Metabolic Profiling of Glycerophospholipid Synthesis in Fibroblasts Loaded with Free Cholesterol and Modified Low Density Lipoproteins *

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Currently, the detailed regulation of major pathways of glycerophospholipid synthesis upon cholesterol loading is largely unknown. Therefore, a detailed lipid metabolic profiling using stable isotope-labeled choline, ethanolamine, and serine was performed by quantitative electrospray ionization tandem mass spectrometry (ESI-MS/MS) in free cholesterol (FC), oxidized (Ox-LDL) and enzymatically modified LDL (E-LDL)-loaded primary human skin fibroblasts. As previously described, an adaptive induction of phosphatidylcholine (PC) synthesis via CDP-choline was found upon FC loading. In contrast to PC, CDP-ethanolamine-mediated phosphatidylethanolamine (PE) synthesis was inhibited by FC incubation. Furthermore, FC induced a shift toward polyunsaturated PE and PC species, which was mediated primarily by PE biosynthesis but not PE remodeling, whereas PC species were shifted mainly by fatty acid (FA) remodeling of existing PC. Modified lipoprotein incubation revealed rather different effects on glycerophospholipid synthesis. E-LDL greatly enhanced PC synthesis, whereas Ox-LDL did not change PC synthesis. Addition of different free FAs (FFA) with and without FC coincubation, as major components of E-LDL, clearly indicated an incorporation of FFA into newly synthesized PC and PE species as well as FFA as important driving force for PC synthesis. Because FC and FFA are known to affect lipid membrane properties including membrane curvature, these data support that CTP:phosphocholine cytidylyltransferase activity and consequently PC synthesis are regulated by modulation of membrane characteristics at the cellular level. In conclusion, the application of high throughput metabolic profiling of major glycerophospholipid pathways by ESI-MS/MS is a powerful tool to unravel mechanisms underlying the regulation of cellular lipid metabolism.

Lipoprotein accumulation and retention in the arterial wall is the first step in a cascade that leads to the development of atherosclerosis (1). Enrichment of lipid-loaded macrophage foam cells in the subendothelium of the vessel wall is a hallmark of atherosclerosis (2). In this process, LDL and its chemically modified forms play a key role. The best characterized modifications is the oxidation of LDL (3). The emerging non-aggregated and non-opsonized minimally oxidized LDL (Ox-LDL) is preferentially internalized via receptor-mediated uptake through clathrin-coated pits (4, 5). Another modification relates to the enzymatic digestion of LDL (E-LDL) retained in the vessel wall. E-LDL consists of liposome like cholesteryl ester core-depleted lipoprotein particles with a high amount of free cholesterol (FC) and free fatty acids (FFA). E-LDL is found in atherosclerotic lesions (6) and is taken up by clathrin-independent and opsonin-driven phagocytosis involving complement and Fcγ receptors leading to macrophage foam cell formation (7, 8).

Advanced atherosclerotic lesion macrophages accumulate cholesteryl ester (CE) and FC (9), which may exert cytotoxic effects (10). FC decreases membrane fluidity (11). A balanced FC/phospholipid ratio resulting in a narrow physiological range of membrane fluidity is necessary for integral membrane protein function as well as formation of lipid membrane microdomains (10, 12). In addition to FC esterification by ACAT (acyl-CoA:cholesterol acyltransferase) and FC efflux, cells are able to induce phospholipid synthesis as an adaptive response to FC accumulation (10). Thus, FC loading increases phosphatidylcholine (PC) synthesis by induction of the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase (CT) in macrophages (13) and human skin fibroblasts (14). Moreover, cholesterol loading induces sphingomyelin (SM) synthesis (15, 16). Another adaptive cellular response is a species shift upon FC loading for PC and phosphatidylethanolamine (PE) from saturated and monounsaturated toward polyunsaturated species (17). This increases membrane fluidity because unsaturated species have a lower gel-to-liquid-crystalline phase transition temperature $T_m$ compared with saturated species (18).

Because no precise information about the influence of cholesterol loading on glycerophospholipid metabolism are available, our aim was to perform a detailed metabolic profiling of major glycerophospholipid metabolism pathways using electrospray ionization tandem mass spectrometry (ESI-MS/MS).
MS) in FC-, Ox-LDL-, and E-LDL-loaded primary human skin fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Reagents and Materials**—Methyl alcohol and chloroform were HPLC grade from Merck (Darmstadt, Germany), ammonium acetate and acetyl chloride were of the highest analytical grade available from Fluka (Buchs, Switzerland). Cholesterol esterase was from Roche Diagnostics (Mannheim, Germany). Bovine serum albumin, trypsin, free fatty acids, cholesteryl ester chloride were from Sigma; the grade available purchased from Fluka (Buchs, Switzerland).

Cholesteryl acetate and acetyl chloride were of the highest analytical grade from Merck (Darmstadt, Germany), ammonium acetate and acetyl chloride were obtained from Cambridge Isotope Laboratories (Andover, MA) with an iso- tope purity higher than 98%. The serine- and choline-depleted Dulbeccoa modified Eagle’s medium was from PAN Biotech (Aidenbach, Germany). Fetal bovine serum was purchased from Biochrom (Berlin, Germany) and Falcon® 6-well plates were used for cell culture (Becton Dickinson Labware).

**Cell Culture**—Fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 1-glutamine, nones- sential amino acids, and 10% fetal calf serum in a humidified 5% CO2 atmosphere at 37 °C. The experiments described were performed with cells at passages 7–14. Mycoplasma contamination of fibroblasts was routinely tested using MycoAlert® Mycoplasma Detection Assay (Cambrex, USA) and only negative tested cells were used for experiments. For lipid analysis, cells were seeded into 6-well plates at a density of 80,000 cells per well. They were grown to confluence and then incubated in serine- and choline-depleted Dulbecco’s modified Eagle’s medium containing 2 mg/ml fatty acid free bovine serum albumin supplemented with 50 μg/ml of [13C3]serine, [D4]ethanolamine, and [D3]choline chloride. In parallel lipid loading was performed using 15 μg/ml FC, 40 μg/ml E-LDL, or Ox-LDL, respectively. At the indicated time points fibroblasts were rinsed twice with phosphate-buffered saline and lysed with 0.2% SDS.

**Lipoprotein Preparation**—LDL (d = 1.019-1.063 g/ml) from sera of normolipidemic volunteers was isolated and enzymati- cally modified as described previously (19) with slight modifi- cations in the preparation of E-LDL. Briefly, for enzymatic modification, LDL was diluted to 2 mg/ml protein in phosphate-buffered saline. Enzyme treatment was performed with trypsin (6.6 μg/ml) and cholesterol esterase (40 μg/ml) for 48 h at 37 °C. Oxidation of LDL was performed according to published protocols (20). Briefly, LDL was diluted to 1 mg/ml pro-tein in phosphate-buffered saline and dialyzed against 5 μM Cu2+ (42 h, 4 °C). The modified lipoproteins were stored at 4 °C and used within a week.

**Protein Determination**—Protein concentrations were mea- sured using bicinchoninic acid as described previously (21). Prior lipid extraction an aliquot of SDS lysed fibroblasts was taken for protein determination.

**Lipid Extraction**—Lipids were extracted according to the procedure described by Bligh and Dyer (22) in the presence of not naturally occurring lipid species as internal standards. The chloroform phase was dried in a vacuum centrifuge and dis- solved as described below for quantitative lipid analysis.

**Mass Spectrometry**—Lipids were quantified by ESI-MS/MS in positive ion mode (as described previously (23–25)). Samples were quantified by direct flow injection analysis using the ana- lytical setup described by Liebisch et al. (24, 25). A precursor ion scan of m/z 184 specific for phosphocholine containing lipids was used for FC, SM (24), and lysophosphatidylcholine (LPC) (26). [D4]Choline-labeled lipids were analyzed by precursor ion scan of m/z 193. Neutral loss scans of m/z 141 and m/z 185 were used for PE and phosphatidylserine (PS), respectively (23). Analogous, neutral loss scans were used for stable isotopelabeled [D4]PE (m/z 145) and [13C3]PS (m/z 188). FC and CE were quantified using a fragment ion of m/z 369 after selective derivatization of FC using acetyl chloride (25). Additionally, lipids present at low concentration were analyzed in a second run by selected reaction monitoring (SRM) to increase preci- sion (especially for stable isotope labeled species at early time points). Correction of isotopic overlap of lipid species as well as data analysis by self-programmed Excel macros was per- formed for all lipid classes according to the principles described previously (24).

To quantify for all lipid classes analyzed non-naturally occur- ring lipid species were used as internal standards (PC 28:0, 44:0, LPC 13:0, 19:0, PE 28:0, 40:0, PS 28:0, 40:0, D6-FC, CE 17:0, 22:0). Quantification was performed by standard addition cali- bration to cell homogenates (lipoproteins) using several naturally occurring lipid species for each lipid class (PC 34:1, 36:2, 38:4, 40:0; SM 16:0, 18:1, 18:0; LPC 16:0, 18:1, 18:0; PE 34:1, 36:2, 38:4, 40:6; PS 34:1, 36:2, 38:4, 40:6; FC, CE 16:0, 18:2, 18:1, 18:0). These calibration lines also were applied for not calibrated species, as follows: Concentrations of saturated, monounsaturated, and polyunsaturated species were calculated using the closest related saturated, monounsaturated and polyunsaturated calibration line slope, respectively. For example PE 36:2 calibration was used for PE 36:1, PE 36:3, PE 36:4; PE 38:4 calibration was used for PE 38:3 and PE 38:5 and so on. Assuming a similar analytical response for stable isotope labeled and unlabeled species, labeled species were quantified using the internal standards and calibration lines described above. The quantitative values were related to the protein amount of the sample.

**RESULTS**

**Lipid Composition of E-LDL and Ox-LDL**—To characterize and compare the lipid composition of LDL, E-LDL, and Ox-LDL ESI-MS/MS was carried out. FC and CE represented the predominant lipid fraction in all three LDL preparations with ~75 mol% of all analyzed lipids (Fig. 1). In contrast to native LDL, which contains about two-thirds of total cholesterol as CE, enzymatic modification of LDL with trypsin and cholesteryl esterase decreased the proportion of CE to about one-third. Mild oxidation of LDL did not significantly influence the FC fraction, whereas the proportion of CE decreased compared with native LDL. Analysis of Ox-LDL using a precursor ion scan of m/z 369 specific for CE (25) revealed peaks absent in native LDL or E-LDL (Fig. 1). These peaks likely arise from oxidative modification of the fatty acid moiety of CE (a detailed lipid
analysis of LDL modifications will be subject of a separate article.

Oxidation of LDL also affected polyunsaturated PC species, reducing the PC fraction from 15 mol% in native LDL to 11 mol% in Ox-LDL. Concomitantly, a strong rise of the LPC fraction was found in Ox-LDL compared with LDL (5-fold to 5 mol%; Fig. 1).

**Cellular Lipid Level upon Lipid Loading**—Primary human skin fibroblasts were loaded with FC, E-LDL, and Ox-LDL, and the lipid loading kinetics were determined by ESI-MS/MS/MS. Cholesterol uptake occurred mainly in the first 24 h, and no major changes in cellular cholesterol levels were observed up to 72 h (data not shown). FC loading increased FC levels to 275%, whereas E-LDL and Ox-LDL increased cellular FC only to 165 and 143% (Table 1), respectively. In contrast to FC loading, where only a marginal increase was observed to 130%, modified lipoproteins induced cellular CE levels of almost 300% compared with unloaded control (Table 1). Interestingly, FC loading did not increase total PC concentration and led to a remarkable decrease of 40% of total PE level. Modified LDL slightly increased cellular PC level (Table 1), which may be caused by the high content of PC in E-LDL and Ox-LDL (Fig. 1). PE and PS are only minor components of modified LDL (Fig. 1), fitting to minor changes observed in cellular PE and PS level upon E-LDL and Ox-LDL loading (Table 1).

**Effects of Lipid Loading on [D₉]PC**—One major goal of the present study was to investigate the effects of FC and lipoprotein loading on cellular glycerophospholipid metabolism. Therefore, parallel to loading with lipids stable isotope-labeled precursors were used to monitor the main pathways of glycerophospholipid metabolism (Fig. 2A). [D₉]Choline, [D₁₀]ethanolamine, and [¹³C₂]serine labels were substituted in medium deficient for the natural compounds. Both unlabeled and stable isotope-labeled phospholipids were analyzed by ESI-MS/MS using specific scan types (see “Experimental Procedures”). As expected, newly synthesized PC was solely derived from the Kennedy pathway via CK, CT, and CPT resulting in [D₉]PC, but no [D₉]PC derived from PE N-methylation was detected (data not shown). Although total PC levels were only marginally influenced (Table 1), pronounced changes were observed in the de novo synthesis of PC upon lipid loading (Fig. 3). Whereas Ox-LDL did not significantly change PC synthesis, FC incubation led to a 40% increase in PC synthesis compared with unloaded control. E-LDL revealed an almost 2-fold increase of PC synthesis compared with control (Fig. 3).

To analyze, whether the lipid species profile is also influenced, as suggested by Blom et al. (17) both detailed species patterns of undeuterated and deuterated [D₉]PC species were investigated in loaded fibroblasts. FC loading did only induce marginal changes in the species profile of undeuterated PC compared with control (Fig. 4A), but [D₉]PC species profile shifted to longer and more unsaturated species, mainly at the expense of PC 32:1 (increase of PC 36:4, PC 36:3, PC 36:2, PC 36:1, PC 38:5, PC 38:4; Fig. 4B). Calculation of total species shift from control to FC-loaded fibroblasts revealed a significantly increased proportion of 13.5% for [D₉]PC compared with 5.0% for undeuterated PC. However, it has to be taken into account that only 10% of total PC was labeled at 24 h (Table 2). Consequently, related to the total PC, it appeared that the de novo synthesized PC contributed the minor proportion with 1.3% compared with 4.5% for unlabeled PC of total species shift.

Similar to FC loading, the [D₉]PC species profile showed more pronounced changes upon E-LDL incubation than undeuterated PC species profiles. Thus, a substantial shift

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**TABLE 1**

| Lipid species | Control | FC | E-LDL | Ox-LDL |
|---------------|---------|----|-------|--------|
| Lipid concentration | % control | % control | % control | % control |
| Cellular FC | 119 ± 11 | 275 ± 27 | 165 ± 14 | 143 ± 13 |
| Cellular CE | 6.3 ± 1.1 | 129 ± 16 | 292 ± 24 | 284 ± 56 |
| Cellular PC | 138 ± 17 | 98 ± 6 | 120 ± 9 | 112 ± 4 |
| Cellular PE | 41 ± 3 | 61 ± 3 | 111 ± 9 | 104 ± 2 |
| Cellular PS | 31 ± 1 | 116 ± 6 | 114 ± 9 | 109 ± 10 |

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**FIGURE 1. Lipid composition of LDL, E-LDL, and Ox-LDL.** LDL and the corresponding enzymatically and oxidatively modified lipoproteins E-LDL and Ox-LDL were prepared as described under “Experimental Procedures.” Lipids were extracted according to Bligh and Dyer (22). Cholesterol and phospholipid fractions were measured by ESI-MS as described under “Experimental Procedures.” Displayed are lipid fractions as percentage of analyzed lipids of LDL (white bars), E-LDL (black bars), and Ox-LDL (dark gray bars). The potentially oxidized cholesteryl ester (CE Ox) represents the sum of not yet identified peaks found after LDL oxidation (analysis was performed by precursor ion scan of m/z 369; peaks were quantified by CE calibration lines). Phospholipids displayed are PC, SM, LPC, PE, and PS. Values are mean ± S.D. from three independent experiments, each performed in triplicate.

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**Phospholipid Metabolic Profiling of Lipid-loaded Fibroblasts**

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A stable isotope labeling of major glycerophospholipid metabolism pathways. Displayed are the incorporation of stable isotope labels into PS by head group transfer either from PE or PC as well as via Kennedy pathway in ethanolamine- and choline-derived PE and PC (A), respectively. B shows pathways of glycerophospholipid species modifications, which occur without label incorporation: On the one hand transacylation of fatty acids from a phospholipid to any lysophospholipid (CoA-dependent or independent). On the other hand phospholipase-mediated lysophospholipid generation with subsequent re-acylation. The following abbreviations were used: Cho, choline; CK, choline kinase; CTP, CDP-choline; CTP: phosphocholine cytidylyltransferase; Etn, ethanolamine; EK, ethanolamine kinase; EPT, CDP-ethanolamine:1, 2-diacylglycerol ethanolaminephosphotransferase; LAT, CDP-ethanolamine:1, 2-diacylglycerol ethanolaminephosphotransferase; PL, phospholipid; PLA, phospholipase A; PSS1, phosphatidylserine synthase 1; PSS2, phosphatidylserine synthase 2; PSD, phosphatidylserine decarboxylase.

Effects of Lipid Loading on \([\text{D}_9\text{]}\text{PC}\) synthesis was about 5-fold than \([\text{D}_9\text{]}\text{PC}\) synthesis at 24 h in unloaded controls (Table 2). In contrast to CDP-ethanolamine-derived PE, no decarboxylation of \([\text{13C}_3]\text{PS}\) to \([\text{D}_9\text{]}\text{PE}\) was observed (data not shown). Whereas incubation of fibroblasts with modified lipoproteins showed no significant changes of \([\text{D}_4]\text{PE}\) levels, FC loading decreased \([\text{D}_4]\text{PE}\) levels to 40–50% compared with the unloaded control (Fig. 5).

The species pattern of undeuterated PE did not reveal major changes upon lipid loading (Fig. 6A). However, significant species shifts were observed upon FC and E-LDL incubation for \([\text{D}_9]\text{PE}\) (Fig. 6B). Thus, \([\text{D}_9]\text{PE}\) species pattern shifted toward long chain and polyunsaturated species at the expense of PE 34:2 and PE 34:1 upon FC incubation (increase of PE 36:1, 38:4, 38:3, 40:6, 40:5, 40:4; Fig. 6B). The overall species shift upon FC loading compared with control was much more pronounced for \([\text{D}_9]\text{PE}\) with 17% of total \([\text{D}_4]\text{PE}\) compared with 4% for undeuterated PE. Taking into account that 23% of total PE were newly synthesized (Table 2), the majority of PE species shift was derived from de novo synthesized \([\text{D}_9]\text{PE}\) (4.0% related to total PE) compared with undeuterated PE (3.0% related to total PE). E-LDL incubation induced a pronounced species shift to polyunsaturated \([\text{D}_9]\text{PE}\) (40:4, 36:4, 36:3, 34:2 (Fig. 6B), whereas Ox-LDL loading did not cause a significant shift of \([\text{D}_9]\text{PE}\) pattern (Fig. 6).

Effects of Lipid Loading on \([\text{13C}_3]\text{PS}\) Lipid loading increased cellular \([\text{13C}_3]\text{PS}\) level at 24 h, whereas after 48 h, only modified lipoprotein incubation led to increased \([\text{13C}_3]\text{PS}\) levels compared with control (Fig. 7). Because the species pattern of \([\text{13C}_3]\text{PS}\) may also depend on a species shift in PC and PE as
substrates for PS synthases (Fig. 2A), the species profile of \([^{13}C_3]PS\) was investigated after 72 h incubation. However, even after 72 h of lipid loading no substantial shift was observed in the species pattern of unabeled PS compared with control (Fig. 8A). \([^{13}C_3]PS\) species profile revealed only minor changes with a slight decrease of \([^{13}C_3]PS\) 36:1 upon lipid loading (Fig. 8B). The fraction of polyunsaturated \([^{13}C_3]PS\) species PS 38:4, 40:6, 40:5 was increased upon FC loading. Modified LDL led to an increased proportion of \([^{13}C_3]PS\) 36:2, whereas increased \([^{13}C_3]PS\) 38:4 and \([^{13}C_3]PS\) 38:3 were observed upon E-LDL and Ox-LDL, respectively.

**Loading of E-LDL Lipid Components and Their Effect on PL Synthesis**—We were also interested in which E-LDL components were responsible for the observed effects on PL synthesis. Thus, E-LDL incubation was compared with lipids extracted from E-LDL and a mixture of FFA, which resembles the composition found in E-LDL (FFA1, 16:1/16:0/18:2/18:1/20:4 = 1:1/1/6/1/1 molar ratio reflecting the esterase-digested CE fraction of E-LDL). Additionally, fibroblasts were incubated with palmitic acid (FFA2) to evaluate the difference between a saturated FFA and an FFA mixture including polyunsaturated FFA. Both FFA incubations were combined with FC loading to investigate the effect of FC on FFA incorporation into glycerophospholipids (Table 3).

Similar to the previous experiments, FC and E-LDL increased \([D_9]PC\) level after 24 h of incubation about 20% and more than 2-fold, respectively (Table 3). Addition of E-LDL-derived lipid extract exhibited an almost similar induction of \([D_9]PC\) level as E-LDL. Moreover, both FFA incubations revealed an increased PC synthesis compared with control, which further was induced by addition of FC. However, the FFA1 mixture showed a more than 50% greater \([D_9]PC\) level compared with the corresponding palmitic acid incubations (FFA2).

Whereas \([D_4]PE\) synthesis was little changed upon E-LDL loading, FC loading strongly decreased \([D_4]PE\) to \(\sim 65\%\) of the control level (Table 3). E-LDL lipid extracts as well as FFA incubation revealed a 40–50% induction of \([D_4]PE\) level compared with control. Strikingly, coincubation of FFA with FC remarkably decreased \([D_4]PE\) concentration.

\([^{13}C_3]PS\) synthesis was increased in all incubations containing FFA including E-LDL and its lipid extract. However, only a minor effect of FC addition was observed on \([^{13}C_3]PS\) level with FFA incubation (Table 3).
Because the main effects of lipid loading were observed on PC and PE synthesis, the species profile of [D9]PC and [D4]PE were analyzed in detail (Fig. 9). The changes in species pattern induced by E-LDL were in accordance with the effects described previously, and incubation with lipids extracted from E-LDL closely resembled changes in the species pattern found upon E-LDL incubation (data not shown). Moreover, the shifts in the species pattern observed after E-LDL loading were similar to those after FFA1 loading (Figs. 4, 6, and 9). All together this clearly indicates an incorporation of FFA either delivered by E-LDL or directly. As expected FFA1 and FFA2 reflect the FFA provided to the media, e.g. the strong increase of newly synthesized 32:0, 32:1, and 34:1 species, which points to cellular desaturation (to 16:1) and elongation (18:0) of palmitic acid (16:0). Interestingly, a clear effect of FC addition on [D9]PC and [D4]PE was observed. Thus, for both FFA1 and FFA2 FC addition increased the proportion of [D3]PC 36:4, 38:6 and 38:4 as well as polyunsaturated [D4]PE species containing 40 carbon atoms.

DISCUSSION

Previously established assays based on ESI-MS/MS for high throughput lipid quantification (24–26) were used to study glycerophospholipid metabolism in fibroblasts loaded with FC, E-LDL, and Ox-LDL, respectively. In general, the used human skin fibroblasts displayed PL de novo synthesis via the so-called Kennedy pathway with direct ethanolamine and choline incorporation into [D4]PE and [D9]PC, as well as [13C3]PS synthesis via PS synthase 1/2 converting PC and PE (27), respectively. As expected PE methylation was not observed, because a relevant contribution to PC synthesis so far only has been described for liver, retina, and brain (27–29). Additionally, no substantial PS decarboxylation forming PE was found, which may be caused by cell type as well as culture conditions especially the ethanolamine supplementation to the culture media.

Although, the applied stable isotope labels allow an accurate monitoring of the major glycerophospholipid synthesis pathways for PC, PE, and PS, it is not possible to assess directly fatty acid remodeling of PLs (Fig. 2B). Therefore, both species pattern of unlabeled and labeled PLs were analyzed. Assuming that all de novo synthesized species are isotope-labeled, a shift in unlabeled species pattern can be interpreted as fatty acid remodeling (Fig. 2B). Stable isotope labeled species pat-
tern results from biosynthesis (Fig. 2A) and potentially fatty acid remodeling of de novo synthesized species (Fig. 2B). This is especially of importance because it has been shown previously that FC loading induces a shift toward polyunsaturated species of PC and PE in the plasma membrane fraction of fibroblasts (17). Moreover, it is known that fatty acid remodeling by phospholipase and reacylation as well as transacylation pathways represent a major pathway for polyunsaturated PL synthesis (30, 31).

Thus, in accordance to previous studies we could demonstrate an increase of the PC synthesis upon FC loading in human skin fibroblasts (14). A novel finding of the present study was a pronounced down-regulation of PE synthesis upon FC loading. Similar to Blom et al. (17), a species shift toward polyunsaturated species was observed for both PC and PE. This represents a mechanism of cells to prevent a decreased membrane fluidity because of the stiffening effect of FC (11), which may even lead to cytotoxicity (9, 32). Interestingly, the observed PC species shift was mainly caused by fatty acid remodeling of existing PC species, whereas de novo synthesized PE contributed the majority to the PE species shift upon FC loading.

In contrast to FC loading, Ox-LDL uptake, which is described to be mediated by clathrin-coated pits (4, 5), did not exhibit a substantial effect on PC and PE synthesis as well as the respective species. Although, cellular FC and CE level were increased reflecting cellular uptake, it seemed as if Ox-LDL derived lipids did not reach cellular compartments involved in the regulation of glycerophospholipid metabolism. A potential explanation may be that oxidized lipids, especially oxidized CE, are resistant to lysosomal degradation, which leads to a trapping of Ox-LDL within lysosomes (33). Additionally, cholesterol derived from Ox-LDL accumulates in lysosomes (34) and consequently may not reach cellular sites involved in the regulation of PC and PE synthesis. The induction of PS synthesis by Ox-LDL is potentially related to Ox-LDL induced cytotoxicity (35), since PS exposure on cell surface is a common feature in apoptosis also observed upon Ox-LDL incubation (36). In addition, it has been shown that newly synthesized PS is preferentially externalized in apoptotic U937 cells (37).

Compared with Ox-LDL, E-LDL led to a massive induction of the de novo PC synthesis (Fig. 3). A potential reason could be a different cellular uptake mechanism for E-LDL, which enters the cell via clathrin independent phagocytosis (7). However, another important factor may be the particular lipid composition of E-LDL, which contains due to CE digestion high FC (Fig. 1) and free FFA levels (38). Accordingly, unphysiologic application of lipids extracted from E-LDL induced PC synthesis to a similar extent as E-LDL (Table 3). Moreover, a FFA mixture resembling those found in E-LDL (FFA1) led to a comparable PC synthesis induction (Table 3). Together with the observed changes in the species pattern of newly synthesized PC, this clearly indicates an incorporation of FFA from E-LDL as well as the major role of FFA in the induction of PC synthesis by E-LDL. According to previous studies in HeLa (39) the induction of PC synthesis depends on the FFA type, because an equimolar concentration of saturated palmitic acid (FFA2) was not able to increase PC synthesis to the level observed for the mixture FFA1 (Table 3). This may be related to a conversion of palmitic acid (FFA2) by elongation and desaturation before incorporation into glycerophospholipids. On the other hand a pronounced increase of the PC synthesis was observed, when both FFA (mixtures) were coincubated with FC (Table 3). These data fit to a recently discussed model describing CT activation by Cornell and Northwood (40). CT is an amphitropic protein, i.e. it interconverts between a soluble inactive form and a membrane-bound active form. Thus, both changes in membrane lipid composition and the phosphorylation state of CT may regulate membrane binding and activation of CT. The changes in membrane lipid composition are described by two classes of lipids: Class I lipids increase negative electrostatic surface potential facilitating CT binding. Class II lipids induce negative curvature strain, which was relieved by CT insertion into the membrane (41, 42). In this model FFAs share features of both classes with their negative charge as well as negative curvature strain increasing lipids. The latter effect should be more pronounced for unsaturated FA than saturated fitting to the higher PC synthesis observed for FFA1 compared with saturated FFA2. Additionally, an increased PC synthesis upon FC incubation either without or in combination with FFAs would

**FIGURE 8.** PS species composition in unloaded fibroblasts and upon lipid loading. Cells were loaded, harvested, and analyzed as described in Figs. 3 and 7. Displayed are mol percentages of either total undeuterated PS (A) or total [13C3]PS (B) after 72 h of treatment. Shown are unloaded (white bars), FC (light gray bars), E-LDL (black bars), and Ox-LDL loaded (dark gray bars) species pattern. Values are mean ± S.D. of one representative experiment from four, each performed in triplicate. Although comparable effects were observed in all experiments major undeuterated PS and [13C3]PS species revealed CVs up to 20% and minor species CVs up to 50% between different experiments.
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TABLE 3
Effects of the E-LDL lipid components on PL synthesis

Cells were loaded, harvested, and analyzed as described in the legend to Fig. 3. Fibroblasts were loaded with FC (15 μg/ml), E-LDL (40 μg/ml), lipid extract derived from E-LDL, E-LDL lipids, and free fatty acid mixture (70 μM) (FFA2: 16:1, 16:0, 18:2, 18:1, 20:4 in a molar ratio of 1:6:1:3 similar to that present in CE present in E-LDL), palmitic acid (70 μM) (FFA2: 16:0) and a combination of FFA1/2 with FC. Shown are the concentration of [D₉]PC, [D₄]PE and [¹³C₃]PS as percent of the unloaded control calculated from nmol/mg cell protein. Values are mean ± S.D. of one representative experiment out of four, each performed in triplicate.

| FC | E-LDL | E-LDL lip | FFA1 | FFA1+FC | FFA2 | FFA2+FC |
|----|-------|-----------|------|---------|------|---------|
| % control | | | | | | |
| [D₉]PC | 121 ± 16 | 239 ± 15 | 186 ± 7 | 239 ± 18 | 313 ± 6 | 140 ± 11 | 207 ± 20 |
| [D₄]PE | 66 ± 14 | 85 ± 8 | 143 ± 3 | 147 ± 11 | 68 ± 6 | 154 ± 7 | 90 ± 15 |
| [¹³C₃]PS | 113 ± 7 | 146 ± 13 | 127 ± 7 | 136 ± 16 | 159 ± 8 | 113 ± 2 | 114 ± 14 |

![Figure 9](image_url)

FIGURE 9. [D₉]PC and [D₄]PE species pattern upon loading with FFA ± FC. Cells were loaded, harvested, and analyzed as described in Table 3. Displayed are mol percentages of total D₉-PC (A) or D₄-PE (B) in unloaded controls (white bars), after loading with FFA1 (gray bars), FFA1 + FC (dark gray bars), FFA2 (light gray bars), and FFA2 + FC (black bars) for 24 h. Values are mean ± S.D. of one representative experiment from four, each performed in triplicate.

be expected from a class II lipid like FC. This model also may provide an explanation, why Ox-LDL loading despite significant elevation of cellular FC level (Table 1) did not induce PC synthesis (Fig. 3). Because Ox-LDL contains large amounts of LPC (Fig. 1), which is known to decrease CT activity by releasing negative curvature strain (41, 42), the stimulating FC effect on CT activity may be balanced by LPC.

In contrast to PC synthesis, PE synthesis seems to be regulated by a different mechanism, because FC loading strongly decreased PE synthesis (Fig. 5, Table 3). However, up to now not much is known about the molecular regulation of CTP:phosphoethanolamine cytidylyltransferase (ET), which is considered to be the rate-limiting enzyme for PE synthesis via Kennedy pathway (27) (Fig. 2A). The difference between E-LDL (no significant regulation) and the lipids extracted from E-LDL on PE synthesis (40% increase compared with control, Table 3), may be explained by a different FC loading efficiency. Thus, E-LDL loading induced a more than 2-fold higher cellular FC level compared with control, whereas E-LDL derived lipid extracts only led to a 50% increase. This argues that PE synthesis is inhibited only above a certain threshold level of cellular FC. Moreover, PE synthesis did not show a strong dependence on the FFA type supplemented (Table 3). One possible explanation for the strong down-regulation of PE synthesis upon FC loading could be that both FC and PE act as class II lipids increasing negative curvature strain in lipid bilayers. Therefore, a decreased PE synthesis does not further increase negative curvature strain and consequently keeps lipid membrane physical properties in a certain range preserving cell function (43). This model is in good agreement with a recent study in yeast, which
presents evidence that intrinsic membrane curvature is maintained in a physiological range by adaptation of lipid physical properties (44).

In Drosophila ET is regulated by the sterol response element binding (SREBP) protein pathway (45). Although SREBP translocation in Drosophila is regulated by PE levels (45) (55% of total PL, Ref. 46)) analogous as FC levels in mammalian cells, there may exist an evolutionary conservation of a sterol response element in the ET promoter. Up to now a contribution of a FC induced down-regulation of ET via SREBP could not be ruled out, even though a recent study investigating the ET promoter in human breast cancer cells MCF-7 did not identify a sterol response element (47). Although less pronounced, PS synthesis seems to be regulated in a similar way as PC synthesis including induction by FFA as well as a dependence on the type of FFA and FC (Table 3).

In summary, the present study could support the idea that changes in lipid membrane composition affecting membrane curvature regulate CT activity and consequently cellular PC synthesis. In addition, an opposite regulation of PC and PE synthesis was found upon FC loading. A species shift toward polyunsaturated PE and PC observed upon FC loading, could be attributed primarily to PE biosynthesis, whereas PC species were shifted to a higher extent by FA remodeling of existing PC. Finally, with the application of high throughput metabolic profiling of major glycerophospholipid pathways by ESI-MS/MS, we could demonstrate that this technique could provide a powerful tool to unravel mechanism underlying the regulation of cellular lipid metabolism.

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