Nutrient Metabolism

Food Restriction Normalizes Chylomicron Remnant Metabolism in Murine Models of Obesity as Assessed by a Novel Stable Isotope Breath Test1,2

Ian J. Martins,3 J.M.L. Tran and Trevor G. Redgrave

Department of Physiology, The University of Western Australia, Crawley, Perth, Australia 6907

ABSTRACT Evidence is increasing that defective metabolism of postprandial remnants of triglyceride-rich lipoproteins contributes to atherogenesis. In obesity, postprandial lipemia is increased by mechanisms that are not currently established. In the present study, a recently developed 13CO2 breath test was used to assess the metabolism of chylomicron remnants (CR) in obese mice. Six murine obese models ob/ob, fat/fat, New Zealand Obese (NZO), db/db, gold thioglucose (GTG)-treated and agouti (A/y) were studied. All obese mice were hyperphagic and their breath test metabolism was markedly impaired (P < 0.01) compared with control, nonobese mice. The breath test was also impaired (P < 0.01) in all obese mice except A/y mice after 24-h food deprivation. However, after restriction to the food intake of paired control mice for 6 wk, the breath test in all obese mice improved to values of control, nonobese mice. The obese NZO, fat/fat and db/db mice had significant (P < 0.02) weight loss when food restricted, whereas A/y GTG, and db/db mice did not. In all obese mice, plasma cholesterol levels decreased (P < 0.02) after the 6-wk period of food restriction. Plasma triglyceride levels significantly decreased (P < 0.02) in NZO, GTG and db/db mice, but not in other obese mice. Plasma glucose levels were significantly decreased (P < 0.02) after the 6-wk period in the obese mice except for the A/y and NZO mice; levels were greater in food-restricted db/db mice. Although some of the obese models such as db/db were diabetic, our data suggest that the defective breath test was independent of diabetes because all obese and diabetic models responded similarly to food restriction. Impaired hepatic catabolism of CR was excluded as a cause of the abnormal breath tests. In summary, the impairment (P < 0.05) in remnant metabolism as assessed by the breath test in obese mice was corrected by food restriction, associated with improvements in plasma glucose, triglyceride and cholesterol levels. J. Nutr. 132: 176–181, 2002.

KEY WORDS: • chylomicron remnant • obese mice • stable isotope • breath test • hyperphagia • food restriction

Chylomicrons (CM)4 are triacylglycerol-rich lipoproteins formed in the intestine during lipid absorption. Absorbed exogenous and bile cholesterol are transported in CM from the intestine to plasma. On entry into the blood, most of the triglycerides in chylomicrons are hydrolyzed by lipoprotein lipase, converting the particles to chylomicron remnants (CR) (1). The remnants transport cholesterol absorbed from the intestine and are normally rapidly removed from the blood plasma by receptor-mediated endocytosis, primarily into the liver. After endocytosis, the lipoproteins are transported into endosomes and eventually into lysosomes where the lipid components are hydrolyzed.

In epidemiologic studies, human obesity is clearly associated with the increased risk for atherosclerosis, contributing to the early onset of coronary artery disease. The metabolic basis for these associations has not been established. CR have the potential to contribute to the development of atherosclerosis, and CR metabolism has been shown to be disordered in obesity. In studies of obese individuals for whom apolipoprotein (apo) B48 and retinyl palmitate were used as tracers for chylomicrons and their remnants, impaired clearance of these lipoproteins was found (2–4).

A previous study of chylomicron clearance in obese Zucker rats (5) concluded that there was an abnormality in the clearance of CR. In genetically obese mice such as the diabetic (db), obese (ob), fat (fat), tubby (tub) and lethal yellow (agouti, A/y), plasma triglyceride and cholesterol levels are elevated. In these obese strains of mice, plasma HDL cholesterol levels are increased sufficiently to protect against atherosclerotic fatty streak lesion development (6).

We recently developed a breath test for CR clearance, which is useful for repeated measurements in mice. In the present study, the effects of obesity on the clearance and metabolism of the remnants of triglyceride-rich lipoproteins were assessed by a 13CO2 breath test in either fed or food-deprived obese mice. CR metabolism was compared among...
various genetic models of obesity and a chemically induced model injected with gold thioglucose (GTG). To assess the contribution of hyperphagia on remnant metabolism in the obese mice, the metabolism of remnant-like emulsion particles was compared in obese mice consuming food freely, with others restricted to the food intake of paired nonobese control mice. To evaluate the role of diabetes on CR clearance and metabolism, breath tests in mice were also conducted after the onset of diabetes in the db/db, GTG and A/+ mice.

MATERIALS AND METHODS

Animals. Mice were obtained at 8 wk of age and weighed ~20 g. Mice of strains C57BL/6J-A/y (A/+), C57BL/6J-db/db (db/db), C57BL/6J-fat/fat (fat/fat), C57BL/6J-ob/ob (ob/ob) and heterozygotes from each strain for controls were obtained from the Jackson Laboratories (Bar Harbor, ME). These mice were fed a D11 diet purchased from Purina Mills (Richmond, IN). The D11 diet contained 19% protein, 10.8% fat, 2.3% crude fiber, 6.68% ash, 1.33% calcium and 0.78% phosphorus. C57BL/6J mice were obtained from the Animal Resources Center, Murdoch, Western Australia and were fed a pelleted diet (Glen Forrest Stockfeeders, Perth, Western Australia) containing 20% protein, 9% fat, 3.0% fiber, 1% calcium and 0.7% phosphorus. C57BL/6J mice were obtained from the Animal Resources Center, Murdoch, Western Australia and were fed rodent pelleted diet (Glen Forrest Stockfeeders, Perth, Western Australia) containing 18.9% protein, 5.2% fat, 5% crude fiber, calcium 0.77%, phosphorus 0.57% and salt 0.41%. C57BL/6J mice (Animal Resources Center) were used as controls for the New Zealand Obese (NZO) mice (Walter and Eliza). The NZO mouse is a polygenic model of noninsulin-dependent diabetes (NIDDM) characterized by obesity and insulin resistance (7). The NZO mice do not have true controls (7). The C57BL/6J mouse has been used as a control for the NZO mouse (7).

CBA mice were obtained from the Blackburn animal house, University of Sydney, Australia and were fed a pelleted diet (Gordon specialty stockfeeds, New South Wales, Australia) containing 23% protein, 6% fat and 5% fiber. The CBA mice were made obese by intraperitoneal injections with GTG (0.5 mg/kg). All mice were housed in an animal holding room (University of Western Australia, Perth, Western Australia) with free access to the standard rodent pelleted diet (Glen Forrest Stockfeeders) and water.

Breath tests were conducted at 2 mo of age in NZO, fat/fat, ob/ob and db/db mice under fed conditions (consumed food ad libitum) and when food intake of the obese mice was restricted (pair-feeding) to the intake of paired nonobese control mice at 3.5 mo of age. At 2 mo of age, db/db mice were diabetic. At 2 mo of age, CBA mice were injected with GTG to induce obesity. At 4 mo of age, CBA-GTG mice were diabetic. Breath tests were conducted at 4 mo of age in control and diabetic CBA-GTG mice (consumed food ad libitum); at 5.5 mo, breath tests were completed in control, diabetic CBA-GTG (pair-fed) and diabetic ob/ob mice (pair-fed). Breath tests were conducted at 6 mo of age in control and obese A/+ mice (consumed food ad libitum) and then at 7.5 mo when obese A/+ mice were pair-fed. At 10.5 mo, pair-feeding studies were conducted in control and diabetic A/+ mice.

In Table 1, the experimental protocol clarifying the rationale for conducting breath tests in mice at different ages is summarized. The care and use of laboratory animals were as allowed by the Animal Ethics and Welfare Committee at the University of Western Australia.

Preparation of remnant-like emulsion particles. Triolein (TO), cholesteryl oleate (CO), cholesterol and egg phosphatidylcholine (PC) were obtained from Nu-Chek Prep (Elysian, MN; each >99% pure). Lipid mixtures containing TO (45 mg), PC (25 mg), either cholesteryl[13C]-oleate (8 mg) or cholesteryl[14C]-oleate (1480 kBq) and cholesterol (8 mg) were emulsified by sonication for 1 h in 8.5 mL of 2.2% glycerol in water as previously described (8). After sonication, the emulsion mixture was centrifuged at 1900 × g for 10 min to remove titanium fragments and then filtered through a 0.22-μm filter into sterile vessels. Uniformly labeled [13C]-oleic acid was purchased from Novachem (Victoria, Australia), and cholesteryl [13C]-oleate was synthesized from cholesterol and [13C]-oleic acid as described previously (9). The remnant-like emulsion particles were of average diameter, 55 ± 3 nm (n = 40), measured by negative-stain electron microscopy. The composition of the injected remnant-like emulsion was as described previously (8).

Assessment of the metabolism of remnants by collection of expired 13CO2. A volume of 50 μL of the remnant emulsion mixture was injected via a tail vein into mice. The mice were placed in small chambers for ~1.5 min and the exhaled breath was collected into evacuated gas sample containers (Europa Scientific, Crewe, UK). Breath samples were collected at 0 min (before injection of emulsion) and then at 10, 20, 30, 45, 60, 90, 120 and 180 min after injection of the emulsion. The enrichment of breath samples with 13CO2 was measured by isotope-ratio mass spectrometry (Breath-Mat™, Finigan MAT, Bremen, Germany). The 13CO2/12CO2 in the breath samples was compared with a reference standard Peeceebeliminite and then the Δ value was calculated for the sample.

Hepatic uptake of radiolabeled CO emulsions. Remnant-like emulsions containing cholesteryl[14C]-oleate were injected via tail vein into mice; after 60 min, mice were killed by an overdose of avertin; the liver was removed for extraction of lipids (10) with

### Table 1

**Experimental protocol for breath tests at different ages in control and obese mice that consumed food ad libitum and after pair-feeding**

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>21</th>
<th>3.52</th>
<th>43</th>
<th>5.54</th>
<th>65</th>
<th>7.56</th>
<th>10.57</th>
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<tbody>
<tr>
<td>Nonobese control mice</td>
<td>Obese mice</td>
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<tr>
<td>C57BL/6/J</td>
<td>NZO</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>fat/+</td>
<td>fat/fat</td>
<td>+</td>
<td>+</td>
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<td>ob/+</td>
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<tr>
<td>C57BL/6J-A/+</td>
<td>A/+</td>
<td>-</td>
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</table>

1 Breath tests in control, New Zealand Obese (NZO), fat, obese (ob/ob) and diabetic (db/db) mice that consumed food ad libitum.
2 Breath tests in control, New Zealand Obese (NZO), fat, obese (ob/ob) and diabetic (db/db) mice after pair-feeding.
3 Breath tests in control CBA and diabetic CBA-GTG mice that consumed food ad libitum.
4 Breath tests in control, diabetic ob/ob and diabetic CBA-GTG mice after pair-feeding.
5 Breath tests in control and A/+ mice after pair-feeding.
6 Breath tests in control and A/+ mice after pair-feeding.
chloroform/methanol (2:1, v/v), and radioactivities in the lipid extracts were measured in 15 mL of scintillant.

**Chemical analysis.** The lipids extracted (10) from either the remnant-like emulsions or organ extracts with chloroform/methanol (2:1, v/v) were separated on silica gel TLC plates of 0.2-mm thickness in a solvent system consisting of petroleum ether 40–60°C/diethyl ether/formic acid (90:10:1, v/v/v). The TC, CO and cholesterol bands were scraped from the plate for assay of triacylglycerol by the chromotropic acid method (11) and free and esterified cholesterol were determined by the o-phthalaldehyde procedure (12). Phospholipid was measured directly on emulsion samples (13). Plasma cholesterol and triglyceride were determined enzymatically with assay kits for cholesterol (CHOD-PAP) and for triglycerides (GPO-PAP) from Randox Laboratories (Antrim, UK). Glucose was assayed by the peroxidase-glucose oxidase method on deproteinized plasma (14).

**Statistical analysis.** The same obese groups consumed food ad libitum and were food restricted so that repeated-measures ANOVA was appropriate for these data. We conducted repeated-measures ANOVA to compare between- and within-group differences in the obese mice. This procedure showed clear differences between the two diets and additionally showed differences in response among strains of mice. Comparison of the control group with the obese group that consumed food ad libitum and with the obese group that was food restricted was conducted using unpaired t-tests. Because this involves two tests we have adjusted the P-values using the Bonferroni correction for multiple tests. The analyses accounted for multiple testing and for correlated error structure of the two sets of data from the obese mice. The t-test for independent means was used to compare differences between means [calculated from area under the curves (AUC)] of 13C-enrichment in breath CO2. The areas under the 13CO2 enrichment curves for the first 45 min of the experiment were calculated for each mouse using a trapezoid algorithm (GraphPad Prism, San Diego, CA).

**RESULTS**

**Body weights, plasma glucose and lipids of obese mice.** Body weights of obese mice that consumed food ad libitum were greater (*P* < 0.002) than the corresponding control mice (Table 2). Plasma glucose concentrations did not differ from controls in NZO, fat/fat, ob/ob and A2 mice but were greater (*P* < 0.002) in GTG obese CBA mice and db/db mice that consumed food ad libitum compared with control mice consuming food ad libitum (Table 3). Plasma triglyceride levels were greater (*P* < 0.002) in NZO and CBA-GTG mice compared with controls before food restriction (consumed food ad libitum), but not in other models before food restriction (Table 4). Plasma cholesterol levels (Table 5) were greater (*P* < 0.002) in all obese mice compared with controls that consumed food ad libitum. The ad libitum food intake of obese mice was greater (*P* < 0.001) than that of corresponding controls (Table 6).

**Measurement of remnant metabolism from CO2 in expired breath of obese mice.** The appearance of 13CO2 in the expired breath of fed control mice (ob/+ and fed obese (ob/ob) mice injected with remnant-like emulsions containing...
The metabolism of remnant-like emulsions containing cholesteryl [13C]-oleate is shown in Figure 1. In ob/+ mice, the enrichment in breath of 13CO2 increased rapidly and peaked at 45 min, subsequently decreasing to near preinjection values by 3 h. In contrast, the enrichment of 13CO2 in the breath of ob/ob mice was markedly less when mice were injected with emulsions. The enrichment of 13CO2 in the expired breath of ob/ob mice was less (P < 0.0001) as calculated from the AUC compared with ob/+ mice.

Figure 2 summarizes the 13CO2 enrichment curves in fed and 24-h food-deprived obese mice. For easier comparison, the data were integrated by calculating the areas under the 13CO2 enrichment curves for the first 45 min of the experiments. The obese mice were separated into Group A (obese nondiabetic mice) and Group B (obese diabetic mice). In Group A, the 13CO2 breath enrichment of fed control mice (ob/+), injected with remnant-like emulsions containing cholesteryl [13C]-oleate was higher (P < 0.001) than in ob/ob mice. In 24-h food-deprived ob/+ and ob/ob mice, the breath enrichments were greater (P < 0.001) than in fed mice. In food-deprived control ob/+ mice, the 13CO2 breath enrichment remained significantly elevated (P < 0.001) compared with ob/ob mice.

The metabolism of remnant-like emulsions was also markedly impaired in fed and food-deprived NZO mice compared with control C57BL/6J mice (Fig. 2, Group A). The enrichment of 13CO2 in the expired breath was also markedly decreased at 12 wk in fed and food-deprived fat/fat mice compared with control mice (P < 0.01). In fed A2 mice at 24 wk of age, the 13CO2 breath enrichment was significantly impaired (P < 0.05) compared with control mice. After food deprivation, no differences were found between control and A2 mice. In fed db/db mice, the 13CO2 breath enrichment was markedly decreased (P < 0.001) compared with control m+/db mice (Fig. 2, Group B). In food-deprived db/db mice, the impairment in enrichment of 13CO2 in the expired breath persisted (P < 0.001). Breath enrichment was decreased in GTG obese CBA mice (8 wk after induction) in both the fed and food-deprived states (Fig. 2, Group B).

**Effects of food restriction on body weight, plasma glucose and lipids in obese mice.** Body weights were significantly decreased in NZO, fat/fat and ob/ob mice but were unchanged in other groups of obese mice 6 wk after food restriction compared with obese mice consuming food freely (Table 2). In the db/db mice, plasma glucose levels were markedly increased and were greater after the 6-wk period of food restriction. In contrast, in the GTG obese CBA, fat/fat and ob/ob mice, plasma glucose levels were decreased after the period of food restriction. Plasma triglyceride levels (Table 4) decreased after food restriction in all mice except for A2, ob/ob and fat/fat mice compared with mice consuming food freely. Plasma cholesterol levels were also markedly reduced by dieting (pair-fed) in all types of obese mice (Table 5).

**Effects of food restriction on CR metabolism in obese mice.** After food restriction, the 13C breath enrichment was similarly increased in obese nondiabetic (Group A) and obese diabetic (Group B) mice, indicating that the impairment in CR metabolism was restored to control values (Fig. 3). In fat/fat, A2 and GTG obese mice, the breath enrichment was greater than (P < 0.01) control values (Fig. 3), indicating that CR metabolism was increased by food restriction.

**CR metabolism in diabetic A2 and ob/ob mice.** After the onset of diabetes in A2 and ob/ob mice, CR metabolism was impaired as assessed by the breath test. The breath enrichments were markedly increased in diabetic A2 mice after a 6-wk restriction of food intake but were not different from controls (data not shown). In diabetic ob/ob mice, breath enrichments were not different from control mice after pair-feeding (data not shown).
Hepatic uptake of radiolabeled CR emulsions. After injection of remnant-like emulsions containing cholesteryl[14C]-oleate into various strains of obese and diabetic mice, the livers were removed and lipids extracted as described in Materials and Methods. In all strains of obese mice, no differences were found in the hepatic uptake of radiolabeled emulsions compared with the respective controls. In control and fat/fat mice, 80% of the injected radioactivity was found in the livers. In control and ob/ob mice liver uptake of radiolabeled emulsions was also 80%. In control and GTG mice, 40% of the injected dose was found in the livers, and hepatic uptake of radiolabeled emulsions was similar in control and db/db mice (40%). In Aβ, db/db and their respective control mice, 60% of the injected dose was found in the livers.

**DISCUSSION**

In previous studies we measured the appearance in breath of 13CO2 after injection of lipid emulsions labeled with cholesteryl [13C]-oleate. Measurement by the breath test provided an assessment of the clearance and metabolism of the remnants of triglyceride-rich lipoproteins and showed the importance of LDL receptors and apo E in the clearance of CR in mice. In the present study, the 13CO2 breath test was used to assess aspects of CR metabolism in various murine models of obesity. In all obese mouse models, remnant metabolism was impaired in the fed state as assessed by the marked delay in appearance of 13CO2 in the expired breath (Fig. 2).

Hyperphagia leads to an increased load of transported fat from the intestine. In all obese models, food intake was significantly increased. In db/db mice, the input of cholesterol from the intestine has been shown to be increased (15). Restricted food intake in obese mice markedly decreased plasma cholesterol and generally improved plasma triglyceride levels. Our results show that prevention of hyperphagia by restricting food intake overcame the impairment in the clearance and metabolism of CR as shown by the improvement in breath enrichments. The defect in CR removal in fed obese mice was possibly associated with the increased competition for clearance of lipoproteins by the liver rather than changes in ligands or receptors, although greater substrate fluxes could...
have diluted breath enrichment with the $^{13}$C-oleate released from the injected emulsion. After 24-h food deprivation, substrate fluxes are comparable to those of mice consuming food ad libitum. The experiments in 24-h food-deprived obese mice showed that CR metabolism was still impaired (Fig. 2).

In a recent study in db/db mice, the plasma triglyceride level was elevated; this was attributed to the impaired clearance of remnants of triglyceride-rich lipoproteins, but CR metabolism was not measured directly (16). In Zucker obese rats, the clearance of CR was shown to be delayed (5). In fed and food-deprived db/db mice in our study, CR metabolism was also markedly abnormal as measured by the breath tests. These findings indicate that the delayed removal of CR in db/db mice may be associated with a defect in the hepatic uptake or catabolism of remnants. In db/db mice, the hepatic uptake of radiolabeled CO emulsions at 60 min was similar to control mice, indicating that the hepatic catabolism of CR was normal. In other obese mice models, the hepatic uptake of radiolabeled CR emulsions was not different, indicating that CR catabolism was normal but CR clearance was impaired in these murine models of obesity.

In this study, after the 6-wk period of food restriction in db/db mice, plasma glucose levels were not improved and remained elevated; however, the metabolism of CR as measured by breath enrichment was normal. In the A$\beta$ mice, which had developed diabetes at a later age, CR metabolism was markedly increased after prevention of the hyperphagia. In diabetic ob/ob mice after food restriction, breath enrichment was lower but not significantly different from control mice. Our data suggest that the defect in CR metabolism was independent of diabetes because all models responded similarly to food restriction.

In conclusion, the metabolism of CR was defective in all fed, obese mice. Prevention of hyperphagia by food restriction markedly improved breath enrichments and lowered plasma glucose and lipid levels in obese mice. The defect in CR metabolism as assessed by the breath test was not connected to the severity of the diabetes but was associated with the marked hyperphagia. This finding can be contrasted with previous measurements in insulin-deficient rats, in which food restriction had no effect on defective remnant clearance (17).

ACKNOWLEDGMENT

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LITERATURE CITED