Effects of arachidonic and docosahexaenoic acids on secretion and degradation of bile salt-dependent lipase in AR4-2J cells

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Abstract In this study we demonstrated that two polyunsaturated fatty acids, arachidonic acid (AA, n–6) and docosahexaenoic acid (DHA, n–3), modulate the secretion of bile salt-dependent lipase (BSDL) by pancreatic AR4-2J cells. The effects of AA and DHA were also compared with that of the monounsaturated fatty acid, oleic acid (OA). Our results showed that the chronic treatment of cells with AA or DHA, that did not affect the biosynthesis rate of BSDL, similarly decreased the amount of secreted BSDL and perturbed the intracellular partitioning of the enzyme, whereas OA had no effect. Particularly, AA and DHA induced the retention of the enzyme in microsomes and lowered its content in the cell cytosol. We have further shown that AA treatment decreased the ubiquitination of the protein, and consequently diminished its export toward the cytosol, a result that might explain the retention of BSDL in microsomes and correlated with membrane phospholipids alteration. The retained protein was further degraded by a nonproteasomal pathway that likely involves ATP-dependent endoplasmic reticulum proteases. These findings concerning the regulation of the pancreatic BSDL secretion by two polyunsaturated acids, AA and DHA, might be of physiological importance in the plasmatic and cellular cholesterol homeostasis.

Polyunsaturated fatty acids (PUFAs) are known as cholesterol-lowering fatty acids (1); particularly that contained in fish oils has prevented the development of atherosclerosis in the rat (2), in part by a decrease in hypercholesterolemia. Also, a reduction of cholesterol absorption by fish oils has been described in the African green monkey (3) and in the rat (4). Moreover, it has been proposed that atherogenesis could be a postprandial phenomenon (5). Another important physiological role attributed to PUFAs is an antitumoral activity observed in vivo and in vitro (6). The type of fat and the consumption may influence the development and the subsequent progression of several common cancers in human population (7). PUFAs significantly inhibit the growth of several human pancreatic cancer cell lines in vitro, at concentrations that can be achieved in vivo, suggesting that administration of such fatty acids may be of therapeutic benefit in cancer (8). Administration of PUFAs has been advanced as cancer therapy and several clinical trials have been initiated particularly for the treatment of patients with pancreatic cancer (9). PUFAs, the effectiveness of which increased as a function of double bond number, induced apoptosis in pancreatic tumoral cells (10).

Bile salt-dependent lipase (BSDL) is a digestive enzyme secreted by the pancreatic acinar cells (11). This enzyme which is able to hydrolyze dietary cholesteryl esters (12) may be also involved in their absorption by intestinal cells (13). Furthermore, it has been suggested that long chain polyene fatty acids of diacylglycerides, formed during the action of colipase-dependent lipase on dietary triacylglycerides containing these fatty acids, may be the physiological substrate of BSDL (14). Clearly, this latter enzyme in vitro preferentially hydrolyses esters of arachidonic and eicosapentaenoic acid, among other PUFAs (15). After a probable transcytosis through the enterocyte (16), a fraction of pancreatic BSDL reached the blood circulation where it forms an equimolar complex with the apolipoprotein B-100 (apoB-100) of low density lipoproteins (LDL) (17). It has been further proposed that BSDL has the capability to modify human LDL composition and structure and to reduce the atherogenicity of oxidized LDL by decreasing its lysophosphatidylcholine content (18). Opposite to this protective effect, BSDL may have deleterious consequences by converting larger and less...
atherogenic LDL to smaller and more atherogenic LDL subspecies (19). Isoforms of the pancreatic BSDL were also detected in many pancreatic tumor and hepatoma cells (20). In pancreatic cells expressing BSDL and in Chinese hamster ovary (CHO) cells transfected with the cDNA of BSDL, the enzyme partitioned between cytosol and microsomes (21). We have proposed that BSDL could be implicated in the cycle of the cellular homeostasis of cholesterol, which is particularly affected in tumoral cells leading to cholesterol ester storage in cytosolic lipid droplets (22).

Therefore, any alteration of the expression or of the behavior of the pancreatic BSDL by PUFAcs could, on the one hand, affect the amount of secreted enzyme, which in fine leads to a variation of the amount of circulating BSDL. On the other hand, PUFAcs could perturb the partitioning of BSDL within tumoral pancreatic cells and, in turn, lead to the modification of intracellular lipid metabolism and to the diminished growth of pancreatic cancer cells and possibly of other cancer cells that also expressed BSDL.

Among polyunsaturated fatty acids, arachidonic acid (AA) and docosahexaenoic acid (DHA), which are highly concentrated in lipid membranes, have been selected in our studies for their particular physiological interest. AA, which is the principal functional form of essential fatty acids leading to eicosanoids, has been shown to modulate hormone secretion in a variety of tissues (23). DHA, which is largely concentrated in fish oils, is to date well known for its beneficial effects on human health, in part by protecting against heart diseases (24). For all the above physiopathologic and therapeutic aspects, we have studied the effects of AA and DHA on the expression of BSDL in relation to its secreted and intracellular levels and compared these effects with that of monounsaturated fatty acids largely found in the human diet such as oleic acid (OA). For this purpose, we have used as a cell model the rat pancreatic cell line AR4-2J, allowing to test the effects of PUFAcs independently of possible secondary effects due, for example, to any variation of circulating hormones arising in in vivo studies.

MATERIALS AND METHODS

Materials

Glutamine, penicillin, trypsin-EDTA and streptomycin were from Life Technologies. Protein-A-Sepharose CL-4B, rabbit polyclonal antibodies to bovine erythrocyte ubiquitin, carboxybenzoyl leu-leu-leucinal (MG132), o-phenanthline, alkaline phosphatase-labeled antibodies to rabbit IgG, 4-nitrophenyl hexanoate, OA, AA, and DHA were from Sigma (St Louis, MO). [35S]methionine (trans35S-label) was from ICN (Costa Mesa, CA). [3H]OA (15 Ci/mmoll and [3H]AA (98 Ci/mmoll) were from NEN (Les Ulis, France). Antibodies specific for BSDL were raised in rabbit using purified rat pancreatic BSDL (25).

Cell culture

The rat pancreatoma AR4-2J cell line was obtained from the European Collection of Animal Cell Culture (No 93,100,618).

The cells were routinely cultured at 37°C in a 5% CO2, 95% air atmosphere in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) containing 10% (v/v) fetal calf serum, 2 mM glutamine, 1 g/l glucose, 100 units/ml of penicillin, 100 µg/ml streptomycin, and 0.1% (v/v) fungizone. When cells reached 80% confluence, they were harvested with 0.25% trypsin and 0.05% EDTA in 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 buffer (PBS) and aliquots of dissociated cells were plated on 100 mm diameter Petri dishes. Cells were then cultured under required conditions.

Fatty acid treatment

Three days after seeding, fresh media containing fatty acids at the required concentration (stock solution 50 mM in ethanol) or not, were added to cells and changed every day during 3 days, a time at which control cells had reached 80% confluence. Cells exposed to fatty acids were morphologically indistinguishable from control cells.

Subcellular fractionation

Subcellular fractionation was performed by serial centrifugations inspired from Jamieson and Palade (26). First, after scraping, cells were homogenized with a polytron in a 20 mM pH 7.4 Tris/HCl buffer (0.25 M sucrose). Cell debris and heavy organelles (nuclei, zymogen granules, mitochondria, and lysosomes) were pelleted by centrifugation (15,000 g for 15 min at 4°C). Lastly, microsomes were separated from cytosol by centrifugation of the post-organites supernatant at 100,000 g for 1 hour at 4°C and were resuspended either in the homogenizing buffer (experiments with intact microsomes) or in an hypotonic buffer (5 mM Tris/HCl, pH 7.4) and sonicated for 15 s at 4°C (4 W, Branson Sonifier). The two isolated subcellular fractions were characterized by an enrichment in cytochrome C reductase and lactate dehydrogenase activities, characteristic for microsomes and cytosol, respectively. When required, protease inhibitors (Complete-EDTA-free inhibitors cocktail from Roche Diagnostic, Meylan, France) were added.

Immunoprecipitation, polyacrylamide gel electrophoresis and Western blot

Aliquots of cell lysate performed by sonication in TETN buffer (Tris/HCl, 5 mM; EDTA, 5 mM; NaCl, 250 mM and Triton X-100, 1%; pH 7.4) or aliquots of cell-free media were incubated overnight at 4°C with specific antibodies against rat BSDL or rabbit pre-immune serum. The antigen-antibody complexes were incubated for 4 h at 4°C under agitation with 6 mg of prewashed protein A-Sepharose. The antigen-antibody protein A complexes were recovered by centrifugation (10,000 g, 15 min, 4°C). The final pellet was washed twice with TETN buffer, then twice with a 10 mM, Tris/HCl, 5 mM, EDTA, pH 7.4 buffer. The immunoprecipitated BSDL was dissociated from the protein A-Sepharose by heating at 95°C for 5 min in Laemmli’s sample buffer (27). After centrifugation, the supernatant was analyzed by electrophoresis on SDS-containing 7.5% polyacrylamide gels (SDS-PAGE) according to Laemmli (27). For Western blots, proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (28) in 0.2 M Tris/HCl, pH 9.2 buffer, at 1 mA/cm² overnight in a cold room. Completeness of transfer was verified by staining polyacrylamide gels with Coomassie blue R250 and destaining. Membranes were then incubated with polyclonal antibodies specific for rat BSDL or ubiquitin. The antigen-antibody reaction was detected with alkaline phosphatase-labeled goat antibodies to rabbit. Finally membranes were quantitated using the NIH Image program (http://rsb.info.nih.gov/nih-image/).
Protein, DNA, and enzyme activities determinations

Proteins were routinely assayed using the biocinchonic acid method (Pierce) using serum albumin as standard. DNA was quantitated by fluorimetric assay according to Rao and Otto (29). The BSDL activity was evaluated by the hydrolysis of 4-nitrophenyl hexanoate measured spectrophotometrically at 400 nm and pH 7.4 (Tris/HC1 0.5 M, NaCl 0.1 M) in a thermostated cell at 30°C as described by Gjellesvik et al. (30) in the presence of 4 mM sodium taurocholate as activator. The α-amylase activity was determined at 410 nm on 4-nitrophenyl maltopentaoside in 0.1 M sodium phosphate, 50 mM NaCl, pH 7.4 buffer in the presence of α-glucosidase (>24 units/ml).

Pulse chase procedure

After 1 h incubation in fresh medium, cells were pulse labeled with [35S]methionine (20 μCi/ml) in methionine-free DMEM; the pulse medium was removed after 30 min incubation followed by two quick washes with PBS. Cells were then chased for the required time period in DMEM. At the end of the chase period, cell-free media were withdrawn and cell lysates prepared as follows: cells of each dish were carefully washed in PBS, harvested by scraping with a rubber policeman and centrifuged at 1,000 g for 5 min at 4°C; the cell pellets were resuspended in TETN buffer, rapidly cooled at 4°C and sonicated (4 W) for 15 s. Then, aliquots of cell lysate and of cell-free medium corresponding to the same amount of cell proteins were subjected to the immunoprecipitation procedure described above.

Quantitation of labeled proteins and BSDL

After separation on SDS-PAGE, immunoprecipitated radiolabeled proteins were fixed by staining with Coomassie R250 and destaining in ethanol-acetic acid-water (2:3:5, v/v/v). Gels were immerged in Amplify (Amersham, Buckinghamshire, UK) for 30–60 min then dried, and [35S]BSDL was localized by autoradiography using Kodak Films Biomax MR (Rochester, NY). Quantitative analyses were performed by cutting out and counting radioactive bands of the gel. For this purpose, excised gel slices were treated with Soluene 350 (Packard Instrument, Meriden, CT) followed by scintillation counting in IONICFluor according to the manufacturer instructions (Packard Instrument).

Total protein synthesis and secretion were determined after a 30-min pulse of cells with [35S]methionine (20 μCi/ml) followed by extensive washings of cells and chased for 45 min. [35S]-labeled proteins of cell lysate and of cell-free medium were then precipitated with 10% trichloroacetic acid and, after three washes, the radioactivity of the precipitate was determined by scintillation counting.

Labeling of cell lipids with [3H]OA and [3H]AA

AR4-2J cells were seeded in 100 mm dishes and grown to subconfluence. Cells were incubated with 50 μM of either [3H]OA or [3H]AA (10 μCi/dish). After 3 h incubation at 37°C, cells were exhaustively washed, scraped in ice cold PBS and pelleted by centrifugation. The pellet was then lysed and lipids were immediately extracted from cell lysate as previously described (31). Radiolabeled phospholipids were separated on silica gel 60 TLC plates (Merck, Darmstadt, Germany) developed in chloroform-methanol-methylamine 40% (68:26:6, v/v/v). Phospholipids were visualized with iodine vapor and radioactive spots were located and analyzed by scanning with a TLC-linear analyzer (Tracemaster, Berthold). Furthermore, 3H-labeled spots were scraped off and the radioactivity was recorded by liquid scintillation counting.

Phospholipids analysis of fatty acid treated cells

After chronic treatment (3 days, 50 μM) with OA, AA, or DHA, cell phospholipids were extracted and separated on TLC plates as described above. Phospholipids were then revealed with Coomassie blue (32) and quantitative analyses were performed by densitometric scanning using the NIH Image program.

Statistical analysis

Significance of data was determined by the Student’s t test and the difference was considered significant when P < 0.05.

RESULTS

Effects of AA and DHA on the level of secreted BSDL activity

When AR4-2J cells were treated with OA, AA, or DHA (10–100 μM) for few hours, no substantial effect on BSDL and α-amylase activities was recorded. Consequently, AR4-2J cells were chronically treated with these unsaturated fatty acids at the required concentration for 3 days, then their incidence on BSDL fates was determined. Fig. 1A (top panel) showed that, at the 10–100 μM concentration range, AA and DHA decreased the level of BSDL activity present in the cell-free medium. The maximal effect was reached for a fatty acid concentration of around 50 μM with a significant decrease in secreted BSDL activity of

![Fig. 1](https://www.jlr.org)

Effect of fatty acid concentration on the BSDL and α-amylase activities during chronic treatment. AR4-2J cells were incubated for 3 days with increasing concentrations of OA (open circles), AA (closed circles), or DHA (open squares). At the end of treatment time, cells were incubated for another 2 h in fresh medium supplemented with the same fatty acid concentration. A: Then the cell-free-medium was withdrawn and saved for BSDL (top) and α-amylase (bottom) activities determination. B: Cells were harvested, lysed, and the BSDL (top) and α-amylase (bottom) activities recorded in cell lysate. Results, expressed in percent, were given relative to control cells (without fatty acid treatment) taken as reference. Values are means (+ SD) of at least three independent experiments, each performed in triplicate.
23\% (n = 17, P < 0.0001) and 20\% (n = 9, P < 0.0001) when cells were incubated with AA and DHA, respectively. Under identical conditions, α-amylase activity was not significantly altered (Fig. 1A, bottom panel), albeit the secretion of this enzyme seemed a little increased by AA. Contrary to AA and DHA, OA at same concentrations did not modify the secretion level of BSDL.

Effects of AA and DHA on intracellular BSDL activity

As shown in Fig. 1B, the intracellular activity of BSDL, determined in the homogenate of AR4-2J cells, was a little more affected than the secreted activity. In cells treated with 50 μM fatty acids, a significant decrease of ~31\% (n = 19, P < 0.0001) and 35\% (n = 10, P < 0.0001) was induced by AA and DHA, respectively. Although the ratio of cellular protein versus DNA content was slightly diminished by these two PUFAs (~15\% for AA, n = 16, P < 0.0001 and DHA, n = 8, P < 0.001), the specific activity of intracellular BSDL (i.e., BSDL activity reported to cellular proteins) was significantly diminished upon AA (~18\%, n = 18, P < 0.0001) and DHA (~23\%, n = 8, P < 0.0001) treatment. As already observed for secreted BSDL activity, OA did not alter the intracellular level of this activity. Also, the intracellular level of α-amylase activity was not significantly modified by AA or DHA treatment (Fig. 1B, bottom panel).

Effect of AA and DHA on subcellular localization of BSDL

In a previous study, we have shown that in AR4-2J cells, BSDL equally partitioned between microsomes and cytosol (33). Therefore, we wondered whether PUFAs, such as AA or DHA, affected this partitioning. We found (Table 1), that the decrease in BSDL specific activity, as above observed in homogenate of cells, consecutive to AA and DHA treatment, was essentially due to a significant decrease in BSDL activity in the cytosolic compartment. This decrease reached some 26\% (n = 6, P < 0.005) and 33\% (n = 6, P < 0.005), in cells treated with AA and DHA, respectively.

Contrary to that found in the cytosol, BSDL activity of microsomes was slightly increased by AA or DHA. Furthermore, OA had no effect on the intracellular partitioning of the enzyme. The modification of the BSDL subcellular localization was not due to a selective alteration of the enzyme activity by these two PUFAs since Western blot analysis performed on control and PUFA treated cells. Equivalent quantities of proteins of each fraction were separated on SDS-PAGE, transferred to nitrocellulose membrane and probed with antibodies specific for rat pancreatic BSDL. The upper panel displays a Western blot representative of four independent experiments, the lower shows the results (means ± SD) of quantitative densitometric analysis of dark intensity of Western blot from four independent experiments performed in duplicate. Effects of PUFAs were expressed relative to control taken as 100\%. Significant differences between PUFAs and control were determined by paired Student’s t-test (* P < 0.05).

Effects of AA and DHA on BSDL biosynthesis

To assess whether the decreased amount of BSDL activity upon AA or DHA treatment was due to a decrease in the enzyme biosynthesis, pulse-chase protocols were performed following PUFA treatment of cells (50 μM, 3 days). Cells were pulse-labeled for 30 min with [35S]methionine, chased in fresh medium for the required time period, and finally lysed. Lysates were then subjected to an immunoprecipitation using antibodies specific to rat pancreatic BSDL, the immunoprecipitated material was consecutively analyzed by SDS-PAGE, autoradiographed to locate [35S]-labeled BSDL (autoradiogram in Fig. 3, top panel) and appropriate radioactive bands were excised and assayed by scintillation counting. As shown in Fig. 3 (bottom panel), the incorporation of [35S]methionine into intracellular BSDL at the end of the pulse (“Cell lysate, 0 min chase”) was similar in control and AA or DHA treated cells, indicating that there was no significant effect of these two PUFAs on the initial rate of BSDL synthesis.

When a chase of 45 min was performed after the 30 min pulse, followed by an immunoprecipitation and quantitation of the intracellular [35S]-labeled BSDL (“Cell lysate, 45 min chase”), one can observe (Fig. 3, bottom panel) a significant decrease of the intracellular [35S]-labeled BSDL in PUFA treated cells, relative to the control. This decrease was of 32 ± 6\%, (n = 4, P < 0.001) and 25 ± 5\%, (n = 4, P < 0.05), after AA and DHA treatment, respectively. This

### Table 1. Effect of chronic unsaturated fatty acid treatment on BSDL activity in subcellular fractions

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>Control</th>
<th>OA</th>
<th>AA</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10⁻³ units/mg protein</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>15,000 g Pellet</td>
<td>192 ± 36</td>
<td>185 ± 25</td>
<td>195 ± 45</td>
<td>200 ± 52</td>
</tr>
<tr>
<td>Microsomes</td>
<td>140 ± 31</td>
<td>132 ± 30</td>
<td>156 ± 42</td>
<td>145 ± 38</td>
</tr>
<tr>
<td>Cytosol</td>
<td>313 ± 75</td>
<td>320 ± 49</td>
<td>231 ± 40***</td>
<td>210 ± 51***</td>
</tr>
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AR4-2J cells were cultured for 3 days in the absence (control) or in the presence of 50 μM fatty acid. Cells were harvested and lysed. Cell lysate was cleared by centrifugation (15,000 g, 15 min, 4°C) leading to the “15,000 g pellet,” then the cleared cell lysate was used to isolate microsomes and cytosol subfractions by a centrifugation at 100,000 g (1 h, 4°C). The BSDL activity was recorded in each subcellular fractions and reported to the amount of protein contained in fractions. Data were mean values (± SD) of at least six independent experiments with assays in triplicate. Significant differences between unsaturated fatty acid-treated and mock-treated (control) cells were determined by paired Student’s t-test (*** P < 0.005).
in intracellular decrease due to AA and DHA treatments was correlated with a significant decrease in secreted $^{35}$S-labeled BSDL. (*Cell-free medium*) that was, relative to control cells, of 17 ± 3% ($n = 4$, $P < 0.05$) for AA and of 14 ± 4% ($n = 4$, $P < 0.05$) for DHA. A simple explanation to conciliate these data is that AA and DHA increased the degradation of BSDL.

When a subcellular fractionation was performed after the 45 min chase, the $^{30}$S-labeled BSDL immunoprecipitated from the cell cytosol (which contains the greatest amount of radiolabeled enzyme) was lower in AA-treated cells (−30 ± 4%, $n = 4$, $P < 0.05$) than in mock-treated cells. This result corroborates the decrease in cytosolic BSDL activity (Table 1).

Taken as a whole, these data suggested that PUFAs such as AA and DHA, that did not modify the initial rate of BSDL biosynthesis, affected the intracellular distribution of BSDL, possibly by retaining the enzyme within vesicular cell compartments, and induced the degradation of the protein.

**Specificity of AA effects on BSDL**

AA, that produced an important effect on AR4-2J cells, has been selected as effector to address the question concerning the specificity of PUFAs effects on BSDL. After 3 days treatment with 50 µM AA, cells were pulse-labeled for 30 min with $[^{35}S]$methionine, washed, chased for 0 or 45 min, and lysed; then $^{35}$S-labeled cell proteins were precipitated with 10% TCA and quantitated by scintillation counting. $^{35}$S-labeled proteins secreted during the chase period were also precipitated with TCA under identical conditions.

Once reported to cell DNA content and compared with mock-treated cells, AA did not affect the amount of cell proteins synthesized during the pulse period (0 min chase). After subcellular fractionation of the cell homogenate into soluble and microsomal fractions, it results that the amount of $^{35}$S-labeled proteins decreased by some 15% in the cytosol with an increase in microsomes (+34%). These results were not affected after the 45 min chase period. However, after this chase period, the amount of TCA precipitable $^{35}$S-labeled proteins secreted in the culture medium of AA-treated cells (that represented about 10% of intracellular radiolabeled proteins), was increased by 95% when compared with mock-treated cells. This suggests a systemic effect of AA on the secretion rate of proteins.

The above-mentioned results showed that the overall protein biosynthesis and the BSDL biosynthesis as well, were not affected by AA treatment. Further, the decrease in BSDL activity (−30%) in the cell cytosol, although relatively more important than that of total proteins (−15%), suggested that AA (and probably DHA) has a systemic lowering effect on the cytosol protein content. This decrease could be due to an inhibition of the export of proteins from the endoplasmic reticulum (ER) to the cytosol, leading to the majored secretion of proteins as observed upon AA treatment. These proteins would otherwise be degraded in the cytosol. However, AA (or DHA but not OA) decreased the secretion of BSDL. (see Fig. 1). Consequently, AA (and probably DHA) had an opposite effect on the bulk secretion of proteins, thus confirming the specificity of AA effects on BSDL. Because of its particular secretory pathway (34), it is possible that BSDL, which was not addressed to the cytosol upon AA treatment, might accumulate in the ER and Golgi compartments. Indeed, a slight increase of the protein was actually observed in microsomes that are representative of these compartments (see Fig. 2). Although AA and DHA did not alter the biosynthesis rate of BSDL, they specifically decreased BSDL in cells, particularly in the cell cytosol and in the culture medium. Because these effects were not balanced by an equivalent retention of the enzyme in microsomes, we addressed the question of the possible degradation of the enzyme upon cell treatment with these PUFAs, in part AA.
Effects of ATP and ATP + MG132 on microsomal BSDL

Because OA had no effect on BSDL secretion and retention within AR4-2J cells and also because DHA had similar effects than AA, we have focused on AA effects. Therefore, we have attempted to define mechanisms leading to the degradation of BSDL that was retained within vesicular compartments upon AA treatment of AR4-2J cells. For this purpose, intact microsomes isolated from control and AA-treated cells were incubated at 37°C in Tris/sucrose buffer for 90 min in the absence or presence of ATP (5 mM) and of the proteasome inhibitor MG132 (50 μM). Following these treatments, a centrifugation (100,000 g, 90 min, 4°C) of microsomes allowed us to separate soluble and membrane fractions. Finally Western blottings, using antibodies to rat BSDL, were used to quantitate the amount of BSDL released in the incubation medium from microsomes (Fig. 4A) and that remaining associated with microsomes (Fig. 4B) during the incubation period. Results are expressed relative to values obtained with microsomes incubated under identical conditions without ATP and MG132. Fig. 4A shows that, in control cells, ATP favored the release of BSDL from microsomes into the soluble fraction, whereas that remaining associated with microsome membranes was only slightly increased (Fig. 4B). This agreed with a well-known effect of ATP that translocates within the ER (35) and that is required for the export of proteins out of the ER. We have also shown that ATP induced a conformational change of BSDL (36) favoring the translocation of the enzyme out of the ER. The proteasome inhibitor MG132 seems to have a slight effect in protecting BSDL that may translocate toward the cytoplasmic face of microsomes suggesting that some proteasome particles might be present at the microsomal surface (37). On the other hand, in microsomes of AA-treated cells, ATP largely decreased the amount of released BSDL (Fig. 4A) and this effect was emphasized by MG132. Interestingly, ATP and ATP + MG132 also decreased, but to a lower extent, the amount of BSDL that remained associated with microsome membranes of AA-treated cells (Fig. 4B).

These results indicated that upon treatment with ATP and ATP + MG132, less BSDL was released in the medium from microsomes of AA-treated cells and suggested that a degradation of the enzyme may occur in microsomes. The major effect of MG132 in AA-treated cells was a little puzzling as this proteasome inhibitor appeared to increase the degradation of BSDL. However, it has been observed that proteasome inhibitors, while inhibiting degradation, did not stimulate the secretion of apoB-100 that was stabilized and accumulated in Golgi membranes (38). Furthermore, in MG132-treated hepatocytes, microsomal apoB-100 was more sensitive to exogenous trypsin than that of mock-treated cells. This result suggests that the proteasome inhibitor modifies the localization of the protein in membranes (39). Therefore, it could be that AA acted, as MG132, on the translocation machinery and on the proteasome functionality. Extrapolated to the in vivo situation, one can hypothesize that BSDL molecules, which upon AA treatment did not move toward the cytosol and accumulate in membrane vesicles, could be degraded by microsomal proteases. As ATP increased the degradation of BSDL in AA-treated cells, it may be that these proteases are ATP-dependent. These ATP-dependent proteases might not be ER-metallopeptases since o-phenanthroline did not reverse the ATP-dependent degradation of BSDL in microsomes of AA-treated cells (not shown). On the contrary, o-phenanthroline heightened the decrease in BSDL observed with ATP alone in soluble as well as in membrane fractions of AA-treated AR4-2J cells. Like MG132, o-phenanthroline was also effective in stabilizing apoB-100, slowed the transport of secretory proteins and blocked membrane fusion (40), promoting the retention of secreted proteins within the ER. Therefore, MG132 and o-phenanthroline might emphasize the primary effect of AA that was to retain BSDL in ER without any compensatory increase in secretion, the retained BSDL being then degraded by an ATP-dependent ER-proteases, the nature of which remains to be determined.
AA lowers the level of ubiquitinated BSDL.

We then attempted to further determine how AA might promote the retention of BSDL in the vesicular compartment. One possibility was that this PUFA impaired the export of BSDL out of this compartment. It is well documented that ubiquitination is a prerequisite for the retrotranslocation of proteins from the ER to the cytosol (41). Therefore, we tried to detect ubiquitinated BSDL and wondered whether AA may alter the ubiquitination of this protein.

Fig. 5 showed a Western blot performed on microsomes and cytosol of control and AA-treated cells and revealed with anti-BSDL and anti-ubiquitin antibodies. We have previously shown that antibodies to ubiquitin used in this study mainly recognized ubiquitinated BSDL (Ub-BSDL) (37) whereas antibodies to rat BSDL recognized two glycoforms of the enzyme (34) in rat pancreatic AR4-2J cells. In microsomes, the BSDL amount was slightly increased by AA treatment of AR4-2J cells as seen on Fig. 2. Furthermore, a high Mr protein (96 kDa), that is reactive with antibodies to BSDL and to ubiquitin, can be detected within microsomes isolated from mock-treated and from AA-treated AR4-2J cells. This 96 kDa protein corresponding to ubiquitinated BSDL referred to as Ub-BSDL (37) was significantly decreased by about 35% upon AA treatment as measured by densitometric analysis of Western blots probed either with antibodies to BSDL or to ubiquitin. We also confirm that, under these conditions, the level of BSDL decreased in the cytosol. Nevertheless, the 96 kDa ubiquitinated form of BSDL cannot be detected in cytosol of control and of AA-treated cells.

These data suggested that PUFAs, such as AA, lowered the ubiquitination of BSDL. This explains the effect of AA that decreased both the amount of Ub-BSDL and the amount of cytosolic BSDL, meaning that ubiquitination of the enzyme could be a prerequisite to its translocation toward the cytosol. However, the absence of Ub-BSDL in the cytosol of either control or AA-treated cells suggested that the ubiquitin-conjugated protein is either rapidly degraded or deubiquitinated.

Incorporation of polyunsaturated fatty acids into lipids of AR4-2J cells

Membrane lipid modifications could be responsible for the effects of AA and DHA observed on the BSDL secretion. Consequently, we have examined on the one hand the incorporation of [3H]OA and of [3H]AA into lipids of AR4-2J cells, and on the other hand, the modifications of membrane major phospholipids. In that concerning the incorporation of radioactive fatty acids, AR4-2J cells were incubated for 3 h with [3H]OA or [3H]AA. Analysis of a same quantity of cells (assessed by DNA measurement) showed that these two labeled fatty acids were incorporated into AR4-2J cell lipids to the same extent as determined by the radioactivity of the chloroformic phase after lipid extraction. After separation on TLC plates, the total radioactivity incorporated in phospholipids (as measured from the sum of the radioactivity of spots representing phospholipids), was identical independently of the fatty acid, [3H]OA or [3H]AA, used in cell incubation. However, the partitioning of the radioactivity in the major classes of phospholipids was slightly different for the two acids. Compared with data obtained with [3H]OA, the amounts of [3H]AA incorporated into phosphatidylinositol (PI) and phosphatidylethanolamine (PE) were largely increased by 66% and 107% respectively. Under our experimental conditions the labeling of sphingomyelin (SM) remained negligible whatever the unsaturated fatty acid tested.

These observations suggest that the ineffectiveness of OA was not due to a lower incorporation of this fatty acid into lipids of AR4-2J cells membranes. However these results, that likely reflect the turnover of radiolabeled fatty acids into lipids, did not exclude modifications of other metabolic processes such de novo lipid biosynthesis leading, for example, to an adaptive response of the cell to the membrane changes in fatty acid profile. Modifications of major membrane phospholipids following AR4-2J cells incubation for 3 days with fatty acids are presented in Fig. 6. For the accurate densitometric determination of spot intensities on TLC plates, aliquots of chloroformic phase laid on plates were larger for the studies of minor phospholipids, PA-PS or PI, than that used for PC, PE, or SM analyses. Although upon cells incubation with OA, AA, or DHA, the amount of cell PC was not modified, different molecular forms of this phospholipid appear following cell treatment with AA or DHA as shown by densitometric profiles presented on Fig. 6A. Furthermore, acid phospholipids PA-PS were dramatically diminished by AA (−56%) or DHA (−64%) but not by OA cell treatment.

Taken as a whole, the observation of qualitative and quantitative modifications of phospholipids upon cell treatment with AA and DHA inclined us to suspect that these alterations might lead to changes in membrane biophysical characteristics (42) that in turn could modulate many metabolic processes and functions (43–45).
DISCUSSION

There are only few papers in the literature examining effects of nutrients on the digestive pancreatic enzyme BSDL. Hui et al. (46) have shown that cholesterol loading of AR4-2J cells modulated the expression of this enzyme and we have reported (33) an increase in BSDL level in rat pancreatic cells chronically treated with ethanol. Dietary PUFAs have long been known as cholesterol lowering fatty acids (1). The postprandial hypercholesterolemia may largely depend upon the amount of dietary cholesteryl esters that are hydrolyzed by BSDL (12) and consequently correlated with the intraduodenal level of this enzyme.

We showed here that chronic treatment of AR4-2J pancreatic cells with AA and DHA concentrations that are easily attained under physiological conditions, decreased the secretion of BSDL. Whether the decrease in BSDL secretion promoted by PUFAs, such as AA and DHA, is sufficient to also reduce the amount of enzyme that is transcytosed through the enterocyte, has to be determined (16). If so, this could also decrease the level of circulating BSDL and, as a consequence, affect the atherogenic potency of LDL, the structure and lipid composition of which can be modified by this enzyme (18, 19). Consequently, it is likely that under physiological conditions lowering the BSDL secretion by some 25%, as observed in this study, could affect the amount of circulating BSDL and, in turn, atherogenic processes. In addition, hypocholesterolemic properties of PUFAs could be due to their effects on BSDL secretion that might impair hydrolysis of dietary cholesteryl esters and absorption of free cholesterol (12, 13). AA and DHA specifically affected the secretion of BSDL suggesting that these fatty acids interfered with the secretion pathway of the enzyme. Although DHA is not further metabolized as AA to lead to eicosanoids (47), it had similar effects on BSDL to those induced by AA. Therefore, one can suggest that the observed effects were not due to fatty acid metabolites but to fatty acids per se.

Quantitatively more important was the decrease in intracellular BSDL specifically found in the cytosol of AA- and DHA-treated cells. In previous studies, we have demonstrated, both in AR4-2J pancreatic cells and in CHO cells transfected with rat pancreatic BSDL cDNA (21, 33), that this enzyme partitioned between microsomes and cytosol and had an intracellular role in lipid metabolism. BSDL, in part, affected the cholesteryl ester turnover in the cytosol of transfected CHO cells (21). This let us to suspect that the cytosolic level of BSDL might be physiologically relevant. Therefore, we have attempted to define mechanisms leading to the decrease in BSDL in the cytosol of AR4-2J cells upon treatment with PUFAs. We have demonstrated that the initial rate of BSDL biosynthesis was not affected by two PUFAs, AA and DHA, but that the newly synthesized BSDL disappeared more rapidly in PUFAtreated than in control cells. This correlated with a decrease in secreted BSDL, showing that the diminished intracellular BSDL was not due to a faster secretion of the enzyme, opposite to that observed with other proteins, which seemed to be more efficiently secreted upon cell treatment with AA. All these observations suggested that the degradation of BSDL was induced by AA. Because DHA treatment of AR4-2J cells led to effects comparable to those produced by AA, whereas a monounsaturated fatty acid, such as OA was ineffective, it is strongly suggested that PUFAs may have a specific effect on BSDL secretion and degradation.

Of particular importance was the finding that an ubiquitinated form of BSDL (Ub-BSDL) can be detected in microsomes of AR4-2J cells (37) but not in the cytosol. Ubiquitination of proteins is a hallmark for proteasome degradation (48). However, it may also be a way to target proteins toward the cytosol (49). At the current stage of results, we are facing two non-exclusive hypotheses.

The first hypothesis considers that Ub-BSDL can be degraded more rapidly by the proteasome-dependent pathway that could be stimulated by PUFAs. Under these conditions, proteasome inhibitor MG132 should reverse the effects of PUFA. However such an effect had never been observed.

The second possibility is that PUFAs lowered the ubiquitination of BSDL, which in turn could be responsible for the lower export of Ub-BSDL to the cytosol. Under control conditions, Ub-BSDL is degraded by the proteasome that, in AR4-2J cells, appeared associated with the cytosolic face of ER membranes (37). This pathway is a part of the quality control machinery that sorts aberrant BSDL
molecules toward degradation (37). We described here, that Ub-BSDL was lowered in microsomes of AA-treated compared with control cells. It is likely that a relationship between the decreased level of Ub-BSDL and that of BSDL in the cytosol of AA-treated cells may exist. In this case, BSDL molecules destined to the cytosol cannot be ubiquitinated, may not be translocated and would be degraded within the ER. This resulted in lower amount of cytosolic BSDL, as observed in the present study. Nevertheless, cytosolic BSDL should be active to exert any role in cell-lipid metabolism as observed in cells transfected with the cDNA coding for this enzyme (21). Because Ub-BSDL appeared inactive (37), the enzyme should be deubiquitinated in the cytosol to recover its activity. A few proteins undergo a reversible ubiquitination-deubiquitination process that seems to regulate their function (50), and interestingly these proteins are not targeted for proteasome degradation. Although this gives a simple explanation to the absence of Ub-BSDL in the cytosol of AR4-2J cells, further studies are necessary to understand this point. In part, one has to define whether ubiquitination drives BSDL to the proteasomal degradation or target the enzyme toward the cytosol or both. For this purpose, Ub-BSDL should be better characterized, as it could be multi-ubiquitinated or poly-ubiquitinated (51). From a functional standpoint, this distinction is of importance because poly-ubiquitinated chains are the most efficient proteasome-targeting signal. Therefore, we hypothesize that BSDL could be poly-ubiquitinated for degradation purpose (this point only refers to aberrant BSDL molecules) and/or multi-ubiquitinated to be exported to the cytosol where it could be deubiquitinated to accomplish its physiological function.

The question now is to delineate how BSDL molecules, which cannot be addressed to the cytosol or secreted, are degraded in PUFA-treated AR4-2J cells. The hypotriglyceremic effect of PUFAs has been attributed, in part, to a decrease in apolipoprotein B (apoB) secretion by the liver, due to a higher intracellular post-translational apoB degradation. In the liver, dietary fish oils inhibit export of apoB-containing precursors out of the ER and target luminal apoB to degradation. This apoB degradation is reversed by the inhibition of the ER luminal proteolysis by the metalloprotease inhibitor o-phenantroline (38). On the other hand, in HepG2 hepatoma cells, DHA decreased apoB secretion with a concomitant increase in apoB degradation. This apoB degradation is reversed by the inhibition of the ER proteasome by ATP-dependent ER proteases. The activity of these ER proteases, the nature of which has to be further defined, seemed to be ATP-dependent. Once extrapolated to an in vivo situation, it could be that AA (or DHA) treatment of cells induced the retention of the enzyme within the ER and inhibited, on the one hand, the translocation of the enzyme toward the cytoplasmic compartment by altering ubiquitination processes, or, on the other hand, promoted the degradation of BSDL by ATP-dependent ER proteases.

How PUFAs affected the sorting of BSDL is still an open question. Although OA and AA were incorporated to the same extent into phospholipids, only PUFAs affected the lipid composition of cell membranes. This, in fine, should alter the physical properties of these membranes, in part ER membranes (53). Inasmuch as BSDL is the only pancreatic enzyme that associates with membranes during its secretion route (34, 54), it is conceivable that BSDL targeting and secretion would depend upon the intracellular membrane composition and would be altered by PUFAs in a different way than other secreted pancreatic proteins or enzymes such as α-amylase (this study and ref. (55, 56)).

The effects of PUFAs on BSDL targeting and degradation may arise not only in the pancreatic cells as above described but also in all cells where BSDL has been detected such as fetal and cancer cells, where it is susceptible to play a metabolic role, particularly concerning their proliferation (57). The decrease in BSDL cytosolic activity observed here was accompanied by a lowered proliferation of AR4-2J cells after 3 days of PUFA treatment, confirming preceding results in different pancreatic cell lines (8) and in vivo on pancreatic cancer (58). The lack of response of oleic acid in our experiments demonstrated that only PUFAs affected BSDL in the pancreatic cell line used. This work is the first to establish that modifications of proteolytic degradation are involved in the down-regulatory effects of PUFAs on BSDL secretion in AR4-2J pancreatic cells.

This study was financed in part by grant No. 9912 awarded by the Association pour la Recherche sur le Cancer (ARC, Villejuif, France). We thank Mrs O. Nobili for her technical assistance and for fruitful discussions.

Manuscript received 13 November 2000 and in revised form 6 March 2001.

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