Intramuscular Triglyceride and Muscle Insulin Sensitivity: Evidence for a Relationship in Nondiabetic Subjects

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Intracellular triglyceride (TG) is an important energy source for skeletal muscle. However, recent evidence suggests that if muscle contains abnormally high TG stores its sensitivity to insulin may be reduced, and this could predispose to type II diabetes. To test this hypothesis, we measured muscle lipid content in 27 women aged 47 to 55 years (mean, 52) and related it to their glucose tolerance, insulin resistance, and muscle insulin sensitivity as measured by insulin activation of glycogen synthase, an insulin-regulated enzyme that is rate-limiting for insulin action in muscle. Both muscle TG content and intracellular lipid determined by Oil red O staining of muscle fibers were negatively associated with glycogen synthase activation ($r = .43$, $P = .03$ and $r = -.47$, $P = .02$, respectively). In addition, intracellular lipid correlated with features of the insulin resistance syndrome, including an increased waist to hip ratio ($r = .47$, $P = .01$) and fasting nonesterified fatty acids ([NEFA] $r = .44$, $P = .04$). These data demonstrate that increased muscle TG stores are associated with decreased insulin-stimulated glycogen synthase activity. Intracellular fat may underlie a major part of the insulin resistance in normal subjects, as well as type II diabetics.

SKELETAL MUSCLE contains large amounts of intracellular triglyceride (TG), which provides an important and readily available energy source with an overall caloric value exceeding that of glycogen stores.[1] However, recent evidence suggests that if muscle contains abnormally high TG stores its sensitivity to insulin may be reduced. Most of the evidence derives from animal experiments that show that a high-fat diet leads to accumulation of TG in muscle, and that the insulin sensitivity of skeletal muscle correlates inversely with muscle TG concentration.[2] In man, evidence for such a link is restricted to the observation of a sixfold excess of TG concentrations in the muscle of subjects with type II diabetes.[3] It is suggested that this linkage is mediated via the glucose–fatty acid cycle, whereby endogenous muscle TG supplies high local concentrations of fatty acids that directly inhibit glucose uptake and utilization.[4] Increased muscle TG would decrease insulin sensitivity and thus predispose to type II diabetes.

However, it is not clear whether the accumulation of intracellular TG is a consequence of the metabolic derangements in type II diabetes or whether there is a physiological link between muscle TG stores and insulin sensitivity. If an association between muscle TG and insulin sensitivity could be demonstrated in normoglycemic subjects, this would be important evidence of a physiological link. The present study was therefore designed to test the hypothesis that high intramuscular TG is associated with impaired insulin action in vivo as measured by whole-body insulin sensitivity and by the ability of insulin to activate glycogen synthase, an insulin-regulated enzyme that is rate-limiting for insulin action in muscle. We tested this hypothesis in a group of women with normal glucose tolerance for whom we have data on the body size at birth and the degree of adult obesity, both of which are important for insulin resistance, enabling us to determine whether one or both of these factors are associated with high intramuscular TG.[5] In this study, muscle TG was assessed by extraction from muscle biopsies and by specific staining to determine intracellular fat.

SUBJECTS AND METHODS

Subjects

The subjects were born in Sharoe Green Hospital (Preston, Lancashire) and still live in and around Preston. They comprise part of an epidemiological study to determine the relationships between fetal growth and subsequent type II diabetes. We have previously shown in this group that low birthweight, or more specifically, thinness at birth, predicts hypertension, glucose intolerance, and insulin resistance independently of the level of adult obesity.[5,6]

Of 266 subjects who had oral glucose tolerance tests, we selected a sample of 40 normoglycemic women to encompass the range of birth and placental weights. Their insulin resistance was measured with a short insulin tolerance test, during which insulin resistance was estimated by the half-life in minutes of the decrease in blood glucose concentration after administration of a bolus of insulin (0.05 IU/kg).[7] Twenty-seven (68%) agreed to undergo a gastrocnemius muscle biopsy. Following an overnight fast, the women consumed a standard meal containing 30 g carbohydrate. One hour later, a biopsy of 100 to 200 mg was obtained from the medial head of the gastrocnemius muscle under local anesthesia (1% lidocaine) using the conchotome technique. The biopsy was trimmed of fat and connective tissue and either immediately frozen in liquid nitrogen (biochemical studies) or transported in buffer (histological studies) before being frozen in liquid nitrogen. There was insufficient muscle for biochemical analysis in one subject.

Height was measured with a portable stadiometer (CMS Weighing Equipment, Camden, London) and weight with a digital scale (SECA, Birmingham, UK) with the subjects wearing light clothing. Body mass index was calculated as weight in kilograms divided by

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height in meters squared. Skinfold thicknesses were measured by a single observer with Harpenden skinfold calipers at the biceps, triceps, subscapular, and suprailiac sites. Body fat percentage was computed by the method of Durnin and Wormsley.[8] Waist circumference was measured at the level of the umbilicus and hip circumference at the level of the greater trochanters. The waist to hip ratio was calculated as an index of central obesity.

Analyses

Plasma glucose level was measured by the hexokinase method and insulin by two-site immunometric assays.[9] Plasma nonesterified fatty acids (NEFA) were determined enzymatically based on acyl-coenzyme A synthetase activity (Boehringer Mannheim, Lewes, Sussex, UK), with the resultant acyl-coenzyme A being oxidized to yield hydrogen peroxide, which was measured colorimetrically. The assay had a between-assay coefficient of variation of 10% at 0.4 mmol/L and 6% between 1.2 and 1.3 mmol/L.[10]

Plasma TG level was measured using the RA 1000 autoanalyzer (Bayer Diagnostics, Basingstoke, Hants, UK) with a standard enzymatic method. Muscle samples were transferred from liquid nitrogen to glass tubes containing 5 mL chloroform/methanol (2:1 vol/vol) and butylated hydroxytoluene (50 mg/mL). After extraction of lipid,[11] the organic phase was removed and the remaining solid material dissolved in 1 mL NaOH (1 mol/L) before protein assay.[12] The organic phase from the extraction was concentrated and lipid species separated by thin-layer chromatography. The TG spot was scraped into a glass tube, and TG was eluted with 5 mL chloroform/methanol (2:1 wt/vol) and then evaporated to dryness under N_2. It was then saponified in ethanolic KOH (0.5 mL, 2.8% wt/vol) at 70°C for 1 hour and neutralized with $MgSO_4$ (0.5 mL, 0.3 mol/L), and glycerol in the supernatant was estimated by the method of Humphreys et al.[13] The overall coefficient of variation of this assay estimated on replicate samples from a pool of muscle powder was 12% to 13%.[14]

Cross-sections of 8-μm muscle sections were cut with a cryotome at −20°C. Sections were stained for fat with Oil red O.[15] The biopsies were assessed for lipid content by a semiquantitative method. An observer blinded to the clinical state of the subjects graded 100 consecutive fibers in each biopsy into one of five categories defined with reference to a series of standard photographs. A histological lipid score was calculated as the mean lipid content weighted by the number of fibers in each of five categories scored from 0 to 4, where the fat score = $\Sigma i \cdot n_i/\Sigma n_i$, i = 0 to 4, and n_i is the number of fibers in category i. Sections were also stained for myosin adenosine triphosphatase activity after preincubation at pH 4.3, 4.5, and 9.4. Approximately 500 fibers were counted in each biopsy and classified as slow-twitch (type 1) and fast-twitch (type 2) fibers. Glycogen synthase activity was assayed by the method of Thomas et al,[16] where 1 U activity is the amount of enzyme that incorporates 1 nmol [^{14}C]glucose from UDP-[^{14}C]glucose into glycogen per minute. The initial level of activity was determined under pseudophysiological conditions (0.1 mmol/L glucose-6-phosphate), the total activity was assayed in the presence of 10 mmol/L glucose-6-phosphate, and the fractional activity was calculated as initial/total.

Statistics

Measurements of glucose, insulin, NEFA, and biopsy TG content (referred to subsequently as muscle TG content) were transformed to normality using logarithms. The biopsy lipid score (referred to subsequently as intracellular lipid) was normalized using a square-root transformation. Statistical significance based on the least-squares correlation coefficient for continuously distributed variables and *t* tests for categorical variables were assessed using two-sided tests.

RESULTS

The 27 women were aged 47 to 55 years (mean, 51.6). They had a mean birthweight of 3.2 kg (range, 2.3 to 4.15) and a mean placental weight of 0.6 kg (range, 0.4 to 0.8). Their mean body mass index was 24.8 kg/m^2 (range, 19.2 to 31.9), waist to hip ratio 0.78 (range, 0.66 to 0.95), and body fat percentage 37.7% (range, 30.4% to 45%). In this group of subjects, insulin resistance as assessed by the short insulin tolerance test was related to the ponderal index at birth ($P = .04$) and the waist to hip ratio ($P = .05$), but not significantly to the body mass index or body fat percentage.

Intracellular lipid assessed histologically ranged from a score of 0 to 1.26 (mean, 0.18) and correlated significantly ($r = .43$, $P = 0.02$) with muscle TG content, which ranged from 2.6 to 404.4 μmol/g protein (mean, 33.9). Figure 1A and B shows that intracellular lipid and muscle TG content are negatively associated with glycogen synthase activation ($r = -.43$, $P = .03$ and $r = -.47$, $P = .02$, respectively).

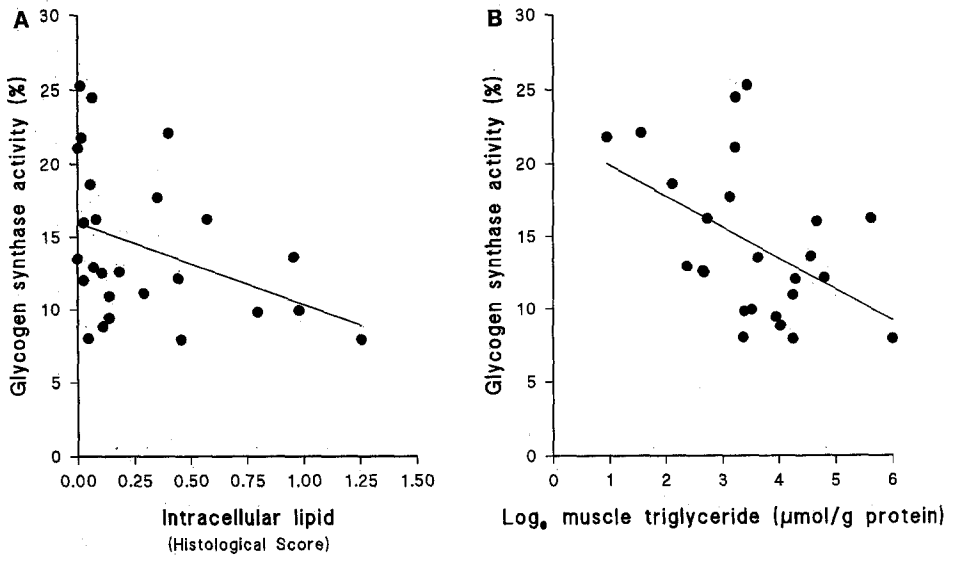

Fig 1. Correlation between glycogen synthase activity and (A) intracellular lipid or (B) muscle TG content.

Table 1 shows that intracellular lipid content correlated with central obesity as measured by the waist to hip ratio ($r = .47$, $P = .01$) and fasting NEFA ($r = .44$, $P = .04$), but not with the body mass index, percentage body fat, biceps, triceps, subscapular, or suprailiac skinfold thicknesses, 2-hour plasma glucose during the oral glucose tolerance test, fasting insulin, TG, or insulin resistance as assessed by the short insulin tolerance test. Although biopsies with a higher proportion of type 1 fibers tended to have more intracellular lipid, this did not reach significance ($r = .34$, $P = .08$). Muscle TG content measured biochemically did not correlate with the variables listed in Table 1.

Table 2 shows intracellular lipid and muscle TG content according to measurements of body size at birth. We used the same divisions of birth measurements as in previous analyses of data from Preston.[5] Women with low birthweight had lower intracellular lipid and TG content. Intracellular lipid and TG contents were not significantly related to the ponderal index, placental weight, or length. They also were not related to the ratio of head circumference to length, which we have previously used to define babies as disproportionately short.[17] However, intracellular lipid and muscle TG contents were lower in adults with a small head circumference at birth.

DISCUSSION

These results show that the accumulation of intracellular muscle lipid in normoglycemic women is associated with features of the insulin resistance syndrome, including central obesity, high fasting NEFA, and reduced insulin activation of glycogen synthase, but not with whole-body insulin resistance as indicated by the fasting insulin level or the insulin tolerance test. Muscle TG content measured biochemically correlated with glycogen synthase activation, but not with glucose tolerance, insulin resistance, central obesity, or NEFA levels (Table 1), possibly because the TG content of the biopsy reflects the amount of interstitial and intracellular fat, which may be deposited as a result of different metabolic processes.

The reasons for the TG accumulation in muscle are unknown. The current study suggests that there are large variations in muscle fat content between subjects that are not explained and do not correlate with the overall level of obesity as indicated by the body mass index. Our results are in agreement with previous studies that show that type 1 muscle fibers have higher intracellular TG content than type 2 fibers.[18] However, we found that increased intracellular lipid was associated with an increased tendency to central obesity as indicated by a high waist to hip ratio and with increased NEFA, suggesting that the intracellular accumulation of lipid could be a result of increased flux of NEFA into muscle from an expanded fat mass in the abdominal area.

Our finding that increased intracellular lipid is associated with reduced glycogen synthase in normoglycemic subjects suggests a pathogenic role for this intracellular fat. Several previous studies have shown that central obesity is related to reduced glycogen synthase activation. The mechanism is unknown, but Felber et al[19] have suggested that muscle oxidation of NEFA leads to accumulation of glycogen, inhibition of glycogen synthase phosphatase, and phosphorylation of glycogen synthase. Our observations are consistent with this hypothesis, but suggest that the immediate availability of fatty acids from hydrolysis and β-oxidation of intracellular TG could be a more immediate and thus more potent source of substrate than circulating NEFA, as suggested originally by Randle et al.[20]

The lack of correlation between in vivo whole-body insulin sensitivity and intramuscular TG content is unlikely to be due to the method of measuring insulin sensitivity. Measurement of insulin sensitivity in this study depended on the short insulin tolerance test, a method of measuring insulin sensitivity that correlates highly ($r = .81$ to $.86$) with estimates of insulin sensitivity using a euglycemic clamp, widely accepted as the reference method.[21-23] We have also demonstrated that this test has adequate reproducibility.[7] In any group of normoglycemic subjects, the range of insulin sensitivity is less than if subjects with impaired glucose tolerance or non–insulin-dependent diabetes mellitus (NIDDM) are included. This would make a demonstration of a true relationship difficult, since even though there is a wide range of insulin sensitivity in people, inclusion of

Table 1. Correlations Between Anthropometric and Biochemical Measurements and Intracellular Lipid Assessed Histologically or Muscle TG Content in 27 Subjects

Measure	Intracellular Lipid (histological score)	Muscle TG Content (μmol/g protein)
Body mass index (kg/m²)	.01	−.16
Body fat (%)	.16	−.05
Waist to hip ratio	.47*	.15
120-min plasma glucose (mmol/L)	.33	.26
Fasting insulin (pmol/L)	−.07	−.38
Insulin resistance ($1/t_{1/2}$, min)	−.01	.06
Fasting serum TG (mmol/L)	.09	.22
Fasting NEFA (μmol/L)	.44*	.25
Type 1 fibers (%)	.34	.00

*$P < .05$.

Table 2. Mean Intracellular Lipid and Muscle TG Content According to Birth or Placental Weight or Body Size at Birth

Measure	Intracellular Lipid (histological score)	Muscle TG Content (μmol/g protein)
Birthweight (kg)		
<3.4	0.10 (15)	22.0 (15)
>3.4	0.32 (12)*	61.3 (11)*
Placental weight (kg)		
<0.7	0.18 (15)	33.5 (14)
>0.7	0.18 (12)	34.5 (12)
Ponderal index (kg/m³)		
<25	0.20 (18)	35.1 (17)
>25	0.14 (9)	31.8 (9)
Head circumference (cm)		
<35.6	0.11 (13)	17.5 (13)
>35.6	0.26 (14)*	65.8 (13)*
Length (cm)		
<50.8	0.19 (15)	28.5 (15)
>50.8	0.17 (12)	42.9 (11)

NOTE. Number of subjects is in parentheses. *$P < .05$.

impaired glucose tolerance or NIDDM subjects markedly increases the number of insulin-resistant subjects. Previous observations suggest markedly increased muscle TG levels in NIDDM subjects, and such subjects have low insulin sensitivity.[3] Thus, it would be anticipated that a correlation may be demonstrated in a group that included both NIDDM and normal glucose tolerance subjects. A further point to be considered is that liver and adipose tissue influence the in vivo assessment of insulin sensitivity, whereas muscle TG levels are likely to affect muscle metabolism only. It is in the latter context that the observed correlation between insulin activation or muscle glycogen synthase and intramuscular TG content is most relevant. It is known that TG content of skeletal muscle is extremely variable both within any one muscle and between different muscles.[14] The failure to demonstrate a correlation could therefore be a result of poor correlation between the biopsy lipid content and the overall skeletal muscle lipid content in the subjects, which would determine insulin sensitivity. This point could be resolved either by a larger study or by the use of imaging techniques to measure muscle lipid content that have shown correlations with insulin sensitivity.[24]

A paradoxical finding of this study is that subjects who had low birthweight or who were small at birth as indicated by a small head circumference had low concentrations of muscle TG and reduced muscle lipid. The reason is unclear. However, it is possible that the mechanism by which small babies become insulin-resistant and glucose-intolerant may differ from the mechanisms involved in insulin resistance associated with central obesity and intracellular lipid accumulation. If this were the case, the lack of association between glucose tolerance or whole-body insulin sensitivity and intracellular lipid content in this study could be explained, because the processes causing insulin resistance in low-birthweight subjects would reduce the lipid content, whereas in centrally obese subjects the increased supply of NEFA to muscle would increase it.

These data demonstrate for the first time that increased muscle TG stores are associated with decreased insulin-stimulated muscle glycogen synthase activity in normoglycemic subjects. Further studies are required to determine whether this physiological association is causative, and whether the pathological excess of muscle TG underlies a major part of the insulin resistance in type II diabetes.

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