Fish oil fatty acids impair VLDL assembly and/or secretion by cultured rat hepatocytes

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Abstract We determined the effect of the two major fish oil fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on VLDL assembly and secretion by cultured rat hepatocytes. The incorporation of [^{3}H]glycerol into total triglyceride (cell plus media) was stimulated eight-fold when hepatocytes were incubated for 2 h with 1 mM EPA, DHA, or oleic acid (OA), suggesting that fish oil fatty acids stimulate hepatic triglyceride synthesis to an extent similar to OA. In contrast, mass quantitation of secreted triglyceride showed impaired triglyceride secretion with EPA and DHA compared to OA. During a 42-h time course, cells stimulated with EPA and DHA progressively accumulated triglyceride compared to cells stimulated with OA. To determine whether fish oil fatty acids impair very low density lipoprotein (VLDL) secretion, cells were labeled with [^{35}S]methionine and the secretion of de novo synthesized apoB was measured. Compared to OA, EPA and DHA significantly impaired the secretion of both molecular weight forms of apoB. The cellular content of apoB was not altered by any of the fatty acids. The concordant decrease in the secretion of both triglyceride and apoB suggests that fish oil fatty acids impair VLDL assembly and/or secretion.—**Lang, C. A., and R. A. Davis.** Fish oil fatty acids impair VLDL assembly and/or secretion by cultured rat hepatocytes. *J. Lipid Res.* 1990. **31:** 2079–2086.

Supplementary key words fish oil • eicosapentaenoic acid • docosahexaenoic acid • triglycerides • apoB • oleic acid

Chronic fish oil feeding lowers fasting plasma triglyceride levels in humans and experimental animals (1–4). Lipoprotein turnover studies suggest that lower triglyceride levels are due to decreased hepatic VLDL triglyceride and apoB secretion (5). The mechanism for decreased VLDL secretion has not been established. Fish oil fatty acids could impair VLDL secretion by decreasing the amount of triglyceride available for packaging as VLDL core lipid (decreased triglyceride synthesis) or by decreasing the number of VLDL particles secreted from hepatocytes (impaired VLDL assembly/secretion).

Several groups have examined the direct effects of the major fish oil fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on VLDL secretion using cultured hepatocytes and perfused livers. Impaired VLDL triglyceride secretion has been observed when cultured rat hepatocytes are incubated with EPA or DHA (6, 7). Since EPA and DHA were not as effective as oleic acid (OA) in stimulating triglyceride synthesis, the impairment of VLDL triglyceride secretion was attributed to inhibition of triglyceride synthesis (6, 7). This hypothesis is supported by data suggesting that fish oil fatty acids decrease the activity of two enzymes involved in triglyceride synthesis: diacylglycerol acyl transferase (8) and phosphatidate phosphohydrolase (9).

However, in subsequent studies using HepG2 cells, Wong, Fisher, and Marsh (10) showed that EPA, DHA, and OA stimulated triglyceride synthesis 6- to 9-fold. Yet, both EPA and DHA impaired triglyceride secretion. While EPA was 24% less effective than OA in stimulating triglyceride synthesis, DHA stimulated triglyceride synthesis as well as OA (10). Thus, in HepG2 cells, decreased triglyceride synthesis cannot explain the decrease in triglyceride secretion observed with DHA. Additionally, in rat hepatoma cells, EPA impaired triglyceride secretion but continued triglyceride synthesis resulted in progressive triglyceride accumulation (11).

To determine whether fish oil fatty acids impair the assembly and/or secretion of VLDL particles, we studied the effects of EPA and DHA on the assembly and secretion of VLDL by cultured rat hepatocytes. The results show that fish oil fatty acids impair VLDL assembly or secretion through a mechanism not involving decreased triglyceride synthesis or availability.

MATERIALS AND METHODS

Essentially fatty acid-free bovine serum albumin (BSA), oleic acid, eicosapentaenoic acid, and docosahexaenoic acid were purchased from Sigma Chemical Company (St. Louis, MO). The radioisotopes, Tran^{35}S-Label (70% L-methionine) and [2-^{3}H]glycerol, were purchased from ICN Radiochemicals (Irvine, CA). The enzymatic kit for measuring triglycer-

Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; VLDL, very low density lipoproteins; OA, oleic acid; TLC, thin-layer chromatography.

ide mass was purchased from Sigma Chemical (Triglyceride UV Procedure No. 334-UV). Thin-layer chromatography (TLC) plates (Silica Gel G, 250 μm) were obtained from Analtech (Newark, DE). Reagents and supplies for hepatocyte culture were obtained from previously described sources (12, 13).

Hepatocyte culture

Hepatocytes were prepared from male Sprague-Dawley rats (200–300 g) (Charles River Breeding Laboratories, Wilmington MA) that had been fed a standard pellet diet. The cells were obtained by collagenase digestion and plated for 4 h in Dulbecco's Modified Eagle's (DME) medium supplemented with 20% newborn calf serum, insulin, glucose, and penicillin and streptomycin as previously described (14). Studies using [^{3}H]glycerol and [^{35}S]methionine were performed in 60-mm dishes; experiments involving measurement of triglyceride mass were performed in 100-mm dishes (8 h incubations) or 60-mm dishes (18 and 42 h incubations).

Experimental protocol

Stock solutions of each fatty acid in benzene were blanketed with nitrogen and stored at −20 °C for subsequent use. On the day of an experiment, a sterile solution of 0.13 mM fatty acid-free albumin in DME was prepared and the pH was adjusted to 10. Aliquots of each fatty acid–benzene solution were added to glass tubes and dried under nitrogen. The DME–albumin solution was added to the dried fatty acid while sonicating vigorously to produce a solution of fatty acid - albumin in the desired concentration (usually 1 mM fatty acid/0.13 mM albumin). The pH was adjusted to 7.5 and insulin and penicillin/streptomycin were added. The media containing fatty acid was warmed to 37 °C before use.

Four hours after initial plating in DME plus 20% calf serum, the medium was changed to serum-free DME containing fatty acid complexed with albumin. The stimulatory effect of each fatty acid on the incorporation of [^{3}H]glycerol into cell and media triglyceride and phospholipid was determined during 2-h incubations with [^{3}H]glycerol (10 μCi/dish, 25 μM). The time course of triglyceride mass accumulation in cells and media was determined by incubating cells with fatty acid for 8, 18, and 42 h. The effect of each fatty acid on the incorporation of [^{35}S]methionine (200 μCi/dish) into immunoprecipitated cell and media apoB during 18-h incubations was determined as described (15, 16). All incubations were performed in triplicate. At the end of each incubation the cells and media were harvested as previously described (14) and frozen at −20 °C for subsequent analysis.

Assay of cell and media triglyceride and total phospholipid

Cells and media were thawed and resuspended by thorough sonication. An aliquot of the cell solution was removed for protein determination by the method of Lowry et al. (17) using BSA as a standard. The cell and media lipids were extracted (13) and triglycerides and total phospholipid were separated by TLC. The silica gel containing ^{3}H-labeled triglyceride or phospholipid was scraped into vials and assayed for radioactivity via scintillation counting. Counts were normalized to the amount of cellular protein per dish.

Triglyceride mass was measured after extracting the silica gel twice with 4 ml of chloroform–methanol 2:1 and twice with 4 ml of chloroform. The extracts were dried under nitrogen, resuspended in 150 μl methanol, and triglyceride mass was assayed by the Sample Start procedure as outlined by the manufacturer of the triglyceride enzymatic kit. The sole modification of the procedure was incubation for 75 rather than 10 min. Recovery of triglyceride after TLC isolation and subsequent extraction was 90–95%. For all conditions used the reaction was linear for time and amount of triglyceride added as standard. Triglyceride mass was calculated from simultaneously performed analysis of triolein standards based on the sample absorbance of 340 nm versus blanks.

Immunoprecipitation of apoB

Immunoprecipitation of cell and media apoB was performed as previously described (15, 16). Briefly, an amount of polyclonal rabbit antiserum that was found to specifically and quantitatively bind apoB was incubated with aliquots of cells and media with shaking overnight at room temperature. The immune complexes were absorbed to protein A Sepharose beads, washed five times with buffer, and boiled with SDS–PAGE buffer containing a standard amount of d < 1.21 g/ml lipoproteins. After SDS–PAGE on linear gradients of 1–20% acrylamide, the Coomassie-stained bands corresponding to apoB-100 and apoB-48 were cut from the gels and assayed for radioactivity (15). TCA precipitation of cell proteins showed that none of the different culture conditions affected the incorporation of [^{35}S]methionine into cell protein.

Assessment of triglyceride synthesis and VLDL secretion

Triglyceride synthetic rates were not measured directly. Triglyceride synthesis was inferred from the ability of each fatty acid to stimulate the incorporation of [^{3}H]glycerol into cell plus media triglyceride and from static measures of cell plus media triglyceride mass at various time points. Since greater than 95% of [^{3}H]triglyceride secreted into the medium of cultured rat hepatocytes is recovered in the VLDL fraction (d < 1.006 g/ml) the accumulation of [^{3}H]triglyceride can be used to estimate VLDL triglyceride secretion (12, 13). Furthermore, since greater than 95% of newly synthesized apoB secreted into the culture medium is recovered in the VLDL fraction, the accumulation of media [^{35}S]apoB can be used to estimate VLDL particle secretion (12). Total phospholipid synthesis was inferred from the ability of each fatty acid to stimulate the incorporation of [^{3}H]glycerol into cell plus media phospholipid.

Statistical analysis

All data are presented as means plus or minus the standard deviation. Each animal contributes an n of 1 which represents the average of the triplicate dishes for that animal in a specific experiment. The data were analyzed using a one-way analysis of variance and contrasts were tested using the Student-Newman-Kuels test (18).

RESULTS

Effect of fatty acids on triglyceride synthesis and secretion

During 2-h incubations all three fatty acids (EPA, DHA, and OA) stimulated the accumulation of cell plus media [^{3}H]triglyceride to a similar extent (**Fig. 1A**) suggesting that there is no difference between fish oil fatty acids and OA in their ability to stimulate triglyceride synthesis. In contrast to the similar effects on triglyceride synthesis, the fish oil fatty acids stimulated triglyceride secretion less than OA (Fig. 1B). Cells stimulated with OA secreted 34 - 57% more [^{3}H]triglyceride than cells stimulated with EPA or DHA, respectively. These data suggest that while fish oil fatty acids were competent substrates for triglyceride synthesis, the triglyceride produced was inefficiently secreted. Accumulation of [^{3}H]triglyceride in cells stimulated with fish oil fatty acids was not observed in these 2-h incubations in which secreted [^{3}H]triglyceride was only 10% of that accumulating in cells (Fig. 1C).

Effect of fatty acids on triglyceride mass accumulation and secretion

The mass of triglyceride assumulating in cells and the media after 18-h incubations with fatty acids was measured. Triglyceride mass accumulated in cells plus media to the same extent with all fatty acids (**Fig. 2A**). Moreover, there was markedly less triglyceride secreted by cells stimulated with EPA and DHA than was secreted by cells stimulated with OA (Fig. 2B). In fact, less triglyceride mass accumulated in the media of cells stimulated with the fish oil fatty acids than in the media of unstimulated cells (i.e., cells incubated with albumin alone). Thus, fish oil fatty acids clearly impaired triglyceride secretion through a mechanism not involving decreased triglyceride availability. While there was a trend toward greater accumulation of triglyceride mass in cells stimulated with EPA and DHA compared to cells stimulated with OA, this difference was not statistically significant with n = 4 (Fig. 2C).

Time course of intracellular triglyceride accumulation and secretion

The relationship between triglyceride accumulation in cells and secretion of (VLDL) triglyceride was elucidated during a time course experiment in which cells were incubated with fatty acid for 8 to 42 h. Cells treated with the fish oil fatty acids accumulated triglyceride progressively in comparison to cells treated with OA (**Fig. 3A**). Despite progressive accumulation of triglyceride mass in cells incubated with EPA or DHA beyond that occurring with OA, significantly less triglyceride was secreted during the entire time course (Fig. 3B). Cells stimulated with EPA and DHA secreted less triglyceride than cells incubated without fatty acid. Thus, EPA and DHA impair triglyceride secretion resulting in its intracellular accumulation.

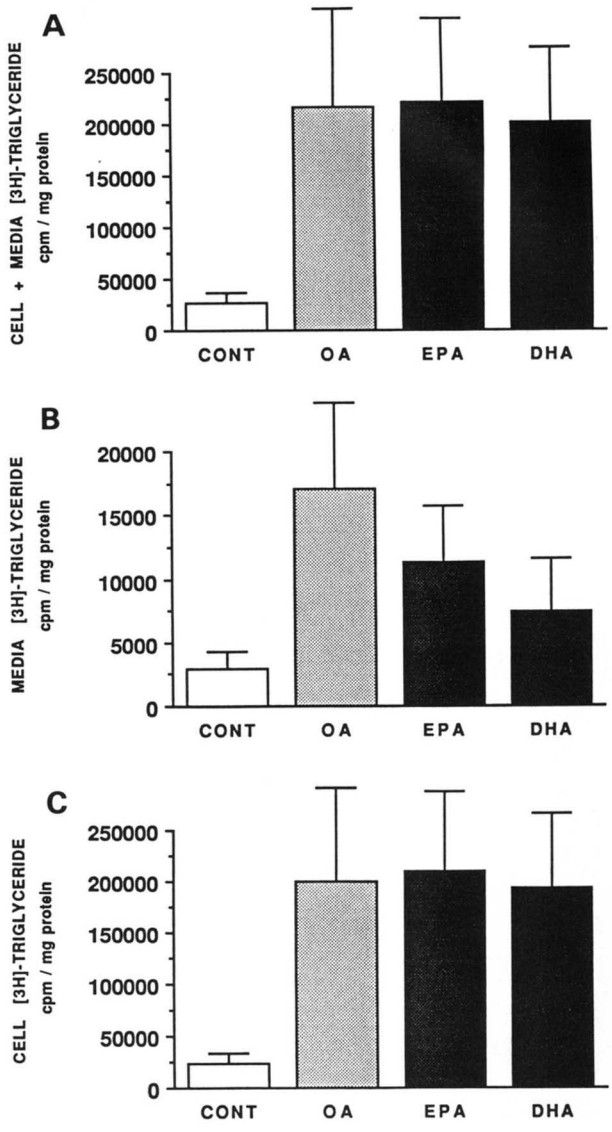

Fig. 1. Stimulation of [^{3}H]glycerol incorporation into cell and media triglyceride by fish oil fatty acids or oleic acid versus albumin alone. Hepatocytes were incubated for 2 h with 1 mM fatty acid (OA, EPA, or DHA) complexed with 0.13 mM albumin or albumin alone (CONT), n = 11. Panel A: CONT versus OA, EPA, or DHA: $P < 0.05$. Differences between OA, EPA, DHA were nonsignificant. Panel B: (note difference in scale compared to panels A and C) CONT versus, OA, EPA, or DHA: $P < 0.05$. OA versus EPA or DHA: $P < 0.05$. Panel C: CONT versus OA, EPA, or DHA: $P < 0.05$. Differences between OA, EPA, DHA were not significant.

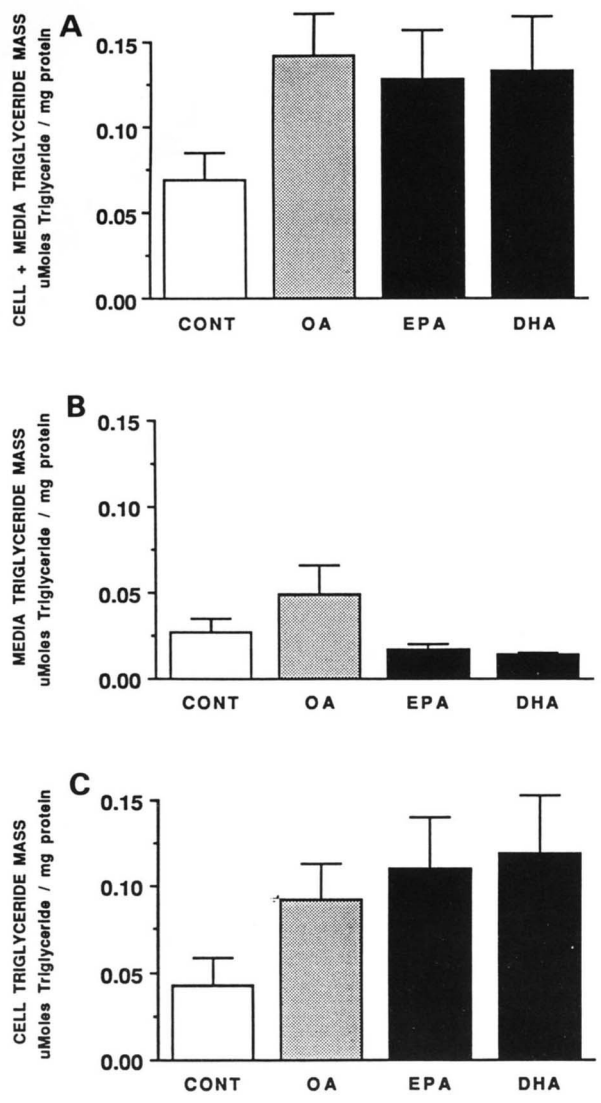

Fig. 2. Stimulation of triglyceride mass accumulation in cells and media by fish oil fatty acids or oleic acid versus albumin alone. Hepatocytes were incubated for 18 h with 1 mM fatty acid (OA, EPA, or DHA) complexed with 0.13 mM albumin or albumin alone (CONT), n = 4. Panel A: CONT versus OA, EPA, DHA: $P < 0.05$. Differences between OA, EPA, DHA were not significant. Panel B: CONT versus OA: $P < 0.05$. OA versus EPA or DHA: $P < 0.05$. Differences between CONT, EPA, and DHA were not significant. Panel C: CONT versus OA, EPA, or DHA: $P < 0.05$. Differences between OA, EPA, DHA were not significant.

Effect of fatty acids on secretion of de novo synthesized apoB

Previous studies in cultured hepatocytes suggest that fatty acid stimulation of triglyceride synthesis and secretion does not alter apoB secretion, indicating that apoB secretion is not regulated by triglyceride availability (15, 16, 19). In contrast, apoB availability does determine the capacity to secrete triglyceride (13, 16). To determine whether decreased triglyceride secretion with EPA and DHA is due to decreased VLDL particle assembly and/or secretion or is the result of decreased triglyceride availability, we measured apoB secretion. Cells were incubated with the fatty acids for 18 h in the presence of [^{35}S]methionine and apoB was immunoprecipitated and its radioactivity was quantitated. The incorporation of [^{35}S]methionine into total cellular protein (i.e., trichloroacetic acid precipitable) was similar for all fatty acids (data not shown) suggesting no differences in protein synthesis or labeling. Cells incubated with EPA and DHA showed a nonstatistically significant trend towards apoB accumulation (**Fig. 4A**). Furthermore, consistent with previous results (13), OA did not affect the synthesis or secretion of apoB (Fig. 4A, B). In marked contrast, both EPA and DHA significantly decreased the secretion of [^{35}S]apoB-48 and apoB-100 (Fig. 4B). The concomitant decrease in the secretion of both triglyceride and apoB suggests that fish oil fatty acids impair VLDL particle assembly and/or secretion.

Effect of fatty acid concentration on triglyceride secretion

To determine whether the concentration of fish oil fatty acid or the molar radio of fatty acid to albumin is related

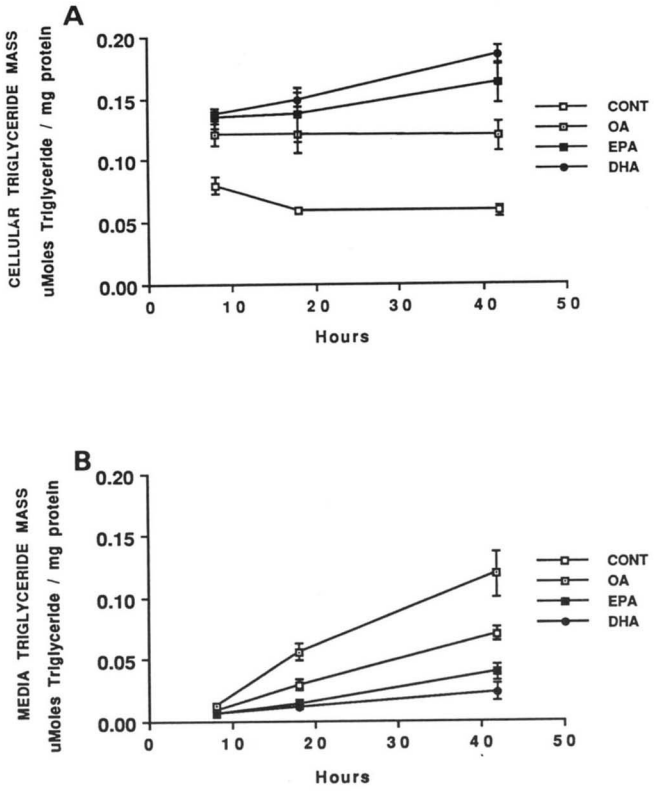

Fig. 3. Time course of triglyceride mass accumulation in cells and media during incubation with fish oil fatty acids or oleic acid versus albumin alone. Hepatocytes were incubated for 8, 18, or 42 h with 1 mM fatty acid (OA, EPA, or DHA) complexed with 0.13 mM albumin or albumin alone (CONT), n = 1. Panel A: CONT versus OA, EPA, DHA: $P < 0.05$. EPA or DHA versus OA: $P < 0.05$. Panel B: CONT or OA versus EPA or DHA: $P < 0.05$. CONT versus OA: $P < 0.05$.

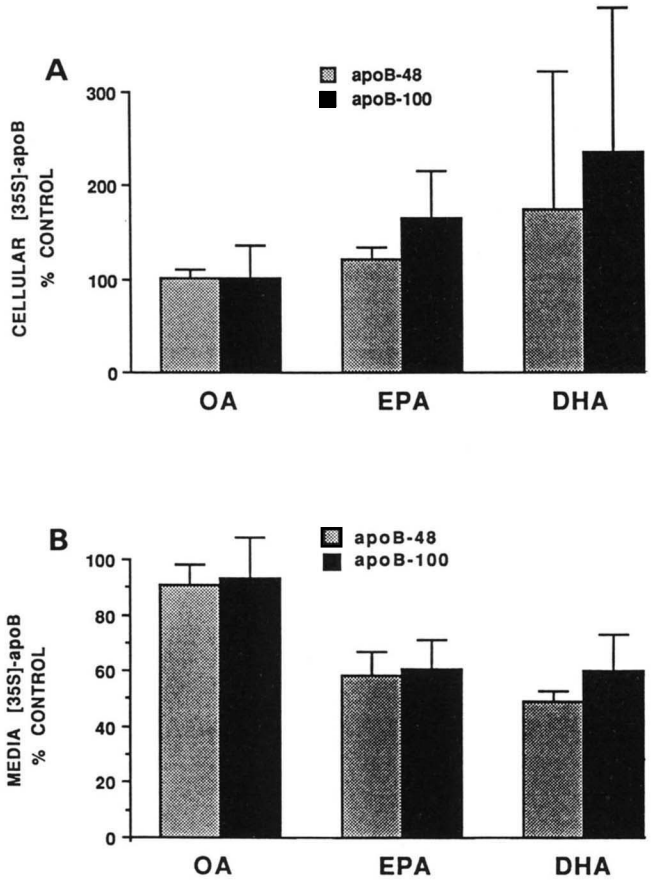

Fig. 4. Inhibition of apoB secretion by fish oil fatty acids versus oleic acid. Hepatocytes were incubated for 18 h with 1 mM fatty acid (OA, EPA, or DHA) complexed with 0.13 mM albumin or albumin alone and the accumulation of [^{35}S]apoB in cells and media was assayed, n = 3. Panel A: Differences between OA, EPA, and DHA were nonsignificant. Panel B: OA versus EPA or DHA: $P < 0.05$.

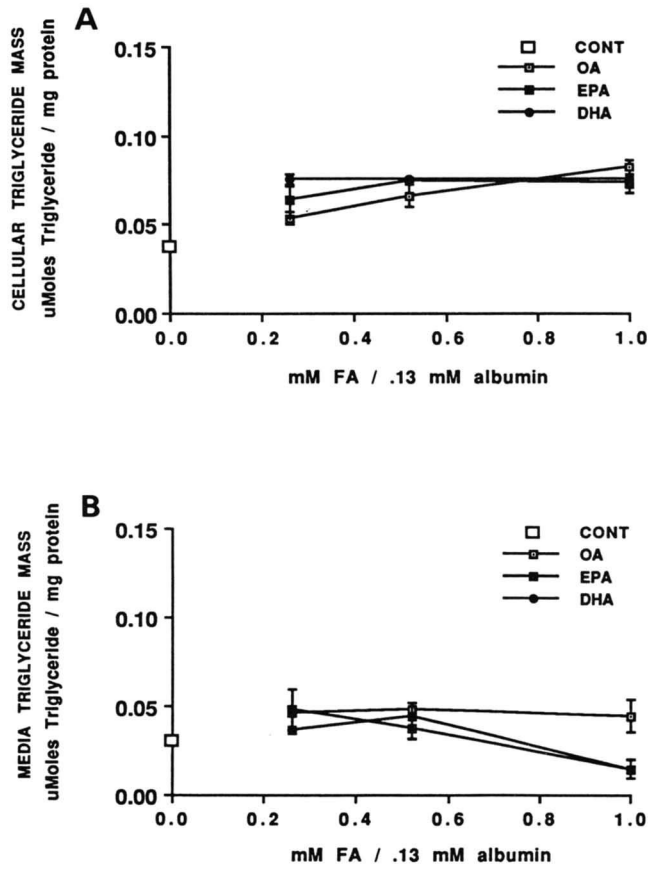

Fig. 5. Effect of fatty acid concentration on triglyceride mass accumulation in cells and media. Hepatocytes were incubated for 18 h with 0.26, 0.52, or 1 mM fatty acid (OA, EPA, or DHA) complexed with 0.13 mM albumin or albumin alone (CONT), n = 1. Panel A: CONT versus OA, EPA, or DHA: $P < 0.05$. Differences between EPA, DHA, and OA were nonsignificant. Panel B: EPA or DHA versus OA at 1 mM fatty acid: $P < 0.05$. Differences between EPA, DHA, and OA were nonsignificant at lower fatty acid concentrations.

to impaired triglyceride secretion, cells were incubated with fatty acids at varying concentrations (0.26 mM fatty acid/0.13 mM albumin, 0.52 mM fatty acid/0.13 mM albumin, and 1 mM fatty acid/0.13 mM albumin) for 18 h and triglyceride mass in cells and media was determined (**Fig. 5**). All three fatty acids stimulated the accumulation of triglyceride in cells to a similar extent regardless of concentration (Fig. 5A). Oleic acid stimulated triglyceride secretion at low and high concentration (Fig. 5B). EPA and DHA inhibited triglyceride secretion only at the highest concentration tested (1 mM fatty acid/0.13 mM albumin) (Fig. 5B). Thus, in this model system, high concentrations (and molar ratios) of fish oil fatty acids were required to inhibit triglyceride secretion. Longer incubations (greater than 18 h) may be required to detect impaired triglyceride secretion when cells are incubated with lower concentrations of fish oil fatty acids.

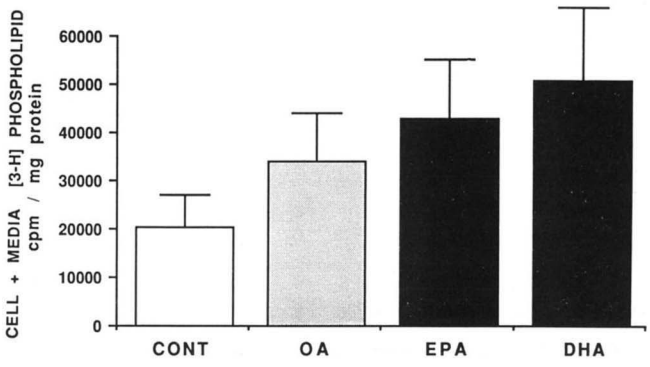

Fig. 6. Stimulation of [^{3}H]glycerol incorporation into cell and media total phospholipid by fish oil fatty acids or oleic acid versus albumin alone. Hepatocytes were incubated for 2 h with 1 mM fatty acid (OA, EPA, DHA) complexed with 0.13 mM albumin or albumin alone (CONT), n = 11. CONT versus OA, EPA, or DHA: $P < 0.05$. OA versus EPA or DHA: $P < 0.05$.

Effect of fatty acids on phospholipid synthesis

During 2-h incubations, all three fatty acids stimulated the accumulation of cell plus media [^{3}H]phospholipid (**Fig. 6**). EPA and DHA stimulated phospholipid synthesis to a greater extent, 110% and 148%, respectively, than oleic acid, 66%. These data reaffirm previous findings of other investigators suggesting that fish oil fatty acids are readily incorporated into cell phospholipid (7).

DISCUSSION

In these studies, both EPA and DHA decreased the secretion of triglyceride while stimulating triglyceride synthesis, resulting in the accumulation of intracellular triglyceride. Fish oil fatty acids impaired the secretion of the major core lipid of VLDL (triglyceride) and the major protein component (apoB) to a similar extent (about 50%) indicating impaired VLDL particle assembly and/or secretion. While these results are similar to those obtained in human HepG2 cells (10) and rat hepatoma cells (11), they are distinctly different from those in rat hepatocytes, which suggests that fish oil fatty acids impair VLDL triglyceride secretion through inhibition of triglyceride synthesis (6, 7). We were unable to resolve these differences by altering reagents or cell culture conditions: experiments performed after overnight plating of hepatocytes versus 4 h, incubations with and without supplemental insulin, or alteration in the methods of preparing fatty acid–albumin complexes (data not shown). The data presented here suggest that the primary mechanism for decreased triglyceride secretion with fish oil fatty acids is unlikely to be direct inhibition of triglyceride synthesis.

EPA and DHA impair apoB secretion in rat hepatocytes (Fig. 4) and in HepG2 cells (10, 20). Cells incubated with EPA and DHA showed a nonstatistically significant trend towards accumulation of apoB (Fig. 4). Other investigators have shown that apoB mRNA levels are unaffected by EPA and DHA using a similar experimental protocol in human HepG2 cells (10). Taken together, these observations suggest that the mechanism for decreased apoB secretion is unlikely to be decreased apoB synthesis. The finding of similar decreases in triglyceride and apoB secretion suggests that the number of VLDL particles secreted is decreased by fish oil fatty acids. Since triglyceride synthesis does not regulate apoB secretion (13), whereas apoB secretion determines the ability to secrete triglyceride (16, 19), impaired triglyceride synthesis by fish oil fatty acids cannot explain decreased apoB secretion. Decreased apoB secretion was not detected in short-term (4 h) liver perfusion experiments in African green monkeys chronically fed fish oil (21). The differences between results in acute incubations in cell culture (this study and references 10 and 20) and those in green monkeys may be due to differences in the experimental models, such as the time frame during which apoB secretion is studied. Thus, we conclude that fish oil fatty acids may impair VLDL particle assembly and/or secretion.

Several lines of evidence suggest that impaired VLDL assembly and/or secretion is unlikely to be due to a nonspecific toxic effect of fish oil fatty acids. First, fish oil fatty acids stimulated triglyceride and phospholipid synthesis. Second, fish oil fatty acids did not impair [^{35}S]methionine incorporation into total cellular protein. Third, we found no obvious microscopic evidence of cellular toxicity such as rounding up of cells or cells lifting off the culture dishes. Finally, triglyceride secretion was impaired but albumin secretion was not affected when HepG2 cells were incubated with fish oil fatty acids using a similar protocol (10). Since high concentrations of fish oil fatty acids were required to inhibit triglyceride secretion, it is possible that the accumulation of a fish oil biosynthetic product or metabolite impairs triglyceride secretion.

The biogenesis of VLDL particles involves several membrane-related events: translocation of nascent apoB across the endoplasmic reticulum membrane, accumulation of triglyceride core lipid in particles with a protein (apoB) and phospholipid surface coat, and vesiculation and transport of nascent VLDL through the Golgi apparatus (12, 13, 15, 16, 19, 21, 22). Fish oil fatty acids delivered to the liver are incorporated into membrane phospholipids (23–26) and may alter the phospholipid composition of cells. Also, since phosphatidylcholine synthesis is required for VLDL secretion (27), it is possible that fish oil fatty acids could impair VLDL secretion by impairing phosphatidylcholine synthesis. In our experiments, fish oil fatty acids stimulated total phospholipid synthesis (Fig. 6). Thus, fish oil fatty acids could alter VLDL particle assembly and/or secretion by altering phospholipid membrane structure and function or phospholipid metabolism. The potent effects of fish oil fatty acids on VLDL assembly and secretion that we describe here may provide a unique model for further study of the molecular events occurring during VLDL biogenesis.

These data raise the possibility that the primary mechanism by which chronic fish oil feeding lowers plasma triglyceride levels is through impaired VLDL assembly and/or secretion. In long term feeding studies, fish oil fatty acids have been shown to divert fatty acids from esterification with glycerol to form triglycerides to beta-oxidation (28–31). This effect may be an adaptive response if triglyceride initially accumulates intracellularly due to impaired secretion. It may also account for the failure to detect triglyceride accumulation in livers during chronic fish oil feeding (23). Additional in vitro studies are needed to further elucidate the molecular mechanism(s) by which fish oil fatty acids inhibit triglyceride secretion. Yet, the data presented here strongly suggest that the mechanism by which fish oil fatty acids impairs triglyceride secretion is not direct inhibition of triglyceride synthesis.

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