, insulin sensitivity indices (Table 2).

Morphometric analyses, electronmicroscopy. The ultrastructure of the muscle fibres appeared normal and there was no qualitative difference between Type II diabetic subjects and obese normoglycaemic subjects. Thus, in both groups, glycogen granules were found either in association with mitochondria or intermyofibrillary dispersed, which suggests that they could also be involved in anaerobic glycolysis [35, 36]. Lipid vacuoles were found almost exclusively in close contact with mitochondria and appeared more prominent both in number and size in the diabetic subjects. Examples of electron microscopic fields with intramyocellular triglyceride are shown in Figure 2A and examples of electron microscopic fields with glycogen are shown in Figure 2B. The morphometric analysis demonstrated an approximately 3.7-fold higher intramyocellular volume fraction of lipids in the diabetic subjects (1.43 ± 0.21 vs 0.39 ± 0.07 %, $p < 0.001$) while the volume fraction occupied by glycogen granules was approximately 2-fold larger in the obese normoglycaemic subjects

Table 2. Rates of glucose and lipid metabolic parameters during clamp studies and insulin sensitivity indices for Si_{RD} and Si_{GF}

	Type II diabetic subjects		Normoglycaemic obese subjects	
	Basal	Insulin	Basal	Insulin
Glucose infusion (GINF)		3.12 (2.21–4–42)		8.43 (7.06–10.1)[d]
Total glucose disposal (Rd)	3.78 ± 0.24	4.96 ± 0.49[a]	3.58 ± 0.15	10.35 ± 0.89[b, d]
Hepatic glucose output	3.66 ± 0.24	1.51 ± 0.31[b]	3.44 ± 0.16	1.70 ± 0.27[b]
Glucose oxidation	2.23 ± 0.21	2.41 ± 0.21	2.41 ± 0.41	5.42 ± 0.45[b, d]
Lipid oxidation	1.59 ± 0.19	1.48 ± 0.20	1.53 ± 0.23	0.51 ± 0.20[b, d]
Non-ox. glucose metab.	1.55 ± 0.22	2.56 ± 0.46[a]	1.17 ± 0.42	4.93 ± 0.74[b, c]
Glycolytic flux	2.58 ± 0.18	3.09 ± 0.18[a]	2.02 ± 0.10	3.76 ± 0.21[b, c]
Glucose storage (GS)		2.03 ± 0.50		6.59 ± 0.83[d]
Si_{RD}		4.31 ± 2.19		22.35 ± 3.22[d]
Si_{GS}		11.98 ± 2.39		21.80 ± 3.40[c]

Steady-state rates of metabolic parameters are expressed as mg · min^{-1} · kg ffm^{-1}

Indices of insulin sensitivity are expressed as μl · min^{-1} · kg ffm^{-1} per pmol/l

[a] $p < 0.05$ vs basal values, [b] $p < 0.001$ vs basal values, [c] $p < 0.05$ vs diabetic subjects, [d] $p < 0.001$ vs diabetic subjects

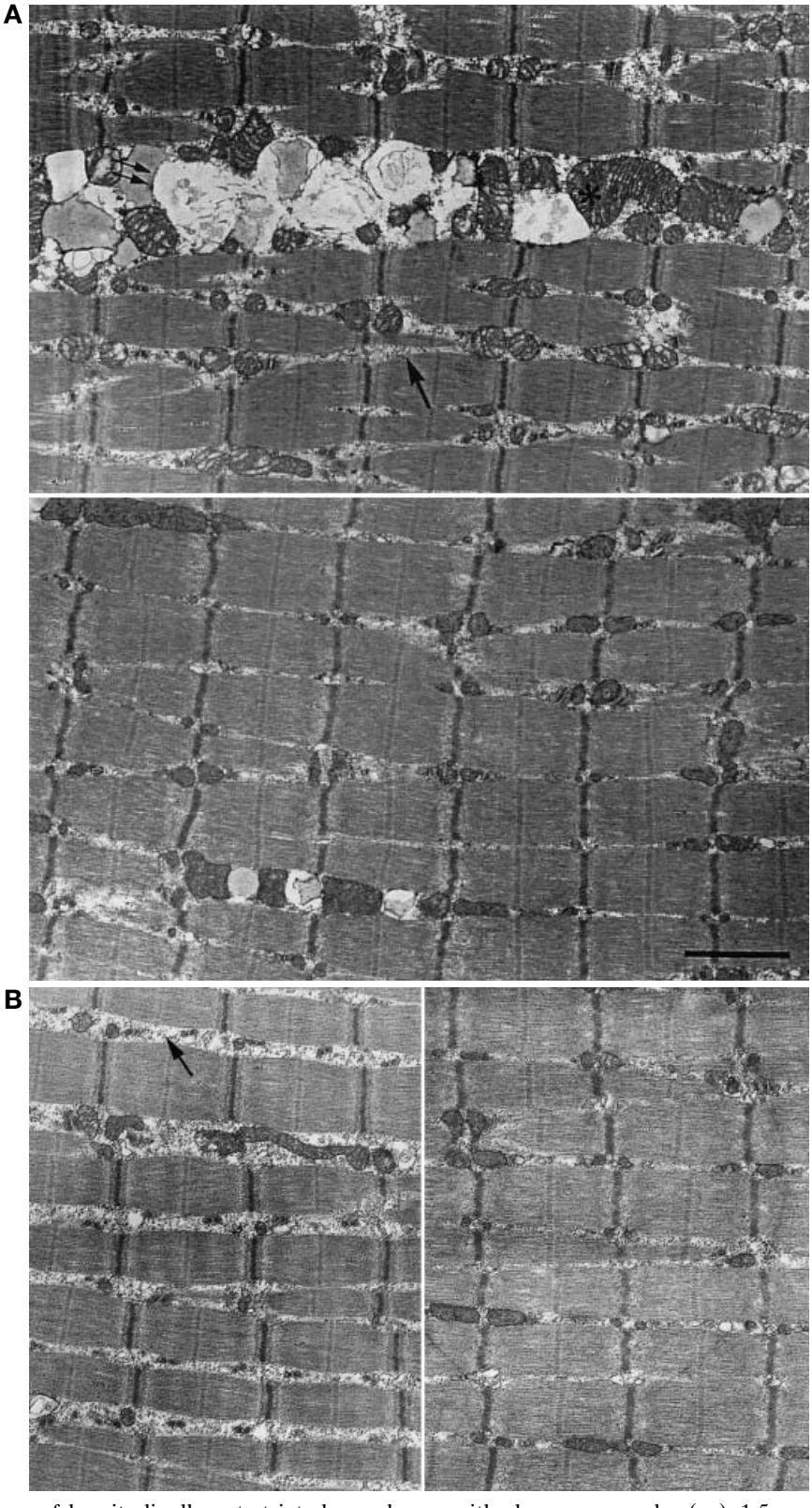

Fig. 2 A. Ultrastructure of longitudinally cut striated muscle from a Type II diabetic subject (upper) and a normoglycaemic obese subject (lower). Prominent lipid vacuoles are seen in the diabetic muscle. Mitochondria morphology in the two pictures are similar. Lipid droplets (⇉), mitochondria (*), area with glycogen granules (→), 1.5 my-meter (—). **B** Ultrastructure of longitudinally cut striated muscle from a Type II diabetic subject (left panel) and a normoglycaemic obese subject (right panel). Glycogen granules (→)

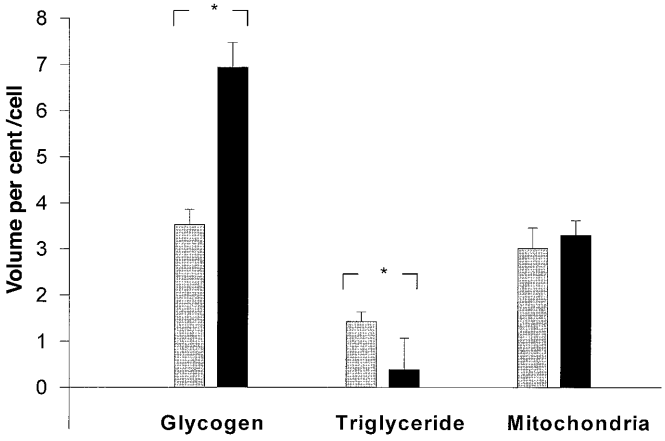

Fig. 3. Intramyocellular volume fractions of glycogen, triglyceride and mitochondria.: Diabetic cohort (▒), normoglycaemic obese cohort (■). *$p < 0.001$

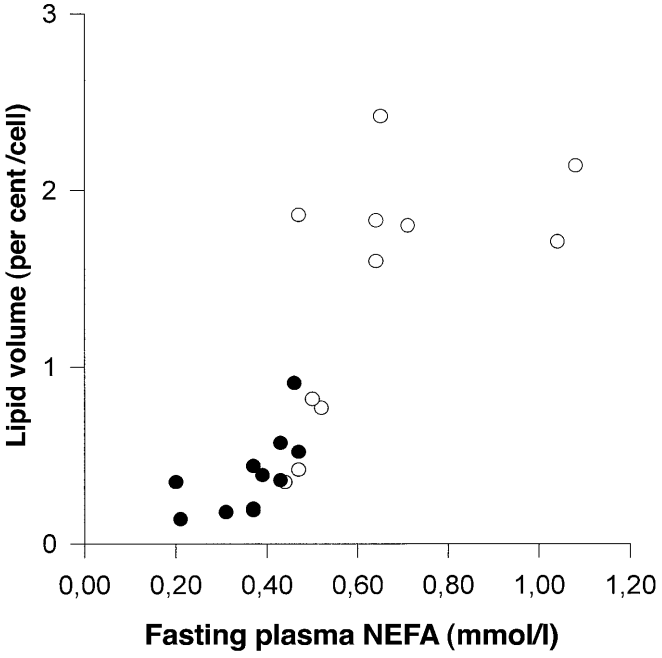

Fig. 4. Relation between fractional intramyocellular lipid volume and the fasting level of NEFA: Diabetic cohort (○), normoglycaemic obese cohort (●)

(3.53 ± 0.33 vs $6.94 \pm 0.54\%$, $p < 0.001$). Mitochondria volume fraction was identical in the two groups (3.01 ± 0.45 vs 3.30 ± 0.32, p = NS), (Fig. 3).

Correlation analysis showed a significant inverse relation between intramyocellular lipid content and Si_{Rd} ($r = -0.71$, $p < 0.02$) as well as SI_{GS} ($r = -0.6$, $p = 0.05$) in the diabetic group whereas no association could be detected in the obese normoglycaemic group ($r = -0.16$, p = NS) and ($r = -0.15$, p = NS, respectively). There were no significant correlations between intramyocellular lipid and BMI ($r = 0.39$, $p = 0.24$ and $r = 0.47$, $p = 0.15$) and fat mass percentage ($r = 0.35$, $p = 0.3$ and $r = 0.27$, $p = 0.42$).

In both the diabetic and obese normoglycaemic group significant positive associations were found between intramyocellular and circulating lipids, that is for fasting NEFA ($r = 0.63$, $p = < 0.05$ and $r = 0.79$, $p = < 0.05$), (Fig. 4) and for TG ($r = 0.74$, $p = 0.01$ and $r = 0.62$, $p < 0.05$).

Discussion

Patients with Type II diabetes have defects in both lipid and carbohydrate metabolism. This study clearly shows that fat accumulates intramyocellulary in big droplets of triglycerides which are located in close proximity to the mitochondria. Moreover, as previously shown in obese non-diabetic subjects [15], the extent of the intramyocellular fat accumulation was related to the degree of insulin insensitivity, in particular Rd and GS, in our obese diabetic subjects. Furthermore, by means of electron microscopy, we provide morphometric documentation for a simultaneous reduction of the amount of intramyocellular glycogen.

In this study we compared obese Type II diabetic patients with obese normoglycaemic subjects. These data are consistent with our earlier studies on insulin stimulated Rd, GS, and lipid and glucose oxidation published by our group for obese normoglycaemic and diabetic subjects [37–39] Thus, the mean glucose infusion rates during the hyperinsulinaemic euglycaemic clamp studies in the present obese normoglycaemic and Type II diabetic subjects and in the three cited studies were 8.4, 8.3 [37],11.4 [38] and 9.2 [39] and 3.1, 3.6 [37], 4.8 [38] and 5.0 $mg \cdot min^{-1} \cdot kg\ ffm^{-1}$ [39], respectively. The mean infusion rate for younger lean subjects was 12.2 $mg \cdot min^{-1} \cdot kg\ ffm^{-1}$ [37]. However, the fasting NEFA concentrations seen in the present obese Type II diabetic and normoglycaemic subjects were somewhat lower than generally observed in similar groups. The reason for this discrepancy is not clear. A major component of the insulin-resistant state noted in Type II diabetes when compared with normoglycaemic obese subjects has been attributed to a "metabolic" insulin resistance or "secondary" insulin resistance due to the various metabolic abnormalities present in the diabetic state itself, such as hyperglycaemia, hyperinsulinaemia and hyperlipidaemia, especially the increased NEFA and triglyceride concentrations [2, 40]. This does not exclude the possibility that a component of an inherited metabolic defect could also contribute to the altered metabolic state found in obese Type II diabetic subjects [41]. Obese diabetic subjects have been shown to accumulate almost four times more triglyceride than normoglycaemic obese subjects. It is therefore possible that the excess triglyceride accumulation could be related to the diabetic state itself and/or the presence of inherited insulin resistance. This is under-

scored by the fact that our obese normoglycaemic subjects seemed to be carrying more fat than our Type II diabetic subjects despite the close matching of BMI ratios.

The contrasting finding of a significant reduction of the glycogen content in skeletal muscle (about 50 %) is surprising given the previous biochemical estimates of glycogen concentrations in skeletal muscle [42, 43]. It is however compatible with data on reduced glycogen synthase activity and glycogen synthesis rate in Type II diabetic patients and their relatives which in turn could be secondary to upstream defects in glucose transport and/or phosphorylation [40, 41; 44–47]. Similar reductions in intramuscular glycogen content have also demonstrated in another study on Type II diabetic subjects using the NMR-technique which measures glycogen content in vivo [48]. More recently, using a specific biochemical extraction method using amyloglucosidase enzymatic degradation of the α-1–4 and α-1–6 glucosidic bonds of glycogen rather than the non-specific technique of acid hydrolysis, investigators showed a significant reduction in muscle glycogen concentration in a group of markedly insulin resistant growth hormone deficient subjects [42, 49, 50]. This discrepancy between the earlier biochemical estimations of intramuscular glycogen [42, 43] and the NMR [48] and our current data could be because the previous biochemical estimations were not sufficiently precise to establish the presence of a difference between intramuscular glycogen content in diabetic and control subjects. (We have now found intramuscular glycogen content to be significantly reduced in insulinopenic modestly hyperglycaemic diabetes in both human beings and dogs. Manuscript in preparation; Christopher, Ward, Alford et al.). Thus, the present morphometric data and the NMR findings seem to indicate that skeletal muscle glycogen content is reduced in Type II diabetes.

The biochemical explanation for increased storage of triglyceride and reduced storage of glycogen is not clear. Subjects with Type II diabetes are characterized by a compensatory hyperglycaemia and hyperinsulinaemia which could lead to normal or slightly increased skeletal muscle glucose uptake because of the mass action of hyperglycaemia itself [51]. The hyperglycaemia results in increased glucose oxidation and reduced fat oxidation across the leg (which consists mainly of skeletal muscle) in obese diabetic subjects [9]. Thus, intramyocellular glucose and NEFA are probably linked in Type II diabetes in such a way that the rate of cellular glucose processing leads to reduced skeletal muscle NEFA oxidation despite normal or increased NEFA uptake by muscles [9, 52]. Non-esterified fatty acids are then believed to be channelled preferentially into triglyceride instead [9]. How this accumulation of triglyceride leads to insulin resistance is not known but one proposal is that increased concentrations of diacyl glycerol (DAG), following the accumulation of muscle triglyceride and long-chain acyl CoA, could lead to increased protein kinase C (PKC) activity with its attended suppression of the insulin action cascade by serine phosphorylation [53, 54] Alternatively, glucose transport activity and glycogen synthesis could be reduced by a direct effect of increased intramyocellular NEFA concentrations (or some NEFA metabolite) on PI-3-kinase activity [55; 56]. Regardless of the exact mechanisms responsible for the fat accumulation, our data clearly shows significant inverse correlations between Si_{RD} and Si_{GS} and the amount of intramyocellular lipid content in skeletal muscle in Type II diabetes. These associations between intramyocellular lipid accumulation and Si_{RD} and Si_{GS} were not, however, evident in the obese normoglycaemic cohort. This could indicate that the intramyocellular lipid accumulation is metabolically important only in diabetic subjects, possibly because of the attended insulin resistance and decrease in activity of the insulin action cascade in muscle. Alternatively, it could indicate that the relation between Si and intramyocellular lipid is present in obese normoglycaemic individuals but cannot be shown because of the small range of scatter of the Si and/or intramyocellular lipid values. Interestingly, the relation between intramyocellular lipid and circulating lipids (NEFA and triglyceride) held for both obese normoglycaemic and obese diabetic subjects.

It could be argued that our whole body calorimetry data which showed identical basal values for lipid oxidation in the two cohorts after the overnight insulin infusion but reduced acute suppression of lipid oxidation in the Type II diabetic group during the hyperinsulinaemic phase of the clamp studies, are inconsistent with our hypothesis of reduced fat oxidation in skeletal muscle. However, a strong cautionary note needs to be added. Whole body calorimetry measurements represent a composite value for liver, adipose tissue and brain as well as skeletal muscle. As has been pointed out, it is not possible to interpolate muscle substrate utilization from whole body measurements [9].

An inverse relation between insulin sensitivity and muscle triglyceride content has previously been shown in normoglycaemic Pima Indians [15], in relatives of Type II diabetic subjects and in subjects without a family history of diabetes [17, 19, 20]. However, a NMR study [57] comparing normoglycaemic European and South Asian men suggested a role of ethnicity and/or other mechanisms for the pathogenesis of intramyocellular lipid accumulation. The authors failed to find a relation between insulin sensitivity and intramyocellular lipid in normoglycaemic South Asians, even though total intramyocellular lipid content and insulin resistance were higher in this cohort. In our study, we too did not find a relation between Si and intramyocellular lipid content in the obese nor-

moglycaemic control cohort. The reason for this is not clear but, as mentioned above, could be related to sample size and/or the narrow range of intramyocellular lipid deposits in the normoglycaemic obese group or activity status of the insulin action cascade. In both the obese normoglycaemic and diabetic cohort we were able, however, to show a positive association between the intramyocellular lipid content and plasma NEFA and triglyceride which is consistent with other studies, including an NMR study which showed fasting NEFA to be an independent predictor of intramyocellular lipid [16, 17]. Moreover, the insulin-resistant relatives previously studied had higher NEFA concentrations [19, 20]. Thus, the NMR studies and the present data, together with previous studies on pre-diabetic subjects suggest that circulating lipids do have an important role for intramyocellular lipid accumulation which in turn could contribute to the development of insulin resistance in the diabetic subjects [3, 4].

Another important issue is the question of accumulation of adipocytes in skeletal muscle (i. e. the extramyocellular component). Adipocytes are encountered only occasionally along the fascia and in the connective tissue of normal muscle and, in the current study, where approximately 500–2000 fibres were counted, no extramyocellular lipid component was found in any of the biopsies [36]. This contrasts with the findings using the NMR technique in which a considerable extramyocellular component has been shown in the calf muscle [17, 19]. There is no ready explanation for this difference but it might be explained partly by the orientation-dependent features of the muscle spectrum which could have reduced the separation between extramyocellular and intramyocellular lipid. In addition, the increased volume of muscle required for NMR measurements could have led to an incorporation of extramyocellular lipid components [58]. We cannot, however, exclude the possibility that some alteration of muscle ultrastructure occurred after osmium fixation leading to a shrinkage in fibre volume or its components and a distortion of in vivo lipid deposits [32].

Increased intramyocellular volume fractions of mitochondria and triglyceride have been shown in both humans after endurance training and in animals adapted to different levels of aerobic performance [35; 59–61]. It is known that larger volumes of mitochondria and triglyceride are seen also in type 1 fibres when compared with type 2 (2A and 2B) muscle fibres [36]. In our study, however, all subjects were characterized by a sedentary lifestyle and mitochondrial volume fractions in the two cohorts were almost identical. Furthermore, intramyocellular triglyceride content, measured by a similar stereological method in untrained young lean subjects was similar to the intramyocellular triglyceride content in our obese older normoglycaemic subjects [60]. This would argue against obesity itself directly influencing intramyocellular triglyceride content.

In summary, this study shows that Type II diabetic patients are prone to store fat intramyocellulary in skeletal muscle and that this might be associated with a simultaneous reduction in glycogen storage and enhanced insulin resistance of key skeletal muscle glucose metabolic pathways. Quantitatively, a major component of the excessive lipid content seems to be secondary in origin, related to the diabetic state itself, although a contribution from an inherited metabolic defect cannot be excluded. Our study suggests that the concentration of circulating lipid does play an important pathogenic role for intramyocellular lipid accumulation in both diabetic and obese normoglycaemic subjects.

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