Abstract The modification of lipoproteins by lipolytic enzymes such as cholesterol esterase (CEase) is assumed to play an important role in the pathogenesis of atherosclerosis. However, details of the activation and inhibition of CEase are still unknown. In this study, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to investigate the extracts of human lipoproteins after treatment with CEase and to monitor the effects of the inhibitor 2-(diethylamino)-6,7-dihydro-4H,5H-cyclopenta[4,5]thieno[2,3-d][1,3]oxazin-4-one (DOT-3). This approach has the advantage that all lipid classes can be independently detected; therefore, conclusions on the mechanism of the applied enzyme are possible. Besides the expected decrease of cholesteryl esters (CEs) in HDL, a significantly enhanced content of lysophosphatidylcholine (LPC) was also detected, confirming the broad substrate specificity of CEase. It was also demonstrated that DOT-3 significantly inhibited the CEase-catalyzed cleavage of CE in HDL. Phospholipid (PL) vesicles prepared from phosphatidylycholine (PC) or PC and cholesteryl linoleate were treated with CEase, and the changes in lipid composition were investigated. From the analysis of the generated LPC species in HDL and in the isolated lipid mixtures, it is evident that CEase catalyzes the cleavage of the fatty acid residues in both the sn-1 and sn-2 positions of the PLs. These effects are obvious in the absence as well as in the presence of detergents.—Zschörnig, O., M. Pietsch, R. Süß, J. Schiller, and M. Gütschow. Cholesterol esterase action on human high density lipoproteins and inhibition studies: detection by MALDI-TOF MS. J. Lipid Res. 2005. 46: 803–811.

Supplementary key words enzyme inhibition • phosphatidylycholine • lysophosphatidylycholine • matrix-assisted laser desorption and ionization time-of-flight mass spectrometry

One important aspect of the development of atherosclerosis is the enzymatic modification of LDL and HDL by lipolytic enzymes that promote arterial wall alterations (1). In this study, the degradation of the lipid moiety of lipoproteins catalyzed by the enzyme cholesterol esterase (CEase) was investigated. LDL and HDL are composed of proteins and varying amounts of cholesteryl esters (CEs), phospholipids (PLs), triacylglycerols (TAGs), and free cholesterol (2). Among the PLs detected in LDL and HDL, phosphatidylycholine (PC) and sphingomyelin (SM) occur most frequently (3). All other PLs represent very minor species.

CEase (EC 3.1.1.13), a rather nonspecific lipolytic enzyme, is capable of hydrolyzing a broad spectrum of substrates, for instance lipophilic compounds with long-chain fatty acid residues such as CE, TAG, PL, and ceramides, requiring the activation by primary bile salts (4–8). In contrast, CEase hydrolysis of water-soluble substrates such as carboxylic esters with short-chain fatty acids or lysophospholipids is not strongly dependent on bile salt activation (8).

The CEase protein is synthesized primarily in the pancreatic acinar cells and lactating glands of higher mammals, but it is also found in the liver, macrophages, endothelial cells, eosinophiles, and circulation (8). Once secreted into the lumen of the duodenum, pancreatic CEase, together with other pancreatic lipolytic enzymes and preduodenal lipase, acts to complete the digestion of dietary lipids (4, 5). However, many observations suggest that the role of CEase extends beyond that of simply hydrolyzing dietary lipids.

In vitro studies have shown that CEase enhances sterol transfer between small unilamellar PL vesicles (9). In addition, the enzyme is involved in plasma lipoprotein metabolism and atherosclerosis (8). CEase has been reported to facilitate the conversion of the larger and less atherogenic LDL particles to smaller and more atherogenic LDL subspecies; thus, the enzyme may be proatherogenic (10).
Moreover, CEase treatment of trypsin-pretreated or Cu²⁺-oxidized LDL particles resulted in their conversion to larger multilamellar and cholesterol-rich vesicles. Applying the same hydrolytic procedure to HDL particles, no morphological change was observed, and only a small portion of HDL CE was hydrolyzed. Untreated LDL particles contain only ~25% of its total cholesterol as free cholesterol. CEase caused the hydrolysis of the CE core of LDLs such that 90% were unesterified (11). These modified lipoprotein species resembled aortic unilamellar and multilamellar lipid particles that accumulate in the extracellular spaces of atherosclerotic lesions (11–13).

In a synergistic action with sphingomyelinase, CEase was also shown to promote cholesterol crystal nucleation and LDL aggregation. Both are regarded as important processes in atherosclerosis (14). In contrast, CEase may be antiatherogenic by promoting the selective uptake of HDL-associating CE by liver cells, thus facilitating reverse cholesterol transport and reducing cholesterol accumulation in peripheral tissues (8, 15). Furthermore, the enzyme decreased the atherogenic potential of oxidized LDL by hydrolyzing the excess lysophosphatidylcholine (LPC) and ceramide generated during the lipoprotein oxidation process (8, 16–18).

In light of the controversial role of CEase in atherosclerosis, analysis of the molecular events associated with changes in lipoprotein composition is a challenge. A new analytical approach, matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS), was used in this study to assess the lipid composition of human lipoproteins and to monitor the changes induced by CEase. MALDI-TOF MS is increasingly used in lipid research because of its fast performance and easy experimental setup (19, 20). Surprisingly, MALDI-TOF MS has rarely been used for the characterization of the lipid moieties of human lipoproteins (21–23) and never for monitoring the effects of the CEase. However, recent reports have shown the potential of MALDI-TOF MS for the determination of enzyme activities (24).

CEase was recently recognized as a target for the development of inhibitors. 2-(diethylamino)-6,7-dihydro-4H,5H-cyclopenta[4,5][1,3]oxazin-4-one (DOT-3) (Fig. 1) is a heterocyclic low molecular weight inhibitor of the enzyme, exhibiting an inhibition constant of 580 nM. Enzyme inhibition may be assayed with p-nitrophenyl butyrate as a chromogenic substrate. It was shown that DOT-3 represents an alternative substrate inhibitor of CEase (25). To date, CEase inhibition by DOT-3 has not been investigated in systems that approximate the physiological situation. In this study, we also addressed the question of whether CEase-induced changes in lipoprotein composition are influenced by DOT-3.

**MATERIALS AND METHODS**

**Materials**

CEase (bovine pancreas) was obtained from Sigma (Deisenhofen, Germany) and used as supplied. Stock solutions of CEase (50 U/ml) were freshly prepared each day in 10 mM HEPES, pH 7.4, containing 100 mM NaCl. DOT-3 (Fig. 1) was synthesized as previously described (26) and was dissolved in chloroform (3 mg/ml).

All chemicals for buffer preparation, lipoprotein isolation, and mass spectrometry [2,5-dihydroxybenzoic acid (DHB) and trifluoroacetic acid (TFA)] as well as all solvents (chloroform and methanol) were obtained in the highest commercially available purity from Fluka Feinchemikalien GmbH (Taufkirchen, Germany). Egg yolk PC, cholesteryl linolate, and sodium cholate were also purchased from Fluka, whereas PC 16:0/18:2 was from Avanti Polar Lipids (Alabaster, AL).

**Preparation of HDLs**

HDLs were isolated from the blood plasma of healthy volunteers by sequential ultracentrifugation according to Havel, Eder, and Brangdon (27) using a Beckman L8-50 ultracentrifuge. The purity of the preparation was checked by Lipidophor gel electrophoresis (Immuno AG, Vienna, Austria). Isolated HDLs were dialyzed overnight at 4°C against 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl. After dialysis, the HDLs were concentrated using ultrafiltration equipment (Amicon, Witten, Germany). The concentrations of apolipoproteins were determined using a commercially available biocinchoninic acid kit (Sigma) and used as a measure of lipoprotein concentration.

**Treatment of lipoproteins and PL vesicles with CEase**

Fifty microliters of HDL (1 mg/ml protein) isolated as described above was treated with CEase at different final concentrations (0.5–2 U/ml) and incubated for 60 min at 37°C (10 mM HEPES and 100 mM NaCl, pH 7.4). After incubation, HDLs were extracted according to the Bligh and Dyer method (28) [methanol-chloroform-water, 1:1:0.9 (v/v/v)], and the water-methanol layer was discarded. Investigations showed that all lipids were present in the chloroform layer under these conditions.

For comparison, liposomes from egg yolk PC were also digested by CEase to check the phospholipase activity of the enzyme. Multilamellar PL vesicles were prepared using the method of Bangham (29). The lipid was initially dried from chloroform, subsequently dispersed in buffer solution (10 mM HEPES, pH 7.4, and 100 mM NaCl), and shaken at a temperature above the gel-to-liquid crystalline transition temperature for 10 min. Large unilamellar vesicles were prepared by five freeze-thaw cycles of multilamellar PL vesicles followed by extrusion (five times) through 0.1 µm Nucleopore filter membranes using an extruder (Lipex Biomembranes, Vancouver, Canada) at 30°C. The PL concentrations was determined by phosphorous content according to Chen, Toribara, and Wanner (30). The PL concentration was adjusted with buffer to be 1 mM in all cases. CEase was added to obtain final enzyme concentrations of 1–5 U/ml, and incubation was performed for 60 min at 37°C. A similar experiment was performed with purified PC 16:0/18:1 in the presence of 100 mM sodium cholate.

Vesicles from selected, purified PC and cholesteryl linolate (both 0.5 mM) were also incubated with CEase. Briefly, aliquots of lipids dissolved in chloroform were evaporated to dryness in a
centrifugal evaporator (Jouan). Vesicles were prepared by dissolving the resulting lipid film in 100 mM sodium cholate (pH 7.4) and digested with CEase (0.6–4.55 U/ml).

**TREATMENT OF HDLS WITH CEASE AND DOT-3**

DOT-3 was dissolved in chloroform, and appropriate amounts of the stock solution (5 mg/ml) were dried on the surface of glass vials. Fifty microliters of HDL (1 mg/ml protein) isolated as described above or the corresponding amounts of isolated lipids were added and incubated under shaking with CEase (final concentration, 2.5 U/ml). Final concentration of DOT-3 ranged between 26 and 260 µg/ml.

**MALDI-TOF MS**

Organic lipid extracts were evaporated to dryness and afterward redissolved directly in the matrix. For all samples, a 0.5 M DHB solution in methanol containing 0.1% TFA was used. Approximately 2 µl of the lipid/matrix mixture was applied to the sample plate. Subsequently, samples were allowed to crystallize. Drying of samples with a moderate, warm stream of air distinctly improved the homogeneity of crystallization (19, 31).

All MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry DE workstation (PerSeptive Biosystems, Framingham, MA). This system uses a pulsed nitrogen laser emitting at 337 nm. The extraction voltage was 20 kV, and the “low-mass gate” was turned on to prevent the saturation of the detector by ions resulting from the matrix (32). One hundred twenty-eight single laser shots were averaged for each mass spectrum. The laser strength was kept at ~10% above the threshold to obtain the best signal-to-noise ratio. To enhance the spectral resolution, all spectra were acquired in the reflector mode. Further details of the MALDI-TOF MS measurements are available elsewhere (33).

**RESULTS**

**COMPOSITIONAL ANALYSIS OF HDLS WITH MALDI-TOF MS**

The suitability of MALDI-TOF MS for the characterization of the lipid extracts of human lipoproteins was recently demonstrated (21). The most important advantages of MALDI-TOF MS are its fast performance and the simple experimental setup. Additionally, even minor PL classes are detectable by MALDI-TOF MS subsequent to separation of the lipid mixture into the individual lipid classes (34). A detailed assignment of the lipids detectable in human HDL extracts has been provided (21).

A typical positive ion MALDI-TOF mass spectrum of an organic extract of HDL is shown in Fig. 2, trace (a). In short, primarily SM and PC that differ considerably in fatty acid composition are detectable in this extract: SM is mainly composed of palmitic acid (m/z = 703.6 for the H⁺ adduct and 725.6 for the Na⁺ adduct), whereas the PC moiety of HDL is characterized by a much broader fatty acid distribution (35).

The PC fraction consists primarily of PC 16:0/18:2 (m/z =

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**Fig. 2.** Positive ion matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectra monitoring the influence of increasing amounts of CEase on human HDLs (1 mg/ml protein). Spectrum (a) corresponds to the lipid extract of HDL in the absence of the enzyme (control); CEase activity was 0.5 U/ml in trace (b), 1 U/ml in trace (c), and 2 U/ml in trace (d). Reactions were carried out in 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl over 60 min at 37°C. All spectra were scaled according to the intensity of the most intense peak (cholesterol), and only some selected peaks are marked according to their \( m/z \) ratios. For more detailed assignments, see the text. The asterisk denotes a typical matrix peak.
758.6 and 780.6 for the H\(^+\) adduct and the Na\(^+\) adduct, respectively) and PC 16:0/18:1 (m/z = 760.6 and 782.6). PC species containing stearic acid instead of palmitic acid are also detectable (the peaks at m/z = 786.6 and 808.6 caused by PC 18:0/18:2). In contrast, cholesterol yields only a single peak at m/z = 369.3 (M+H\(^+\)-H\(_2\)O), whereas CEs are exclusively detectable as the corresponding sodium adducts. The peak at m/z = 671.6 corresponds to the sodium adduct of cholesteryl linoleate. The contribution of TAG is not considered in this study, and the corresponding high-mass region is not shown.

Finally, there are also some smaller peaks of LPC containing a palmitic acid or a stearic acid residue in the region between m/z = 480 and 550 (for assignments, see below).

**Monitoring of the C\(\text{E}\)ase-catalyzed changes in HDLs by MALDI-TOF MS**

In Fig. 2, the influence of increasing amounts of the enzyme C\(\text{E}\)ase on the positive ion MALDI-TOF mass spectra of organic extracts of human HDL is shown. Trace (a) was recorded in the absence of C\(\text{E}\)ase (control sample). In traces (b) to (d), increasing amounts of C\(\text{E}\)ase were used (0.5, 1, and 2 U/ml). It is obvious that there are considerable changes in the peak intensities of the CE (m/z = 671.6) in the presence of the C\(\text{E}\)ase as a result of its enzymatic hydrolysis. The peak of CE is completely missing when higher concentrations of C\(\text{E}\)ase (>0.5 U/ml) are used. The increase of the cholesterol peak as the product of this hydrolysis at m/z = 369.3 is less evident because large amounts of cholesterol are already present in the applied HDL sample in the absence of C\(\text{E}\)ase.

The loss of the cholesteryl linoleate peak is not surprising because this corresponds to the prime activity of the C\(\text{E}\)ase. However, CE hydrolysis is obviously not the only reaction catalyzed by C\(\text{E}\)ase. LPCs are also generated under the influence of C\(\text{E}\)ase [compare the mass region between approximately m/z = 480 and 550; Fig. 2, trace (d)]. It is remarkable that besides the generation of LPC 16:0 (m/z = 496.3 and 518.3) and LPC 18:0 (m/z = 524.3 and 546.3), there are also LPC species with unsaturated fatty acid residues, such as oleic acid (m/z = 522.3 and 544.3) and linoleic acid (m/z = 520.3 and 542.3). This finding indicates that C\(\text{E}\)ase exhibits both phospholipase A\(_1\) and phospholipase A\(_2\) activity and thus hydrolyzes the fatty acid esters located in the sn-1 and sn-2 positions of lipids. Note that there are no major changes when the digestion is performed in the presence of cholate as a detergent (data not shown).

The intensities of the SM peaks (m/z = 703.6 and 725.6) remain constant. Therefore, it is obvious that C\(\text{E}\)ase does not possess sphingomyelinase activity.

**Studies on artificial lipid mixtures**

C\(\text{E}\)ase-induced hydrolysis of isolated PCs. To confirm the results obtained with the lipoproteins, PC from egg yolk was also digested by C\(\text{E}\)ase. In Fig. 3, positive ion MALDI-TOF

![Fig. 3. Positive ion MALDI-TOF mass spectra indicating the influence of increasing amounts of C\(\text{E}\)ase on pure phosphatidylycholine (PC) vesicles (1 mM phospholipid). Spectra were separated into the lysophosphatidylycholine (left) and the PC (right) mass regions. Spectrum (a) corresponds to the lipid extract of the pure PC vesicles in the absence of the enzyme (control). Reactions were carried out in 10 mM HEPES buffer, pH 7.4, containing 100 mM NaCl over 60 min at 37°C. Note that there are only extremely weak peaks of lysophospholipids. C\(\text{E}\)ase activity was 1 U/ml in trace (b), 2.5 U/ml in trace (c), and 5 U/ml in trace (d). Peaks are marked according to their m/z ratios. For more detailed assignments, see the text.](image-url)
mass spectra of PC from egg yolk are shown. Trace (a) corresponds to the control sample (no addition of CEase); spectra at left represent the LPC moiety, and spectra at right correspond to the intact PC (1 mM). CEase activity was 1 U/ml in trace (b), 2.5 U/ml in trace (c), and 5 U/ml in trace (d). Spectra of LPC are scaled to a higher extent than spectra of PC. However, this scaling factor was the same in all spectra. Therefore, intensities between LPC and PC cannot be compared, whereas LPC spectra are comparable with each other with respect to peak intensities (32).

Although there are only very slight changes in the spectra at right, it is evident that the content of LPC increases when the concentration of CEase is increased. The same LPC species observed in HDL extracts are also observed in the pure PC vesicles. Besides LPC 16:0 (m/z = 496.3 and 518.3), which provides the most intense peak as a result of the excess of palmitic acid residues in PC (3), LPC 18:1 (m/z = 522.3 and 544.3) is a very abundant compound, accompanied by LPC 18:0 (m/z = 524.3 and 546.3) and LPC 18:2 (m/z = 520.3 and 542.3). We are currently performing further investigations to clarify the influence of the PC fatty acid composition on the extent of LPC generation and to clarify positional effects.

It is generally accepted that the activity of CEase is significantly enhanced in the presence of a suitable detergent. Therefore, the same experiment shown in Fig. 3 was performed in the presence of detergent (Fig. 4). Because it is most suitable for our experimental setup (33), sodium cholate at a concentration of 100 mM was applied. Purified PC 16:0/18:1 was used in this case to give a highly defined fatty acid composition. Figure 4, trace (a) was recorded in the absence of CEase. Besides the expected peaks of the PC at m/z = 760.6, 782.6, and 798.6, corre-

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**Fig. 4.** Positive ion MALDI-TOF mass spectra indicating the influence of increasing amounts of CEase on pure PC 16:0/18:1 (1 mM) dispersed in sodium cholate. Spectrum (a) corresponds to the lipid extract of the pure PC in the absence of the enzyme (control). Reactions were carried out in 10 mM HEPES buffer, pH 7.4, containing 100 mM NaCl over 60 min at 37°C. CEase activity was 1 U/ml in trace (b), 2.5 U/ml in trace (c), and 5 U/ml in trace (d). Peaks are marked according to their m/z ratios. For more detailed assignments, see the text. Peaks labeled with asterisks are caused by the DHB matrix.
sponding to the H+, Na+, and K+ adducts, respectively, of PC 16:0/18:1, there are only very small signals of LPC.

Although the majority of the sodium cholate (a compound well soluble in water) may be removed by the extraction process, sodium cholate gives a strong signal at \( m/z = 431.3 \) (H+ adduct). This spectral region is not shown for clarity. However, the peaks at \( m/z = 607.3 \) and \( 629.3 \) correspond to the addition of one DHB matrix molecule (+177 or +199) to the neutral form of the sodium cholate. The intensity of these peaks is nearly constant in all cases. The peak at \( m/z = 567.3 \) is assumed to be caused by NaOH elimination from the cholate-DHB adduct.

In the presence of CEase [Fig. 4, traces (b) to (d)], the content of LPC drastically increases, whereas the intensities of the peaks of the original material decrease. Considering the peaks at \( m/z = 496.3 \) and 518.3 on the one hand and at \( m/z = 522.3 \) and 544.3 on the other hand, it is obvious that the generation of LPC 18:1 is favored compared with LPC 16:0. Accordingly, CEase prefers the sn-1 to the sn-2 position and hydrolyzes this ester bond approximately three times faster.

CEase-induced hydrolysis of PC/CE mixtures. From the experiments described above, it is difficult to draw any conclusions regarding to what extent CE hydrolysis is preferred compared with PC hydrolysis. Therefore, in subsequent experiments, a mixture of PC and CE was subjected to enzymatic hydrolysis to determine the individual extents of hydrolysis.

In Fig. 5, the influence of increasing amounts of CEase on the positive ion MALDI-TOF mass spectra of a 1:1 mixture of cholesteryl linoleate (\( m/z = 671.6 \)) and PC 16:0/18:2 (\( m/z = 758.6 \) and 780.6) is shown. Trace (a) corresponds to the control sample (without CEase), whereas CEase activity was 0.6 U/ml in trace (b), 1.66 U/ml in trace (c), and 4.55 U/ml in trace (d). Peaks are marked according to their \( m/z \) ratios. Peaks labeled with asterisks correspond to the applied DHB matrix.

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**Fig. 5.** Influence of CEase on the positive ion MALDI-TOF mass spectra of a mixture of 0.5 mM cholesteryl linoleate and 0.5 mM PC 16:0/18:2. Spectrum (a) corresponds to the lipid extract of the pure lipids in the absence of the enzyme (control). Note that the peaks at \( m/z = 607.3 \) and 629.3 are caused by 2,5-dihydroxybenzoic acid (DHB) matrix adducts of sodium cholate that was added as a detergent. The mass region of the proton adduct of sodium cholate (\( m/z = 431.3 \)) is not shown for clarity. Reactions were carried out in 100 mM sodium cholate, pH 7.4, over 60 min at 37°C. CEase activity was 0.6 U/ml in trace (b), 1.66 U/ml in trace (c), and 4.55 U/ml in trace (d). Peaks are marked according to their \( m/z \) ratios. Peaks labeled with asterisks correspond to the applied DHB matrix.
trace (c), and 4.55 U/ml in trace (d). It should be noted that the addition of sodium cholate is a must when CE concentrations similar to those of the PC are to be digested. This reflects the different structures of PC and CE in an aqueous environment.

The intensity of the cholesteryl linoleate signal ($m/z = 671.6$) is lower than the intensity of PC ($m/z = 758.6$ and 780.6), although both compounds are present in equal amounts. This is caused by the presence of the quaternary ammonium group in PC that makes PC more sensitively detectable (33). It is obvious that CE is faster (and, therefore, preferentially) hydrolyzed by the enzyme compared with PC. When cholesteryl linoleate ($m/z = 671.6$) is already completely hydrolyzed, there are still clearly detectable peaks of PC ($m/z = 758.6$ and 780.6).

The same LPC species are detected as in the case of the lipoproteins and the isolated PC. However, it is obvious that hydrolysis of PC is not a random process, because in that case the same amounts of LPC 16:0 ($m/z = 496.3$ and 518.3) and LPC 18:2 ($m/z = 520.3$ and 542.3) would be generated. Because the generation of LPC 18:2 is obviously preferred compared with LPC 16:0, we assume that the favored cleavage site at the substrate is the sn-1 position and, therefore, that CEase primarily exhibits phospholipase A$_1$ activity.

**Influence of DOT-3 on lipoprotein hydrolysis**

The influence of the CEase inhibitor DOT-3 is shown in Fig. 6. In trace (a), the influence of CEase (2.5 U/ml) on human HDL in the absence of the inhibitor is shown. In traces (b) to (d), increasing amounts of the inhibitor DOT-3 were used [26 μg/ml (trace b), 130 μg/ml (trace c), and 260 μg/ml (trace d)] to decrease the effects of the CEase. It was clearly demonstrated that in the presence of DOT-3, the CE is not digested. This is most evident in trace (d), where the highest concentration of the inhibitor was used.

![Fig. 6. Positive ion MALDI-TOF mass spectra indicating the influence of increasing amounts of the CEase inhibitor 2-(diethylamino)-6,7-dihydro-4H,5H-cyclopenta[4,5]thieno[2,3-d][1,3]oxazin-4-one (DOT-3) on CEase activity. Human HDLs (1 mg/ml protein) were incubated in all cases with a fixed amount of CEase (2.5 U/ml) in 10 mM phosphate buffer, pH 7.4, containing 100 mM NaCl over 60 min at 37°C. Trace (a) is the control sample in the absence of the inhibitor. Concentrations of the DOT-3 inhibitor were 26 μg/ml in trace (b), 130 μg/ml in trace (c), and 260 μg/ml in trace (d). The inhibitor was added before CEase addition. All spectra were scaled according to the intensity of the most intense peak. The peak of cholesterol linoleate at $m/z = 671.6$ is marked with a vertical dotted line. For details, see the text. The asterisk in trace (a) denotes the matrix peak.](https://www.jlr.org/content/fig/6.png)
However, there is only a very small effect on the yield of LPC. Because the peak intensities of LPC species remain nearly constant even in the presence of a significant amount of the inhibitor, one must conclude that DOT-3 is capable of reducing the hydrolysis of CEs but not the cleavage of the PC. This tendency is not altered when the digestion is performed in the presence of sodium cholate (data not shown).

DISCUSSION

In this paper, the interaction between CEase and three artificial lipid systems (PC, PC in sodium cholate, and PC in a mixture with CE in sodium cholate) as well as HDL isolated from the blood of healthy volunteers was investigated. To the best of our knowledge, this was the first investigation that used MALDI-TOF MS to explore the effects of CEase on lipids and to monitor the influence of the inhibitor DOT-3.

It is known that DOT-3 decreases the capability of CEase to hydrolyze p-nitrophenyl butyrate; however, the effects of the inhibitor on activity toward components of lipoproteins and lipid vesicles had not been investigated. There is growing evidence that MALDI-TOF MS is a useful approach to determine the activities of enzymes (36). In contrast to established biochemical assays that are used to determine selectively the concentration of a certain metabolite (e.g., cholesterol), MALDI-TOF MS offers the significant advantage that different metabolites can be detected in a single experiment. Although individual compounds may be characterized by different detectabilities (with compounds containing quaternary ammonia groups such as PC or SM being most sensitively detectable), MALDI-TOF MS is very useful because unexpected products can also be determined (33). For example, using human HDL, we show in this study that CEase, besides the degradation of CEs, is also capable of hydrolyzing PC present in lipoproteins. This information could be obtained in a single experiment.

In this study, MALDI-TOF mass spectra were not quantitatively analyzed because only a commercially available CEase preparation was used, which was not carefully characterized with respect to its absolute activity. Therefore, the experiments we performed are only comparable relative to each other.

Although it is often stated that detergents such as Triton or sodium cholate are essential for CEase activity, experiments with HDL could be performed without such detergents. It is assumed that in the lipoproteins, the lipids are sufficiently solubilized and, thus, accessible for CEase. In contrast, however, suspensions of pure CE or even CE/PC mixtures are not hydrolyzed by the enzyme in the absence of a detergent, whereas isolated PC is easily hydrolyzed because of its amphiphilic properties.

Additionally, it became evident from our studies that the capability of CEase to hydrolyze CE as well as PC is influenced by the inhibitor DOT-3. Whereas relatively moderate concentrations of DOT-3 led to a complete loss of CE hydrolysis by CEase, no significant effects were observed on PC hydrolysis. This might indicate an incomplete inhibition of CEase under the conditions of a comparable high enzyme concentration used in this study. The reasons for the different influence of DOT-3 on CE or PC hydrolysis need to be elucidated in future experiments.

An additional advantage of MALDI-TOF MS in lipid research is the possibility of easily differentiating individual lysophospholipids. Under physiologically relevant conditions and in the majority of lipids, a saturated fatty acid (mainly palmitic acid or stearic acid) is located at the sn-1 position, whereas an unsaturated fatty acid (e.g., oleic acid or linoleic acid) is normally found at the sn-2 position (32). Therefore, the discrimination of the generated lysophospholipids allows for an estimation of phospholipase A₁ or phospholipase A₂ activity. However, MALDI-TOF MS of the CEase (31) did not yield any indication of further proteins present in the enzyme preparation as impurities.

Our experiments clearly indicate that CEase catalyzes the cleavage of both fatty acid residues of PL, in agreement with literature data (4, 37, 38). Because the yield of unsaturated LPC exceeded that of saturated LPC, it is obvious that cleavage at the sn-1 position is preferred. This confirms a previous study in which ¹⁴C-labeled PLs were used to show that CEase from rat exocrine pancreas predominantly exhibits phospholipase A₁ activity (38). The molecular reasons for these differences in the reaction course are unknown. We are currently performing experiments to determine whether this behavior may be caused by an enhanced access of the enzyme to the fatty acid residue at the sn-1 position or, consequently, the steric shielding of the ester linkage at the sn-2 position.

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