A $^{13}CO_2$ breath test to assess the metabolism of triglyceride-rich lipoprotein remnants in mice

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Abstract Remnant-like emulsions were prepared with lipid compositions similar to remnants derived from triacylglycerol-rich lipoproteins. When injected into the bloodstream of conscious mice the remnant-like emulsions labeled with cholesteryl[^{13}C]oleate were metabolized in the liver and the appearance of $^{13}CO_2$ in the breath was measured. In control mice injected with remnant-like emulsions labeled with cholesteryl[^{13}C]oleate, enrichment of $^{13}CO_2$ in the breath peaked at 45 min and then decreased markedly by 3 h. In apoE-deficient (−/−) mice no enrichment was found and in low density lipoprotein receptor (LDLr)-deficient (−/−) mice the appearance of $^{13}CO_2$ in the breath was markedly decreased. These findings were consistent with the ability of the breath test to detect defects in remnant metabolism. The breath test was useful in detecting a defect in remnant metabolism in LDLr heterozygote (+/−) mice, in which the appearance of $^{13}CO_2$ in the breath was less by 45 min but remained elevated for the duration of the experiment when compared with control mice. In hepatic lipase-deficient (−/−) mice no defect in remnant metabolism was found. Under fasting conditions, the enrichment of $^{13}CO_2$ in the breath after injection of emulsion was markedly increased when compared with fed mice, indicating that the metabolism of the injected remnant-like emulsion was probably competed for by post-prandial particles under fed conditions. Our findings show that a ^{13}C breath test can be used to assess the metabolism of remnants. The test provides a useful and sensitive method for non-invasive testing of remnant metabolism in experimental animals.**—Martins, I. J., and T. G. Redgrave.** A $^{13}CO_2$ breath test to assess the metabolism of triglyceride-rich lipoprotein remnants in mice. *J. Lipid Res.* 1998. **39:** 691–698.

Supplementary key words cholesteryl oleate • emulsion • triolein • triglyceride-rich lipoproteins • remnants • cholesterol

Fat absorbed from the intestine is transported in chylomicrons. On entry into the circulation, the chylomicrons come in contact with the enzyme lipoprotein lipase. Lipoprotein lipase, which is located on the surface of capillary endothelial cells of adipose tissue, skeletal and cardiac muscle, and other sites, hydrolyzes the triacylglycerols of chylomicrons producing fatty acids and glycerol. Simultaneously, the apolipoproteins (apo) and phospholipids of chylomicrons are released into the circulation and transferred to other lipoproteins, particularly high density lipoproteins (1).

When lipolysis by lipoprotein lipase has removed 70–90% of triacylglycerol, a cholesterol-rich residual lipoprotein, called the chylomicron remnant, returns into the circulation (2). The remnant lipoprotein particle is removed from the plasma, predominantly by the liver by a process requiring apoE as a ligand for binding to the low density lipoprotein receptor and possibly other receptors (3, 4). After endocytosis of remnants into hepatocytes, the lipoproteins are transported to endosomes and then into lysosomes, where the lipid components are degraded and the fatty acids become available for oxidative metabolism to form carbon dioxide.

In previous studies we measured the appearance in breath of either labeled $^{14}CO_2$ or $^{13}CO_2$ after injection of lipid emulsions, labeled with cholesteryl [1-^{14}C]oleate (3, 4) or cholesteryl[^{13}C]oleate (5), respectively. Measurement by these breath tests provided an assessment of the clearance and metabolism of the remnants of triglyceride-rich lipoproteins. The procedure is non-invasive and simple to perform and provides quantitative information of an animal's ability to metabolize remnants derived from triacylglycerol-rich lipoproteins. In the present study we have used the $^{13}CO_2$ breath test to assess aspects of the physiology of the metabolism of remnants in mice.

Abbreviations: AUC, area under the curve; CO, cholesteryl oleate; apoE, apolipoprotein E; PC, phosphatidylcholine; PL, phospholipid; TO, triolein; apo, apolipoprotein; LDLr, low density lipoprotein receptor; CR, chylomicron remnants.

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MATERIALS AND METHODS

Preparation of remnant-like emulsion particles

Lipid mixtures containing TO (45 mg), PC (25 mg), either cholesteryl[^{13}C]oleate (8 mg) or cholesteryl[^{13}C]palmitate (10 mg), and cholesterol (8 mg) were emulsified by sonication for 1 h in 8.5 ml of 2.2% glycerol in water. After sonication the emulsion mixture was centrifuged at 3000 rpm for 10 min to remove titanium fragments and then filtered through a 0.22-μm filter into sterile vessels. Triolein, cholesteryl oleate, cholesterol, and PC were obtained from Nu-Chek Prep, Elysian, MN (each more than 99% pure). Uniformly labeled [^{13}C]oleic acid and [^{13}C]palmitic acid were purchased from Novachem Pty. Ltd., Victoria, Australia, and cholesteryl[^{13}C]oleate and cholesteryl[^{13}C]palmitate were synthesized from cholesterol and [^{13}C]oleic acid or [^{13}C]palmitic acid as described previously (6). 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 (% by weight, n = 4) TO, 55.9 ± 1.6; CO, 8.2 ± 0.7; cholesterol, 7.7 ± 0.7; and PC, 28.2 ± 1.8.

Animals

C57BL/6J (control) male or female mice were obtained from the Animal Resources Centre, Murdoch, Western Australia. Mice were obtained at 8 weeks of age and weighed approximately 20 g. Colonies of apoE-, LDLr-, and hepatic lipase homozygous-deficient mice, where the genes for apoE, LDLr, or hepatic lipase were nullified by homologous recombination, were established from progenitor stocks obtained from the Jackson Laboratories (Bar Harbor, ME). LDLr and apoE heterozygote deficient mice were obtained by crossbreeding C57BL/6J mice with LDLr and apoE homozygous deficient mice, respectively. In feeding experiments, one group of C57BL/6J mice was injected with the emulsion after a 24-h fast. Mice in a second group were given access to food for 3 h after a 24-h fast, and then injected with emulsion for the breath test.

Assessment of the metabolism of remnants by collection of expired $^{13}CO_2$

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 approximately 1.5 min and the exhaled breath was collected into evacuated gas sample containers (Europa Scientific Ltd, Crewe, U.K.). Breath samples were collected at 0 (prior to injection of emulsion) and then at 10, 20, 30, 45, 60, 90, 120, 150, and 180 min after injection of the emulsion. In other experiments, after injection of [^{13}C]oleic acid complexed with albumin, breath samples were collected at 5, 10, 20, 30, 60, 90, and 120 min. The enrichment of breath samples with $^{13}CO_2$ was measured by isotope-ratio mass spectrometry (ABCA, Europa Scientific, Crewe, U.K.). The $^{13}CO_2/^{12}CO_2$ in the breath samples was compared with a reference standard Peedeebelimnite (PDB_1) and then the delta value was calculated for the sample.

Measurements of particle diameters

Remnant-like emulsion particle size was determined by electron microscopy. Emulsion samples on formvar-coated grids were negatively stained with 2% phosphotungstic acid at room temperature and viewed on a Joel 2000 FX transmission electron microscope.

Chemical analysis

The lipids extracted from the remnant-like emulsions 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 (by volume). The TO, CO, and cholesterol bands were scraped from the plate for assay of triacylglycerol by the chromotropic acid method (7) and free and esterified cholesterol were determined by the *o*-phthaldialdehyde procedure (8). Phospholipid was measured directly on emulsion samples (9).

Statistical analysis

The *t*-test for independent means was used to compare differences between means (calculated from area under the curves).

RESULTS

Measurement of remnant metabolism from CO_2 in expired breath of mice

Figure 1 shows the appearance of $^{13}CO_2$ in the expired breath of mice injected with emulsions containing either cholesteryl[^{13}C]oleate or cholesteryl [^{13}C]palmitate. When mice were injected with emulsions containing cholesteryl[^{13}C]oleate the enrichment in breath of $^{13}CO_2$ increased rapidly and peaked at 45 min, subsequently decreasing to near pre-injection values by 3 h. In contrast, the enrichment of $^{13}CO_2$ in the breath of mice was markedly less when mice were injected with emulsions containing cholesteryl[^{13}C]palmitate. The enrichment of $^{13}CO_2$ in the expired breath of mice injected with emulsions containing choles-

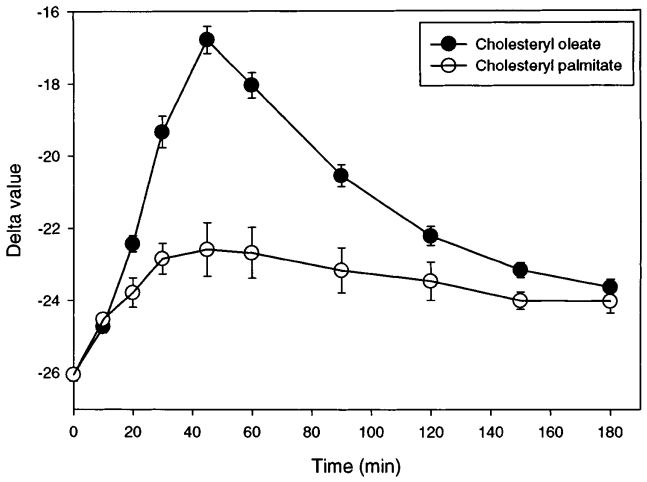

Fig. 1. The enrichment of $^{13}CO_2$ in the breath of mice after injection with remnant-like emulsions containing either cholesteryl[^{13}C]oleate (CO) or cholesteryl[^{13}C]palmitate (CP). Mice were injected with 50 μl of remnant-like emulsions containing cholesteryl[^{13}C]oleate via the tail vein and expired breath was collected as described under Methods. It is apparent from the graph that the enrichment of $^{13}CO_2$ in the breath was markedly lower in mice injected with remnant-like emulsions containing (CP) when compared with mice injected with remnant-like emulsions containing CO. The results are means ± SEM for 30 mice in the CO group and 5 mice in the CP group.

teryl[^{13}C]palmitate was significantly less ($P < 0.0001$) as calculated from the area under the curve (AUC) when compared with mice injected with emulsions containing cholesteryl[^{13}C]oleate.

Remnant metabolism in mice homozygous or heterozygous for deficiencies in LDLr, apoE, or hepatic lipase

Figure 2 (panel A) compares the enrichment in the breath of $^{13}CO_2$ in the expired breath of control, homozygous LDLr-deficient and homozygous apoE-deficient mice after injection of emulsions containing cholesteryl[^{13}C]oleate. In apoE-deficient mice there was no enrichment of $^{13}CO_2$ in the expired breath. In LDLr-deficient mice the rate of expiration of $^{13}CO_2$ in expired breath was decreased compared with controls, indicating a defect in the clearance or metabolism of remnants. The appearance of $^{13}CO_2$ in expired breath was significantly less ($P < 0.0001$) for LDLr-deficient mice when compared with control mice as calculated by AUC.

In heterozygote LDLr-deficient mice (Fig. 2, panel B), the enrichment of $^{13}CO_2$ in the expired breath was similar to control mice as calculated by AUC over the 3-h experiment. However, the patterns of appearance differed in that appearance was slower in the deficient mice. The AUC calculated over the first 45 min was significantly less ($P < 0.0001$) in enrichment of $^{13}CO_2$ in

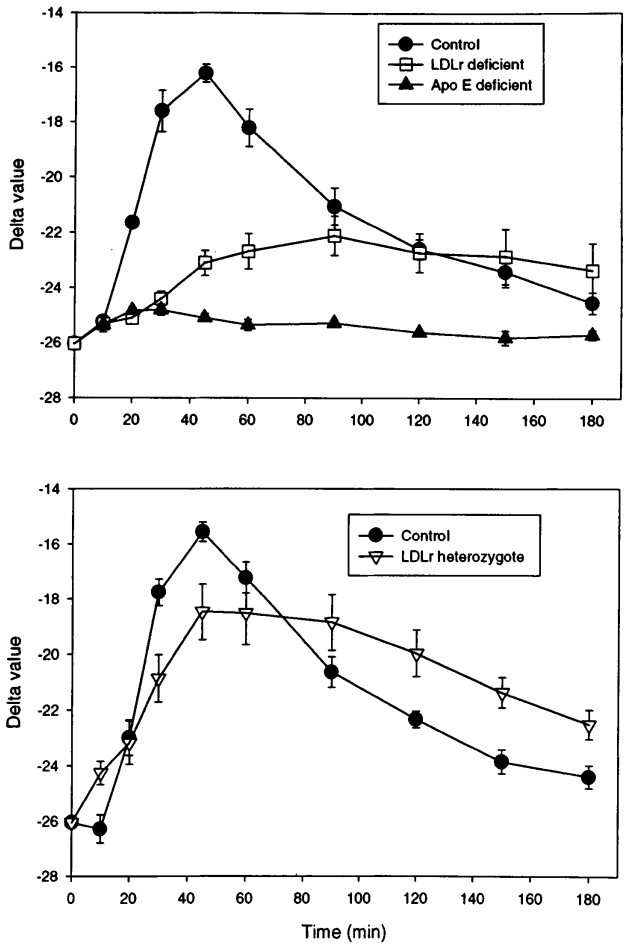

Fig. 2. The upper panel shows the appearance of $^{13}CO_2$ in the breath of control C57BL/6, LDLr-deficient, and apoE-deficient mice. There was no enrichment in $^{13}CO_2$ in the breath of apoE-deficient mice. The appearance of $^{13}CO_2$ in the breath of LDLr-deficient mice was significantly less when compared with controls. The results are means ± SEM for 30 mice in the control group, 4 mice in the LDLr-deficient group, and 5 mice in the apoE-deficient group. In the lower panel the enrichment of $^{13}CO_2$ in the expired breath of control and LDLr heterozygote mice are compared. The graph shows that the enrichment in the breath of LDLr heterozygote was less within the first 45 min but remained elevated for the duration of the experiment when compared with control mice. The results are means ± SEM for 9 mice in the control group and 11 mice in the LDLr heterozygote group.

the breath of the heterozygote LDLr mice. Beyond 90 min the enrichment of $^{13}CO_2$ in the breath of the LDLr heterozygote mice became significantly higher ($P < 0.005$) when compared with control mice, in which levels had returned to background values (calculated by AUC).

Figure 3 (panel A) shows that in heterozygote apoE-deficient mice, the appearance of expired $^{13}CO_2$ from emulsion remnants was slightly less when compared with controls. The difference although small was found

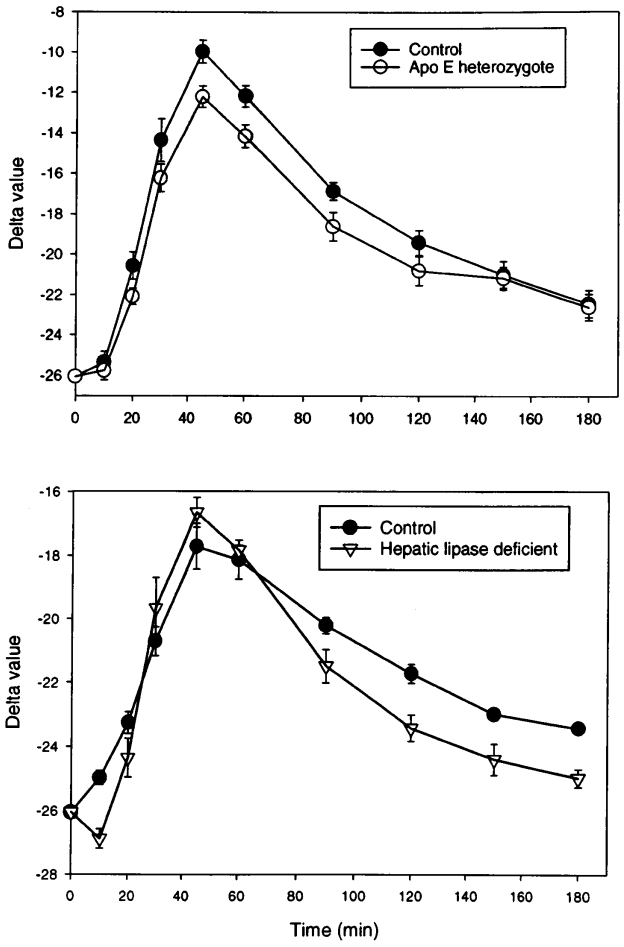

Fig. 3. The appearance of $^{13}CO_2$ in the expired breath of apoE heterozygote and hepatic lipase knockout mice after injection with remnant-like emulsions containing cholesteryl[^{13}C]oleate (CO). In the upper panel, the graph shows that the enrichment of $^{13}CO_2$ in the breath of apoE heterozygote mice was less when compared with control mice. The results are means ± SEM for 9 mice in the control group and 8 mice in the apoE heterozygote group. The lower panel shows that there was no defect in the metabolism of emulsion remnants in hepatic lipase knockout mice. The results are means ± SEM from at least 6 mice in each group.

to be significant ($P < 0.04$) when compared with controls as calculated by the AUC between 45 and 90 min.

In hepatic lipase-deficient mice (Fig. 3, panel B), after the injection of remnant-like emulsions, the areas under the enrichment curves of $^{13}CO_2$ in the breath were similar in hepatic lipase-deficient mice when compared with control mice (calculated by AUC). However, the pattern of appearance of the label in hepatic lipase-deficient mice was slightly different being faster when compared with controls ($P < 0.01$) as calculated as AUC beyond 60 min. It should be noted that in panel A the control and apoE heterozygote mice were female, whereas in panel B male mice were used.

To determine whether the delayed appearance of $^{13}CO_2$ from emulsions labeled in the fatty acid moiety of cholesteryl ester was specific for defects in remnant clearance or metabolism of fatty acids, measurements were made in control, LDLr-deficient, and apoE-deficient mice with [^{13}C]oleic acid complexed with albumin. **Figure 4** compares the enrichment of $^{13}CO_2$ in the breath of control, LDLr-deficient, and apoE-deficient mice injected with [^{13}C]oleic acid complexed with albumin. It is obvious that the patterns of appearance of $^{13}CO_2$ in the breath of LDLr-deficient mice and apoE-deficient mice were not different from controls. In these experiments, although oleic acid was probably metabolized by many tissues including the liver, the results clearly show that the metabolism of fatty acids was unaffected and not related to defects in remnant metabolism found in the LDLr-deficient mice and apoE-deficient mice.

Effects of fasting, feeding, gender, and diurnal variation on remnant metabolism

As shown in **Fig. 5**, panel A, the appearance of $^{13}CO_2$ in the expired breath of mice after a 24-h fast was increased between 30 and 90 min when compared with mice that had been fed for 3 h after a 24-h fast. The enrichment of $^{13}CO_2$ in the expired breath of mice after a 24-h fast was significantly elevated ($P < 0.0001$) as calculated by AUC when compared with mice that had been fed for 3 h after a 24-h fast. The delta value at 0 min (prior to injection of the emulsion) was signifi-

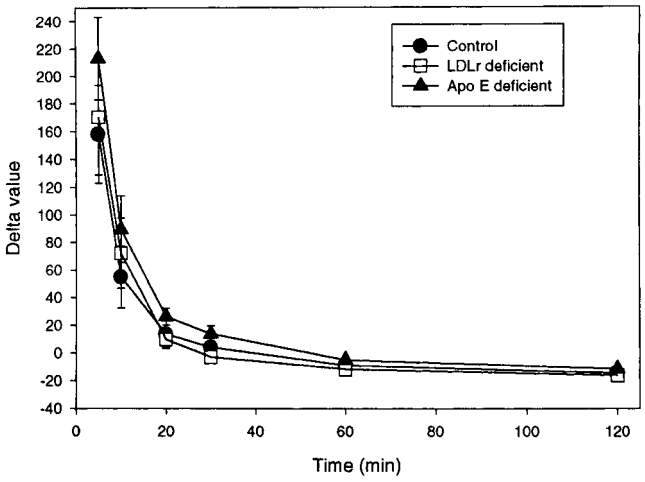

Fig. 4. The figure shows the disappearance of $^{13}CO_2$ after the injection of [^{13}C]oleic acid complexed with albumin in the expired breath of control C57BL/6, LDLr-deficient, and apoE-deficient mice. The disappearance of $^{13}CO_2$ in the breath was similar in control, LDLr-deficient, and apoE-deficient mice. The results are means ± SEM for 2 mice in each group.

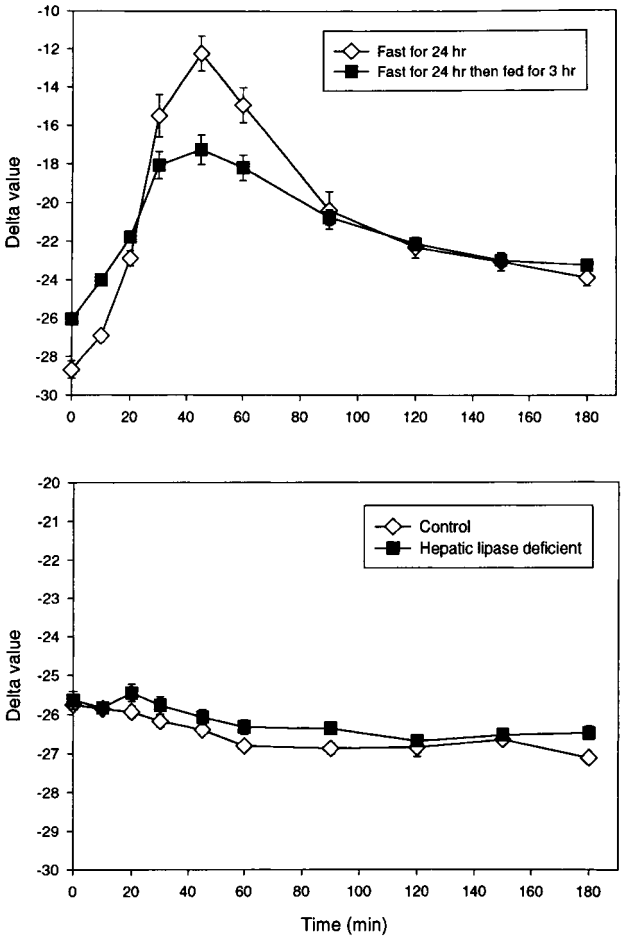

Fig. 5. The enrichment of $^{13}CO_2$ in the breath of mice after injection with remnant-like emulsions containing cholesteryl [^{13}C]oleate (CO). In the upper panel, the graph shows that the enrichment of $^{13}CO_2$ was markedly higher in the breath of mice after a 24-h fast when compared with mice that had access to food for 3 h after a 24-h fast. The results are means ± SEM for 10 mice in the 24-h fast group and 10 mice in the group fed for 3 h after the 24-h fast. The lower panel shows that there was no change in the delta values of either control or hepatic lipase-deficient mice (injected with saline) during the course of the 3-h experiment. The results are means ± SEM for 6 mice in each group.

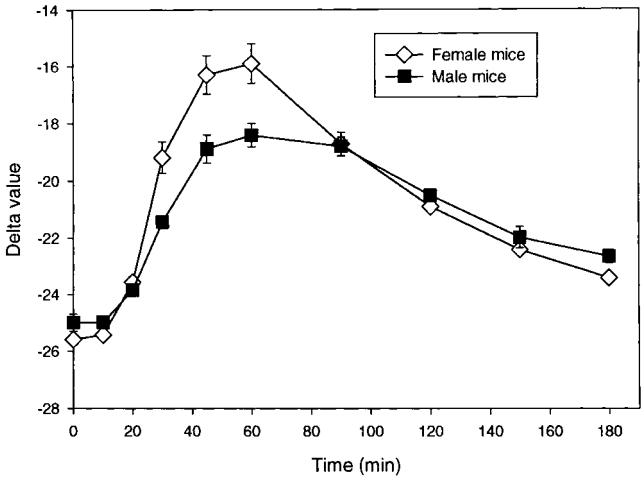

Fig. 6. This figure shows the enrichment of $^{13}CO_2$ in the expired breath of male and female mice after injection of remnant-like emulsions containing cholesteryl[^{13}C]oleate (CO). The appearance of $^{13}CO_2$ in the breath of female mice (body weight 17 g) was significantly more ($P < 0.01$) when compared with male mice (body weight 23 g) as calculated by AUC. The results are means ± SEM for 5–6 mice in each group.

cantly higher in mice that had been fed when compared with mice after a 24-h fast. In mice fed ad libitum, the delta value is approximately 26 delta units. In a control experiment, the delta values in both control and hepatic lipase-deficient mice remained close to 26 delta units over 3 h after the injection of saline (Fig. 5, panel B).

In fed female mice the enrichment of $^{13}CO_2$ in the expired breath was higher between 20 and 90 min after injection of the emulsion when compared with fed male mice (**Fig. 6**), indicating that remnant metabolism was faster in female mice than in male mice. The female mice had expired significantly more $^{13}CO_2$ ($P < 0.01$) when compared with male mice as calculated by AUC (experiments were conducted at 9 am).

To establish whether there was a diurnal variation in remnant metabolism, experiments were conducted in male mice at different times throughout the day. All mice were fed ad libitum, so the usual nocturnal feeding behaviour was evident. In **Fig. 7**, compared with experiments conducted in male mice at either 6 am (panel A), midday (panel B), or at 6 pm (panel C) as calculated by AUC, animals injected with emulsions at midnight (panel D), showed less enrichment in the expired breath with $^{13}CO_2$ ($P < 0.02$). At 6 am (panel A) the metabolism of remnant particles was slightly delayed when compared with remnant metabolism at midday (panel B) or at 6 pm (panel C).

DISCUSSION

In previous studies we have shown that the metabolism of emulsion remnants can be measured by the appearance in breath of either labeled $^{14}CO_2$ or $^{13}CO_2$ after injection of lipid emulsions labeled with cholesteryl [1-^{14}C]oleate (3, 4) or cholesteryl[^{13}C]oleate (5), respectively. In the present studies, to determine the specificity and sensitivity of the ^{13}C breath test in detecting changes in remnant metabolism, experiments were performed with different ^{13}C-labeled cholesteryl

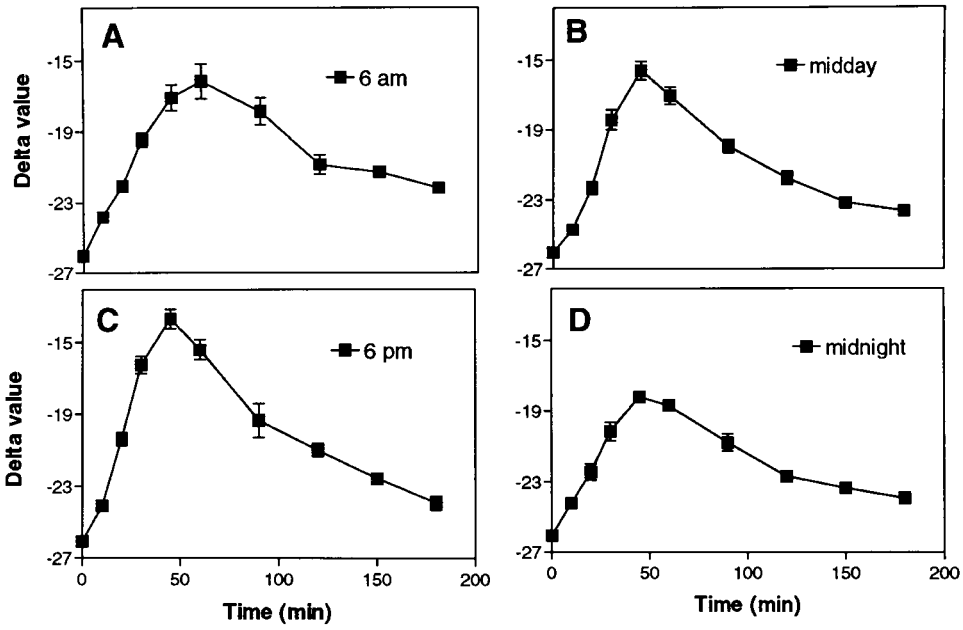

Fig. 7. The appearance of $^{13}CO_2$ in the expired breath of control C57BL/6 mice injected with remnant-like emulsions containing cholesteryl[^{13}C]oleate at 6 am, midday, 6 pm, or at midnight. At midnight the enrichment of $^{13}CO_2$ in the breath of mice was significantly less ($P < 0.02$) when compared with the experiments conducted at 6 am, midday, or 6 pm (panels A, B, C) as calculated by AUC. The results are means ± SEM for 6 mice in the 6 am group, 30 mice in the midday group, 7 mice in the 6 pm group, and 4 mice in the midnight group.

esters, with various transgenic mice, and the effects of feeding and of diurnal variation were established.

The metabolism of remnant-like emulsions prepared with cholesteryl[^{13}C]palmitate was found to be markedly slower than metabolism of emulsions prepared with cholesteryl[^{13}C]oleate (Fig. 1). This finding is consistent with slower hepatic metabolism of cholesteryl palmitate previously reported (10). The marked differences in hydrolysis between the two cholesteryl esters was related to the specificity of cholesteryl ester hydrolase (10). In vitro studies showed that unsaturated cholesteryl esters (cholesteryl oleate and linoleate) were hydrolyzed more rapidly than saturated ones (cholesteryl palmitate and cholesteryl stearate) (11).

In homozygous apoE-deficient mice as measured by the ^{13}C breath test, there was no metabolism of remnants (Fig. 2, upper panel). In homozygous LDLr-deficient mice as measured by the ^{13}C breath test, the clearance and metabolism of remnant-like emulsions were markedly decreased. These results are consistent with our previous findings (3, 4). The breath test was also useful in detecting a defect in remnant metabolism in LDLr heterozygote deficient mice. The metabolism of emulsion remnants was found to be significantly slower in the LDLr heterozygote mice when compared with control mice. In apoE heterozygote deficient mice the metabolism of remnants was slowed to a small but significant extent when compared with controls (Fig. 3). The presence of only one functional apoE allele was shown to increase susceptibility of the mice to diet-induced hyperlipidemia (12). The analysis of intimal lipid accumulation in these apoE-deficient heterozygote mice revealed the development of atherosclerosis when fed a semi-synthetic hypercholesterolemic diet (12, 13).

Hepatic lipase belongs to the lipase gene family (14). In various cell cultures, perfused liver systems and intact animals, evidence for a role of hepatic lipase in mediating the hepatic uptake of chylomicron remnants has been provided (15–20). In contrast, in hepatic lipase-deficient mice, the clearance of chylomicrons and triglyceride-rich lipoproteins has been shown to be normal (21). Our present studies with the breath test in the hepatic lipase-deficient mice confirm that clearance and metabolism of particles were similar to that of normal control mice 60 min after injection of remnant-like emulsions (Fig. 3, panel B). After 60 min, remnant metabolism was faster in hepatic lipase knockout mice when compared with control mice. In the absence of hepatic lipase, possibly lipoprotein lipase or other lipases substitute for hepatic lipase in mediating the clearance of triglyceride-rich particles.

The effect of a 24-h fast on mice resulted in a markedly higher rate of metabolism of remnant-like particles when compared to the fed state as assessed by the $^{13}CO_2$ breath test (Fig. 5). This finding may be explained by the competition of the injected emulsion particles with those produced by feeding. At midnight or 6 am remnant metabolism was found to be decreased when compared with breath tests conducted at midday or at 6 pm (Fig. 7). These findings may also reflect feeding during the dark cycle (midnight and 6 am) when compared with the light cycle (midday and 6 pm).

The results obtained in the fasted mice with remnant-like lipid emulsions were consistent with those found in rats using cholesteryl [1-^{14}C]oleate-labeled remnants prepared from lymph chylomicrons (22). In fasted rats it was previously shown that the appearance in the breath of $^{14}CO_2$ was elevated when compared with fed animals (23, 24). These results were explained by the rapid change in hepatic fatty acid metabolism from oxidation in the starved state to esterification of phospholipids and triacylglycerols in the fed state (23). In our study, in fasted mice the delta value was significantly lower at 0 min (prior to injection of the emulsion) when compared with the delta value in the fed mice (Fig. 5, panel A). This finding may be explained by the ^{13}C content of the diet consumed by the mice. Enrichment with ^{13}C in foods depends on their ultimate origin from C_3 or C_4 plants (25). After a 24-h fast the metabolism of carbohydrates would be markedly decreased and fats would be the major fuel presumably extensively derived from C_3 plants accounting for a lower $^{13}C/^{12}C$ ratio in expired breath.

Chylomicron remnants (CR) are known to contribute to the development of atherosclerosis. In situations of extreme defects in CR metabolism it is clear that CR have the potential to contribute to the development of atherosclerosis. The metabolism of CR may be a sensitive index of susceptibility to atherosclerosis, but at the present time there is no simple method for assessment of an individual's capacity to metabolize chylomicron remnants. Previous methods have used postprandial assays of lipids, apoB-48, and retinyl esters, but these methods are complex and insensitive to small defects in CR clearance.

In conclusion, a $^{13}CO_2$ breath test has been described to assess the metabolism of remnants. The test provides a useful and sensitive method for non-invasive testing of susceptibility to atherosclerosis in experimental animals. Clinical trials in humans are needed to establish the predictive value of the breath test in the diagnosis of individuals susceptible to atherosclerosis.

This work was supported by Inovax Ltd. We thank Allan Light for the electron microscopy.

Manuscript received 28 July 1997 and in revised form 10 November 1997.

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