Dietary fish oils inhibit early events in the assembly of very low density lipoproteins and target apoB for degradation within the rough endoplasmic reticulum of hamster hepatocytes

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Abstract **Dietary fish oils inhibited secretion and stimulated intracellular degradation of apolipoprotein (apo)B in hamster hepatocytes, while dietary sunflower oils stimulated secretion and had no effect on degradation of apoB. To investigate the intracellular site at which fish oils act, we have made use of our previous observations that inhibition of degradation by N-acetyl-leucyl-leucyl-norleucinal (ALLN) results in accumulation of apoB in the *trans*-Golgi membrane and does not stimulate secretion, while inhibition of degradation by *o*-phenanthroline results in accumulation of apoB in the rough endoplasmic reticulum membrane and stimulates secretion. Thus, ALLN protects apoB which has been diverted from secretion and *o*-phenanthroline protects apoB which is targetted for secretion. Addition of *o*-phenantholine to the incubation medium of hepatocytes from fish oil-fed hamsters inhibited degradation of apoB and stimulated its secretion in particles of the density of VLDL, while addition of ALLN had no effect. These observations suggest that dietary fish oils reversibly inhibit early steps in the assembly of very low density lipoprotein precursors and target apoB for degradation in the rough endoplasmic reticulum.**—Kendrick, J. S., and J. A. Higgins. **Dietary fish oils inhibit early events in the assembly of very low density lipoproteins and target apoB for degradation within the rough endoplasmic reticulum of hamster hepatocytes. *J. Lipid Res.* 1999.** 40: **504–514.**

Supplementary key words liver hepatocytes • hamster • diet • fish oil • sunflower oil • apolipoprotein B • degradation • secretion • *o*-phenanthroline • ALLN

Many studies have shown that increased consumption of oily fish or fish oils protects against cardiovascular disease: this effect has been attributed to the content of n–3 fatty acids (reviewed in refs. 1 and 2). Although, multiple mechanisms are probably involved, one effect of an increased intake of fish oil is a reduction in the level of serum triacylglycerol in humans and experimental animals (1, 2). Prefeeding fish oils or addition of fish oil fatty acids (eicosapentaenoic acid, EPA, and docosahexaenaoic acid, DHA) to the incubation medium of isolated rat hepatocytes inhibits secretion of apolipoprotein B (apoB) and very low density lipoproteins (VLDL) and increases degradation of newly synthesized apoB (3–7). As VLDL are the direct precursors of the atherogenic low density lipoprotein (LDL) this presents one mechanism by which increased dietary fish oils may exert their effect.

Studies of VLDL assembly in rat and rabbit hepatocytes have shown that newly synthesized apoB is either translocated into the lumen of the rough endoplasmic reticulum (RER) or remains membrane bound (8–15). Most of the newly synthesized membrane-bound apoB is degraded in the RER (13–16). However, significant amounts of apoB are found in all the membranes of the secretory pathway (10–12, 17, 18), and, when degradation of the membrane-bound form of apoB is inhibited, the protein accumulates in the *trans*-Golgi membrane (13–15). In the RER lumen, apoB is incorporated into lipid-poor VLDL-precursor particles. A large fraction of this pool of apoB is degraded in the RER lumen (13, 15) presumably because it has not acquired the correct complement of lipids, is not properly folded, or is prevented in some way from moving on through the secretory pathway. Most of the VLDL lipids are transferred to the lumen of the smooth endoplasmic reticulum (SER) and are assembled with the apoB-containing VLDL precursors between the SER and the *cis*-Golgi (14, 19). The initial events in the intracellular transit of apoB and the assembly of precursor particles in the lumen of the RER are similar in HepG2 cells and hepatocytes (20, 21). Degradation of apoB in the ER membrane and the ER lumen also

Abbreviations: apoB, apolipoprotein B; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; VLDL, very low density lipoprotein; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LDL, low density lipoprotein; ALLN, N-acetyl-leucyl-leucyl-norleucinal; DMEM, Dulbecco's minimal essential medium.

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occurs in HepG2 cells (reviewed in refs. 22, 23). However, in these cells apoB is not fully lipidated and denser lipoprotein particles are secreted.

There are several steps at which intracellular transit of apoB and assembly and secretion of VLDL may be regulated. These include *i*) translocation of apoB across the RER membrane; *ii*) incorporation into a secretion-competent precursor in the lumen; and *iii*) acquisition of the full complement of lipids. At each of the first two steps, apoB may be diverted from the secretory to a degradative pathway, either at the cytosolic side of the RER membrane or in the lumen of the RER. The third step is probably not essential for secretion of apoB, as hepatoma cells and cells transfected with truncated cDNAs secrete apoB in dense particles (24, 25). However, in liver, this step may be important in determining the composition of the secreted VLDL. The aim of this investigation was to identify the intracellular site of action of fish oils in inhibiting VLDL assembly. Our hypothesis was that dietary fish oil fatty acids modify one or more of the putative regulatory steps indicated above. To relate this work as far as possible to humans, we have used hamsters for these studies. Hamster liver secretes only apoB-100, as does human liver, and a number of recent reports have suggested that the hamster is a good model for studies of the secretion and metabolism of VLDL (26, 27). Our results demonstrate that dietary fish oils inhibit VLDL secretion by inhibiting the initial assembly of apoB to form VLDL precursors in the lumen of the RER. Moreover, the inhibition of secretion produced by fish oil is reversed when apoB degradation in the RER lumen is inhibited by *o*-phenanthroline.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise indicated materials were as described previously (10–15, 17, 19).

Animals and diets

Male golden Syrian hamsters (91.16 ± 3.36 g [SD] initial body weight) were used for these studies. They were maintained in the University of Sheffield Field Laboratories on a 12-h light/dark cycle and allowed free access to chow and water. After preliminary studies in which oils mixed with chow in pastes or pellets were presented to the animals, gavage was selected as the best way to ensure reproducibility of dietary intake. A dose of 1.5 ml of either fish oil (Maxepa, a gift from Dr. F. W. Vas-Dias, Seven Seas Ltd, Marfleet, Hull, UK) or sunflower oil was administered daily, for 3 weeks, by personnel in the University Field Laboratories, who also monitored food intake and the weight and health of the hamsters. Fish oil was stored in sealed drums at 4°C. After the drums had been opened they were flushed with nitrogen and resealed. The cholesterol content of the fish oil was determined as 1.5% (w/v) by quantitative high performance thin-layer chromatography and the cholesterol content of the sunflower oil was adjusted to that of the fish oil. To minimize oxidative damage, oils were taken into 2-ml syringes excluding air; the syringes were stored at 0°C for up to 1 week and were warmed to room temperature immediately before use.

The body weights of the hamsters increased between 1 and 2 g weekly over the period of the diet and there was no significant difference among the three dietary groups. In the chow-fed groups, food intake was 11.06 ± 2.52 g [SD] a day; this contributed 174.4 ± 33.7 kJ [SD] to the dietary energy. In the sunflower oil-fed animals, food intake was 7.18 ± 1.19 g [SD] of chow per day, The total energy of this diet was 174.4 ± 17.6 kJ [SD] per day of which chow contributed 115.3 ± 17.6 kJ [SD] and oil 58.8 kJ. In the fish oil-fed group, food intake was 7.09 ± 1.84 g [SD] of chow per day. The energy of this diet was 163.6 ± 26.9 kJ [SD] per day, of which chow contributed 104.8 ± 26.9 kJ [SD] and oil 58.8 kJ. The contribution of fat to the total dietary calories was 7% for the chow-fed animals, 39% for the sunflower oil-fed animals, and 40% for the fish oil-fed animals.

Preparation and incubation of hepatocytes

Isolated hepatocytes were prepared by perfusion of hamster livers with collagenase as described previously for the preparation of rabbit or rat hepatocytes (13–15). Cell viability was assessed by exclusion of trypan blue and by the ability of the isolated hepatocytes to incorporate [^{35}S]methionine into proteins. Incubation conditions were as described previously (14, 15). Briefly, the cells were resuspended (50% w/v) in methionine-free Dulbecco's minimal essential medium (DMEM), containing bovine serum albumin (BSA) (final concentration 1%), which had been gassed with 95% oxygen/5% carbon dioxide. Aliquots containing 2 g of hepatocytes in a total volume of 4 ml were incubated at 37°C in 50-ml siliconized flasks with [^{35}S]methionine (70–150 μCi) to radiolabel apoB. Depending on the experiment, oleate (1 mm final concentration, bound to fatty acid-free BSA), ALLN (final concentration 42 μg/ml), or *o*-phenanthroline (final concentration 200 μg/ml) was added to the incubation media. These concentrations of inhibitors were selected after preliminary experiments to determine the concentration required to give maximum inhibition. The incubation flasks were gassed with 95% oxygen/5% carbon dioxide for a few seconds at approximately 15-min intervals throughout the incubation. In some experiments the hepatocytes were incubated for a series of times; in other experiments, the cells were incubated with radiolabeled substrate for 30 min, isolated by centrifugation, and reincubated with unlabeled substrate (45 mm) for up to 120 min to follow the fate of the radiolabeled apoB (13–15, 19). At each time interval the cells were pelleted by centrifugation at 1000 *g* for 5 min. In some experiments hepatocytes were homogenized and subcellular fractions were isolated (13–15) at the beginning and end of the reincubation step. The subcellular fractions were characterized by assay of NADPH-cytochrome C reductase as a marker for the ER and UDP-galactosyltransferase as a marker for *trans*-Golgi (13–15). The fractions were similar in their degree of enrichment and recovery to those prepared from rat or rabbit hepatocytes described previously (**Table 1**). A cocktail of protease inhibitors was added to homogenized cells, subcellular fractions, and supernatants before further analysis (10, 13).

The supernatants from the incubations, which contained the secreted material, were recentrifuged to remove any cell debris (17,500 *g* for 20 min) and the lipoproteins were adsorbed onto PMH-Liposorb (Calbiochem) (14, 15). In some experiments, the density of the secreted apoB was determined by sequential centrifugation after adjusting the density of the supernatants to 1.019, 1.063, and 1.21 g/ml with potassium bromide (10). In each case the top 1 ml of the sample was removed after centrifugation and the radiolabeled apoB was determined. The efficacy of Liposorb, as a method for adsorption of radiolabeled apoB-containing lipoproteins from the incubation media, was checked by comparison with concentration using Centricon 30 centrifugation tubes (Amicon) and by comparison with flotation of the lipoproteins on the salt gradients. As reported previously (14, 15), more than 95% of the ^{35}S-labeled apoB (measured by two-dimensional counting after SDS-PAGE) in the incubation me-

TABLE 1. Distribution of marker enzymes in subcellular fractions

Fraction	NADPH-Cytochrome C Reductase	Recovery	UDP-Galactose-galactosyltransferase	Recovery
	nmol/min/mg protein	*%*	*pmol/min/mg protein*	*%*
Homogenate	9.74 ± 2.39		6.49 ± 0.78	
RER	54.80 ± 2.20	14.23	4.72 ± 0.01	0.21
SER	54.59 ± 1.12	14.95	5.24 ± 0.12	0.32
trans-Golgi	10.08 ± 2.42	0.28	300.56 ± 15.07	7.10

Subcellular fractions were prepared from hamster hepatocytes and the specific activities of UDP-galactose galactosyltransferase and NADPH cytochrome C reductase were determined. Results are the mean of four determinations ± standard deviation. Recoveries are the total marker enzyme activities recovered in each subcellular fraction as a % of that in the total homogenate.

dium was recovered in the Liposorb pellet, compared with apoB recovered by concentration or flotation. No radiolabeled apoB was detected in the supernatant from the Liposorb pellet when this was concentrated and assayed.

Determination of the incorporation of [^{35}S]methionine into cellular and secreted apoB

Hepatocytes and subcellular fractions were resuspended and aliquots were dissolved in sample buffer. Secreted lipoproteins adsorbed onto Liposorb were also dissolved in sample buffer. Aliquots were separated by SDS-PAGE on 3–20% gradients, as described previously (13). In early experiments the radiolabel was determined by excising and solubilizing the apoB band (13, 15, 17) and in later experiments the radiolabeled bands were detected and quantified in the intact gels using a Packard InstantImager (two-dimensional counter). An LDL–apoB standard, prepared from hamster plasma as described previously for rabbit plasma (10), was included on all gels in addition to molecular weight markers. ApoB was also identified by immunoblotting, using a polyclonal anti-hamster LDL antibody raised in rabbits, and using a commercially available anti-human apoB antibody (Boehringer-Mannheim), which we have shown cross-reacts with hamster LDL. Radiolabeled apoB was also identified in the gels using [^{35}S]methionine-labeled VLDL, isolated by flotation from the incubation medium, as a marker. The InstantImager is a two-dimensional counter that directly measures the radioactivity in protein bands in SDS-PAGE gels with time. An image is also generated at any selected time point, which is a guide to the position of radiolabeled bands on the gels. This image is not used for quantitation; however, for illustrative purposes, we have included black and white print-outs of the images in some figures.

In initial experiments, hepatocytes were solubilized, apoB was immunoprecipitated, the immunoprecipitates were separated by SDS-PAGE, and the radiolabel in the apoB bands was determined as described previously (17). However, to minimize experimental manipulations, during which apoB may be lost or degraded, despite addition of a cocktail of protease inhibitors, we investigated the possibility of determination of the incorporation of [^{35}S]methionine into apoB after separation of the total cell proteins on 3–20% gradient gels. We have successfully used this method previously in studies of rabbit or rat hepatocytes (13–15, 19). Essentially the same results were obtained using either immunoprecipitation or counting of apoB bands on SDS-PAGE gels (**Fig. 1**). This method of assay is possible because isolated hepatocytes incorporate sufficient [^{35}S]methionine into apoB and because

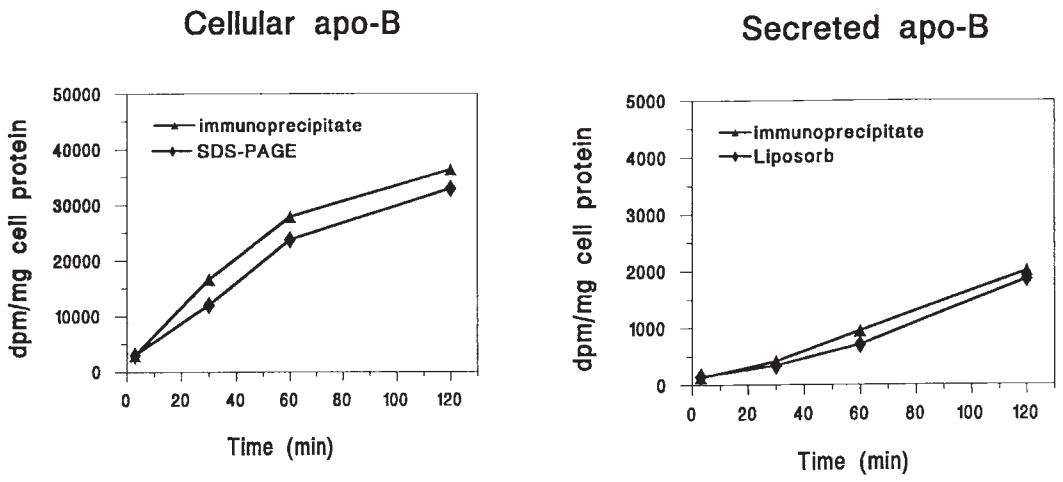


Fig. 1. Determination of [^{35}S]methionine incorporation into apoB measured by immunoprecipitation and direct assay of apoB after separation on SDS-PAGE. Hepatocytes were incubated with [^{35}S]methionine as described in Experimental Procedures and the cells and media were separated by centrifugation. Hepatocytes were either solubilized or apoB immunoprecipitated and the immunoprecipitate was dissolved in sample buffer (17) or cells were dissolved in sample buffer. ApoB in the media were immunoprecipitated (17), or apoB-containing lipoproteins were adsorbed onto Liposorb as described in Experimental Procedures. Immunoprecipitates and Liposorb pellets were solubilized in sample buffer and aliquots of all samples were separated on 3–20% gradient gels with apoB markers as described in Methods. ApoB bands were excised, solubilized, and counted. Dpm in apoB/mg hepatocyte protein are plotted against time of incubation.

apoB-100, found in hamster liver, runs close to the top of the gel and separates from the other cell proteins.

Determination of the incorporation of [^{35}S]methionine into total proteins

Aliquots of the resuspended hepatocytes and the incubation media were pipetted onto 2.5-cm glass microfiber discs, which were allowed to dry and then dropped into ice-cold 10% trichloroacetic acid (TCA). The discs were washed successively with boiling 10% TCA (3 min), 5% TCA (3 × 5 min), alcohol–ether 1:1 (v/v) (2 min), and finally with ether before drying and counting in a Packard scintillation counter.

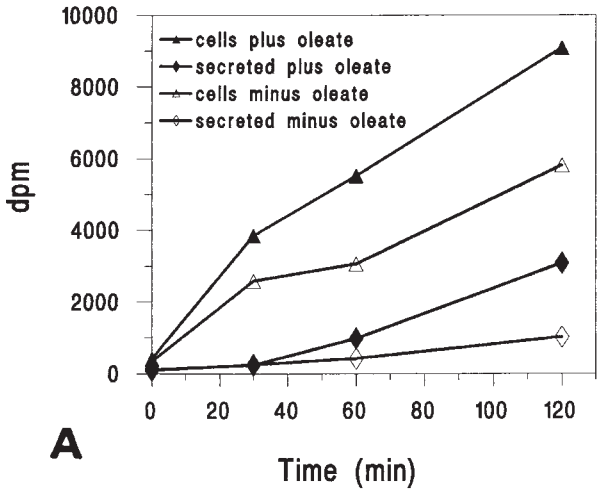
RESULTS

Isolated hamster hepatocytes synthesize and secrete apoB-100 in particles of the density of VLDL

Incorporation of [^{35}S]methionine into apoB-100 by hamster hepatocytes was linear for the 120-min incubation and was approximately 50% higher in the presence of oleate (**Fig. 2**). In the absence of oleate, secretion of apoB-100 was low and after 120 min incubation was approximately 25% of that in the presence of oleate (Fig. 2). Ninety-eight percent of the radiolabeled apoB secreted was in particles that floated at a density <1.019 g/ml. Previous studies of rabbit and rat hepatocytes have shown that there is considerable intracellular degradation of apoB in the absence of oleate (13–15). The radiolabel accumulating in the cells at each time point is therefore a balance between synthesis and degradation. This may account for the apparent stimulatory effect of oleate on synthesis of ^{35}S-labeled apoB. However, the results demonstrate that hamster hepatocytes synthesize and secrete apoB-containing VLDL particles and are therefore appropriate for further studies. In parallel experiments, incorporation of [^{35}S]methionine into total TCA-insoluble protein was measured as an internal check of the viability of the hepatocytes, and to determine whether variations in protein synthesis occurred in different experiments or on addition of oleate. Synthesis of total TCA-precipitatable protein was linear for 60 min incubation and slowed during the next 60 min, and secretion increased linearly after a lag period of about 30 min (data not shown). Neither synthesis nor secretion was affected by addition of oleate to the incubation medium.

Relative proportion of apoB degraded or secreted is altered by provision of oleate during and/or after synthesis of apoB

When hepatocytes were incubated with [^{35}S]methionine, isolated by centrifugation, and reincubated in the



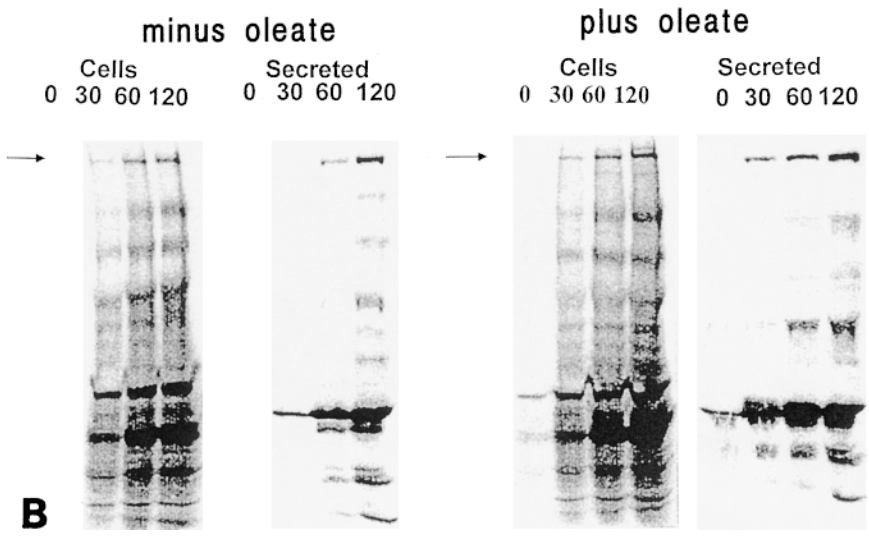


Fig. 2. Incorporation of [^{35}S]methionine into cellular and secreted apoB of hamster hepatocytes. Hepatocytes were incubated with [^{35}S]methionine in the presence and absence of oleate as described in Experimental Procedures. The cells and media (secreted apoB) were separated by centrifugation and aliquots (equivalent to 1/5 of the media and 1/100 of the cells) were separated by SDS-PAGE and the radiolabel in the apoB band was determined using the Packard InstantImager. In (A) dpm/mg of cell protein are plotted. Data from one experiment performed in duplicate are plotted. Similar patterns of incorporation were obtained in four repeated experiments. In (B) print-outs of images from one set of experiments are shown. These images were not used for quantitation and are included to illustrate the location of radiolabeled apoB-100-containing bands on the gel. The arrow indicates the position of apoB.

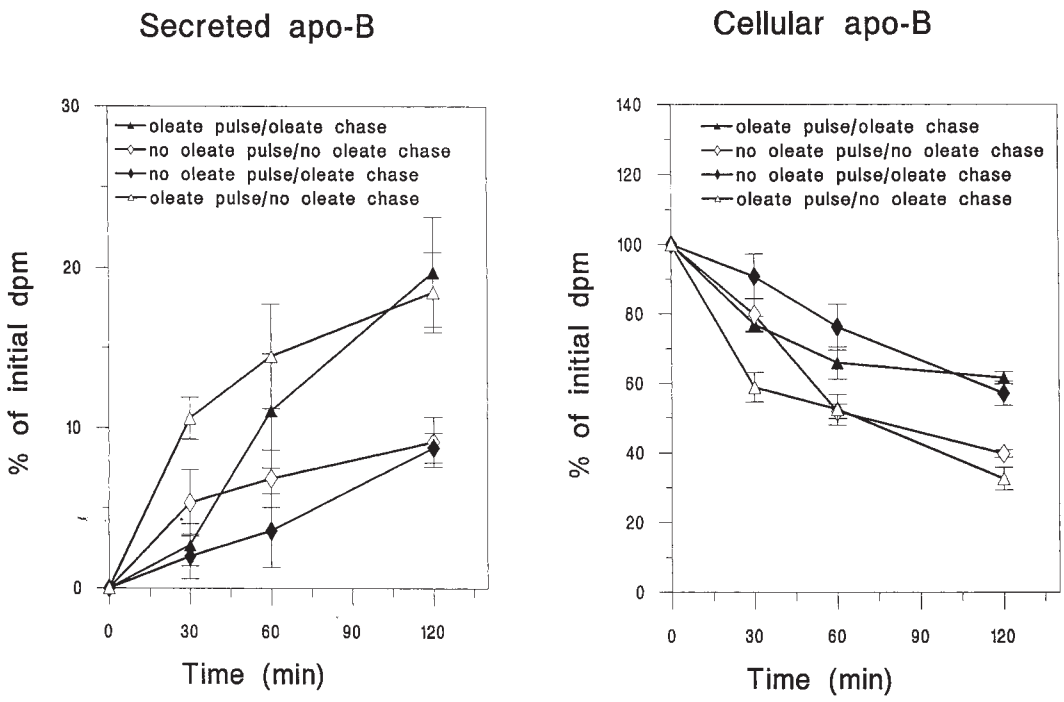


Fig. 3. Effect of addition of oleate to the pulse and/or chase on the fate of newly synthesized apoB in hamster hepatocytes. Hepatocytes were incubated with [^{35}S]methionine for 30 min. The cells were isolated and reincubated for a series of times with an excess of unlabeled methionine as described in Experimental Procedures. Incubations included oleate in the labeling step, in the chase step, in both steps, or in neither step The hepatocytes and media were separated by centrifugation and aliquots were separated by SDS-PAGE. The apoB-containing bands were excised and solubilized before counting. The results plotted are the mean of three separate experiments and, in order to directly compare different experiments, are expressed as the % change in radioactivity in apoB during the incubation. The initial activity (100%) ranged from 13,000 to 26,000 dpm per mg of cell protein. The error bars indicate the standard deviations.

presence of excess unlabeled methionine, a significant fraction of the newly synthesized radiolabeled cellular apoB was degraded (**Fig. 3**). Inclusion of oleate during synthesis of radiolabeled apoB, with or without oleate in the chase step, resulted in secretion of a larger fraction (~20%) of the initial radiolabeled apoB, than when oleate was omitted in the labeling step (~8%) (Fig. 3). Omission of oleate from the reincubation step resulted in loss of a larger fraction of the initial newly synthesized intracellular apoB (~62%) than when oleate was present (~35%) (Fig. 3). Thus, overall degradation of apoB (cellular plus secreted) was greatest (~50%) when oleate was omitted from pulse and chase and least when oleate was present in both pulse and chase (~15%). Under all experimental conditions, there was no significant degradation of total TCA-insoluble radiolabeled proteins and the rate of secretion of total proteins was independent of the presence of oleate in either the labeling or chase incubation steps (data not shown).

Dietary fish oils inhibit secretion and stimulate degradation of newly synthesized apoB and dietary sunflower oils stimulate secretion of newly synthesized apoB

Intracellular degradation of newly synthesized apoB was increased in hepatocytes prepared from fish oil-fed hamsters compared with those from chow-fed animals (**Fig. 4**). Secretion was decreased in cells from fish oil-fed animals, so that after 120-min chase 90% of the total newly synthesized apoB was degraded (Fig. 4). In contrast, secretion of newly synthesized apoB was stimulated approximately 2-fold in cells from sunflower oil-fed hamsters compared with those from chow-fed hamsters (**Fig. 5**). Loss of radiolabeled apoB from hepatocytes of sunflower oil-fed hamsters was more rapid than from hepatocytes of chow-fed hamsters. However, this could be largely accounted for by the increased secretion, so that overall degradation of apoB was similar (~35%). There was no loss of total TCA-insoluble radiolabeled proteins and secretion of total proteins was similar in all three groups of hepatocytes, indicating that the effect of diet on newly synthesized apoB was not due to changes in protein synthesis or secretion (data not shown).

Inhibition of proteases by *o*-phenanthroline, but not by ALLN, reverses the effect of dietary fish oils on the degradation and secretion of newly synthesized apoB

We have previously shown that *o*-phenanthroline, a metalloprotease inhibitor, inhibits degradation of apoB resulting in accumulation of apoB at the lumenal side of the RER membrane, and stimulates secretion of apoB (15). ALLN, a cysteine protease inhibitor, inhibits degradation of apoB, resulting in accumulation of apoB in the Golgi membranes. ALLN has no effect on apoB secretion. Our

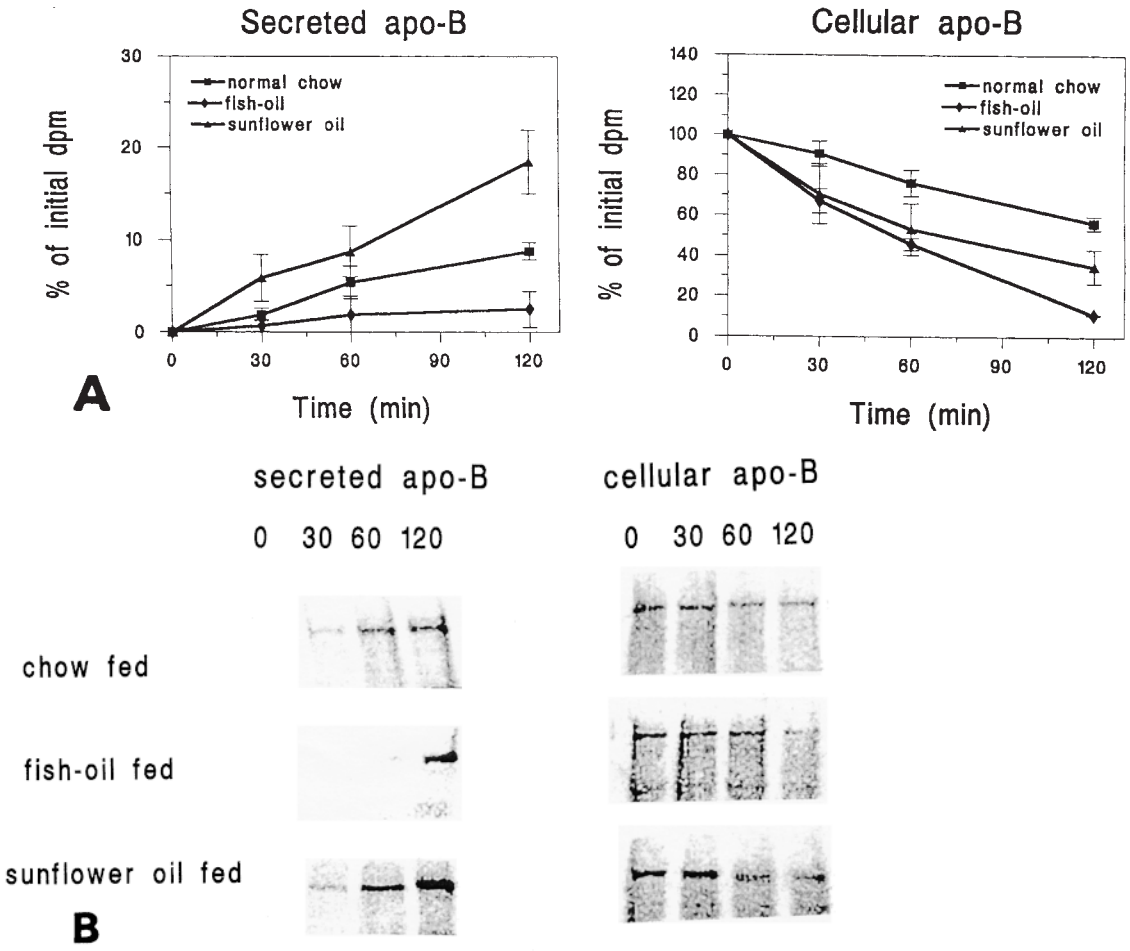


Fig. 4. Fate of newly synthesized apoB in hepatocytes from chow-, sunflower oil-, or fish oil-fed hamsters. Hepatocytes prepared from chow-fed, fish oil-fed, or sunflower oil-fed hamsters were incubated with [^{35}S]methionine for 30 min without oleate. The cells were isolated and reincubated for a series of times with an excess of unlabeled methionine, with addition of oleate, as described in Experimental Procedures. The hepatocytes and media were separated by centrifugation and aliquots (equivalent to 1/5 of the media and 1/100 of the cells) were separated by SDS-PAGE. The radiolabel in apoB was determined using the Instant-Imager. (A) The results plotted are the mean of three separate experiments and, in order to directly compare different experiments, are expressed as the % change in radioactivity in apoB during the incubation. The error bars indicate the standard deviations. The initial radiolabel (100%) varied between 4,000 and 10,000 cpm/mg cell, and showed a similar spread in isolated hepatocytes from each of the three dietary groups. (B) Illustrates one image obtained from a time course for each set of cells. These images are black and white print-outs and were not used for quantitation but to illustrate the qualitative change observed in the radiolabel apoB in each experiment. Because the initial radioactivity at the start of the chase period varied between experiments, it is not possible to directly compare the density of bands in different gels.

interpretation of these observations is that ALLN protects apoB from degradation at the cytosolic side of the RER and after it has been targetted away from the secretory pathway. This is consistent with the fact that ALLN is an inhibitor of the cytosolic proteosomes, which have been implicated in the degradation of membrane-bound apoB in HepG2 cells (28, 29). In contrast, *o*-phenanthroline protects apoB when it is still in a pool that is secretion competent. ALLN and *o*-phenanthroline thus provide a tool for probing the intracellular sites at which fish oil feeding targets apoB to degradation.

In the present study, both *o*-phenanthroline and ALLN protected apoB from degradation in isolated hepatocytes from chow-fed hamsters. ALLN had no significant effect on secretion and *o*-phenanthroline stimulated secretion (Fig. 5). *o*-Phenanthroline inhibited intracellular degradation of newly synthesized apoB and stimulated secretion of apoB in hepatocytes from fish oil-fed hamsters and thus reversed the effect of fish oil feeding, so that the hepatocytes behaved like those from chow-fed animals (Fig. 5). The total recovery of radiolabeled apoB, when degradation was inhibited by *o*-phenanthroline, was about 110–115%. This is probably due to completion, in the chase period, of apoB molecules partly synthesized during the pulse period. This has been reported previously by ourselves and others in similar experiments on adult hepatocytes (6, 15). In contrast, ALLN had no significant effect on either degradation or secretion of newly synthesized apoB in hepatocytes from

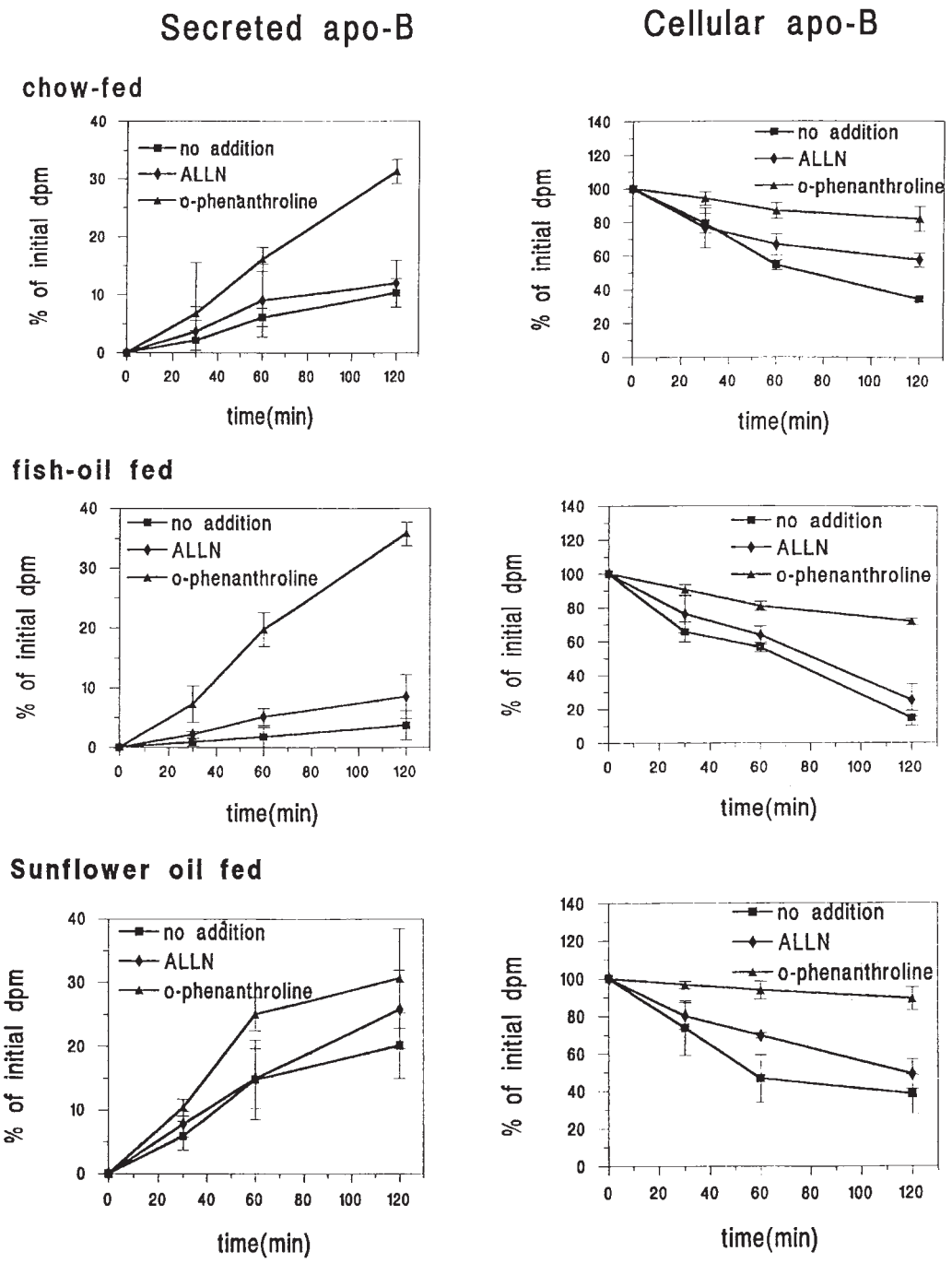


Fig. 5. Effect of protease inhibitors on the fate of newly synthesized apoB in hepatocytes from chow-fed, sunflower oil-fed, or fish oil-fed hamsters. Experiments were conducted as in Fig. 3 except that ALLN, *o*-phenanthroline, or no inhibitor was added in the chase step and oleate was omitted from the pulse and the chase steps. The initial activity (100%) varied between 5,000 and 14,000 cpm/mg cell protein. The results plotted are the mean of three experiments and the error bars indicate the standard deviations.

fish oil-fed hamsters. Hepatocytes from sunflower oil-fed hamsters are stimulated to secrete apoB, compared with those from chow-fed hamsters: the rate of secretion was increased in the presence of *o*-phenanthroline, while ALLN had no significant effect. Addition of *o*-phenanthroline also protected newly synthesized apoB from degradation, while ALLN slowed the rate of degradation in hepatocytes from sunflower oil-fed hamsters (Fig. 5). Radiolabeled apoB secreted by hepatocytes from fish oil-fed hamsters in the presence of *o*-phenanthroline was in particles of density <1.016 g/ml and showed the same density distribution as lipoproteins secreted by hepatocytes from chow-fed or sunflower oil-fed hamsters (**Fig. 6**).

In hepatocytes from chow, fish oil, or sunflower oil-fed hamsters, most of the radiolabeled apoB was in the RER fraction at the beginning of the chase step (**Fig. 7**). In the

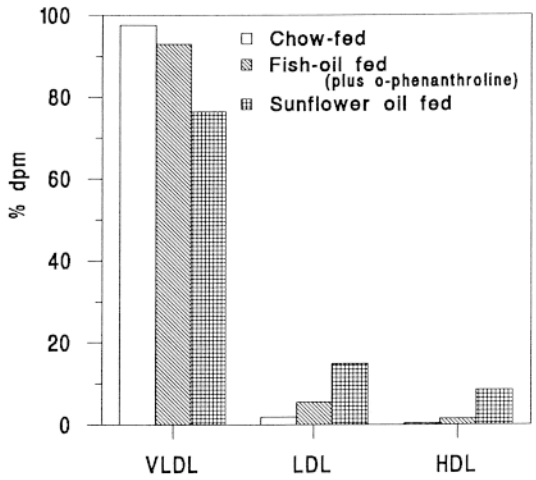


Fig. 6. Density of apoB-containing lipoproteins secreted by hepatocytes from chow-, fish oil-, or sunflower oil-fed hamsters. Isolated hepatocytes from chow-fed, sunflower oil-fed, or fish oil-fed hamsters were incubated with [^{35}S]methionine for 120 min as in Fig. 4. *o*-Phenanthroline was included in the medium of the hepatocytes from fish oil-fed animals. The hepatocytes and media were separated by centrifugation and the density of the media was adjusted sequentially to 1.019 (VLDL), 1.063 (LDL) and 1.21 g/ml (HDL) followed by centrifugation as described in Experimental Procedures. Aliquots of the media were separated by SDS-PAGE and the radiolabel in the apoB was determined using the InstantImager. The mean distribution of dpm from two experiments performed in duplicate is plotted as a % of the total dpm, which ranged from 150,000 to 250,000 cpm.

absence of ALLN or *o*-phenanthroline, radiolabeled apoB was lost from all fractions. However, when *o*-phenanthroline was added to the incubation medium of hepatocytes from all three dietary groups, radiolabeled apoB accumulated in the RER (Fig. 7). When ALLN was added to the media of hepatocytes from chow-fed or sunflower oil-fed hamsters, radiolabeled apoB was lost from the RER and accumulated in the Golgi. However, ALLN did not protect apoB from degradation in the hepatocytes from fish oil-fed hamsters. There are two explanations for this: dietary fish oils modify the ALLN-sensitive protease so that it becomes insensitive to the inhibitor, or dietary fish oils target apoB into a pool that is not accessible to the ALLN-sensitive protease. As *o*-phenanthroline completely reversed the effect of feeding fish oils and stimulated secretion of apoB, the latter explanation is more likely. Thus, dietary fish oils inhibit the movement of apoB from the lumen of the RER and target apoB for degradation.

DISCUSSION

A number of studies have shown that fish oils, or n–3 fatty acids, inhibit secretion of VLDL by hepatocytes and stimulate degradation of apoB. Our results confirm these findings in hamster hepatocytes and demonstrate that, in contrast, a diet enriched in n–6 fatty acids stimulates apoB secretion. On the basis of our earlier work on hepatocytes, we hypothesized that fish oils modify one of the key steps in the assembly of VLDL. This would then target apoB for degradation. Several recent studies support a two-stage model for apoB degradation in rabbit hepatocytes and HepG2 cells (15, 21, 30). The first stage of degradation involves the ubiquitin–proteosome pathway (28, 29), is inhibited by ALLN (31–35), and the apoB is associated with hsp-70 presumably at the cytosolic side of the RER (30). The second stage of degradation is inhibited by *o*-phenanthroline in hepatocytes (15) and by DTT, but not ALLN, in HepG2 cells and is assumed to take place in the lumen or at the lumenal side of the RER membrane (15, 30). It has also been reported recently that the ER resident cysteine–protease, ER-60, is associated with apoB in HepG2 cells (36). ER-60 is inhibited by ALLN, ALLM, E64, and leupeptin and is therefore distinct from the DTT-sensitive protease (37), suggesting that further lumenal proteases may be involved in apoB degradation. To determine the stage at which dietary fish oils target apoB into degradation, we have made use of our previous observations, confirmed in the present study, that ALLN inhibits degradation of apoB that has been diverted from the secretory pathway, while *o*-phenanthroline inhibits degradation of apoB that is still secretion competent. The present findings indicate that *o*-phenanthroline reverses the effect of the dietary fish oils, while ALLN has no effect. The effect of fish oil is thus to modify the assembly of the VLDL precursor particle after apoB has been committed to secretion, while it is still in the RER lumen. Wang, Yao, and Fisher (38) have also concluded from studies of McArdle cells transfected with truncated cDNAs for apoB that the ability of n–3 fatty acids to promote apoB degradation is correlated with lipidation and occurs after apoB has been targetted into the secretory pathway.

A further interesting observation from the present studies is that, under conditions in which apoB degradation is inhibited and secretion of VLDL is stimulated by *o*-phenanthroline, apoB remains concentrated in the RER. This was also observed in rabbit hepatocytes (15). The kinetics of transit of apoB through the secretory compartment in both rat and rabbit hepatocytes is also consistent with slow exit of newly synthesized apoB from the RER lumen (13–15). Thus, movement of apoB-containing VLDL-precursors from the RER lumen may be regulated, in addition to translocation of apoB across the RER membrane.

ALLN, at concentrations from 1.8–40 μg/ml, has been shown to inhibit intracellular degradation of apoB in transfected CHO cells and HepG2 cells (28–31, 34, 39–43). Secretion of apoB is not stimulated and the apoB that accumulates is membrane bound, with the bulk of the protein exposed at the cytosolic side of the membrane (30, 40, 42). In our studies, ALLN at a similar concentration (40 μg/ml) inhibits apoB degradation in rat, rabbit, and hamster hepatocytes, resulting in accumulation of apoB in the Golgi. At first sight, this observation is at variance with results from HepG2 cells, which suggest that apoB is degraded in the RER. However, in rabbit or hamster hepatocytes in the absence of ALLN, the RER is the major site of degradation of newly synthesized apoB (13,

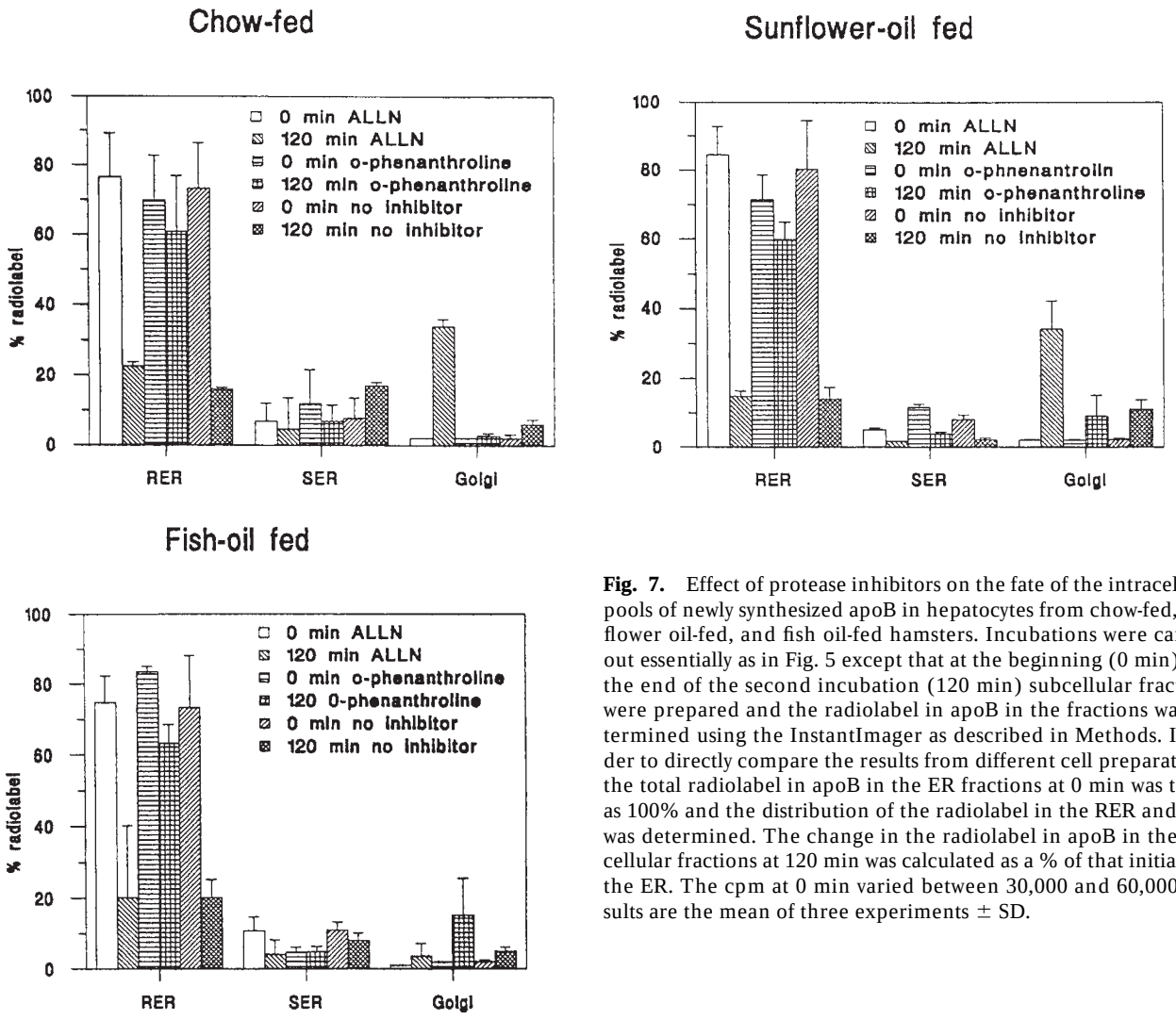


Fig. 7. Effect of protease inhibitors on the fate of the intracellular pools of newly synthesized apoB in hepatocytes from chow-fed, sunflower oil-fed, and fish oil-fed hamsters. Incubations were carried out essentially as in Fig. 5 except that at the beginning (0 min) and the end of the second incubation (120 min) subcellular fractions were prepared and the radiolabel in apoB in the fractions was determined using the InstantImager as described in Methods. In order to directly compare the results from different cell preparations, the total radiolabel in apoB in the ER fractions at 0 min was taken as 100% and the distribution of the radiolabel in the RER and SER was determined. The change in the radiolabel in apoB in the subcellular fractions at 120 min was calculated as a % of that initially in the ER. The cpm at 0 min varied between 30,000 and 60,000. Results are the mean of three experiments ± SD.

15). When this degradation is inhibited by ALLN or by feeding orotic acid (13, 15), apoB accumulates in the Golgi membranes. Measurement of the mass of apoB in subcellular fractions from adult hepatocytes has also shown that apoB is present throughout the ER/Golgi secretory compartment (10, 17). This finding is consistent with a recent morphological study of permeabilized HepG2 cells which showed that cytosolic apoB is distributed through the whole secretory pathway (44). In rat hepatocytes, ALLN (100 μg/ml) inhibited insulin-stimulated degradation of apoB (45), although other studies of rat hepatocytes have reported that ALLN has no effect on apoB degradation (18). The reasons for these differences are not clear, although rat liver secretes both apoB-48 in addition to apoB-100 and the intracellular transit of these proteins differs.

Translocation of apoB across the RER membrane is facilitated by provision of oleate, which also inhibits intracellular degradation and stimulates secretion of apoB (13–15, 46, 47). In the present study, we observed that, when oleate is present during synthesis, a greater fraction of newly synthesized apoB is secreted, suggesting that oleate facilitates translocation co-translationally. Addition of oleate, after apoB has been synthesized, protects the newly synthesized protein against degradation but does not stimulate secretion. Oleate may thus act in two separate ways, by first stabilizing membrane-bound apoB from degradation and secondly, by facilitating translocation of apoB across the RER membrane. It has not been determined whether other fatty acids act in the same way as oleate. However, in the present study we found that feeding sunflower oil, enriched in linoleic acid, stimulates secretion of apoB to an extent similar to that observed after addition of oleate. Translocation and initial lipidation of apoB is dependent on MTP (48, 49) and specific lipids, cholesteryl ester, triacylglycerol, and phospholipids have also been implicated (50–57). Oleate may act as a precursor for all of these lipids.

The observation that *o*-phenanthroline reverses the effect of dietary fish oils and stimulates secretion of VLDL, while ALLN, which protects against degradation of apoB at the cytosolic side of the membrane, has no effect, strongly suggests that the pool of apoB, which is degraded in fish oil-fed hamster hepatocytes, is at the lumenal side of the RER. In rat and rabbit hepatocytes, apoB in the lumen of the RER is associated with particles of d ~1.21 g/ml and as the particles move to the Golgi they acquire lipids and become lighter (13, 14). We have recently shown

that dietary fish oils inhibit movement of apoB-containing VLDL-precursors from the lumen of the RER and their assembly with lipids in rabbit liver (58). This suggests that dietary fish oils either perturb the initial lipidation of apoB and thus target the protein to degradation, or prevent VLDL-precursors from leaving the RER lumen. As inhibition of degradation by *o*-phenanthroline reverses the effect of dietary fish oil, the first mechanism seems most likely. Lipids containing n–3 fatty acids may be poor substrates for MTP or may bind with high affinity to lipid binding sites on MTP and thus inhibit lipid transfer to apoB. Because their effect is reversible, fish oils may only slow the lipidation of apoB so that, when degradation of apoB is inhibited, sufficient time for proper lipidation and assembly of VLDL precursors take place.

We believe that ours are the first studies of the effect of dietary fish oils on intracellular events in VLDL assembly in normal hepatocytes, which secrete only apoB-100. They show that fish oils interfere with the assembly of the initial VLDL precursor particles in the RER lumen and thus target apoB for degradation, and that the effect can be reversed by inhibition of degradation. The specific molecular target of fish oils is under investigation and may lead to novel pharmaceutical strategies for lowering plasma levels of the atherogenic LDL.

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