Superior role of apolipoprotein B48 over apolipoprotein B100 in chylomicron assembly and fat absorption: an investigation of apobec-1 knock-out and wild-type mice

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Editing of apolipoprotein (apo)-B100 mRNA to yield apo-B48 is a specific and developmentally regulated step in enterocytes of mammals. However, the functional significance of this step is not known. Since mice containing only apo-B100 have not been documented to exhibit any difference in intestinal fat absorption from wild-type mice, the evolutionary advantage of apoB mRNA editing has been questioned. In the present study, we have compared fat absorption and chylomicron assembly in apobec-1 knock-out (KO) or wild-type (WT) mice subjected to different dietary manipulations: low-fat chow, a fat-enriched ‘Western’ diet and overnight fasting. Experiments in vivo and in vitro revealed differences in the ability of KO and WT enterocytes to assemble and secrete chylomicrons under different dietary conditions. After overnight fasting, chylomicron secretion is reduced considerably in KO compared with WT enterocytes. This is not due to reduced synthesis of apo-B or triacylglycerol (TAG), but appears to be a result of impaired assembly of chylomicrons, so that triacylglycerol accumulates in the enterocytes. After feeding with fat, secretion of chylomicrons enriched in pre-existing TAG is stimulated in KO compared with WT mice. In the present study, we have documented for the first time that apo-B100 is considerably less efficient than apo-B48 in exerting its role in the early stage of chylomicron assembly, which is rate-limiting under conditions of low dietary fat. However, this impairment is overcome by increased TAG stores that stimulate later stages in assembly, which are rate-limiting in the fat-fed state. apo-B mRNA editing may result in more efficient fat absorption, specifically under conditions of food shortage or low-fat content, and thus provide an evolutionary advantage.

Key words: electron microscopy, intracellular events, isolated enterocytes, mRNA editing, triacylglycerol.

INTRODUCTION

Chylomicrons are assembled in the enterocytes, and consist of droplets of non-polar lipid, triacylglycerol (TAG) and cholesterol esters, stabilized by a shell of phospholipid, cholesterol and protein. The major protein is apolipoprotein (apo)-B48, which is essential for the assembly, secretion and subsequent metabolism of chylomicrons (reviewed in [1,2]). apo-B48 is a truncated form of apo-B100, and is produced by post-transcriptional editing of the mRNA for apo-B100. Cytidine 6666 is deaminated by an RNA-editing enzyme, apobec-1, resulting in the production of a stop codon after 48 % of the mRNA has been translated [3,4]. The editing enzyme is expressed in the enterocytes of all mammals investigated, and is regulated developmentally. apo-B48 is not obligatory for chylomicron assembly, since apobec-1 knock-out (KO) mice, which produce only apo-B100, absorb dietary fat. However, the specific and regulated nature of the editing step suggests that it plays a fundamental role in the assembly of chylomicrons. As yet, the functional significance of the editing remains obscure. To date, no laboratory has reported any difference in intestinal fat absorption or chylomicron production between wild-type (WT) and apobec-1 KO mice. The rationale of the present study was that differences in the role of apo-B100 in relation to apo-B48 in chylomicron assembly and secretion by enterocytes would be revealed by subjecting apobec-1 KO mice and WT mice to dietary manipulation and examination of these processes, both in isolated enterocytes in vitro and in mice in vivo.

MATERIALS AND METHODS

Animals and diets

Homozygous apobec-1 (KO) and WT mice [5] were housed in the University of Sheffield Field Laboratories. The phenotype was confirmed by immunoblotting of the apo-B100 or apo-B48 proteins in plasma after performing SDS/PAGE. Mice were fed on: (i) low-fat chow [fat content of 4.7 % (w/w), comprising 9.6 % of the dietary calories]; (b) chow supplemented with lipids of the composition of a ‘Western’ diet [6] [fat content of 24.7 % (w/w); 35 % of the dietary calories]; or (c) chow followed by fasting for 16 h.

Plasma lipoprotein analysis

Blood was collected by cardiac puncture from several mice, pooled, and red blood cells were removed by centrifugation. Chylomicrons were floated by centrifugation at 100000 g for 15 min. The infranatant was removed, and plasma lipoproteins were separated by centrifugation in self-generating gradients of iodixanol [7]. Gradients were collected in fractions; the lipo-
proteins were identified by agarose-gel electrophoresis, and the cholesterol and TAG content was determined [7]. The diameters of chylomicrons were determined by electron microscopy after negative staining with phosphotungstic acid.

Figure 1  Diameters of chylomicrons isolated from plasma of KO and WT mice

Chylomicrons from plasma of chow- or fat-fed WT and KO mice were examined in the electron microscope after staining with phosphotungstic acid. Diameters of 200 particles were measured, and the frequency as a percentage is plotted against apparent diameter: ▲, chow-fed KO mice; △, fat-fed KO mice; ●, chow-fed WT mice; ○, fat-fed WT mice.

Determination of the rate of absorption of fat in vivo

For each experiment, 12 mice subjected to each dietary treatment were injected with tyloxytop (20 mg in 100 μl; Sigma) [8], followed by gavage with a bolus of triolein containing [3H]oleic acid (10 μCi). At time intervals of 30 min after gavage, two mice were killed, and blood was removed by cardiac puncture before determination of the TAG content (in mM). Chylomicrons were floated, as above, and the lipids of both the chylomicrons and the infranatant were extracted, separated by HPTLC [9], and the rate of appearance of radiolabelled TAG and the mass of TAG in the chylomicrons and the chylomicron-free plasma were determined.

Isolation and incubation of enterocytes

This was essentially as reported previously for rabbit enterocytes [10]. In some experiments, lipids were isolated from freshly isolated enterocytes and subsequently separated and quantified by HPTLC [9–12]. In other experiments, isolated enterocytes were resuspended in Dulbecco’s modified Eagle’s medium (‘DMEM’) and incubated with bile salt/lipid micelles (final concentrations: sodium taurocholate, 0.4 mM; sodium taurodeoxycholate, 0.54 mM; phosphatidylcholine, 0.3 mM; oleic acid).

Figure 2  Accumulation of newly synthesized TAG and TAG mass in plasma of KO and WT mice treated with tyloxytop

KO and WT mice were treated with tyloxytop followed by a bolus of olive oil containing [3H]oleic acid, as described in the Materials and methods section. Plasma TAG (concentrations in mM) and the incorporation of the radiolabel into chylomicron TAG were determined as described in the Materials and methods section. Values shown are the means, and the bars represent ± S.D. (n = 4). In some cases, the bars are obscured by the symbols. ●, WT mice; ○, KO mice.
acid, 0.45 mM; and glycerol mono-olein, 0.26 mM), prepared as described previously [6,10,11], containing either \[^{14}\text{C}]\text{oleate} (1 \mu\text{Ci}) to radiolabel newly synthesized TAG or \[^{35}\text{S}]\text{methionine} (100 \mu\text{Ci}) to radiolabel apo-B48. At timed intervals, the enterocytes and incubation media were separated by centrifugation, and the secreted chylomicrons were isolated by binding to Liposorb, after preliminary experiments showed that all of the radiolabelled apo-B and TAG were bound [10,11]. Incorporation of \[^{35}\text{S}]\text{methionine} into cellular and secreted apo-B48 or apo-B100, \[^{14}\text{C}]\text{oleate} into cellular and secreted TAG, and the mass of TAG were determined as described previously [6,9–11].

**Cell fractionation**

Isolated enterocytes were homogenized after osmotic shock, the total microsomes were isolated and the cytosol (membrane-free supernatant after isolation of microsomes) was retained [11,12]. Homogenate, microsomal and cytosolic proteins were assayed, and the lipids were extracted from all fractions and analysed by HPTLC [9].

**Electron microscopy**

Segments of small intestine were fixed in 2 \% (v/v) glutaraldehyde in cacodylate buffer, followed by 1 \% (w/v) osmium tetroxide. These sections were cut and stained with uranyl acetate and lead citrate, and viewed under a Phillips CM10 electron microscope.

**RESULTS**

**Fat absorption and chylomicron secretion in KO and WT mice**

The plasma lipoprotein cholesterol and TAG profiles of KO and WT mice were essentially the same under the different dietary regimens (results not shown). The only marked difference in the lipoproteins was that, after fat feeding, the diameters of chylomicrons isolated from KO mouse plasma were approx. 66 % larger than those isolated from WT mice (Figure 1). This was a reproducible finding using several sets of pooled plasma preparations.

To investigate the rate of fat absorption, mice subjected to the different dietary regimens were treated with tyloxapol to inhibit chylomicron clearing, followed by a bolus of triolein containing \[^{3}\text{H}\text{triolein}. More than 95 \% of the radiolabel and the TAG in the plasma was found in the chylomicron fraction. The accumulation of TAG mass in the chylomicron fraction of the plasma was greater in the chow-fed and fat-fed KO mice than in WT mice (Figures 2b and 2f). However, after fasting, the accumulation of chylomicron TAG in the KO mice was approximately half that in the WT mice (Figure 2d). This was a fall in TAG mass accumulation after 120 min of \approx 70 \% and \approx 85 \% compared with that in chow-fed and fat-fed KO mouse respectively. In chow-fed and fat-fed WT and KO mice, the rate of appearance of radiolabelled TAG in the plasma chylomicrons was similar (Figures 2a and 2e); however, in the fasted KO mouse the
accumulation of radiolabelled TAG was very low compared with that of the WT, and after 120 min was \( \approx 80\% \) that found in the WT (Figure 2c). \([\text{H}]\text{TAG}\) is a marker of the appearance in the plasma of chylomicrons containing newly absorbed dietary fat, whereas the mass of TAG in the plasma is a marker of total chylomicron secretion, which might include TAG in the process of absorption (e.g. in the enterocytes, the lamina propria or the lymph) at the beginning of the experiment. Thus chow or fat-fed KO mice exhibit increased chylomicron production from pre-existing lipid and a similar absorption of lumenal TAG relative to the chow-fed mice. However, after fasting, which presumably allows pre-existing TAG to be cleared from the intestine, the absorption of TAG from the lumen and secretion of chylomicrons is reduced considerably in the KO mice.

These observations led us to formulate the following hypothesis to explain the complex differences in fat absorption between KO and WT mice. In the KO mice, chylomicron assembly and secretion is less efficient than in the WT mice, resulting in accumulation of TAG derived from dietary lipid in the enterocytes. This TAG is a substrate for chylomicron formation and, as it accumulates, stimulates chylomicron secretion. After a period of overnight fasting, the TAG pool is cleared and chylomicron secretion is then reduced in the KO mice. To test this hypothesis, we first investigated the TAG content of isolated enterocytes.

Enterocyte preparations from KO or WT mice consisted of single, and groups of, cells with typical morphology (Figure 3). The enterocytes from chow-fed KO mice contained three times as much TAG as the cells from chow-fed WT mice (Figure 4). However, after fasting, the TAG content was reduced and there was no significant difference between the enterocytes from WT and KO mice. After fat feeding, the amount of TAG increased approx. 2- and 3-fold in enterocytes from KO and WT mice respectively. We also prepared enterocytes from KO and WT mice that had been fed \([\text{H}]\text{triolein}\) to determine whether newly absorbed TAG is indeed retained within the enterocytes. Under the same dietary conditions, 90 min after administration of a bolus of \([\text{H}]\text{triolein}\), the radiolabelled TAG in enterocytes from KO animals was \( \approx 25\% \) greater than in those from WT mice.
Thus TAG accumulates in enterocytes from KO mice relative to WT mice, the TAG pool is related to the fat content of the diet, and the accumulated TAG is cleared by overnight fasting.

To investigate the intracellular location of the TAG, total microsomes, which consist mainly of the endoplasmic reticulum [10], were prepared from enterocytes of KO and WT mice subjected to the different dietary regimens. The TAG content of the microsomes varied, with the lowest level in those from fasted mice and the highest level in those from fat-fed mice (Figure 4). However, there were no significant differences between preparations from enterocytes of WT and KO mice, and similar recoveries of microsomal protein were obtained from all enterocyte preparations. TAG recovered in the cytosolic fraction of the enterocytes was increased in the fed KO mice compared with that recovered from the WT. This suggests that the excess TAG that accumulates in the enterocytes of KO mice over that in WT mice is located in the cytosol. Consistent with this, NADPH–cytochrome c reductase, a marker of endoplasmic reticulum membranes, was scarcely detectable in the cytosolic fraction (results not shown).

Electron micrographs of chow-fed intestine from KO mice revealed many lipid particles (chylomicrons) between the interdigitating lateral surfaces of the enterocytes and lipid particles of a similar size in both membrane-bound vesicles and in the cytoplasm (Figure 5). Lipid particles were also observed in the same locations in enterocytes of WT mice, although these appeared fewer in number. Particles at all sites were reduced after fasting and increased after fat feeding (results not shown). During enterocyte isolation, the intracellular chylomicrons would be lost, so that accumulation of TAG in KO mice could be due to cytosolic lipid droplets and/or membrane-bound lipid droplets. Biochemical analysis suggests that accumulation is not...
in the microsomes; however, it is possible that some membrane-bound vesicles containing lipid droplets would remain in the supernatant even after high-speed centrifugation. Therefore, at present, we cannot exclude the possibility that some of the TAG that accumulates in the enterocytes of KO mice is in a membrane-bound compartment poor in NADPH-cytochrome c, rather than in the cytosol.

Synthesis and secretion of apo-B and TAG by isolated enterocytes

To determine whether the effects of fasting and feeding on chylomicron appearance in the plasma were a consequence of changes in chylomicron production at the cellular level, isolated enterocytes were prepared from fasted and fat-fed KO and WT mice. As we have previously reported for rabbit enterocytes [6], synthesis and secretion of apo-B and TAG was dependent on addition of lipid/bile salt micelles to the incubation medium. There was no difference between the incorporation of \(^{14}C\)oleate and \(^{3}H\)methionine into apo-B48 or apo-B100 by enterocytes from fasted or fed KO mice (Figure 6a). TAG synthesis from \(^{14}C\)oleate was also similar in enterocyte preparations, although the mass of TAG in the cells differed as described above (Figure 6c). The effect of fasting and feeding on the secretion of TAG by enterocytes showed a similar pattern, albeit with quantitative differences, to the observations in vivo. Fasting reduced the secretion of radiolabelled TAG and TAG mass compared with fat feeding in enterocytes from both WT and KO mice (Figures 6d and 6f). After an incubation period of 120 min, the mass of secreted TAG was 0.4 and 116 \(\mu g/mg\) protein for KO mice and 3.8 and 8.7 \(\mu g/mg\) protein for WT mice (Figure 6d). Thus, as found in vivo, secretion of radiolabelled TAG was considerably reduced in the fasted KO mice, and secretion of unlabelled (pre-existing) cellular TAG was increased in the fat-fed KO mice.

**DISCUSSION**

ApoBec-1 KO mice have been produced in four different laboratories [5,13–15]. Studies to date in these laboratories have either not commented on any abnormality in fat absorption [5,15], or have concluded that intestinal fat absorption was not different in mice containing only apo-B100 and WT mice [13,14]. This conclusion is counterintuitive, because apoB mRNA editing occurs in the intestine of all mammals examined and it is hard to imagine that such a universal evolutionary process has no physiological consequence. Our present in-depth analysis has revealed that, indeed, WT mice display more efficient fat absorption than KO mice, especially when they are fed a regular (low-fat) chow diet.

Enterocytes of KO and WT mice secrete chylomicrons in response to fat feeding. However, dietary manipulations have revealed subtle differences between KO and WT mice. Both in vivo and in vitro, when intra-enterocyte stores of TAG are depleted by overnight fasting, secretion of chylomicrons from KO mice is reduced considerably compared with WT mice. This is not due to differences in the ability of enterocytes from KO and WT mice to synthesize apo-B or TAG. Neither, the assembly and secretion of chylomicrons containing apo-B100 appears to be less efficient than that of chylomicrons containing apo-B48. However, in chow-fed or fat-fed KO mice, TAG accumulates in the enterocytes, secretion is markedly stimulated and the stored TAG is used for chylomicron assembly. A less marked accumulation of intracellular TAG and stimulation of secretion also occurs in fat-fed WT mice, and has been shown previously in rabbit enterocytes [6].

There have been few investigations of the molecular details of assembly of chylomicrons in normal enterocytes. However, these are consistent with a model for chylomicron assembly in which apo-B-rich, lipid-poor particles and TAG-rich particles are transferred separately into the lumen of the smooth endoplasmic reticulum, followed by fusion of the two types of particle to form chylomicrons [2,6,11,16–20]. When dietary TAG is low (chow diet), the rate of secretion of chylomicrons is regulated by movement of apo-B-containing particles into the lumen of the smooth endoplasmic reticulum. However, when dietary TAG is high (Western diet), the rate-limiting step appears to be the addition of TAG to the apo-B-containing particles [6,11]. This step also determines the size of the chylomicrons produced. Within this context, the observations reported here suggest that, in the KO mouse, apo-B100 assembly in chylomicrons is inefficient in the early steps of chylomicron assembly, but that intracellular TAG accumulation (due to a high-fat diet) overcomes the inefficient step and increases chylomicron secretion at the later stage in assembly. This might also account for the observation that chylomicrons from fat-fed KO mice are > 66\% larger than those from WT mice fed the same diet. The accumulated TAG in the enterocytes might produce larger TAG particles for assembly with the apo-B100 containing precursors. The proteins involved in regulation of chylomicron assembly have not been fully elucidated. It might be that apo-B100 and apo-B48 interact with regulatory proteins to different extents, thus modulating different stages of chylomicron assembly. The KO mice will provide a useful tool for probing the regulation of fat absorption.

Editing of apo-B in the intestinal enterocytes is specific, precise and regulated. However, the reason for editing is obscure. The present observations suggest that under conditions of restricted food intake or low-fat diets, editing of apo-B might provide an evolutionary advantage by improving the efficiency of fat absorption.

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