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The intestine as a source of apolipoprotein A_I

(high density lipoprotein/chylomicron/mesenteric lymph/lipid absorption)

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ABSTRACT **The major apoprotein of rat mesenteric lymph chylomicrons has been isolated and characterized and shown to be identical to apoprotein A_I (apo A_I) isolated from serum high density lipoprotein (HDL). During intestinal lipid absorption, active synthesis of apo A_I was demonstrated by radioactive amino acid incorporation into lymph chylomicron A_I as well as lymph HDL. Immunofluorescence studies of intestinal epithelium demonstrated a marked increase in apo A_I fluorescence, confirming an active synthesis of this apoprotein during lipid absorption. Quantitative immunoelectrophoretic methods were used to measure apo A_I in lymph and peripheral blood during various conditions designed to estimate the quantitative importance of intestinal apo A_I to the levels of circulating lipoproteins. During lipid feeding there was an increase in lymph apo A_I that was associated with lymph lipoproteins (50%) of density <1.006 g/ml whereas in basal lymph most apo A_I (85%) was in the lipoproteins of density >1.006 g/ml. Lipid feeding in animals without lymph fistulas resulted in a significant increase in serum apo A_I levels; biliary diversion, designed to eliminate intestinal lipoproteins of density <1.006 g/ml, resulted in a significant decrease in serum apo A_I levels. These studies demonstrate that the intestine actively synthesizes apo A_I and is a significant source of this apoprotein for circulating lipoproteins.**

There is ample evidence that the intestine is an important source of chylomicrons and very low density lipoproteins (1, 2), but less is known concerning the role of the intestine in metabolism of high density lipoprotein (HDL). The available evidence has shown that the liver actively synthesizes nascent HDL which is secreted as disc-shaped particles (3). In addition, studies with isolated perfused rat livers indicate that nascent hepatic HDL is deficient in the major apoprotein of circulating HDL, apoprotein A_I (apo A_I) (3, 4), apparently acquiring this apoprotein after secretion from the liver. Some question, therefore, exists as to whether there are significant extrahepatic sites of apo A_I synthesis. The sites of synthesis of apo A_I assume increasing importance in view of the recent recognition that this apoprotein is an activator of lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43) (5) and may be important in the regulation of cellular cholesterol content (6).

Data from several laboratories (7, 8) have shown that the intestinal lipoproteins of density <1.006 g/ml [chylomicrons and very low density lipoproteins (VLDL)] contain apoproteins in common with circulating HDL. Previous data from this laboratory (7) demonstrated that chylomicrons isolated from rat mesenteric lymph contained an apoprotein, immunologically related to HDL, with a molecular weight of approximately 25,000 and comprising 40–50% of chylomicron protein. This apoprotein, tentatively identified as apo A, however, has not been fully characterized. Therefore, although apoproteins such as apo A_I have been measured in human thoracic duct lymph chylomicrons (9) and rat mesenteric lymph lipoproteins (10), the recent knowledge that rapid interchange of apoproteins may occur among lipoproteins (11) makes it unclear that the intestine actively synthesizes apoproteins such as apo A_I.

In the present study, we isolated and characterized the major apoprotein of rat mesenteric chylomicrons and showed that it is identical to apo A_I isolated from rat serum HDL. Monospecific antibodies to apo A_I have been developed and used to demonstrate apo A_I within rat intestinal epithelial cells during lipid absorption. Quantitative immunoelectrophoretic methods were used to measure apo A_I in lymph and serum under various physiological conditions. The results of these studies indicate that the intestine synthesizes significant amounts of apo A_I destined for lymph and serum lipoproteins.

METHODS

Animals and Operative Technique. Male rats (150–200 g) of the CD strain (Charles River Laboratories, Boston, MA) were used for all studies. Cannulation of the main mesenteric lymphatic duct and duodenum and maintenance of the animals have been described (12). All animals were studied 16–24 hr after surgery. They were maintained in restraining cages and allowed free access to 0.9% (wt/vol) saline and 5% (wt/vol) dextrose. When indicated, Intralipid (Cutter Medical Laboratories, Berkeley, CA) was infused intraduodenally by a constant-infusion pump at a rate of 1 ml/hr. Bile diversion was performed by ligating the common bile duct near the duodenum and cannulating the duct, to permit complete external diversion of bile. Sham-operated rats underwent an abdominal incision and were then allowed access to water and food.

Isolation of Lipoprotein Subfractions and apo A_I Purification. Lymph was defibrinated, and chylomicrons were isolated by ultracentrifugation at 3×10^6 *g* min. Chylomicrons were separated from the subnatant fraction by means of a tube slicer and purified by passage through agarose columns (12). In some cases, lipoproteins of density <1.006 g/ml were isolated from lymph by ultracentrifugation at 10^8 *g* min. HDL (1.063–1.210 g/ml) was isolated from mesenteric lymph of lymph-fistula rats and from pooled fresh serum obtained from 12 rats by ultracentrifugation (3.6×10^6 *g* hr) (13). The HDL fraction was recentrifuged at density 1.21 g/ml and then dialyzed exhaustively (48–72 hr) against 0.15 M NaCl.

Purified chylomicrons were delipidated overnight at 4° with 20 volumes of ethanol/ether, 3:2 (vol/vol), and the protein precipitate was washed three times with ether. Delipidated chylomicron apoproteins were solubilized in 0.01 M Tris buffer/8 M urea/1 mM EDTA, pH 8.2, and dialyzed against the same buffer for 24 hr. apo A_I was isolated from the chylomicron apoproteins by chromatography in 8 M urea on a Sephadex G-200 column (2.5 × 90 cm) (14). Column fractions

Abbreviations: HDL, high density lipoprotein; apo A_I, apoprotein A_I; VLDL, very low density lipoprotein; $NaDodSO_4$, sodium dodecyl sulfate.

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were monitored by ultraviolet absorption at 280 nm and sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis (7). HDL was similarly delipidated and subjected to column chromatography as for the chylomicron apoproteins.

Immunological Studies. Antiserum to apo A_1 was initially prepared by immunizing with individual unfixed and unstained acrylamide gel slices representing individual chylomicron apoproteins. The desired apoprotein was located by its relative mobility as determined on duplicate stained gels scanned densitometrically (7). Individual gel slices were homogenized between glass slides, mixed with an equal volume of complete Freund's adjuvant, and injected at multiple sites intracutaneously along the backs of New Zealand White rabbits. Animals were given booster injections weekly, beginning 2 weeks after the initial immunization.

Antisera were characterized by immunodiffusion and immunoelectrophoresis in agarose by standard methods. Additional antisera to apo A_1 were prepared by using apo A_1 isolated by column chromatography; these gave equivalent results (described below).

Immunofluorescence Studies of apo A_1 in Isolated Intestinal Cells. Isolated intestinal epithelial cells were prepared from segments of rat jejunum according to the method of Weiser (15). Some animals were given lipid intragastrically via a small tube at varying time intervals prior to sacrifice; in others, biliary diversion was performed 3 days previously.

Isolated cells, after preparation, were dried on glass slides and then fixed by immersion in cold methanol for 4 min and in acetone for 2 min (16). Indirect immunofluorescence studies with antiserum to apo A_1 were carried out as described (16). Nonimmune sera and antisera that had been absorbed with pure apo A_1 served as controls. Slides were mounted in glycerol/phosphate-buffered saline, 9:1 (vol/vol), pH 10, and viewed under a Zeiss fluorescent microscope equipped with a filter specific for fluoroscein isothiocyanate. Slides were photographed with Agfachrome 64 film and a ×40 oil-immersion objective.

Quantitative Electrophoresis of apo A_1. Quantitation of apo A_1 was performed by the rocket immunoelectrophoresis technique of Laurell (17). A sample (usually 5–10 μl) of serum, plasma fraction, whole lymph, or lymph subfraction was mixed with an equal volume of 1,1,3,3-tetramethylurea at 30° as described by Albers *et al.* (18); 0.01 M Tris buffer in 8 M urea, pH 8.0, was added to a final dilution of 1:8 for whole serum and 1:4 for lymph. Concentrated lipoprotein subfractions (density <1.006 g/ml) from lymph harvested after fat feeding were diluted in 0.15 M NaCl after tube slicing to permit even dispersion of lipoproteins before delipidation with tetramethylurea.

Samples were applied to antibody-containing plates of 1% agarose in 0.05 M barbital buffer, pH 8.8. Agarose was heated to 56°, mixed with antisera (2%, vol/vol), and then poured onto glass plates (20 × 10 cm) that had been precoated with 0.3% agarose. Electrophoresis was performed in a water-cooled electrophoresis apparatus (MRA Corp.) with 0.05 M barbital buffer, pH 8.8; 2 μl of each diluted sample was applied to the wells with the current running. A current of 50 mA was applied for 3 hr. Plates were washed overnight in phosphate-buffered saline and then water, dried, and stained with Buffalo black. Rocket height was measured from the center of the well.

Purified apo A_1 was treated identically as samples (dilutions of pooled rat serum served as a standard). Standard curves were plotted from dilutions of whole sera and also purified apo A_1.

Isotope Incorporation Studies. The incorporation of [^{3}H]leucine into lymph chylomicron apo A_1 was studied in rats pretreated with 4-aminopyrazolopyrimidine (Aldrich Chemical Co., Milwaukee, WI) administered intraperitoneally in saline daily at a dosage of 40–50 mg/kg for 3 days. These rats were allowed free access to water and food. Twenty-four hours after the last dose, the main mesenteric lymph duct and duodenum were cannulated. After a constant duodenal infusion of Intralipid had been established, a single dose of 200 μCi of [^{3}H]leucine was administered intraduodenally to lymph-fistula rats. Lymph was collected over the indicated time intervals, and the chylomicrons were purified, delipidated, and electrophoresed on $NaDodSO_4$/polyacrylamide gels. Radioactivity incorporated into individual proteins on polyacrylamide gels was determined by cutting the gel into 1-mm slices with a lateral gel slicer. Individual slices were related to specific protein bands by comparison with duplicate gels that were stained and scanned densitometrically. The specific activity of leucine incorporation into apoprotein bands was determined by previously described methods (7). Other rats that had received the same drug were sacrificed after receiving Intralipid intragastrically, and isolated jejunal epithelial cells were prepared for indirect immunofluorescence identification of apo A_1.

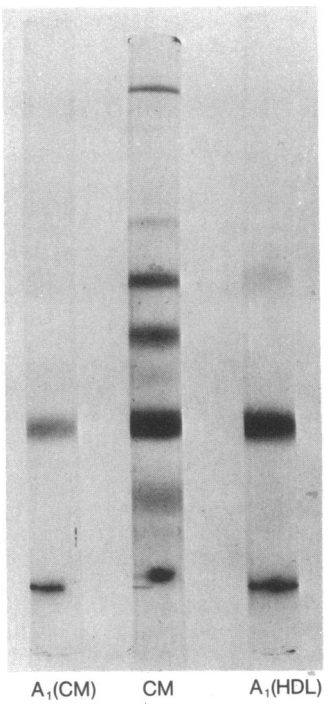

FIG. 1. $NaDodSO_4$/polyacrylamide electrophoresis of apo A_1 isolated from mesenteric chylomicrons (CM) and serum HDL by Sephadex column chromatography. The electrophoresis front is marked at the bottom of each gel.

RESULTS

Purification and Characterization of apo A_1 from Chylomicrons and HDL. When solubilized chylomicron apoproteins and HDL apoproteins were subjected to chromatography on Sephadex G-200 columns, the apoproteins were eluted in three major peaks. The descending portion of the second peak contained pure apo A_1 as determined by $NaDodSO_4$/gel electrophoresis (Fig. 1). The elution volume of apo A_1 corresponded to a molecular weight of approximately 28,000. When subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis, purified chylomicron apo A_1 gave a single band corresponding to the major band seen on the gel of delipidated whole chylomicrons.

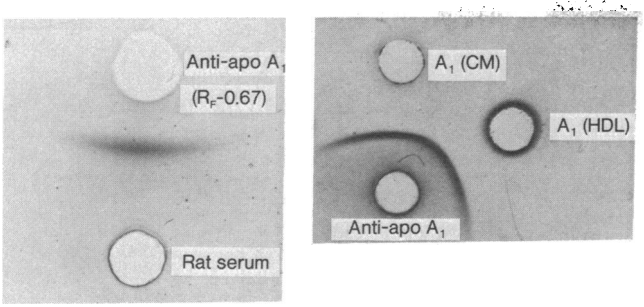

FIG. 2. Ouchterlony analysis of antisera to chylomicron apo A_1, prepared and reacted against whole rat serum (*Left*) and apo A_1 prepared from chylomicrons and HDL (*Right*). A single precipitin arc against whole serum is evident, as well as a reaction of identity between apo A_1 from chylomicrons and HDL (*Right*). Stained with Buffalo black.

apo A_1 prepared from HDL showed a mobility identical to that of chylomicron apo A_1. The molecular weights of apo A_1 from chylomicrons and HDL were identical when determined by $NaDodSO_4$/polyacrylamide gel electrophoresis. The value, 25,000, corresponds to the molecular weight of this apoprotein as determined previously in our laboratory (7) as well as that obtained by Marsh (4). Amino acid analyses carried out on chylomicron apo A_1 revealed a pattern similar to that of HDL apo A_1, both proteins being rich in aspartate, glutamate, leucine, and lysine, as previously reported (14, 19).

Further evidence for the identity of chylomicron apo A_1 with apo A_1 prepared from HDL was provided by immunologic studies of the two proteins. Fig. 2 demonstrates that an antiserum prepared to chylomicron apo A_1 was monospecific when tested against whole serum. This antibody reacted with apo A_1 from chylomicrons and HDL in a reaction of identity, further confirming the similarity of apo A_1 from both sources.

Turnover Studies of Lymph apo A_1. In order to minimize the potential transfer of apo A_1 to lymph lipoproteins from nonintestinal sources, animals were treated for 72 hr with 4-aminopyrazolopyrimidine. We confirmed the previous observations (20, 21) that the dose used in the present study was associated with the development of a visibly fatty liver and a decrease in serum cholesterol to 10% of control values. In addition, electrophoresis of serum lipoproteins from treated animals revealed greatly decreased amounts of HDL and VLDL, as described by others (20, 22). Evidence for the active intestinal synthesis of apo A_1 was provided by [^{3}H]leucine incorporation into lymph lipoproteins in the treated animals. In treated animals with mesenteric lymph fistulas, a constant duodenal infusion of Intralipid was established and then a single 200-μCi dose of [^{3}H]leucine was administered intraduodenally. Hourly lymph samples were collected and chylomicron and HDL fractions were prepared. Fig. 3 illustrates that, within the first hour after its administration, there was a rapid incorporation of the isotope into apo A_1 in chylomicrons as well as lymph HDL. Because a constant lipid infusion was maintained for the duration of the experiment (5 hr) and the isotope was administered as a "pulse," the progressive decline in specific activity of apo A_1 in both chylomicrons and HDL is consistent with continued active synthesis of apo A_1.

Immunofluorescent Studies of apo A_1 in Isolated Intestinal Epithelial Cells. With methods developed in our laboratory (16), isolated jejunal intestinal epithelial cells were prepared from animals that had undergone biliary diversion for 72 hr or from 4-aminopyrazolopyrimidine-treated animals. Fig. 4 *left* illustrates that, despite biliary diversion, a procedure known to deplete the intestinal mucosa of lipoproteins of density

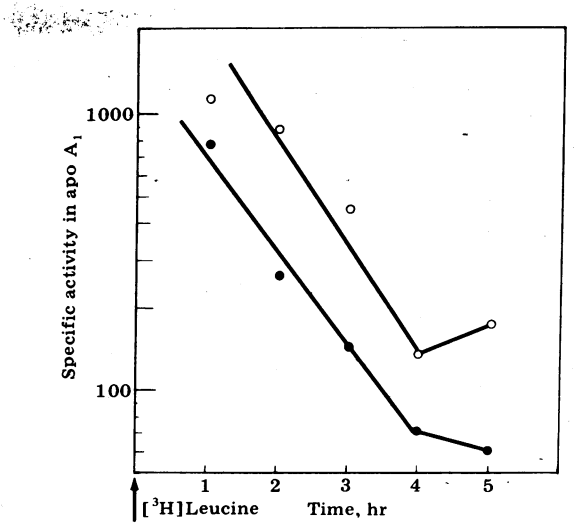

FIG. 3. [^{3}H]Leucine incorporation into apo A_1 of mesenteric lymph chylomicrons (○) and HDL (●). Animals were pretreated with 4-aminopyrazolopyrimidine and lymph fistulas were created. After constant duodenal infusion of lipid was established, a pulse dose of [^{3}H]leucine was given intraduodenally. Lymph was collected at hourly intervals, and chylomicrons and HDL were isolated and purified. Specific activity of apo A_1 was determined from polyacrylamide gels (see *Methods*).

<1.006 g/ml (23), apo A_1 fluorescence is present as a fine pattern beneath the microvillus membrane, suggesting that a small pool of apo A_1 is present in the nonabsorptive cell. Lipid absorption in the treated animals was associated with a marked increase in immunofluorescence, filling the entire apex of the cell (Fig. 4 *right*). This pattern of immunofluorescence was identical to that seen in nontreated animals during lipid absorption. All fluorescence was specifically blocked by prior absorption of the antiserum with purified apo A_1. Together with the data on isotope incorporation into lymph lipoproteins, the immunofluorescence results indicate that there is an active synthesis of apo A_1 by the intestine during lipid absorption.

Contribution of Intestinal apo A_1 to Plasma Levels. The intestinal contribution to the plasma level of apo A_1 was assessed by quantitation of the apoprotein by rocket electrophoresis.

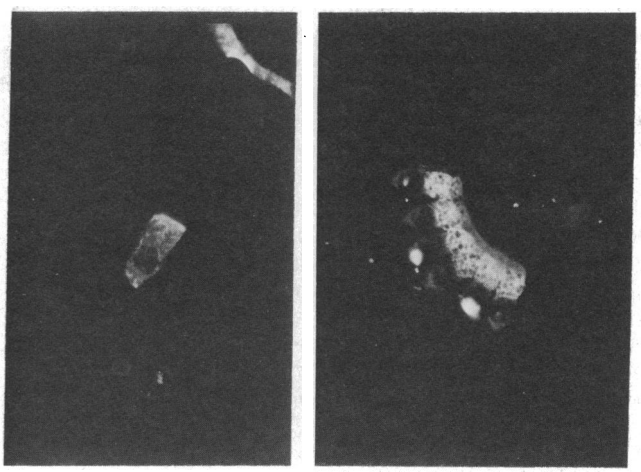

FIG. 4. Immunofluorescence of apo A_1 within isolated intestinal epithelial cells. Isolated jejunal cells were prepared from animals with prior biliary diversion (*Left*) and, during lipid absorption, from animals treated with 4-aminopyrazolopyrimidine (*Right*). A fine pattern of immunofluorescence is present in the nonabsorptive cell (*Left*) and markedly increases during lipid absorption (*Right*). Similar results were obtained in nontreated animals. (×40.)

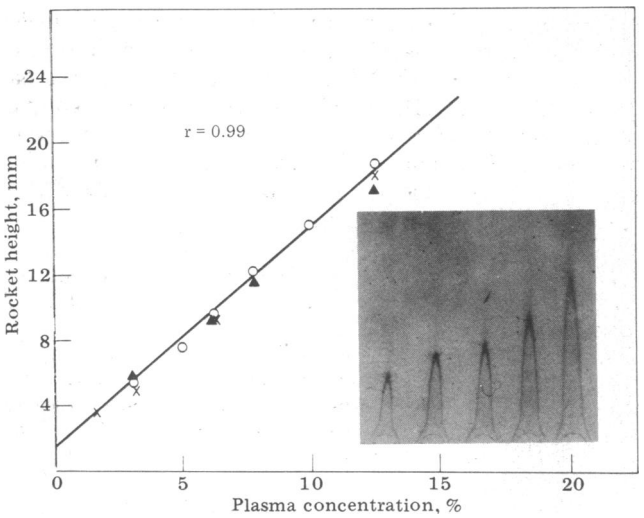

FIG. 5. Quantitative immunoelectrophoresis of rat apo A_1. A linear relationship between rocket height and concentration of apo A_1 in dilutions of rat serum is evident. Typical rockets are illustrated. Symbols indicate separate standard curves.

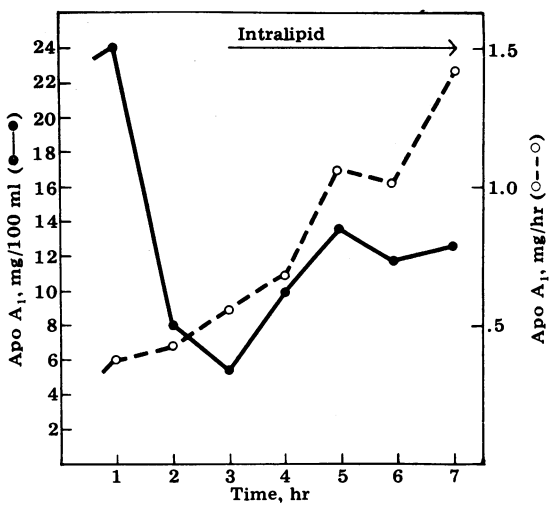

FIG. 6. Output of apo A_1 in mesenteric lymph. Lymph was collected on ice from lymph-fistula rats during the basal state and during a constant intraduodenal infusion of Intralipid. The hourly lymph volumes were 1.4, 5.5, 10.5, 6.5, 8, 9, and 12.2 ml, respectively. Apo A_1 was measured by quantitative immunoelectrophoresis.

Pooled rat serum served as a standard. Fig. 5 demonstrates a linear relationship between rocket height and protein concentration. The intraassay and interassay coefficients of variation were 2.3 and 3.1%, respectively. Immunochemically pure apo A_1 of known protein content, solubilized in 0.01 M Tris buffer/8 M urea, pH 8.0, also gave a linear response in this assay. The addition of tetramethylurea to the standard did not alter the rocket height. Delipidation of serum in ethanol/ether overnight resulted in a rocket height similar to that of tetramethylurea-treated serum, which was greater than that of native serum. All serum samples were therefore delipidated with tetramethylurea prior to electrophoresis. The normal value (mean ± SEM) for serum was 50.9 ± 1.2 mg/100 ml (n = 23 animals).

In order to assess the contribution of intestinal lipoproteins of density <1.006 g/ml to serum apo A_1 levels, blood was taken before and after 3 days of external biliary diversion (Table 1). The biliary diversion resulted in a decrease in the serum apo A_1 level of 17%. Sham-operated animals had no such decrease. Because bile diversion is known to deplete intestinal lymph of lipoproteins of density <1.006 g/ml (2), these data are consistent with a significant contribution of chylomicrons and intestinal VLDL to total circulating apo A_1 levels. Further support for this was derived from measurements of plasma apo A_1 levels after acute fat feeding in nonlymph-diverted animals. Plasma apo A_1 was significantly elevated ($P < 0.005$) after fat feeding, at a time when the serum was lipemic and the serum triglyceride level was elevated.

In order to explore the possibility that the intestine contributes apo A_1 to the peripheral circulation in lipoproteins other than chylomicrons and VLDL, the effect of total lymph diversion on plasma apo A_1 levels was determined. In two animals, external lymph drainage for 72 hr resulted in a greater decrease in plasma apo A_1 than did biliary diversion (42% versus 17%), suggesting the possibility that apo A_1 is present in lymph in the fraction of density >1.006 g/ml. Studies were therefore performed on the distribution of apo A_1 in mesenteric lymph. From lymph-fistula rats that were allowed free access to dextrose/saline, lymph was collected on ice overnight and hourly for 3 hr. The animals then received a constant intraduodenal infusion of Intralipid, and lymph was collected for an additional 4 hr. Although the lymph concentration of apo A_1 varied with lymph flow, the hourly content of lymph apo A_1 was relatively constant (Fig. 6). The output of apo A_1 over a 12-hr basal period was approximately 6 mg. Lipid infusion was associated with a marked increase in lymphatic content of apo A_1 above basal levels. The distribution of apo A_1 was then determined in basal and absorptive lymph. Lymph was centrifuged at its own density for 10^8 g min to separate the chylomicrons and VLDL from lipoproteins of density >1.006 g/ml. apo A_1 was measured in both the light and heavy fractions. In basal lymph, 15.7 ± 1.9% of the apo A_1 was in the light fraction and 84.5 ± 2.0% was present in the heavy fraction (n = 6). In absorptive lymph, 46.2 ± 3.7% of apo A_1 was in the light fraction and 53.8 ± 3.7% was in the heavy fraction. In other studies, chylomicrons and VLDL were isolated separately from the fraction of density <1.006 g/ml and apo A_1 was detected in the VLDL fraction as well as in the chylomicrons.

Table 1. Effect of lipid feeding and biliary diversion on serum apo A_1 levels

	apo A_1, mg/100 ml		
	Before	After	P
Biliary diversion, n = 7	51.6 ± 2.2	42.6 ± 1.8	<0.005
Fat feeding, n = 7	52.3 ± 2.1	56.6 ± 2.0	<0.005

Serum apo A_1 was measured by quantitative immunoelectrophoresis (see *Methods*) in animals with prior external biliary diversion or after acute fat feeding. Results are expressed as mean ± SEM.

DISCUSSION

Although data from many laboratories have established that the intestine is an active site of chylomicron and VLDL synthesis, less is known concerning the role of the intestine in a HDL metabolism. Qualitative demonstration of intestinal HDL synthesis in the rat has been provided by studies using an isolated perfused intestine (24); however, quantitative studies of intestinal HDL synthesis are not available.

That the intestine may influence HDL metabolism was suggested by the finding that mesenteric lymph chylomicrons and VLDL contained apoproteins immunologically related to

HDL (7, 8); thus, the possibility arose that intestinal lipoproteins may represent a source of HDL apoproteins. The recent finding that nascent HDL isolated from perfused rat liver is relatively deficient in apo A_1 (3, 4) provides further indirect evidence that significant extrahepatic sites of synthesis of HDL apoproteins may exist. The present studies were therefore undertaken to characterize the major apoprotein of rat mesenteric lymph chylomicrons and to provide evidence that intestinal synthesis is a significant source of this apoprotein for circulating lipoproteins.

We have isolated the major apoprotein of rat mesenteric lymph chylomicrons and shown that it is identical to apo A_1 isolated from serum HDL. Both apoproteins had identical molecular weights (25,000) and similar amino acid compositions and were immunologically identical (Fig. 2).

To demonstrate that the apo A_1 found in mesenteric lymph chylomicrons was actively synthesized by the intestine and not merely transfered to lymph lipoproteins from circulating lipoproteins, animals were pretreated with 4-aminopyrazolopyrimidine, a drug known to produce in rats a fatty liver and consequently decrease the serum concentration of circulating VLDL and HDL (20–22) (a finding corroborated in the present study). No previous data were available, however, on the effect of this drug on intestinal lipoprotein formation. The present study provided qualitative evidence that, despite impaired hepatic release of lipoproteins, intestinal chylomicron formation and release occurred. Despite the drug treatment, there was active incorporation of [^{3}H]leucine in chylomicron apo A_1. In addition, in treated animals the apo A_1 was localized (by indirect immunofluorescence) within absorptive intestinal epithelial cells, further supporting an active synthesis of this apoprotein by the intestinal epithelium. These results coupled with the increase in lymph apo A_1 content during lipid feeding (Fig. 6) indicate that the intestine actively synthesizes chylomicron apo A_1.

Further evidence that the intestine may contribute to circulating levels of apo A_1 was provided by changes in serum apo A_1 levels after fat feeding and biliary diversion (Table 1). Acute lipid feeding in non-lymph-diverted animals produced a transient but modest increase in plasma apo A_1 levels during alimentary lipemia, a result consistent with a bolus of chylomicron apo A_1 acutely entering the peripheral circulation. Additional evidence that mesenteric lipoproteins of density <1.006 g/ml contribute to plasma apo A_1 levels was provided by the 17% decrease in plasma apo A_1 levels after the fasting and biliary diversion. This procedure is known to deplete the intestinal mucosa and lymph of chylomicrons and VLDL within 6 hr (23, 25). Although it is possible that biliary diversion may have resulted in unappreciated changes in hepatic lipoprotein production, it is probable that this estimate of intestinal apo A_1 production is conservative. The demonstration that 85% of basal lymph apo A_1 is present in the lipoproteins of density >1.006 g/ml suggests that biliary diversion may not have decreased lymph apo A_1 present in these lipoproteins. In several animals, after biliary diversion lymph contained significant amounts of apo A_1. Because HDL may be filtered from peripheral blood into lymph (24), it is not clear what proportion of apo A_1 present in density >1.006 g/ml is of intestinal origin. As shown in Fig. 3, there was active synthesis of apo A_1 present in lymph HDL in the drug-treated animals. These results are in agreement with studies, using the isolated perfused intestine, which demonstrated active synthesis of HDL apoproteins (26). It is clear that a proportion of the HDL found in lymph is of intestinal origin, but the present studies do not permit a precise quantitation of intestinal HDL production. The results of the present studies suggest, however, that the intestine contributes significant amounts of apo A_1 to circulating lipoproteins.

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