Preliminary experience with a new stable isotope breath test for chylomicron remnant metabolism: a study in central obesity

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ABSTRACT

We aimed to investigate the metabolism of chylomicron remnants in the postabsorptive state employing a new stable isotope breath test in centrally obese men without overt hyperlipidaemia. Groups of 12 centrally obese and 12 non-obese men of similar age and with similar plasma cholesterol and triacylglycerol (triglyceride) levels were studied. The catabolism of chylomicron remnants was measured using an intravenous injection of a remnant-like emulsion containing cholesteryl [13C]oleate. Isotopic enrichment of 13CO2 in breath was determined using isotope-ratio mass spectrometry, and a multi-compartmental model (SAAM II program) was used to estimate the fractional catabolic rate (FCR) of the chylomicron remnant-like particles. The plasma concentrations of low-density lipoprotein (LDL)-cholesterol, non-high-density lipoprotein (HDL)-cholesterol and insulin were significantly higher (P < 0.05) in the obese than the control subjects. The obese subjects had significantly lower HDL-cholesterol (P < 0.05) and, in particular, a decreased FCR of the remnant-like particles compared with lean subjects (0.061 ± 0.014 and 0.201 ± 0.048 pools/h respectively; P = 0.016). In the obese group, the FCR of remnant-like particles was inversely associated with the waist/hip ratio, and with plasma triacylglycerol, cholesterol, LDL-cholesterol and non-HDL-cholesterol levels. In multiple regression analysis, the waist/hip ratio was the best predictor of the FCR of the emulsion. In conclusion, this new test suggests that postabsorptive chylomicron remnant catabolism is impaired in centrally obese subjects without overt hyperlipidaemia. This defect may be due to the degree of adiposity.

INTRODUCTION

The precise reason for the increased risk of cardiovascular disease in central obesity remains unclear, but may relate to dyslipoproteinaemia [1]. Hypertriglyceridaemia is the most consistent lipid disorder in central obesity, but its status as a risk factor for coronary disease is dependent on whether it reflects elevated plasma concentrations of triacylglycerol (triglyceride)-rich lipoproteins (TRLs) that are potentially atherogenic [2]. The notion that the postprandial accumulation in plasma of chylomicron and very-low-density lipoprotein (VLDL) remnants is

Key words: breath test, chylomicron remnants, obesity, stable isotope.

Abbreviations: apoE (etc.), apolipoprotein E (etc.); BMI, body mass index; CETP, cholesteryl ester transfer protein; FCR, fractional catabolic rate; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; LDL, low-density lipoprotein; NEFA, non-esterified fatty acid; TRL, triacylglycerol (triglyceride)-rich lipoprotein; VLDL, very-low-density lipoprotein; WHR, waist/hip ratio.

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causally related to the development of atherosclerosis is now well supported by experimental, genetic and clinical studies [3–5].

The metabolism of postprandial lipoproteins is controlled by genetic, environmental and hormonal factors [5]. Insulin exerts a powerful homeostatic effect postprandially by enhancing the clearance of TRLs by both endothelial-bound lipoprotein lipase and hepatic lipoprotein receptors, as well as by decreasing the hepatic output of VLDL particles [6,7]. There are reports of postprandial dyslipidaemia in subjects with central obesity [8–10] and with Type II diabetes [11,12], the magnitude and duration of the lipoprotein responses being directly related to the fasting plasma triacylglycerol concentration. The kinetic defects involved may be due to oversecretion of intestinally derived lipoproteins and/or a reduction in the clearance of these lipoproteins and their remnants, but these mechanisms have not yet been specifically identified. Whether kinetic defects in postprandial lipoproteins, particularly chylomicron remnants, occur in the absence of hypertriglyceridaemia in obesity also remains to be examined.

Postprandial lipaemia may be quantified by measuring the response of plasma triacylglycerol, retinyl esters or apolipoprotein B-48 (apoB48) to a standardized oral fat load, but none of these tests provides an accurate estimate of chylomicron remnant metabolism [5,13,14]. Chylomicron remnant particles are removed from plasma by the liver by a process involving the binding of apolipoprotein E (apoE) to heparan sulphate proteoglycans, hydrolysis by hepatic lipase and lipoprotein lipase, and finally endocytosis mediated by the low-density-lipoprotein (LDL) receptor and the LDL-receptor-related protein [15]. The function of this pathway has not yet been examined in humans, and particularly not in subjects with visceral obesity and insulin resistance.

We have previously described and validated a ‘breath test’ [16], based on the injection of a remnant-like emulsion labelled with cholesteryl [13C]oleate and subsequent measurement of [13C]O2 enrichment in breath, that provides a functional assessment of chylomicron remnant metabolism in the postabsorptive state [17–20]. In a set of early studies, we have employed this technique to address whether centrally obese subjects with normal plasma cholesterol and triacylglycerol concentrations have a kinetic defect in the catabolism of chylomicron remnants, and whether this is related to the degree of visceral adiposity and insulin resistance.

**MATERIALS AND METHODS**

**Subjects**

We studied 12 men with central obesity and 12 age- and sex-matched non-obese subjects. All subjects were normolipidaemic, with plasma triacylglycerol < 2.0 mmol/l and cholesterol < 6.0 mmol/l [21] while consuming *ad libitum*, weight-maintaining diets. Central obesity was defined as waist circumference > 94 cm, waist/hip ratio (WHR) > 1.0 and body mass index (BMI) > 29 kg/m² [22]. The non-obese group was defined as waist circumference < 92 cm, WHR < 0.9 and BMI < 28 kg/m². The subjects were screened and selected from the community via newspaper advertisement. None of the subjects had diabetes mellitus, proteinuria, creatinemia (> 120 µmol/l), hypothyroidism, hepatomegaly or elevated liver enzymes (alanine aminotransferase, γ-glutamyltransferase), or consumed more than 30 g of alcohol/day. None reported a history of cardiovascular disease, or were taking medication or other agents known to affect lipid metabolism. All subjects provided written consent, and the study was approved by the Ethics Committee of the Royal Perth Hospital.

**Experimental protocol**

Weight and height were measured without shoes and in light clothing. Waist circumference (cm) was measured at the point midway between the costal margin and iliac crest in the mid-axillary line. Hip circumference (cm) was measured at the widest point around the greater trochanter. BMI, WHR and plasma volume [23] were calculated. Arterial blood pressure was recorded after 3 min in the supine position using a Dinamap Instrument (Critikon).

All subjects were admitted to the metabolic ward in the morning after a 14 h fast. They were studied in a semi-recumbent position and allowed water only. Venous blood was collected for measurements of plasma concentrations of triacylglycerol, cholesterol, high-density lipoprotein (HDL)-cholesterol, glucose and insulin, and for assessment of apoE genotype.

The isotopically labelled remnant-like emulsion (14 ml) was injected intravenously within a 2 min period into an antecubital vein via a 21 G butterfly needle. Breath samples were taken at baseline and at time intervals over 24 h. Volunteers were asked to breathe quietly and collect an end-expiratory breath sample via a plastic tube into a Vacutainer (Labco Ltd) that was subsequently and promptly sealed with a screw-cap. After injection of the emulsion, they were requested to collect breath samples every 10 min for the first 1 h, every 30 min for the next 6 h and hourly for another 3 h. After this they were given a snack and allowed to go home. On the following day, subjects provided two additional breath samples. During the first 10 h of the breath sample collection the subjects sat quietly in a chair and were allowed water only. After leaving the metabolic ward, they were requested to refrain from vigorous activity and to take a light supper prior to termination of the period of breath sample collection.
**Dietary analysis**

Dietary intake was assessed for energy and major nutrients using at least two 24 h dietary diaries which were subsequently analysed using DIET 4 Nutrient Calculation Software (Xyris Software, Highgate Hill, Queensland, Australia). Alcohol consumption was also recorded prior to the studies.

**Preparation of stable-isotope-labelled remnant-like emulsion**

Pure lipid mixtures containing triolein (135 mg), phosphatidylcholine (75 mg), cholesteryl [13C]oleate (70 mg) and cholesterol (24 mg), all > 99% pure, were emulsified by sonication for 1 h in 8.5 ml of 2.2% (v/v) glycerol in water, as described previously [17,18]. After sonication the mixture was centrifuged at 2500 g for 10 min to remove titanium fragments and then filtered through a 0.22 μm filter into sterile vessels. All emulsion preparations were confirmed to be sterile and pyrogen-free (Pharmacy Department, Royal Perth Hospital). The emulsion preparation (approx. 14 ml) was placed into a sterile, plastic syringe, stored frozen at −20 °C and thawed 30 min prior to administration. Uniformly labelled [13C]oleate was purchased from Novachem Pty. Ltd. (South Yarra, Victoria, Australia), and cholesteryl [13C]oleate was synthesized from cholesterol and [13C]oleic acid as described previously [16,18]. The remnant-like emulsion particles had an average diameter of 55 ± 3 nm (n = 40), measured by negative-stain electron microscopy. The composition of the injected remnant-like emulsion (% by weight) was: triolein, 55.9 ± 1.6%; cholesteryl oleate, 8.2 ± 0.7%; cholesterol, 7.7 ± 0.7%; phosphatidylcholine, 28.2 ± 1.8%.

**Biochemical analyses**

Plasma triacylglycerol and cholesterol concentrations were determined by standard enzymic methods using a Cobas Mira Analyser (Roche Diagnostics, Basel, Switzerland). HDL-cholesterol was estimated after precipitation of apoB-containing lipoproteins with heparin/manganese. LDL-cholesterol was calculated using the Friedewald equation. Genomic DNA was extracted by the standard Triton X-100 procedure, and the genotype for apoE was determined as described by Hixson and Vernier [24]. Plasma non-esterified fatty acids (NEFAs) were measured by an enzymic, colorimetric method using a commercial kit (Boehringer Mannheim, Catle Hill, NSW, Australia). Plasma insulin was measured by RIA (DiaSorini s.r.l., Saluggia, Italy). Plasma glucose concentration was measured by an enzymic hexokinase reaction method using a Technicon Axon analyser (Bayer Diagnostics, New York, NY, U.S.A.). Insulin resistance was estimated by homeostasis model assessment (HOMA) that employs the formula:

\[ \text{Insulin resistance} = \frac{\text{fasting insulin (m-units/l)} \times \text{fasting plasma glucose (mmol/l)}}{22.5} \]

as described by Matthews et al. [25]. The expired CO₂ in the exhaled breath samples was analysed at each time point by isotope-ratio MS using a Finnigan BreathPlus machine (Thermoquest Systems Pty Ltd, Sydney, NSW, Australia). The \(^{13}\text{CO}_2/^{12}\text{CO}_2\) ratio was referenced to PeeDeebelemnite standard values, and the delta unit value was calculated using the Breathmat software. The delta units reference a sample of limestone, a standard in the \(^{13}\text{C}\) isotope ratio field, and basal (non-enriched) values correspond approximately to 1% \(^{13}\text{C}\).

**Kinetic analysis**

A compartmental model describing the appearance of labelled \(^{13}\text{CO}_2\) in breath was developed using the SAAM II program (SAAM Institute, Seattle, WA, U.S.A.). The model was developed assuming that the fractional rate constants \([k(i,j)]\) were time invariant and first order. The model used to fit the tracer data is shown in Figure 1.

Compartment 1 of the model represents the plasma compartment into which the labelled remnant-like emulsion is injected. Material in compartment 1 can be lost from plasma to compartment 2 or 3. Compartment 2 represents labelled \(^{13}\text{CO}_2\) in breath and is sampled during the course of the study. The primary pathway for clearance of emulsion and appearance of labelled \(^{13}\text{CO}_2\) is via compartment 3. This compartment may include the hepatic processes associated with the uptake, hydrolysis and oxidation of the labelled oleate.

The compartmental model was fitted to the observed \(^{13}\text{CO}_2\) breath data, and estimates of the fractional catabolic rate (FCR) were determined as the sum of the two rate constants for movement out of compartment 1 shown in Figure 1, i.e. \(k(2,1) + k(3,1)\).
Statistical analysis

All analyses were carried out using SPSS software. Group characteristics were compared by unpaired t-tests. Skewed variables were examined after logarithmic transformation. Associations were examined by simple and multivariate linear regression methods. In multiple regression models, we selected variables that approached statistical significance in univariate analysis and could *a priori* be associated (causally or consequently) with remnant lipoprotein clearance or catabolism. Statistical significance was defined at the 5% level using a two-tailed test.

RESULTS

Table 1 shows the clinical characteristics of the obese and lean men. Age and blood pressure were not significantly different between the groups. As anticipated, the obese group had a significantly higher body weight, BMI, waist circumference, WHR and surface area (*P* < 0.001) compared with the lean group, but there were no significant differences in plasma volume. In the obese group, reported daily dietary energy intake was 10181 kJ (S.E.M. 2488 kJ), of which 28.6% (3.6%) was derived from fat, 52.8% (4.4%) from carbohydrate, 16.5% (0.8%) from protein and 2.3% (0.8%) from alcohol. This was not significantly different from the reported intake in the controls.

Table 2 shows the plasma concentrations of lipids, lipoproteins, glucose, insulin and NEFAs, and insulin resistance (estimated by HOMA), in the subjects studied. Plasma cholesterol and triacylglycerol levels were not significantly different in the obese compared with the lean men. The obese men had significantly higher plasma LDL-cholesterol (*P* = 0.012) and non-HDL-cholesterol (*P* = 0.012) levels, but lower HDL-cholesterol (*P* = 0.004), compared with lean men. Although plasma glucose and NEFAs were not significant different between the groups, the obese subjects had significantly higher plasma insulin concentrations and calculated insulin resistance. With regard to apoE genotype, 10 of the obese subjects were E3/E3 homozygotes, one was an E2/E3 heterozygote and one was an E3/E4 heterozygote. In the lean group, six subjects were E3/E3 homozygotes, two were E2/E3 heterozygotes, two were E3/E4 heterozygotes and one was an E2/E2 homozygote; consent was not obtained for DNA analysis for the other lean subject. There were no statistically significant differences in the frequency distribution of apoE alleles between the groups.

Table 2 Plasma lipid, lipoprotein, glucose, insulin and NEFA levels and insulin resistance in the obese and lean subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Obese (n = 12)</th>
<th>Lean (n = 12)</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerol (mmol/l)</td>
<td>1.36 (0.12)</td>
<td>1.10 (0.08)</td>
<td>0.078</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.12 (0.14)</td>
<td>4.94 (0.15)</td>
<td>0.412</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.03 (0.07)</td>
<td>1.50 (0.13)</td>
<td>0.005</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.45 (0.13)</td>
<td>2.89 (0.16)</td>
<td>0.012</td>
</tr>
<tr>
<td>Non-HDL-cholesterol (mmol/l)</td>
<td>4.09 (0.17)</td>
<td>3.44 (0.16)</td>
<td>0.012</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.01 (0.16)</td>
<td>4.90 (0.21)</td>
<td>0.389</td>
</tr>
<tr>
<td>Insulin (i.u./l)</td>
<td>13.0 (1.4)</td>
<td>4.95 (0.63)</td>
<td>0.001</td>
</tr>
<tr>
<td>Insulin resistance (HOMA score)</td>
<td>2.96 (0.37)</td>
<td>1.06 (0.14)</td>
<td>0.001</td>
</tr>
<tr>
<td>NEFAs (mmol/l)</td>
<td>0.31 (0.04)</td>
<td>0.47 (0.09)</td>
<td>0.077</td>
</tr>
</tbody>
</table>

Figure 2 Enrichment of $^{13}$CO$_2$ in breath in the lean (■) and obese (Δ) subjects after injection of the chylomicron remnant-like particle emulsion

Values are means ± S.E.M.
DISCUSSION

We present new data suggesting that normotriglyceridaemic men with visceral obesity have decreased catabolism of chylomicron remnants. In multiple linear regression analysis, decreased catabolism of the remnant-like emulsion in the obese subjects was positively associated with the degree of visceral adiposity, as measured by the WHR, and not with the normal or near-normal plasma lipid levels [21]. Our observation is based on the use of a novel and acceptable stable isotope breath test that has been extensively studied and validated in experimental animals, and more recently in human models of genetic hyperlipidaemias [17–20].

That central obesity is associated with disturbances in lipoprotein metabolism is well recognized [1,22,26]. The principal abnormalities include increases in plasma TRLs and to a lesser extent LDL, with a reciprocal decrease in HDL. The increase in plasma TRLs is due to an elevation of heptatically derived VLDLs and their remnants, as well as to intestinally derived remnant lipoproteins [26]. Postprandial lipaemia, as reflected by an increased response of TRLs to a fat load, has been described previously in obese men [1,9] and is directly related to the fasting plasma triacylglycerol concentration [27]. Fasting levels and postprandial responses of both apoB-48- and apoB-100-containing TRLs have also been shown to be increased in viscerally obese subjects without hypertriglyceridaemia compared with controls [10]. In the study of Mekki et al. [10], postprandial responses were inversely related to the plasma concentration of apoC-III and positively related to fasting insulin levels. Postprandial lipaemia has also been demonstrated in obese patients with Type II diabetes with and without hypertriglyceridaemia [11,12]. Not all findings based on the postprandial triacylglycerol response have been consistent [28], however, and this could be due to methodological considerations. Whether the results of the aforementioned studies reflect the accumulation of chylomicrons, VLDL or their remnants remains uncertain [27,29], as existing methods for assessing postprandial lipaemia rely on the measurement of apoB-48 in plasma or the postprandial changes in ingested vitamin A, which do not distinguish clearly between defective lipolysis and problems of remnant clearance [14]. The present findings therefore extend previous observations by demonstrating that, in the postabsorptive state, delayed catabolism or
clearance of chylomicron remnants is a specific feature of viscerally obese subjects who are not ostensibly hyperlipidaemic.

Chylomicron remnants are cleared by the liver by a three-stage process [15]. Central to this is the binding of apoE on the remnant particle to the hepatic LDL/apoE receptor. The functional uptake of chylomicron remnants by this pathway may be disturbed in visceral obesity because of hepatic insulin resistance, which downregulates receptor activity [30,31], and also because of competition for hepatic receptors between chylomicron remnant and VLDL particles [15,32]. We have shown previously that hypertriglyceridaemic, insulin-resistant men with visceral adiposity have an increased hepatic output of VLDL [33]. Since the obese subjects in the present study had normal or near-normal plasma triacylglycerol levels, it is unlikely that there would have been significant competition between VLDL and the injected chylomicron remnant-like particles for hepatic receptors, especially since the study was carried out in the postabsorptive state. This is also supported by the absence of a significant correlation between the FCR of the chylomicron remnant-like emulsion and the plasma triacylglycerol level in multiple regression analysis. Furthermore, an analysis of obese patients in our study with plasma triacylglycerol levels of < 1.7 mmol/l [21] also confirmed that they had impaired catabolism of the emulsion compared with lean subjects. Increased plasma levels of apoC-III have been demonstrated in obesity and may also relate to insulin resistance [10]. Association of apoC-III with the injected remnants might explain the delayed clearance of these particles from plasma [35] and thus the delayed appearance of 13CO2 in the breath. That we showed no correlation between chylomicron remnant catabolism and insulin resistance, as measured by the HOMA model [25], does not exclude the possibility that hepatic insulin resistance may contribute to the delayed clearance of the injected particles [36], and to a lesser extent to recycling of labelled oleate into VLDL. The inverse correlation noted between the WHR and delayed fractional clearance of the chylomicron remnants suggests involvement of hepatic, as opposed to peripheral, insulin resistance [26,37]. The lack of a significant inverse association between plasma NEFAs and fractional catabolism of the emulsion is still compatible with this notion. Insulin has been demonstrated to regulate triacylglycerol kinetics in normotriglyceridaemic subjects [38]. An inverse correlation between plasma concentrations of HDL-cholesterol and the postprandial triacylglycerol response has also been demonstrated previously [39,40]. This may be attributed to decreased clearance of TRLs by adipose tissue with a reduced shift of cholesterol to HDL [41], as well to increased transfer of cholesteryl esters from HDL to TRLs by cholesteryl ester transfer protein (CETP) [42]. Our finding of delayed clearance of chylomicron remnant particles in normotriglyceridaemic obese patients with low HDL levels may be more compatible with the second possibility. Further studies of the impact of CETP activity and apoE transfer on the breath test are required. In patients with isolated low plasma HDL levels, a subtle defect in chylomicron remnant clearance may be clinically significant by enhancing the risk of coronary disease, as suggested elsewhere [40].

A major assumption in inferring impaired chylomicron remnant removal from the breath test result is that the appearance of 13CO2 in the breath reflects remnant particle clearance by the liver. The breath test depends not only on removal of the emulsion remnant particles into the liver for catabolism, but also on the subsequent metabolism of fatty acids hydrolysed from the emulsion of cholesteryl ester. Increased cytosolic triacylglycerol stores in central obesity may delay the appearance of 13CO2 from cholesteryl oleate. Although Figure 2 suggested delayed global catabolism of the chylomicron remnant-like emulsion, the rate constants in Table 3 suggest that this was due chiefly to reduced uptake by the liver. Moreover, none of our obese patients had abnormal liver enzymes, hepatomegaly, markedly elevated triacylglycerols, diabetes or a high alcohol intake, factors that could have potentially confounded the intrahepatic processing of the remnant-like particles. Increased release of NEFAs by adipose tissue has been described in obese insulin-resistant subjects [7,43], and might have been predicted to dilute the appearance of the label in the breath. However, we did not find a significant correlation between plasma NEFAs and the fractional clearance of the emulsion in a combined or subgroup analysis, nor in a larger group of patient in another study (D. Chan and G. F. Watts, unpublished work). Hence it is unlikely that potential expansion of the plasma NEFA pool contributed to the flatter breath test in the obese patients. Hepatic lipase activity is increased in obese patients with insulin resistance [44], and this could have increased lipolysis and receptor-mediated uptake of the injected emulsion [15,45], thereby confounding our results by enhancing the apparent appearance of 13CO2 in the breath. However, in spite of this possibility, the FCR of the remnant particles remained lower in the obese patients. The confounding effect of enhanced CETP activity in transferring the emulsion cholesteryl ester to other plasma lipoproteins also needs to be acknowledged, although in a study of lipoprotein lipase-deficient subjects with markedly elevated triacylglycerol and low HDL levels, the results of the breath test were not different from controls [20].

Support for the validity of the breath test as an estimate of remnant clearance from plasma derives from experiments in which the delayed appearance of 13CO2 in the breath following the injection of the labelled emulsion has been described in animals deficient in apoE, LDL receptor or LDL-related protein [18,19]. Consistent with
whether this metabolic defect in obesity is correctable in men without significant elevations in plasma lipid levels. Chylomicron remnant-like particles in viscerally obese individuals have delayed plasma clearance, showing decreased fractional catabolism in the breath test, confirming concordance in results. One of the advantages of the modelling is that the need for correction of emulsion dose for body size is obviated, since our kinetic end-point is fractional catabolism, which is independent of body size and volume of distribution. This is supported in the present study by the lack of correlation between the FCR of the emulsion and BMI, body weight or plasma volume. Carrying out the test in the postabsorptive state and restricting physical activity is important to prevent competition of TRLs for clearance with the emulsion and artefactual dilution of label due to accelerated oxidation of unlabelled substrate respectively.

In summary, we have communicated our preliminary experience with a new breath test, showing decreased catabolism and, by extension, delayed clearance of chylomicron remnant-like particles in viscerally obese men without significant elevations in plasma lipid levels. Whether this metabolic defect in obesity is correctable with weight reduction and/or with insulin sensitizers warrants examination. Furthermore, whether an abnormality in the breath test is a more sensitive and specific predictor of cardiovascular events than conventional measures of disordered postprandial lipoprotein metabolism also requires investigation, particularly given the uncertainty regarding hypertriglyceridaemia as a cardiovascular risk factor [2].

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