Rapid Communication

A novel antiserum specific to apolipoprotein B-48: application in the investigation of postprandial lipidaemia in humans

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1. Apolipoprotein B-48, the transport protein for chylomicrons, is identical with apolipoprotein B-100 for the first 48% of its sequence. No antiserum has yet been reported that can recognize apolipoprotein B-48, but not apolipoprotein B-100.

2. In the present study an antiserum was raised to the C-terminal sequence of apolipoprotein B-48, using specific chemical reactions to ensure that the charged carboxyl group of the C-terminal isoleucine residue was free. In a Western blot the antiserum was shown to bind to a protein band having the characteristics of apolipoprotein B-48, but not to apolipoprotein B-100.

3. In the early evening 11 subjects were given a test meal which contained 40 g of mixed oil and retinyl palmitate. Blood samples were collected over 9 h. Chylomicron-enriched fractions were prepared and analysed for triacylglycerol, retinyl palmitate and apolipoprotein B-48, the latter after separation using SDS/PAGE and visualization by chemiluminescence on a Western blot. Both triacylglycerol and apolipoprotein B-48 showed an early peak at 1 h, which was not seen with retinyl palmitate. All three substances gave a broader peak between 5 and 6 h postprandially. Retinyl palmitate concentrations declined rapidly during the late (6–9 h) postprandial period, but apolipoprotein B-48 concentrations remained elevated.

4. This study has shown that an antiserum has been produced which is specific for apolipoprotein B-48. This has enabled measurement of postprandial concentrations of the protein that revealed features of chylomicron metabolism which have not been reported previously.

INTRODUCTION

At present there is no simple rapid assay that can be used to distinguish between lipids that are derived from the diet, which can circulate in the form of chylomicrons, and those that are synthesized in the liver and are initially secreted as very-low-density lipoproteins (VLDL). Ultracentrifugation only achieves partial separation of the two lipoprotein classes, and the laborious procedure precludes its use for the routine analysis of large numbers of samples. The inclusion of retinyl palmitate in the diet, which is subsequently incorporated into the core of chylomicrons, has been used to follow chylomicron metabolism [1]. The method, which includes organic extraction followed by h.p.l.c. separation, is lengthy and lacks good reproducibility. Recently, the validity of retinyl palmitate as a tracer of intestinal lipoproteins has been questioned due to the demonstration of significant transfer of retinyl palmitate between lipoprotein classes in the circulation [2].

An alternative approach is to assay apolipoprotein B-48 (apo B-48), which is uniquely associated with chylomicrons in humans [3]. The most common approach to the routine assay of specific proteins is to use an immunoassay. However, apo B-48 has an amino acid sequence (2152 amino acids) identical with the N-terminal 48% of apolipoprotein B-100 (apo B-100), which is present in VLDL, intermediate-density lipoproteins and low-density lipoproteins (LDL). As a consequence of this structural similarity, antisera that recognize apo B-48, but not apo B-100, have not been produced [4]. A possible solution to this problem is to exploit the fact that the C-terminal isoleucine residue of apo B-48 is free with its α-carboxyl group being negatively charged, whereas this same residue in apo B-100 is fixed in the protein structure with its α-carbonyl group being part of a peptide bond.

We have made use of this approach to produce

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Abbreviations: apo B-48, apolipoprotein B-48; apo B-100, apolipoprotein B-100; ECL, enhanced chemiluminescence; HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very-low-density lipoproteins.

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an antiserum to apo B-48. We report the use of this antiserum in an immunoblot analysis of apo B-48 in chylomicron-enriched samples obtained from subjects after a standard test meal, and we compare the data with that obtained from analysis of retinyl palmitate.

MATERIALS AND METHODS

Raising of antiserum

A heptapeptide consisting of an N-terminal cysteine residue attached to the C-terminal hexapeptide (H$_2$N$^+$-Cys-Leu-Gln-Thr-Tyr-Met-Ile-COO$^-$), equivalent to residues 2147-2152 of apo B-48, was custom-synthesized by the National Institute of Medical Research (Mill Hill, London, U.K.). The peptide was conjugated to ovalbumin using N-succinyl-6-maleimidocaproate (Fluka Biochimika, Buchs, Switzerland) and on average 11 peptide molecules were attached to each ovalbumin carrier molecule [5]. Conjugate, containing the equivalent of 200 μg of peptide, was emulsified in non-sterile Freund's incomplete adjuvant (Guilhay Antisera, Guildford, Surrey, U.K.) and injected both subcutaneously and intramuscularly into a New Zealand White rabbit. After 6 weeks the animal was boosted with conjugate containing the equivalent of 100 μg of peptide and blood was collected at 8, 11, 13 and 15 days for measurement of the titre using a competitive e.l.i.s.a.

Subjects for the postprandial study

The subjects were 11 healthy male volunteers with an average age of 22.5 years (range 21-25 years) and a body mass index of 23.8 kg/m$^2$ (range 19.6-26.2 kg/m$^2$). Habitual fat intake, assessed by completion of 4-day dietary diaries, range from 33% to 45% of total energy intake as fat. None of the subjects had a history of endocrine or liver disease. Fasting LDL and high-density-lipoprotein (HDL) cholesterol and triacylglycerol concentrations were within the normal reference range. All subjects gave their written consent to participate, and ethical consent for the study was obtained from Ethics Committees of the University of Surrey and the Royal Surrey County Hospital, Guildford, Surrey, U.K.

Study protocol

On the study day all the subjects attended the Investigation Unit for the collection of a fasting blood sample. In order to standardize food intake during the day before the evening test meal, subjects were served breakfast in the Unit (2.43 MJ; 11 g of fat) and given a packed lunch (3.69 MJ; 32 g of fat) and snacks (2.24 MJ; 23 g of fat), to be eaten at stated times. Subjects continued with their normal activities and returned to the Unit at 18.30 hours, where a venous cannula was inserted into the antecubital vein for the purpose of serial blood sampling and two baseline blood samples were collected. At 19.00 hours subjects consumed a test meal (see below) and postprandial blood samples were collected at 30 min, 60 min and at hourly intervals until 9 h after the meal. Subjects remained in the Unit throughout the evening and overnight carrying out only normal light activities until 23.00 hours when they retired to bed. They were allowed water, but no other food or drink was permitted during the period of blood collection.

Test meal

The meal was a fat-free rice dish and salad, 40 g of the test oil and a fat-free milk shake (Carnation Ltd, Croydon, Surrey, U.K.). The test oil was a mixed oil formulated so that the fatty acid composition closely approximated that of the current U.K. fatty acid intake (44% saturated fatty acids, 42% monounsaturated fatty acids, 14% polyunsaturated fatty acids). The test oil was administered as 20 g in the form of a salad dressing and 20 g added with water to the powdered milk shake. Retinyl palmitate (700 i.u./kg body weight) was also added to the milk shake. Subjects consumed the meal within 20 min.

Chylomicron preparation and analyses

Blood samples, 10 ml in heparinized tubes, were spun immediately for 7000 g min in a bench centrifuge and the plasma was transferred to 10 ml glass tubes containing no anticoagulant. A chylomicron-enriched fraction was prepared by centrifugation for 2.1 x 10$^6$ g min using the method of Grundy and Mok [6], which allows the flotation of larger triacylglycerol-rich particles of densities <1.006 g/ml. This fraction was mixed and aliquots were placed into separate tubes which were stored frozen at -20°C for the later analysis of triacylglycerol, retinyl palmitate and apo B-48. A preservative mixture [7] was added to the chylomicron-enriched fraction used for apo B-48 analysis. Triacylglycerol concentrations were determined on a Cobas Bio centrifugal analyser using a kit supplied by Roche (Welwyn Garden City, Herts, U.K.). Retinyl palmitate was analysed by reversed phase h.p.l.c. using the procedure described by De Ruyter and De Leeuwer [8]. For analysis of apo B-48, chylomicron-enriched samples were loaded on to an SDS/polyacrylamide gel with a linear gradient from 5% to 20%, which was subsequently blotted on to a nitrocellulose membrane. The membrane was incubated with the anti-(apo B-48) antiserum at a dilution of 1:1000 and visualized using an enhanced chemiluminescence (ECL) system according to the manufacturer's instructions (Amersham International plc, Little Chalfont, Bucks, U.K.). The bands were scanned with a flying-spot densitometer at 550 nm (Shimadzu CS-9001PC, from V.A. Howe, A. S. Pool et al.
Banbury, Oxon, U.K.). The intra- and inter-assay coefficients of variation were 28.3% and 22.3%, respectively. An aliquot of the same chylomicron sample was run on each gel and used as an internal standard. The densitometry value for this was arbitrarily set at 100% and the other apo B-48 values were expressed as a percentage of this value. When different quantities of the internal standard were used, a linear response was obtained.

Values for triacylglycerol (mmol/l), retinyl palmitate (µg/ml) and apo B-48 (%) are given as means ±SEM for 11 subjects for each time period.

RESULTS

Protein staining and Western blotting of chylomicron-enriched fractions

Fig. 1 shows the pattern obtained after separation by SDS/PAGE of the chylomicron-enriched fractions from one of the subjects. Fig. 1(a) shows the result after staining for protein with Coomassie Blue. The prominent high-molecular-mass band, apo B-100, was clearly visible at all time points (lanes 3–15). The fainter band, marked apo B-48, with a molecular mass of slightly greater than 205 kDa, was just visible in lanes 2, 7, 10 and 11 of the original gel. However, when these same fractions were visualized using the antiserum raised against the heptapeptide corresponding to the C-terminal sequence of apo B-48, the apo B-48 band was visualized but apo B-100 was not (Fig. 1b). No bands were seen for any of the other proteins from this subject. Some of the protein bands in the molecular mass markers were stained more diffusely, due to non-specific interactions with secondary and tertiary antibodies.

Postprandial response of triacylglycerol, retinyl palmitate and apo B-48 to a mixed oil meal

Triacylglycerol concentrations in the chylomicron-enriched fraction showed a marked rise in response to meal ingestion with an increase from preprandial concentrations of 1.6 ± 0.4 mmol/l to an initial peak value of 2.5 ± 0.3 mmol/l 1 h after consumption of the test meal (Fig. 2a). A second and more sustained rise in triacylglycerol was seen with a peak value of 2.4 ± 0.3 mmol/l 5 h after the meal. Triacylglycerol concentrations then returned to preprandial levels 8–9 h after the meal. A similar pattern of response was seen for apo B-48 concentrations, with a sharp peak response at 1 h (64 ± 18%), representing a 3-fold rise in circulating apo B-48 (Fig. 2b). A second, more sustained, rise to 91 ± 21% was observed between 5 and 6 h postprandially, with values remaining around this level up to 9 h after the meal. The pattern of response with retinyl palmitate differed from that described for triacylglycerol and apo B-48 (Fig. 2c). A rise from zero concentrations in retinyl palmitate was first observed at 3 h postprandially with no evidence for an early peak at 1 h. The major increase in retinyl palmitate occurred between 5 and 7 h postprandially, reaching concentrations of 1.2 ± 0.3 and 1.3 ± 0.4 µg/ml, with values declining sharply thereafter.

DISCUSSION

We have produced antiserum to the C-terminal sequence of apo B-48 by using an appropriate peptide as a hapten. The peptide was attached specifically to the carrier protein through the sulphydryl group of the N-terminal cysteine residue of the heptapeptide to ensure that its C-terminal end was freely available. This antiserum was used in a Western blot procedure where it produced only one
meal, because retinyl palmitate (fed with the test meal) is not detectable in the sample collected 1 h postprandially. Since an early sharp rise in triacylglycerol concentrations is not seen in subjects given a test meal after an overnight fast [11], we suggest that in the present study, where subjects were not in a fasted state, this early peak represents chylomicrons formed from previously ingested food. The pattern of response implies that meal ingestion triggers secretion of pre-formed chylomicrons from a storage pool. Their exact origin and the signal for their release require further investigation.

Our main reason for producing an antiserum to apo B-48 is to follow the effect of diet on the metabolism of chylomicrons and in particular to enable us to study chylomicron remnants and their possible role in atherosclerosis [12]. To do this we are developing a more sensitive e.l.i.s.a. This will allow apo B-48 to be measured in small sample volumes with better reproducibility than the semi-quantitative ECL assay described here.

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REFERENCES