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ORIGINAL CONTRIBUTION

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Meal ingestion provokes entry of lipoproteins containing fat from the previous meal: possible metabolic implications

Summary *Background* Prolonged and exaggerated postprandial plasma triacylglycerol (TAG) concentrations are considered as an independent risk factor for coronary artery disease. Western populations eat many meals at regular intervals, and can be in a postprandial state for at least 17h of a 24h period. After consuming 2 meals an early plasma TAG peak has been observed after the second meal, the origin of which is unclear. *Aim of the study* To test the hypothesis that the early TAG peak observed following sequential meals was of intestinal origin and represented fat derived from the previous meal. *Methods* Postprandial plasma lipaemic responses of 17 healthy postmenopausal women were studied by giving a test breakfast followed by a lunch. Water-miscible retinyl palmitate (RP) was added to the breakfast, but not the lunch test meal. Plasma TAG, retinyl esters (RE) and apo B-48 were determined for a 10h period following breakfast. *Results* In response to the test meals, RE, apo B-48 and TAG showed multiple peaks. Despite omission of RP from the lunch, RE showed an early peak response after ingestion of lunch in 15 of 17 subjects. The peak response after lunch of all three markers appeared significantly earlier compared with their respective peak responses after the breakfast ($P < 0.0001$). The area of RE response after lunch was significantly correlated with the RE lipaemic response to the breakfast ($r = 0.67$; $P < 0.004$) and to the fasting TAG concentration ($r = 0.48$; $P < 0.05$). *Conclusions* Since the lunch did not contain RP, the distinctive second influx of RE after lunch was believed to have originated from the breakfast. This, together with the fact that all three markers showed an earlier response to the lunch than the breakfast, supports the view that ingestion of a second meal provokes entry of fat from the previous meal, from an as yet unidentified site (gut, enterocytes, lymph). The results indicate that the degree of TAG "storage" from previous meals might be a function of TAG tolerance and provide a possible site of regulation of the entry of fat into the systemic circulation.

Key words triacylglycerol – retinyl ester – apolipoprotein B-48 – postprandial lipaemia – chylomicron – triacylglycerol tolerance

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Introduction

Prolonged and exaggerated postprandial plasma triacylglycerol (TAG) concentrations are considered as an independent risk factor for coronary artery disease (CAD) [1]. The atherogenic potential of postprandial TAG-rich lipoproteins (TRL) both directly and indirectly, by influencing the metabolism of other lipoproteins, is now well established [2–4]. In order to investigate the contribution of chylomicrons (CM) and chylomicron remnants (CMR) to CAD a technique which distinguishes CM and CMR from hepatically derived TRL is required. Retinyl ester labelling of CM by the administration of retinyl palmitate (RP) with a test meal is one method which has been used as a marker for CM and their remnants, although limitations have been raised about this technique [5]. Another important

marker is apolipoprotein (apo) B-48, the unique structural apolipoprotein present in CM and CMR, which when quantified, gives an accurate measure of CM particles [6, 7]. Use of more than one marker allows a more complete picture of postprandial events to be formed.

Western populations eat many meals at regular intervals, and they are usually in a postprandial state for at least 17h of a 24h period [8]. Following intestinal absorption, fats ingested in the meal appear in the blood predominantly as TAG, and reach a peak around 3–4h and then gradually decrease. Although many studies have shown a single peak response of TAG after a fat-rich meal, Cohn et al. [9] reported multiple peaks of TAG in a 12h study period following the ingestion of a fat-rich meal given after 14h of overnight fast. They suggested that TRL from both the liver and intestine contribute to the earlier and later peaks in postprandial triglyceridaemia. Williams et al. [10] and Peel et al. [11] also reported a marked biphasic TAG response to an evening meal given 5 h after a standard lunch, in normal subjects. The highly associated apo B-48 and TAG multiple peaks obtained in the latter study suggested that both peaks were of intestinal origin. RP which was given with the evening meal as a marker in that study showed only one peak response associated with the second peak of apo B-48 and TAG. Therefore, it was concluded that the earlier TAG and apo B-48 peaks represented fat consumed from the previous meal (lunch). Another study that investigated the origin of the early TAG peak seen when successive meals have been consumed, used a breakfast and a lunch containing very different fatty acid compositions [12]. Data from this study suggested that the CM released into the circulation immediately after consuming the lunch contained fatty acids derived from the breakfast. Overall, these data support the possibility of TAG retention within the gut lumen, enterocyte or lymphatics, which on subsequent food ingestion is released into the circulation. A rise in arterial concentration of TAG, 30–60 min after the ingestion of 75 g of oral glucose in normal subjects suggested that there is a possibility of releasing pre-formed CM following ingestion of carbohydrate as well as mixed meals [13].

In the present study, using an aqueous source of RP as a marker of ingested fat for the first meal of two sequential meals [14], we have investigated the hypothesis that the early TAG peak observed after the second meal was of intestinal origin and represented fat entering from the previous meal. Data from this study will increase our understanding of the mechanisms of lipid absorption and metabolism. We have also investigated the concordance of plasma TAG, RE and apo B-48 responses.

Subjects and methods

Subjects

The subjects were a group of seventeen healthy, post-menopausal women with an average age of 62 years (range 52–76) and BMI of 26.6 kg/m^2 (range 21.5–35.2). Post-menopausal women were chosen as they represent a group with high risk of CHD development, and in whom plasma TAG is an important CHD risk factor, due to their gender and menopausal status [8]. None of the subjects had a history of endocrine or liver disease, none took hormone replacement therapy or smoked. Mean baseline biochemical characteristics of the volunteers are shown in Table 1.

Study protocol

The study was approved by the Ethics Committee of the University of Reading and all subjects gave informed consent to participate in the study. On the study day, each subject reported to the Investigation unit at 0800 hours after a 12h overnight fast. Subjects were asked to refrain from alcohol and strenuous activities during the previous day. A cannula was inserted into a forearm vein and two baseline fasting blood samples were collected over a period of 10 minutes. At 0830 hours (time 0 min) all subjects consumed the standard test breakfast (see below) and at time 270 min the standard lunch (see below) was eaten. Blood samples were collected every 30 min for the first 90 min after each meal and thereafter hourly until 600 min. The volunteers were allowed water, sugar-free drinks and decaffeinated beverages, without sugar or milk, but no other food or drink was permitted during the period of blood collection.

Table 1 Baseline anthropometric and biochemical characteristics of post-menopausal women

	Mean	SD	Range
Age (years)	62	8	52–76
BMI (kg/m^2)	26.6	4.0	21.5–35.2
TAG (mmol/l)	1.3	0.5	0.8–2.5
Cholesterol (mmol/l)	6.0	1.0	3.8–7.6
LDL-cholesterol (mmol/l)	3.9	0.9	2–5.3
HDL-cholesterol (mmol/l)	1.4	0.4	0.9–2.2
Glucose (mmol/l)	5.1	0.3	4.4–5.5
Insulin (pmol/l)	34.1	19.3	13–87.5

Test meals

The standard breakfast contained croissant, butter, jam, semi-skimmed milk and orange juice (2.4 kJ energy, 46 % energy as fat: 27 % energy as saturated, 12 % energy as monounsaturated, 5 % energy as polyunsaturated, 2 % energy as trans; 6 % energy as protein and 48 % energy as carbohydrate: 25 % energy as starch, 25 % energy as sugar) and a standard lunch contained vegetable casserole and dumplings, strawberry and cream sundae and orange juice (3.1 kJ energy, 52 % energy as fat: 27 % energy as saturated, 18 % energy as monounsaturated, 5 % energy as polyunsaturated, 2 % energy as trans; 6 % energy as protein and 42 % energy as carbohydrates:15 % energy as starch, 27 % sugar). In addition 200,000 IU of water miscible RP (Roche Vitamins Europe Limited, Switzerland) was added to the orange juice consumed at the breakfast. Subjects consumed each test meal within 20 min.

Biochemical analysis

Blood samples were collected into 10 ml EDTA tubes and were spun immediately at 3000 rpm for 15 min in a bench top centrifuge. Plasma was removed and aliquoted and stored at –20 °C. A preservative mixture 5 % (v/v) [15] was added to the plasma samples used for apo B-48. The preservative contained: EDTA, sodium azide, sodium chloride (Merck, Lutterworth, UK), aprotinin, benzamidine, chloramphenicol and gentamicin sulphate (Sigma, Poole, UK). Samples for the RE analysis were covered with silver foil to avoid degradation due to exposure to the sunlight. Plasma TAG, TC, HDL-C and glucose analysis was performed using an IL Monarch centrifugal analyser (Instrumentation Laboratory, Warrington, UK) using enzymatic colorimetric kits (Instrumentation Laboratory, Warrington, UK). HDL-C concentrations were determined following precipitation of the fresh plasma with dextran-magnesium chloride reagent [16]. LDL-C was calculated using the Friedewald formula [17]. Plasma insulin was measured using a specific enzyme-linked immunosorbant assay (Dako, Cambridge, UK). Retinyl esters were estimated by normal phase high performance liquid chromatography (HPLC) procedure described by Ruotolo et al. [18]. Apo B-48 was quantitated by a competitive enzyme linked immunosorbant assay (ELISA) [19]. The mean intra- and inter-assay CVs for TC, TAG, glucose, insulin, apo B-48 and retinyl ester were 2.1, 1.9, 2.0, 4.0, 5.0 and 2 % and 4.0, 3.1, 4.7, 5.5, 9, and 3 % respectively.

Statistical analysis

The median time to the peak responses of each marker was calculated by averaging the individual time of peak concentration from the time of ingestion of a given meal. All peaks, before and after the lunch, were identified visually. The first peak was defined as the highest concentration during the period 0–270 min, while the second peak was defined as the highest concentration of a risen peak response during 270–600 min. To determine whether the measured concentration of the second peak represented a significant increase in concentration, a Wilcoxon Sign Rank test was performed on the second RE peak concentration and the RE concentration at the previous time point. Areas under the time response curve (AUC) were calculated using the trapezoidal rule [20]. Repeated-measures analysis using the general linear mixed model procedure [21] was performed to test for the significance of changes in plasma concentrations with time. The degree of significance of difference between means was calculated using the Paired t-test where the data were normally distributed and using the Wilcoxon Sign Rank test for the distribution free data. Correlation analysis was performed calculating Pearson correlation coefficients. All analyses were performed using SAS statistical package version 6.12 (SAS Institute, Cary, NC, USA). P values of < 0.05 were taken as significant. To improve clarity and as median values were plotted no error bars were included in Fig. 1.

Results

The median plasma TAG concentration increased gradually after breakfast from baseline to give a peak concentration between 210–270 min (Table 2, Fig. 1). The concentration fell prior to lunch, but following lunch, it increased again showing another peak concentration around 60–90 min after the lunch. The calculated me-

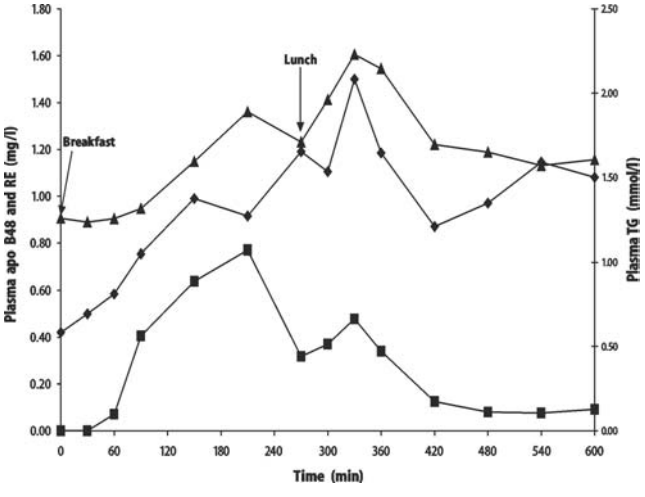

Fig. 1 Median plasma triacylglycerol (TAG) (▲), apolipoprotein (apo) B-48 (◆) and retinyl ester (RE) (■) responses of postmenopausal women to sequential meals given at 0 min and 270 min (n = 17)

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Table 2 Median times of peak responses, AUC1 (0–270 min) and AUC 2 (270–600 min) of apolipoprotein B-48, triacylglycerols and retinyl esters[1]

	AUC1 (0–270 min)	AUC2 (270–600 min)	Peak response time (min)	
			After breakfast (0–270 min)	After Lunch (270–600 min)
TAG (mmol/l.min)	374±66	563±116	210±19[a]	60±12*
Apo B48 (µg/ml.min)	254±52	376±87	210±21[a]	60±12*
RE (µg/ml.min)	120±36	53±66	150±27[b]	60±25*

[1] Median ± CI 95%, n = 17
* Significantly different from breakfast, P < 0.0001
[a, b] Values with unlike superscripts were significantly different (apo B48 vs RE, P < 0.003; TAG vs RE, P < 0.002)
apo apolipoprotein; *TAG* triacylglycerol; *RE* retinyl esters

dian times of the TAG peaks after the breakfast (210, range 150–270 min) and lunch (60, range 30–150 min) were significantly different (Wilcoxon Sign Rank test, $P < 0.0001$; Table 2).

Apo B-48 concentration rose from the baseline after the breakfast and reached an initial peak around 210–270 min (Table 2, Fig. 1). In response to the lunch a median apo B-48 peak was observed at around 60 min after lunch (330 min from study commencement). The calculated median times of the apo B48 peaks after the breakfast (210, range 150–270 min) and lunch (60, range 60–150 min) were significantly different from one another (Wilcoxon Sign Rank test, $P < 0.0001$; Table 2).

The exogenous marker, RE, appeared in the plasma after the breakfast and a median peak occurred around 150–210 min (Table 2, Fig. 1). The RE concentration fell rapidly from the peak up to 300 min after the breakfast. In response to lunch (given at 270 min) which did not contain RP, another small, less sustained peak of RE occurred around 60–90 min after the lunch. Repeated-measures ANOVA showed significant changes of plasma RE concentrations between 270–600 min after breakfast ($P < 0.01$). There was found to be a statistically significant increase in RE concentrations at the second RE peak compared to the value at 270 min, when the second meal was given (Wilcoxon Sign Rank test, $P < 0.0001$). The median times of the RE peaks after the breakfast (150, range 90–270 min) and lunch (60, range 30–210 min) were significantly different from one another ($P < 0.0001$; Table 2). Although there was a considerable between-subject variability in the second RE response, only two subjects out of 17 failed to show a second RE peak in response to lunch. The RE peak after lunch occurred significantly earlier than the RE peak after breakfast ($P < 0.0001$; Table 2). In addition the RE peak after breakfast appeared significantly earlier than the apo B48 (paired t-test, $P < 0.003$) and TAG (Wilcoxon Sign Rank test, $P < 0.002$) peaks after breakfast.

Correlation analysis was carried out in order to understand factors associated with variability in the second RE response. There was a significant correlation ($r = 0.67$; $P < 0.004$) between the extent of RE lipaemia to the breakfast (AUC1 (AUC for RE from 0–270 min)) and the extent of the second RE response to lunch (AUC2 (AUC for RE 270–600 min)). AUC2 for RE was also significantly correlated with the fasting TAG concentration ($r = 0.48$; $P < 0.05$).

Discussion

In the present study, the response of dietary-derived lipoproteins in post-menopausal women who ingested two sequential meals, was investigated using apo B-48, RP and plasma TAG as markers. RP was specifically used to label the fat consumed in the breakfast and to evaluate the effect of giving a lunch, given within a few hours after the breakfast, on overall RE response.

Fifteen out of the seventeen subjects demonstrated multiple distinguishable RE peaks, but of varying degrees, in response to the two sequential meal. Since the lunch did not contain RP, the RE appearing as a peak response after lunch (and therefore deviating from the declining point of the RE response to breakfast) almost certainly originated from the first meal. This, together with the fact that all three markers showed an earlier peak response to the lunch than to breakfast, supports the view that CM in the early postprandial period after the lunch may originate from the breakfast [10–12, 22]. The exact mechanism of the carry over of fat from one meal to the postprandial lipaemic profile of the next meal is not clear. The ingestion of the second meal could trigger the release of CM stored in the enterocytes or possibly in the lymphatic system following the previous meal, into the circulation. The increase in blood circulation to the small intestine following food ingestion may increase the outflow of pre-formed CM from the enterocytes. Also an increase in lymphatic flow rate and volume could cause a release of CM particles in lymphatic system or lacteals. Increase in the lymphatic flow and volume after a meal has been identified by early investigators [23]. A displacement of pre-formed CM by newly formed CM is another possible explanation. But the fact that even a low fat meal, or a glucose drink can cause a

similar effect opposes the hypothesis that this process is fat-mediated [13, 22, 24–26]. Sham feeding has been shown to be as effective as food ingestion in activation of vitamin A and lipid entering the blood stream [27, 28], although there are inconsistencies in the literature as a recent study showed no effect of sham feeding on release of stored TAG from a high fat meal eaten 5 hours previously [29]. Other studies showed significant differences in postprandial lipaemic responses when volunteers were subjected to different oral fat exposures following the ingestion of safflower oil capsules and suggested that chemosensory or tactile mechanism can affect postprandial lipid metabolism, including the possibility of release of pre-formed CM [28, 30]. A recent study reported that there was a significantly earlier time to reach peak concentrations of TAG, RE and apo B-48 following the second low-fat meal compared with the first high fat breakfast, containing different fatty composition, given 5 hours earlier [22].

A two meal protocol was used in the current study which mimics the eating patterns of free-living individuals. Both meals were high in fat to provoke a high lipaemic stress, with the lunch containing a higher energy, carbohydrate and fat content compared with the breakfast, as is typically found in free-living individuals. Postprandial insulin release is dependant on the quantity and type of nutrients within the meal, especially that of carbohydrate. Plasma insulin concentrations of the subjects in the current study have been published previously and were typically higher after the second meal, which reflected the higher carbohydrate content of lunch [31]. It has been proposed that insulin could be involved with displacement or transformation of preformed chylomicrons from the enterocyte [26]. It was reported that after a high fat meal, lipid was retained in the jejunal tissue and released into he plasma following insulin secretion after glucose ingestion [26]. In addition to carbohydrate, the saturation of fatty acids within a meal has been reported to influence the insulin sensitivity with 'carry-over' effects for the second meal [32]. Further research needs to be completed to determine the significance of plasma insulin concentrations and whole body insulin sensitivity on chylomicron formation and release, and its contribution to the second meal effect.

Care should be taken in interpreting conclusions from test meal studies in which pharmacological doses of RP are used since saturation of normal esterification processes (via lecithin: retinol acyl transferase) [33, 34] may result in retention of RE in cytoplasmic droplets, with the possibility of release of RE long after TAG absorption has been completed. Cohn et al. [9], who used much lower dose of RP (~10000 IU), also observed small but corresponding multiple peaks of RE and TAG and concluded that multiple peaks in plasma RE were not a feature of the amount of vitamin A ingested. Overall, concordance of the present data with those of other workers who have used different approaches, leads us to conclude that the RE second peak marks the entry of fat from the first meal.

Considerable inter-individual variation in the degree and timing of the second RE response and the observation that the second RE response was correlated with both the extent of lipaemia to the first meal and the fasting TAG concentrations, raises the possibility that the degree of TAG storage, in the enterocyte, from previous meals (reflected here in the area under the second RE response) is a function of triacylglycerol tolerance. We speculate that this relationship may arise as a result of a feedback mechanism for fat entry into the circulation, with the rate of entry determined in part by prevailing TAG concentrations. This possibility is supported by a recent comparison of "second meal" responses in young versus middle aged subjects in which stable isotopically-labelled fatty acids were given with a first meal and their labelling of CM after a second meal was used as a measure of TAG retention in the gut after the first meal [35]. In middle aged subjects, with significantly higher fasting and postprandial TAG responses, the isotopic labelling of CM after the second meal was found to be markedly higher than in younger subjects [35].

An alternative explanation for the second RE peak after lunch is that this represents accumulation of RE-rich remnant lipoproteins from the first meal, accumulating due to competition between these remnant particles and those being produced following hydrolysis of chylomicrons formed from the second meal. However, the concordance of the RE second peak with a peak in plasma TAG supports the view that the lipoproteins that are accumulating at this point are TAG-rich and unlikely to represent remnant particles. In the absence of data on RE concentrations in the lipoprotein fractions, including the S_f 60–400 and S_f 20–60 fractions, which include the large and small chylomicron remnant fractions, further speculation on this alternative explanation is not possible.

Similar postprandial patterns and similar peak times in TAG (derived from exogenous and endogenous lipoprotein particles) and apo B-48 observed in the present study support previous work which indicates the predominant contribution to the postprandial rise in TAG concentrations by the apo B-48 containing lipoproteins, especially during the initial postprandial phase [6, 22, 36]. Although the RE response to the breakfast differed from that of apo B-48 and TAG, this study did not show the delayed RE/RP response relative to TAG and apo B-48, which has been reported by other authors [5, 9, 11, 37] including our own studies [7]. Indeed the individual peak responses of the RE after breakfast occurred significantly earlier relative to apo B-48 and plasma TAG peaks responses (Table 2) which was reported by our group previously [14]. A delayed RE peak

response relative to TAG and apo B-48 has been explained by differences in the incorporation of RE into the CM, and differences in the catabolism of TAG and RE in the CM [6, 38]. During the lipolytic cascade, TAG associated with CM are hydrolysed and removed from the particles (and from the plasma), whereas RE remains with the CMR until they are removed from plasma by the liver. Delayed absorption and different contents of RE in the CM particles which enter the circulation have also been suggested as reasons for the reported delayed RE peak response [5]. However, an important explanation for the variations in the reports of discordance between apo B-48, TAG and RE/RP peak responses [18, 39–41] may be the nature of the RE used to label the meals. RP in the oily preparations which was used by investigators who obtained a delayed RE response, has to be hydrolysed to retinol and solubilized by bile salts before uptake by the enterocytes whereas RP in water-miscible form can be taken up by the enterocytes more rapidly.

The use of water-miscible RP in the present study may be the reason for obtaining an early peak response of RE relative to apo B-48 and TAG peaks. Data from the present study and a previously published study [14] suggest that limitations in the use of RE as a marker of CM particles, particularly delayed peak response, may have been exaggerated because of widespread previous use of the oil-based RP. However, the problems of transfer of RE onto other lower density lipoproteins remains a major difficulty, since during prolonged postprandial follow-up, this may result in misinterpretation of the degree of CMR retention following meal ingestion.

Therefore, despite some limitations of the use of RE/RP as an exogenous marker of intestinally derived TRL, the present investigation has revealed that RE/RP can be used as a useful tool to understand some important aspects of postprandial lipid metabolism in human studies. The significant feature of the response of plasma RE to sequential meals in the present study is the appearance of a small, sharp second peak after ingestion of a lunch in which RP was omitted. The RE response to the second meal was more pronounced in subjects with elevated fasting TAG and who showed elevated postprandial lipaemia after breakfast. These data support the conclusion that unless all the fat ingested with an earlier meal enters the circulation within a few hours of ingestion, subsequent meal ingestion may result in an early entry of previously ingested fat. This early entry of TAG following the second meal may have adverse consequences for the regulation of circulating TAG in individuals with impaired triacylglycerol tolerance.

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