We previously showed that \( \Omega-3 \) fatty acids reduce secretion of apolipoprotein B (apoB) from cultured hepatocytes by stimulating post-translational degradation. In this report, we now characterize this process, particularly in regard to the two known processes that degrade newly synthesized apoB, endoplasmic reticulum (ER)-associated degradation and re-uptake from the cell surface. First, we found that \( \Omega-3 \)-induced degradation preferentially reduces the secretion of large, assembled apoB-lipoprotein particles, and apoB polypeptide length is not a determinant. Second, based on several experimental approaches, ER-associated degradation is not involved. Third, re-uptake, the only process known to destroy fully assembled nascent lipoproteins, was clearly active in primary hepatocytes, but \( \Omega-3 \)-induced degradation of apoB continued even when re-uptake was blocked. Cell fractionation showed that \( \Omega-3 \) fatty acids induced a striking loss of apoB\(_{100} \) from the Golgi, while sparing apoB\(_{100} \) in the ER, indicating a post-ER process. To determine the signaling involved, we used wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor, which blocked most, if not all, of the \( \Omega-3 \) fatty acid effect. Therefore, nascent apoB is subject to ER-associated degradation, re-uptake, and a third distinct degradative pathway that appears to target lipoproteins after considerable assembly and involves a post-ER compartment and PI3K signaling. Physiologic, pathophysiologic, and pharmacologic regulation of net apoB secretion may involve alterations in any of these three degradative steps.

Apolipoprotein B (apoB), the major protein of atherogenic lipoproteins, is synthesized primarily by hepatic and intestinal cells. Most studies have focused on apoB metabolism in the liver, given the greater contribution to the plasma apoB pool made by that organ and the availability of relatively convenient primary and transformed hepatic cell models. The apoB message level and translational rate in hepatic cells are largely constitutive, and so secretory control is achieved primarily through co- and post-translational degradation of the protein (e.g. see Refs. 2–4 for recent reviews).

Two specific mechanisms for the destruction of newly synthesized apoB in hepatic cells have been characterized. The first is endoplasmic reticulum-associated degradation (ERAD). Newly synthesized apoB in the endoplasmic reticulum (ER) is initially complexed with small amounts of lipid that are thought to be shuttled by the microsomal triglyceride transfer protein (MTP) (5). During severe lipid deprivation (6, 7) or MTP deficiency (8, 9), this initial lipidation fails, and the apoB becomes ubiquitylated, which targets it for degradation by proteasomes (10–14).

The second mechanism for degradation of newly synthesized apoB is the re-uptake pathway. Re-uptake can occur after fully assembled apoB-containing particles have been exported across the plasma membrane but before they have diffused away from the vicinity of the cell by traversing the unstirred water layer that is adjacent to the plasma membrane (15) (see also Refs. 16, 17). Surprisingly, these nascent apoB-containing particles are quite capable of binding cell surface receptors, such as LDL receptors (15) or specific heparan sulfate proteoglycans (18–21) that then bring them back into the cell. Delivery to lysosomes and proteolytic degradation follows. The re-uptake pathway is stimulated by sterol deprivation (15), which induces LDL receptor expression (22), or by molecules that can bridge between apoB-containing particles and cell surface proteoglycans (23, 24). The architecture of the liver may favor re-uptake, owing to the presence of diffusional impediments, such as the space of Disse and the fenestrated endothelial barrier, through which nascent lipoproteins must pass before escape into the circulation. Because apoB and cell surface li-
MATERIALS AND METHODS

General—Male Harlan Sprague-Dawley rats (Ace Animals, Boyertown, PA) weighing 200–225 g were used to obtain hepatocytes by a protocol approved by the institutional animal care committee. All reagents, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO). [14C]Eicosapentaenoic acid, [35S]methionine, and [3H]triolein, and ENHANCE Fluor solution were purchased from PerkinElmer Life Sciences (Boston, MA). [14C]oleic acid and 1-palmitoyl-2-[14C]oleoyl phosphatidyl choline (POPC) were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). Immunoprecipitin (staph A cells) was purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase was purchased from Type Tissue Collection (Manassas, VA). Rabbit polyclonal antiserum to rat apoB or apoE were included in each gel.

Cell Culture Techniques—Rats fed ad libitum were sacrificed in the morning, and liver cells were isolated by collagenase perfusion using 0.225 mg of collagenase/ml dissolved in Krebs-Ringer buffer containing 1.66 mM calcium. Hepatocytes were purified by differential centrifugation through a 45% Percoll solution, and the viability of the isolated cells was determined by trypan blue dye exclusion. The isolated hepatocytes were suspended in the BR buffer (150 mM NaCl, 25 mM HEPES, 0.1% bovine serum albumin, and 0.1 mM phenylmethylsulfonyl fluoride) and the total protein was determined using the Lowry method.

Cell Viability Assay—Hepatocytes, isolated as in preparation for electrophoresis, were applied to each lane of the gel and the relative signal intensity of each was determined by densitometry. Using the total radioactivity applied to each lane could be determined. Also, protein size standards were included in each gel.

To immunoprecipitate apoB or apoE from unfractionated conditioned medium, monospecific rabbit antiserum to rat apoB or apoE was used. Briefly, an aliquot of the medium was mixed with an equal volume of diluted antiserum (1:100 in PBS, pH 7.5; 0.1% BSA) and incubated overnight at 4 °C. Staph A was added to the form of the immunoprecipitate. The resultant precipitate containing the immune complexes was washed extensively, the staph A cells were removed, and the isolated apoprotein was dissolved in gel sample buffer (0.0625 M Tris/Cl, pH 6.8, 20% glycerol, 2% SDS). Electrophoretic separation of apo-VLDL, apoIDL, and immunoprecipitates was accomplished using 3.5% acrylamide-18% polyacrylamide gels as described previously (38). The samples were included in each gel.

To determine the differential effects of MTP inhibition on the secretion of labeled apoB and apoE, some experiments, McArdle RH-7777 cells were pretreated for 30 min with MTP inhibitor BMS-200150 (10 μM dissolved in Me2SO (39)) or Me2SO alone (final concentration 0.5%) before adding radiolabel. The cells were further incubated for 4 h, and the conditioned media contents of apoB and apoE were determined by immunoprecipitation and SDS-PAGE analysis as described above. To determine the effects of inhibiting PI3K on apoB secretion, rat primary hepatocytes were pretreated for 30 min with 1 μM wortmannin (dissolved in Me2SO) or Me2SO alone (final concentration 0.5%), which were maintained throughout the experiment. BSA, or BSA complexed with OA, was then added at 10 μg/ml and the cells were incubated for 5 h. The total apoB contents of the conditioned media samples were determined by radioimmunossay (35). In some experiments, [35S]methionine was used to pulse label apoB. The effect of wortmannin on labeled apoB recovery from cell lysate and conditioned medium at the 15- and 90-min time points of the chase period was determined by immunoprecipitation and SDS-PAGE analysis (29).
To determine whether the proteasome mediated Ω-3 fatty acid-induced apoB degradation, McA hepatoma cells were incubated at 37 °C for 4 h in [35S]methionine-containing medium supplemented with either BSA or EPA-BSA complexes in the absence or presence of the proteasome inhibitor, lactacystin (10 μM; purchased from the laboratory of Dr. E. J. Corey, Harvard University, Cambridge, MA). Samples of cell lysates and conditioned media were subjected to immunoprecipitation analysis with anti-apoB antiserum, followed by SDS-PAGE and fluorography. To determine whether Ω-3 fatty acids targeted apoB to lysosomal degradation, a similar experiment was performed substituting ammonium chloride (40 mM) for lactacystin. At this concentration of ammonium chloride, there was no evidence of cell toxicity as assessed by trichloroacetic acid-phosphotungstic acid precipitation analysis, but there was >80% inhibition of the degradation of exogenously added [125I]-LDL (gift of Dr. Ira Tabas, Columbia University, New York, NY).

Effects on Re-uptake of Nascent VLDL—To evaluate whether there were differential effects of fish oil fatty acids and OA on the re-uptake of newly synthesized VLDL, we employed an experimental design identical to that described above, except heparin (0.1 or 10 mg/ml) was added to the treatment media to block LDL receptor-dependent and proteoglycan-mediated re-uptake (18). Control experiments were performed showing that the flotation properties of VLDL were unchanged in the presence of heparin.

Effects on Microsomal Triglyceride Transfer Protein Activity—Three methods were used to assess possible effects of Ω-3 fatty acids on cellular MTP function. First, rat primary hepatocytes were incubated with BSA, OA, or EPA, and then MTP activity in cell lysates was measured using a fluorescent assay (Roar Biomedical, New York, NY). Second, to determine if lipid classes synthesized in the presence of OA or Ω-3 fatty acid are equivalent substrates for MTP, primary rat hepatocytes or HepG2 cells were incubated with tritiated OA or EPA, total cellular lipids were extracted into isopropanol, and bioseynthetically labeled cholesteryl ester, triglyceride, and phospholipids were separated by preparative thin layer chromatography. Each of these isolated lipids was incorporated into vesicles that were used as donors in a transfer reaction with acceptor vesicles and 50 μg of purified bovine MTP, as described previously (40). As an internal control for lipid transfer, each donor vesicle also contained [14C]triolein. To determine if Ω-3 lipids inhibit MTP-mediated transfer of non-Ω-3 lipids, artificial donor vesicles were prepared containing either labeled triolein or labeled POPC. The majority of lipid mass in these vesicles was unlabeled phosphatidylcholine that contained either 100% oleate esterified at the sn-2 position (control), or a mixture of 70% oleate and 30% DHA or 30% EPA. Transfer of the labeled lipids to acceptor vesicles in the presence of 50 μg of purified bovine MTP was measured as described previously (40).

Subcellular Fractionation of Rat Primary Hepatocytes—Rat primary hepatocytes were isolated as above, allowed to recover overnight, and then incubated for 4 h in Dulbecco’s modified Eagle’s medium supplemented with [35S]methionine (300 μCi/ml), 0.16 mM BSA, and either no fatty acids, 0.8 mM OA, or 0.8 mM DHA. The monolayers were washed three times, and the cell lysates and post-nuclear supernatants were prepared as described (41). The cell lysates were mixed with sucrose to a final concentration of 8.58% then placed onto discontinuous sucrose gradients. The density layers were 56% sucrose (0.46 ml), 50% (0.92 ml), 45% (1.38 ml), 40% (2.5 ml), 35% (2.3 ml), 30% (1.38 ml), 20% (0.46 ml), and 8.58% (2.3 ml of the post-nuclear supernatant). After ultracentrifugation (SW41 rotor, 4 °C, 39,000 rpm, 18 h), 23 fractions of 0.5 ml each were collected from the top of the tube. From each of the top 22 fractions, 420 μl was subjected to immunoprecipitation/SDS-PAGE analysis using rabbit anti-rat apoB antiserum, followed by fluorography then densitometric quantification; 4 μl was used to assay the Golgi marker enzyme α-mannosidase II (42); 11 μl was used for Western blot analysis of the transmembrane protein palmitoyl-CoA:diacylglycerol acyltransferase (using an antibody kindly supplied by Calbiochem) followed by densitometric quantification by densitometry.

Statistical Analyses—Results are displayed as mean ± S.E., n ≥ 3. Absent error bars in the figures indicate S.E. values smaller than the drawn symbols. For comparisons between a single experimental group and a control, the unpaired, two-tailed t test was used. For comparisons involving several groups simultaneously, analysis of variance (ANOVA) was initially used. When the ANOVA indicated differences among the groups, pairwise comparisons of each experimental group versus the control group were performed using the Dunnett’ q statistic. Analyses were performed with PRISM (GraphPad Software, San Diego, CA).

RESULTS

Nature of the Target for Ω-3 Fatty Acid-induced Degradation—Previous studies using McA hepatoma cell clones that express a range of artificially truncated human apoB constructs have suggested that buoyant lipoproteins, regardless of the precise length of the transacted apoB construct, are the most susceptible to degradation induced by Ω-3 fatty acids (29, 33). We now used two approaches to test this suggestion with native, rather than artificially truncated, forms of apoB. First, we studied rat primary hepatocytes, a non-transformed cell that secretes apoB100 almost exclusively in the form of VLDL, while dividing its production of apoB48 between particles with the density of VLDL (~2/3 of secreted apoB48) and HDL (~1/3 of secreted apoB48) (43). Thus, this pattern of apoB48 secretion allows us to compare the effect of Ω-3 fatty acids on particles with different buoyant densities but containing the same naturally occurring form of apoB.

Rat primary hepatocytes were incubated at 37 °C for 4 h with either OA or DHA (0.8 mM, complexed to 0.16 mM BSA) in the presence of [35S]methionine. Conditioned media samples were subjected to density gradient fractionation, and the labeled apoB48 content of each density class was determined by immunoprecipitation followed by SDS-PAGE and then scintillation counting of the excised gel bands containing apoB48. Results shown are the mean ± S.E. (n = 6).

To pursue the implication that the apoB sequence per se is not a determinant of Ω-3 fatty acid-induced degradation, we turned to a different cell culture model, the human hepatocarcinoma HepG2, which produces apoB100, but no apoB48 whatsoever. A small, but easily measurable amount of HepG2 apoB100 is secreted as part of lipoproteins with the density of VLDL, with the majority appearing in the denser IDL/LDL (1.006 < d < 1.065 g/ml) and HDL (1.065 < d < 1.21) fractions. Thus, this pattern of apoB100 secretion allows us to compare the effect of Ω-3 fatty acids on particles with different buoyant densities but containing the same naturally occurring form of apoB.
apoB. In previous studies (44), we found that EPA or DHA treatment significantly reduced the secretion of newly synthesized VLDL-apoB₁₀₀ by HepG2 cells, but we did not examine effects on the denser apoB-lipoproteins. Therefore, HepG2 cells were incubated 4 h with [³⁵S]methionine and either OA or DHA, and the conditioned media were separated by centrifugation into fractions of d < 1.006 g/ml (VLDL) and 1.006 < d < 1.21 g/ml (LDL + HDL). The recoveries of apoB₁₀₀ from each density fraction are summarized in Fig. 2. Note the different scales in the two axes, which reflects the limited ability of HepG2 cells to secrete VLDL.

As expected, a small amount of labeled apoB₁₀₀ was secreted into the VLDL fraction in either treatment group (Fig. 2, left two bars). Nevertheless, the relative recovery of newly secreted VLDL-apoB₁₀₀ was considerably lower after DHA treatment (~35% of that in the OA group; p < 0.01, consistent with Fig. 1). In contrast, DHA treatment only mildly affected the relative recovery of labeled apoB₁₀₀ from the higher density LDL + HDL class (~75% of that in the OA group; Fig. 2, right two bars). Thus, a single type of apoB, apoB₁₀₀, was differentially affected by DHA depending entirely on the density of the associated lipoprotein.

Overall, the separate results from the primary hepatocytes, in which apoB₄₈ appears in several different density fractions, and HepG2 cells, in which it is apoB₁₀₀ that appears in several different density fractions, strongly suggest that it is a property of the lipoprotein particle, not the primary amino acid sequence of apoB, that is the critical factor in determining susceptibility to degradation induced by Ω-3 fatty acids. Similar results have been obtained in McA hepatoma cell clones expressing a range of artificially truncated human apoB constructs (33). These previous and current results suggest a robust phenomenon independent of the primary sequence of apoB.

There are two possible explanations for the preferential loss of large, buoyant apoB-lipoproteins in the presence of Ω-3 fatty acids: either Ω-3 fatty acids prevent these particles from being assembled but without allowing the unused apoB to be secreted as higher density particles or Ω-3 fatty acids permit the buoyant particles to be assembled but then selectively induce their destruction. Importantly, these two possibilities would have different consequences for other components of the buoyant particles. When apoB is lost without being assembled into large particles, the total cellular secretion of other components, particularly apoE, would not be expected to be significantly affected (as implied, for example, by the results in Refs. 45, 46). In contrast, if entire VLDL particles are removed from the secretory pathway upon Ω-3 fatty acid treatment, then all VLDL apoproteins would be destroyed in parallel. To examine the fate of all of the apoproteins normally associated with VLDL, we treated rat primary hepatocytes with BSA, OA, or Ω-3 fatty acids in the presence of [³⁵S]methionine and then collected the conditioned media. After centrifugation to isolate the d < 1.006 g/ml fraction, samples were delipidated, and the incorporation of radiolabel into individual species of apoproteins was determined by SDS-PAGE followed by fluorography. As shown in Fig. 3, the secretion of total labeled VLDL-apoproteins was significantly reduced in the Ω-3 fatty acid-treated groups relative to the results from the BSA and OA groups (p < 0.001). Visual inspection of the fluorograms, such as the one shown in Fig. 4A, indicated that treatment with DHA or EPA produced substantial decreases in the signal intensities of labeled apoB₁₀₀, apoB₄₈, apoE, and apoCs secreted into the medium on VLDL particles. The quantification of the relative signal intensities is summarized in Table I, except that the results for the apoCs were not included, because such a small fraction of radioactivity was attributable to these apoproteins (<5% in any lane), consistent with their being a minor component of VLDL secreted by rat hepatocytes (43, 47).

The results for apoE are particularly informative, because its addition to nascent VLDL most likely occurs after that of apoB, based, in part, on the following: 1) the earliest event in VLDL assembly is the co-translational lipidation of apoB (as reviewed in Ref. 5); 2) in chicken hepatocytes, VLDL is sequentially assembled from its components and non-apoB apoproteins associate with VLDL at different times than does apoB (47–49); 3) in HepG2 and McA cells, the secretions of apoE and apoB are independent of each other until lipogenesis is stimulated, which results in the recruitment of apoE to apoB-containing lipoproteins (45, 46). Consistent with this last point are the results in Fig. 4, A and B. Although apoE can be secreted as a lipid-poor protein (i.e. d > 1.21) or as a component of a particle with HDL density, we have previously shown (29, 33, 44) that Ω-3 fatty acids are potent stimulators of lipogenesis in all three hepatic cell types studied in the present report, which means that, with EPA or DHA incubation, the majority of apoE should be associated with VLDL particles. If so, then the reduction in apoE recovery from unfractionated medium (Fig. 4B) is predicted to be comparable to the reduction in the VLDL fraction (Table I and Fig. 4A), which is exactly what was found.

Thus, the simplest interpretation of our results is that Ω-3 fatty acids induce a global loss of all VLDL-associated apoprotein.
teins, including apoe, because of an effect in the secretory pathway that occurs after at least partial assembly of the lipoproteins.

**Relationship between Ω-3 Fatty Acid-induced ApoB Degradation and ERAD—**ApoB-lipoprotein biogenesis involves an early, regulated degradative process that is associated with the endoplasmic reticulum (14), provoked by inadequate MTP-mediated initial lipidation of newly synthesized apoB (12, 13), and mediated by proteasomes (10–12). Although our data point toward events in the secretory pathway later than the involvement of MTP or the proteasome, we nevertheless sought to directly examine whether Ω-3 fatty acids act at these early steps.

We first tested the possibility that Ω-3 fatty acids stimulate apoB degradation by impeding MTP-dependent early lipidation, thereby targeting apoB to proteasomes. Rat primary hepatocytes were pretreated for 6 h with BSA, OA, or EPA, and then the MTP activity in lysates of these cells was assessed using a fluorescent assay. No difference in MTP-mediated lipid transfer was seen (data not shown). Because an intracellular abundance of relatively poor lipid substrates might be functionally equivalent to MTP inhibition, we next determined how well DHA- or EPA-enriched lipids are transferred by MTP. HepG2 cells were incubated with [3H]OA or [3H]EPA to allow incorporation of these fatty acids into lipid esters. Total cellular lipids were extracted, and then 3H-labeled triglycerides, cholesterol ester, and phospholipids were each isolated by preparative thin layer chromatography and reconstituted into donor vesicles composed primarily of unlabeled phosphatidylcholine. Transfer of the tritiated lipids to acceptor vesicles was then assessed in the presence of purified bovine MTP (“Materials and Methods”). No decrease was seen in MTP-mediated transfer of 3H-labeled triglycerides harvested from EPA-treated cells (rate = 75% ± 10% of [14C]triolein transfer) versus 3H-triglycerides from OA-treated cells (rate = 60% ± 8% of [14C]triolein transfer; p = 0.3). Likewise, no inhibition was seen for transfer of 3H-cholesterol esters (EPA: 25% ± 2% versus OA: 20% ± 4%; p = 0.07), phosphatidylcholines (EPA: 2.6% ± 0.7% versus OA: 2.3% ± 1%; p = 0.7), or phosphatidylethanolamines (EPA: 1.1% ± 0.2% versus OA: 1.1% ± 0.1%; p = 0.6). Essentially identical results were obtained with 3H-lipids derived from rat primary hepatocytes treated with [3H]OA versus [3H]EPA for 6 h (data not shown). Finally, we determined whether lipids containing Ω-3 fatty acyl groups inhibit the transfer of non-Ω-3 lipids. We compared donor vesicles prepared from three different mixtures of unlabeled phosphatidylcholines: either 100% oleate esterified at the sn-2 position (control); 70% oleate plus 30% DHA; or 70% oleate plus 30% EPA. Importantly, the unlabeled Ω-3-phosphatidylcholines did not significantly limit the transfer of labeled triolein or POPC to acceptor vesicles in the presence of purified MTP (e.g., transfer of [14C]triolein from vesicles containing 30% EPA-phosphatidylcholine was 123% ± 16% of the rate of [14C]triolein transfer from control vesicles containing only OA-phosphatidylcholine (p > 0.5). Overall, our data on MTP activity in these cell-free assays contradict the hypothesis that Ω-3 fatty acids interfere with the initial, MTP-dependent phase of VLDL assembly.

To examine this issue in intact, living cells, we assessed the pattern of apoprotein secretion after MTP inhibition. Upon treatment of McA hepatoma cells with BMS compound 200150, an inhibitor of MTP (39), the secretion of newly synthesized apoB and apoe was measured. As seen in Fig. 5, MTP inhibition almost completely abolished the secretion of apoB<sub>100</sub>, as expected (39), whereas there was no significant effect on apoe secretion, consistent with prior work (46). This pattern is unlike the effects of Ω-3 fatty acids, which reduce the secretion of both apoproteins together (Fig. 4 and Table 1).

Further proof in living cells that Ω-3 fatty acids affect a step distal to MTP-dependent lipoprotein assembly comes from examining the role of the proteasome, which mediates apoB degradation after inhibition of either lipid synthesis or MTP-mediated lipid transfer (e.g., Refs. 13, 14). McA hepatoma cells were treated with either BSA or with EPA-BSA complexes in the absence or presence of the proteasomal inhibitor, lactacystin (“Materials and Methods”). Typical data are shown in Fig. 6, in which lactacystin produced little if any inhibition of EPA-induced degradation (Fig. 6A) at the same time it increased...
apoB100 recovery in the absence of \( \text{\textOmega-3} \) fatty acids (Fig. 6B). Thus, involvement of MTP or ERAD cannot explain four key characteristics of \( \text{\textOmega-3} \) fatty acid-induced degradation: it is specific to buoyant lipoproteins; it occurs without any inhibition of MTP; there is collateral loss of other VLDL apoproteins, particularly apoE; and it is independent from proteasomes.

**Relationship between \( \text{\textOmega-3} \) Fatty Acid-induced Degradation and Re-uptake**—To demonstrate re-uptake of nascent VLDL and to evaluate its potential contribution to the effects of \( \text{\textOmega-3} \) fatty acids, rat primary hepatocytes were treated with either OA or DHA, with or without the addition of heparin to the culture medium. The concentration of heparin was 10 mg/ml, which blocks lipoprotein binding to both LDL receptors and HSPGs (Ref. 18 and citations therein). As shown in Fig. 7, the net secretion of newly synthesized VLDL-apoproteins during treatment with either OA or DHA was increased approximately 2-fold \((p < 0.01)\) by the addition of 10 mg of heparin/ml of culture medium. Thus, there is substantial re-uptake of nascent VLDL at the surface of primary hepatocytes in the presence of either fatty acid. Nevertheless, blocking cell surface re-uptake with 10 mg of heparin/ml did not affect the ability of DHA to reduce VLDL apoprotein output: secretion of VLDL apoproteins in the presence of DHA was \(-50\%\) of the OA control, independent of heparin treatment. Thus, re-uptake of newly exported apoB cannot explain the effect of \( \text{\textOmega-3} \) fatty acids on lipoprotein secretion. A similar experiment was conducted with a low concentration of heparin \((0.1 \text{ mg/ml})\) that blocks lipoprotein binding to HSPGs without affecting LDL receptor binding \((18)\). No “bridging molecules,” such as lipoprotein lipase, were added to enhance lipoprotein-HSPG interactions. Under these conditions, the low concentration of heparin failed to increase apoB output in the presence of either fatty acid (data not shown; cf. Fig. 8 and its accompanying text in Ref. 18). Thus, under these specific conditions, re-uptake of nascent VLDL is substantial; it is mediated primarily by the binding of apoB100 or apoE to the LDL receptor, without significant involvement of cell surface HSPGs; and the inhibitory effect of \( \text{\textOmega-3} \) fatty acids persists during blockade of re-uptake at the cell surface.

More evidence against the involvement of re-uptake in \( \text{\textOmega-3} \) fatty acid-induced degradation of apoB is based on the knowledge that lipoproteins captured by either LDL receptors \((22)\) or heparan sulfate proteoglycans \((18–20)\) are directed to lysosomes. Thus, rat primary hepatocytes cells were treated with either OA or DHA in the absence or presence of ammonium chloride, a lysosomal inhibitor. Under these conditions, ammonium chloride decreased the degradation of \( ^{125\text{I}}\)-LDL (“Materials and Methods”), but there was no decrease in DHA-induced degradation of newly synthesized apoB (data not shown). Overall, involvement of re-uptake at the cell surface cannot explain two key characteristics of \( \text{\textOmega-3} \) fatty acid-induced degradation: it continues even in the presence of high concentrations of hepa-
DHA. Based on the distribution of the ER and Golgi markers, the present data do not support a model in which apoB induced Degradation of ApoB—

The ERAD-proteasome and cell surface re-uptake processes represent the initial and the final opportunities, respectively, for a hepatic cell to regulate the net surface re-uptake. This third “threat” to nascent apoB falls somewhere between the other processes, which are at the two temporal extremes of the secretory pathway. The discussion to follow will focus on the intrinsic characteristics of the Ω-3 fatty acid-induced process and its potential to regulate apoB-lipoprotein production in different physiologic and pathophysiologic states.

What is the substrate of Ω-3 fatty acid-induced degradation? Recent data (summarized in Refs. 3, 52) support the model that a small apoB-containing lipoprotein of HDL density is the “primordial” particle that results from the MTP-dependent production of initial lipidation in the ER. These primordial particles can be secreted directly as lipoproteins in the HDL density range or they can be further lipidated within the cell to VLDL.

DISCUSSION

The present results establish that the treatment of hepatic cells with Ω-3 fatty acids induces the degradation of newly synthesized apoB through a process that is distinct from the two previously described degradative pathways, ERAD and re-uptake. This third “threat” to nascent apoB falls somewhere between the other processes, which are at the two temporal extremes of the secretory pathway. The discussion to follow will focus on the intrinsic characteristics of the Ω-3 fatty acid-induced process and its potential to regulate apoB-lipoprotein production in different physiologic and pathophysiologic states.

What is the substrate of Ω-3 fatty acid-induced degradation? Recent data (summarized in Refs. 3, 52) support the model that a small apoB-containing lipoprotein of HDL density is the “primordial” particle that results from the MTP-dependent phase of initial lipidation in the ER. These primordial particles can be secreted directly as lipoproteins in the HDL density range or they can be further lipidated within the cell to VLDL.
density and then secreted. Because apoE most likely associates with nascent VLDL particles after apoB does (for the reasons summarized under “Results”) and may promote VLDL triglyceride secretion (53, 54), this association probably occurs during the conversion of primordial apoB-lipoproteins to more lipiddated particles. Thus, the relative refractoriness of apoB associated with lipoproteins more dense than VLDL to degradation by proteases in that organelle. The apoB in Golgi fractions isolated from rabbit hepatocytes, consistent with degradation by proteases in that organelle. The proteases are present in post-ER acid as “post-ER pre-secretory proteolysis” or PERPP (4). The data indicate that the signal responsible for targeting apoB-containing lipoproteins with receptors on the plasma membrane (Fig. 7). Thus, our data indicate that the Ω-3 effect occurs after the ER but before the particles exit the cell. For convenience, we will refer to the pathway of hepatic apoB degradation induced by Ω-3 fatty acids as “post-ER pre-secretory proteolysis” or PERPP (4).

What are the likely mechanisms involved in post-ER proteolysis of apoB? Certainly, proteases are present in post-ER compartments (55), including the Golgi apparatus (56). Moreover, Carright et al. (57) have demonstrated degradation of apoB in Golgi fractions isolated from rabbit hepatocytes, consistent with degradation by proteases in that organelle. The actual signal that identifies apoB100 from the Golgi (Fig. 5) and the protection afforded by brefeldin A (“Results”). In contrast to cell surface re-uptake, Ω-3 fatty acids do not exert their effects through an interaction of newly exported lipoproteins with receptors on the plasma membrane (Fig. 7). Thus, our data indicate that the Ω-3 effect occurs after the ER but before the particles exit the cell. For convenience, we will refer to the pathway of hepatic apoB degradation induced by Ω-3 fatty acids as “post-ER pre-secretory proteolysis” or PERPP (4).

In support of PERPP, Ω-3 fatty acid-enriched diets consistently lower VLDL levels in human subjects (30), and the mechanism appears to be decreased hepatic VLDL production (75). Given the similarities outlined above between PERPP stimulated by Ω-3 fatty acids and apoB degradation stimulated by insulin, we speculate that syndromes of insulin resistance should reduce PERPP in vivo, thereby resulting in the overproduction of large buoyant apoB-lipoproteins. Thus, PERPP may contribute to the pathogenesis of familial combined hyperlipidemia, syndrome X, and the metabolic syndrome (for recent reviews, see Refs. 2–4, 28), as well as the syndrome of hyperlipidemia and insulin resistance seen after administration of HIV protease inhibitors (78–80). Consistent with this model, the acute post-prandial rise in insulin levels is associated with a specific decrease in hepatic VLDL production in vivo (81), and insulin-resistant animals were recently reported to exhibit decreased degradation of newly synthesized apoB (82).

In conclusion, PERPP is a distinct step in the regulation of apoB secretion from hepatocytes in vitro, with a plausible role in vivo as well. Identification of physiologic stimuli for PERPP, the cellular mechanisms for targeting of apoB to this degradative pathway, and the protease(s) involved should advance our understanding of the regulation of hepatic lipoprotein production in both normal and pathophysiologic states and may provide new targets for pharmacologic intervention.

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The Triple Threat to Nascent Apolipoprotein B: EVIDENCE FOR MULTIPLE, DISTINCT DEGRADATIVE PATHWAYS
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