Published in final edited form as:
Lipids. 2008 March ; 43(3): 243–249. doi:10.1007/s11745-008-3151-z.

Identification of lysophosphatidylcholine-chlorohydrin in human atherosclerotic lesions

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Abstract

Lysophosphatidylcholine (LPC) levels are elevated in sera in patients with atherosclerosisand in atherosclerotic tissue. Previous studies have shown that reactive chlorinating species attack plasmalogens in human coronary artery endothelial cells (HCAEC), forming LPC and LPC-chlorohydrin (LPC-ClOH). The results herein demonstrate for the first time that LPC-ClOH is elevated over 60-fold in human atherosclerotic lesions. In cultured HCAEC, LPC-ClOH led to a statistically significant increase in P-selectin cell-surface expression. These data show that LPC-ClOH is elevated in atherosclerotic tissue and may have unique pro-atherogenic properties compared to LPC.

Keywords

Phospholipid analysis; Analytical Techniques, Atherosclerosis; Physiology, Coronary artery disease; Physiology, Inflammations; Physiology, Plasmalogens; Specific Lipids; Myeloperoxidase; reactive chlorinating species (RCS); plasmalogen; P-selectin; chlorohydrins; lysophosphatidylcholine

Introduction

Activated monocytes and a subpopulation of macrophages release myeloperoxidase (MPO), which is an enzyme that catalyzes the production of reactive chlorinating species (RCS) such as hypochlorous acid (HOCl) (1). Levels of MPO are elevated in atherosclerotic plaques (2), as well as MPO-derived oxidation products such as 3-chlorotyrosine (3) and 2-chlorohexadecanal (4). Plasmenylcholine is a subclass of glycerophospholipid enriched in the cells of the cardiovascular system, and is thought to play an important role in normal heart function, as well as serve as an endogenous lipoprotein antioxidant (5). Recently, our lab and others have shown that RCS attack of plasmenylcholine in liposomes and in human coronary artery endothelial cells (HCAEC) yields LPC (6,7) and LPC-chlorohydrin (LPC-ClOH) (7–9). Additionally, we have shown that HOCl preferentially targets LPC in comparison to PC (8). Since LPC is elevated in atherosclerotic lesions and is preferentially targeted by RCS *in vitro*, the present study was designed to determine that LPC-ClOH levels increase in atherosclerotic lesions. We show here, for the first time, that LPC-ClOH is elevated in atherosclerotic lesions, and that LPC-ClOH induces P-selectin cell-surface expression on HCAEC.

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Experimental Procedures

Plasmenylcholine (PC) 1-*O*-hexadec-1'-enyl-2-heptadec-10'-enoyl-GPC (16:0–17:1 PC), 1-O-hexadec-1'-enyl-2-octadec-9'-enoyl-GPC (16:0-18:1 PC), and 1-*O*-hexadec-1'-enyl-2-nonadec-10'-enoyl-GPC (16:0–19:1 PC) were synthesized by an anhydrous reaction utilizing 1-*O*-hexadec-1'-enyl-GPC and heptadec-10'-enoyl chloride, octadec-9'-enoyl chloride, and nonadec-10'-enoyl chloride, respectively, as precursors and dimethylaminopyridine as a catalyst. 16:0–17:1 and 16:0–19:1 plasmenylcholine were treated with 10-fold molar excess HOCl (10,11), which simultaneously cleaved the vinyl ether bond and formed 17:0 and 19:0 LPC–ClOH, respectively. The desired reaction product, LPC-ClOH, was purified by HPLC, quantified by Bartlett (12), extracted in chloroform and stored at −20 °C under N_2.

Human tissue

Atherosclerotic and normal aortic tissue was harvested from human postmortem autopsy specimens, rinsed, and submerged in PBS supplemented with 100 μM diethylenetriaminepentaacetic acid and 100 μM butylhydroxytoluene, frozen in liquid N_2, and stored at −80 °C until analysis. Frozen tissue was weighed and then crushed in a liquid nitrogen-chilled mortar and pestle. Lipids and internal standards 17:0 and 19:0 LPC-ClOH were added to the crushed tissue in methanol/chloroform/saline (2.5:1.5:1 v/v/v). After 5 min, chloroform was added resulting in a 1:1:0.8 methanol/chloroform/saline ratio (13). The extracted organic phase was stored in chloroform at −20 °C under N_2 until analysis.

Electrospray ionization – tandem mass spectrometry

Lipid extracts were reconstituted in methanol containing 10 μM NaOH and analyzed by ESI-MS in the direct infusion mode at a flow rate of 1–3 μL/min using Thermo Electron TSQ Quantum Ultra instrumentation. The choline glycerophospholipids yield intense [M-Na]+ adduct ions by electrospray ionization in the positive ion mode. Tandem mass spectrometry was performed on selected ions (collision energies were ~ 32–38 eV). To detect LPC and LPC–ClOH molecular species, total ion current (TIC) and neutral loss scanning of 95 a.m.u. (NL 95) was employed. Spectra were averaged at 3 to 5 minutes and processed utilizing Xcalibur software (Thermo), and molecular species were quantified by comparing the ion intensity of individual molecular species to that of the internal standards. The differences in ^{13}C isotope effects (Z_1) between the endogenous 16:0 and 18:0 LPC-ClOH and the internal standards 17:0 and 19:0 LPC-ClOH were corrected (14).

P-selectin surface expression

HCAEC, grown to confluence in 24-well plates, were incubated with indicated lipids in Hanks' buffer for 5 min at 37 °C in 95 % O_2/5 % CO_2 and P-selectin surface expression was determined as previously described (4).

Data analysis

Data were normalized to the respective control mean values and are expressed as means +/− SEM. Statistical analyses of data were performed by t-test and $P \leq 0.05$ was considered statistically significant.

Results

Lipid extracts from both atherosclerotic and normal human aorta samples were analyzed by ESI-MS to assess LPC-ClOH accumulation in atherosclerotic lesions. Initial inspection of the spectra from normal and atherosclerotic tissue in the positive ion mode show noticeable differences, qualitatively revealing an increase in LPC molecular species, which include *m/z*

518, 542, 544, 546, and 566, corresponding to 16:0, 18:2, 18:1, 18:0, and 20:4 LPC, respectively (Fig 1A). Neutral loss scanning of 95 amu, which corresponds to the combined neutral loss of the trimethylamine and HCl (8), was utilized to detect chlorinated choline glycerophospholipid species. Figure 1B (traces 1 and 2) show molecular ions with NL 95 scanning of molecular ions from normal tissue extracted in the presence of synthetic internal standards 17:0 and 19:0 LPC-ClOH at *m/z* 582 and 610, respectively. Figure 1B (traces 3 and 4) show molecular ions with NL 95 scanning from atherosclerotic tissue extracted in the presence of synthetic internal standards (e.g., *m/z* 582 and 610) and demonstrate the appearance of 18:0 LPC-ClOH at *m/z* 596. Atherosclerotic tissue also contains 16:0 LPC-ClOH (*m/z* 568).

Utilizing neutral loss scanning of 95 amu, the relative abundance of 18:0 and 16:0 LPC-ClOH were compared to the relative intensities of the internal standards for quantification. Atherosclerotic tissue contains approximately 67-fold more 16:0 LPC-ClOH and 82-fold more 18:0 LPC-ClOH than normal tissue (Fig 2). However, 18:0 LPC-ClOH is about 10.7-fold more abundant than 16:0 LPC - ClOH. This likely reflects the abundance of 18:1 LPC compared to 16:1 LPC in atherosclerotic tissue (see Figure 1A, spectra 2). Other unsaturated LPC molecular species, such as 18:2 and 20:4 LPC, are elevated in atherosclerotic tissue compared to control tissue (Figure 1A), however chlorohydrin molecular species of LPC were not observed as derivatives of those LPCs. It should be noted that others have shown that chlorohydrins of phospholipids containing polyunsaturated fatty acids are unstable and are not readily detectable (15).

Endothelial activation, and eventual dysfunction, is recognized as a major hallmark in the development and progression of atherosclerosis (16). LPC induces a wide range of pro-atherogenic effects (17), however the effects of LPC-ClOHs are unknown. Two critical events in the development of atherosclerosis are cell-surface expression of proteins that tether blood monocytes to inflamed endothelium and release of chemotactic molecules (18). Figure 3 shows that 10 μM 18:0 LPC-ClOH elicits HCAEC surface expression of P-selectin at levels similar to that elicited by platelet activating factor (4). The results suggest that LPC-ClOH may function to recruit leukocytes to the endothelium by inducing an increase in coronary artery endothelial cell surface expression of P-selectin, an adhesion factor implicated in the development of vascular inflammation (19).

Discussion

RCS attack of plasmalogens leads to the formation of LPC (6,7) and chlorinated lipids such as 2-chloro-hexadecanal (2-ClHDA) (20,21), and LPC-ClOH (7,8). RCS attack of phospholipid alkene bonds occurs at a significantly lower rate than attack of protein tyrosines (22) and phospholipid vinyl ether bonds (8). ClOH formation in PCs occurs at an even lower rate than LPC (8). Additionally, with the exception of α-chloro fatty aldehydes (4), no studies to date have provided direct evidence of resident chlorinated lipids within atherosclerotic lesions. In this study, we utilized a soft ionization technique, ESI-MS/MS, and determined that human atherosclerotic tissue contains 67-fold more 16:0 and 82-fold more 18:0 LPC-ClOH than normal tissue.

LPC is elevated in atherosclerotic lesions (4) and in sera of people with atherosclerosis (23). In cultured endothelial cells, depending on concentration and cell culture conditions, LPC increases permeability (24,25), induces chemotactic factors (26,27), enhances production of ROS (28–30), decreases the synthesis of tissue factor pathway inhibitor (31) increases the secretion of matrix metalloproteinase 2 (32) and leads to death (33,34). The LPC from oxidized LDL has been shown to impair endothelium-dependent relaxation in aortic ring preparations from several mammals (35,36), and only long-chain LPCs are effective in impairing endothelium-dependent relaxation (37). The effect of LPC in certain cases, however, may be

protective. LPC serum levels decrease in patients with advanced cancer (38) and sepsis (39), and administration of LPC led to decreased mortality in rodent models of sepsis from both Gram-positive and Gram-negative bacteria (40,41). Additionally, it has been shown that extracellular application of LPC suppressed tissue factor expression in human monocytes (42) and prevented injury by activated neutrophils in isolated perfused lungs (43).

RCS attack of plasmenylcholine leads to the production of a chlorinated aldehyde (2-ClHDA), LPC, and LPC-ClOH (7–9,20). Previous studies have demonstrated that 2-ClHDA is a potent neutrophil chemoattractant, while unsaturated LPC increases P-selectin surface expression on HCAEC (4,6). The results from this study show that 18:0 LPC-ClOH also leads to increased P-selectin surface expression on HCAEC, which may function to recruit more MPO-containing leukocytes to sites of inflammation. RCS attack of plasmenylcholine may lead to the production of molecular species that lead to both leukocyte attraction and adhesion. In conclusion, the studies herein demonstrate that lysophosphatidylcholine-chlorohydrins are present in human atherosclerotic plaques. These data also explore the potential biological roles of LPC-ClOH, showing increased P-selectin surface expression on HCAEC, and the data provide additional evidence for the involvement of MPO in atherosclerosis. Further studies will be essential to delineate the complex biological properties of the chlorinated lipidome.

Abbreviations

RCS, reactive chlorinating species; LPC, lysophosphatidylcholine; ClOH, chlorohydrin; LPC-ClOH, lysophosphatidylcholine-chlorohydrin; PC-ClOH, phosphatidylcholine-chlorohydrin; FFA-ClOH, free fatty acid chlorohydrin; HCAEC, human coronary artery endothelial cells; MPO, myeloperoxidase; GC–MS, gas chromatography–mass spectrometry; ESI-MS, electrospray ionization-mass spectrometry.

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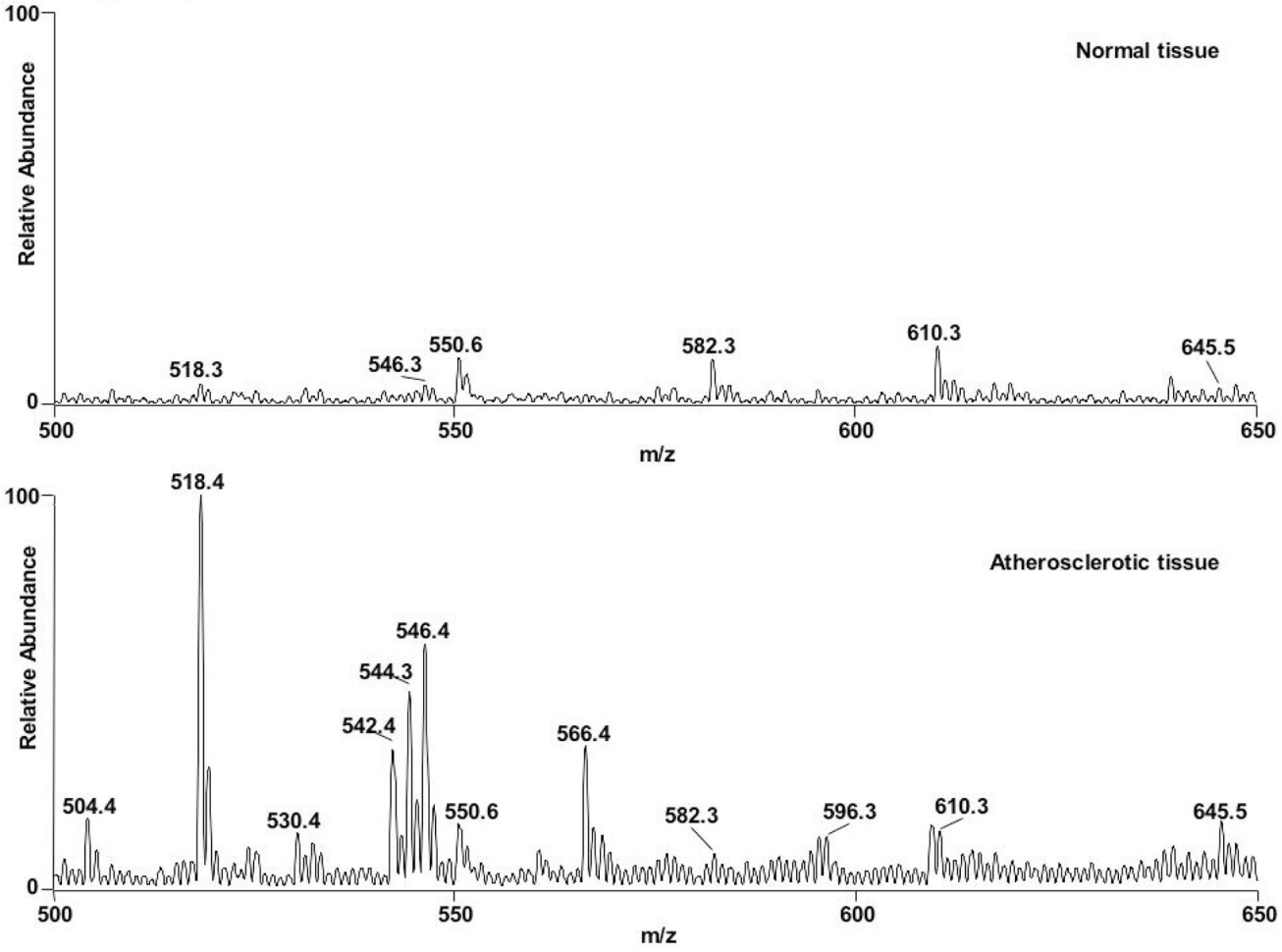
Figure 1, Panel A



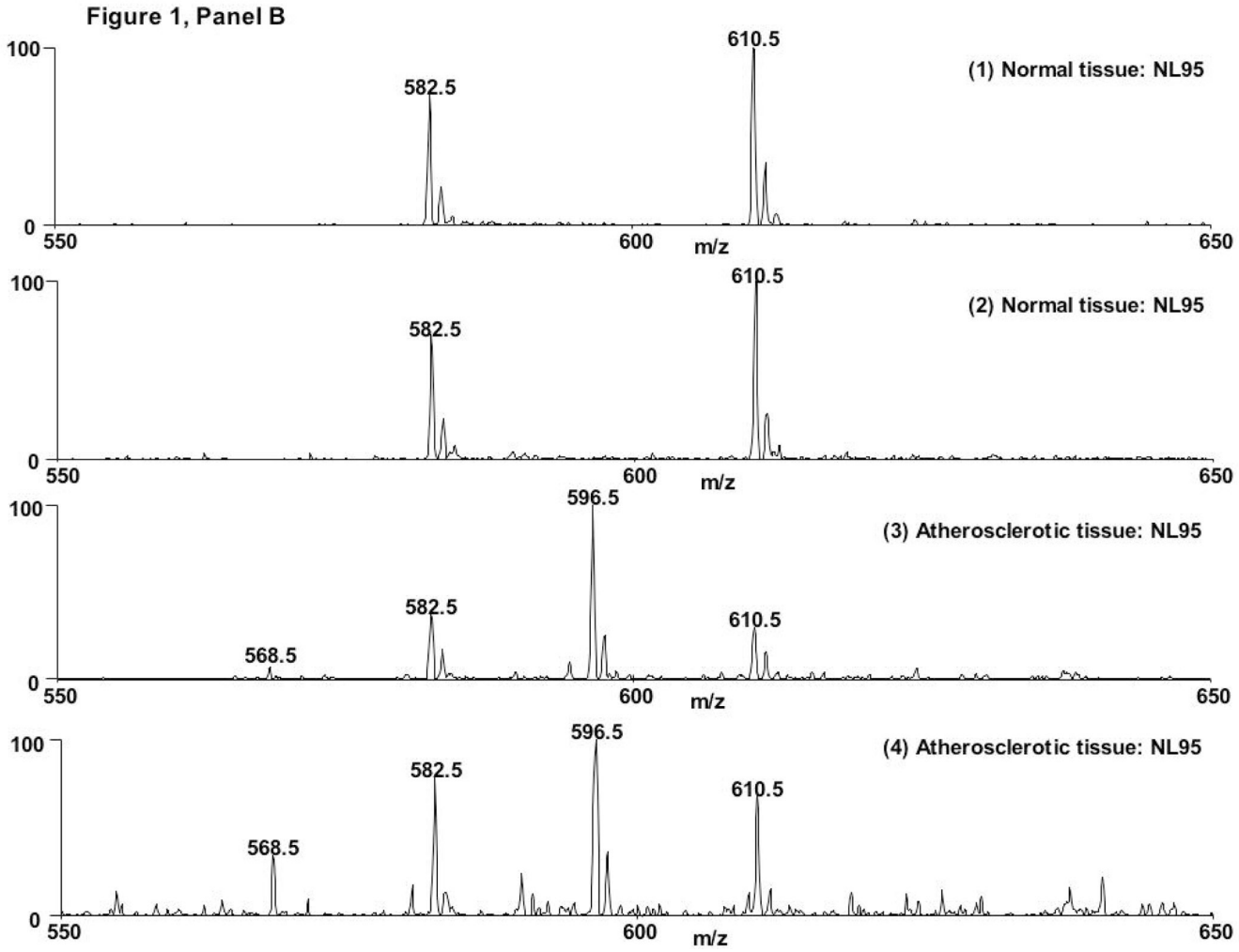


Figure 1. 18:0 LPC-ClOH is detected in atherosclerotic tissue

Lipids from normal and atherosclerotic aorta were analyzed by ESI-MS in positive ion mode with spectra from total ion current shown in (A) and neutral loss scanning of 95 amu (B). The spectra of molecular ions from normal tissue (B, spectra 1 and 2) show the synthetic internal standards 17:0 and 19:0 LPC-ClOH at *m/z* 582 and 610, respectively. The spectra of molecular ions atherosclerotic tissue contain 16:0 LPC-ClOH at *m/z* 568, 18:0 LPC-ClOH at *m/z* 596, and the synthetic internal standards (B, spectra 3 and 4).

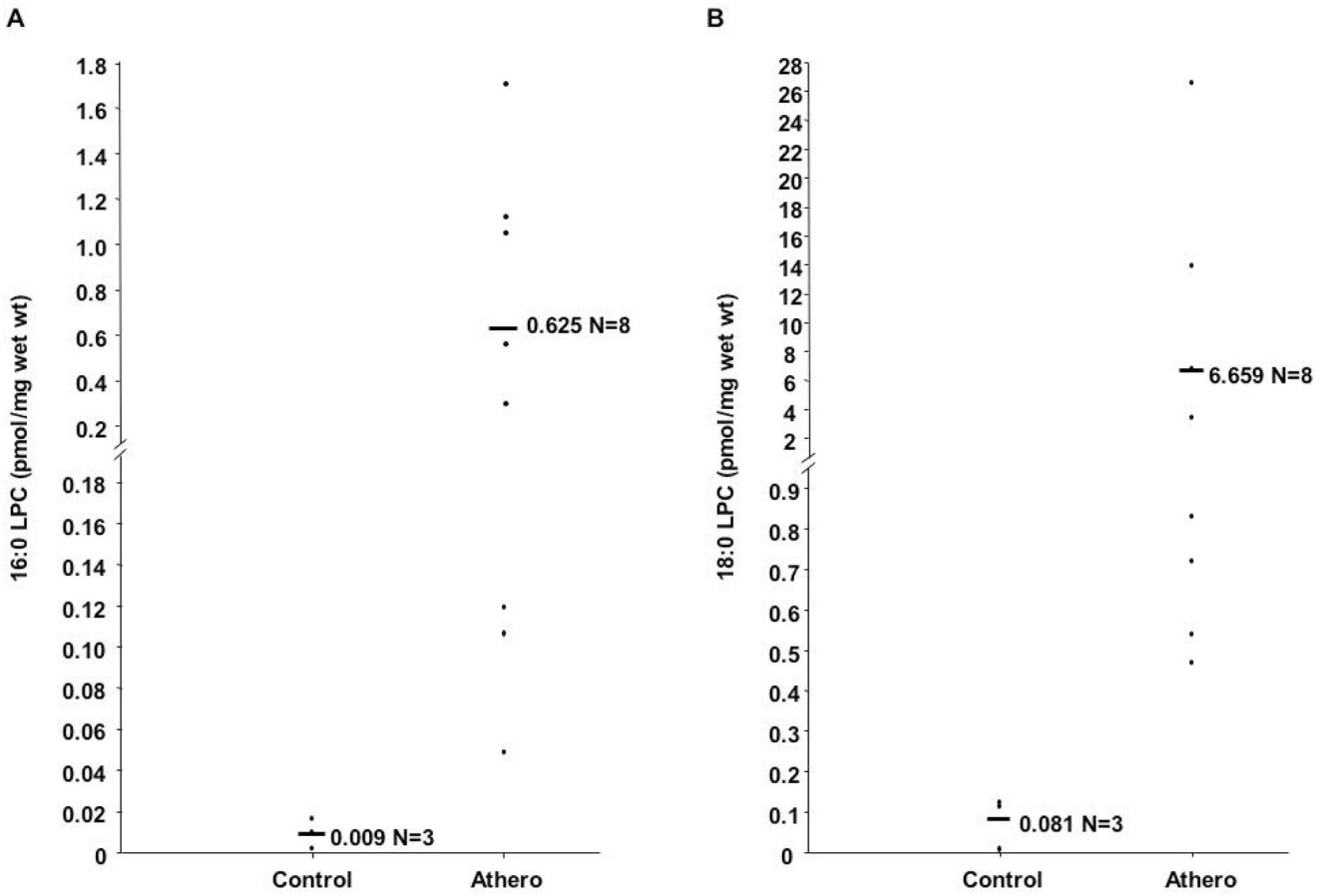


Figure 2. 16:0 and 18:0 LPC-ClOH molecular species are elevated in atherosclerotic tissue

Lipids from normal and atherosclerotic aorta tissue were extracted and analyzed by direct infusion ESI-MS using neutral loss scanning of 95 amu in positive ion mode. Quantification of 16:0 and 18:0 LPC-ClOH was performed by comparison to the relative intensities of the internal standards 17:0 and 19:0 LPC-ClOH, respectively, as described in “Materials and Methods.”

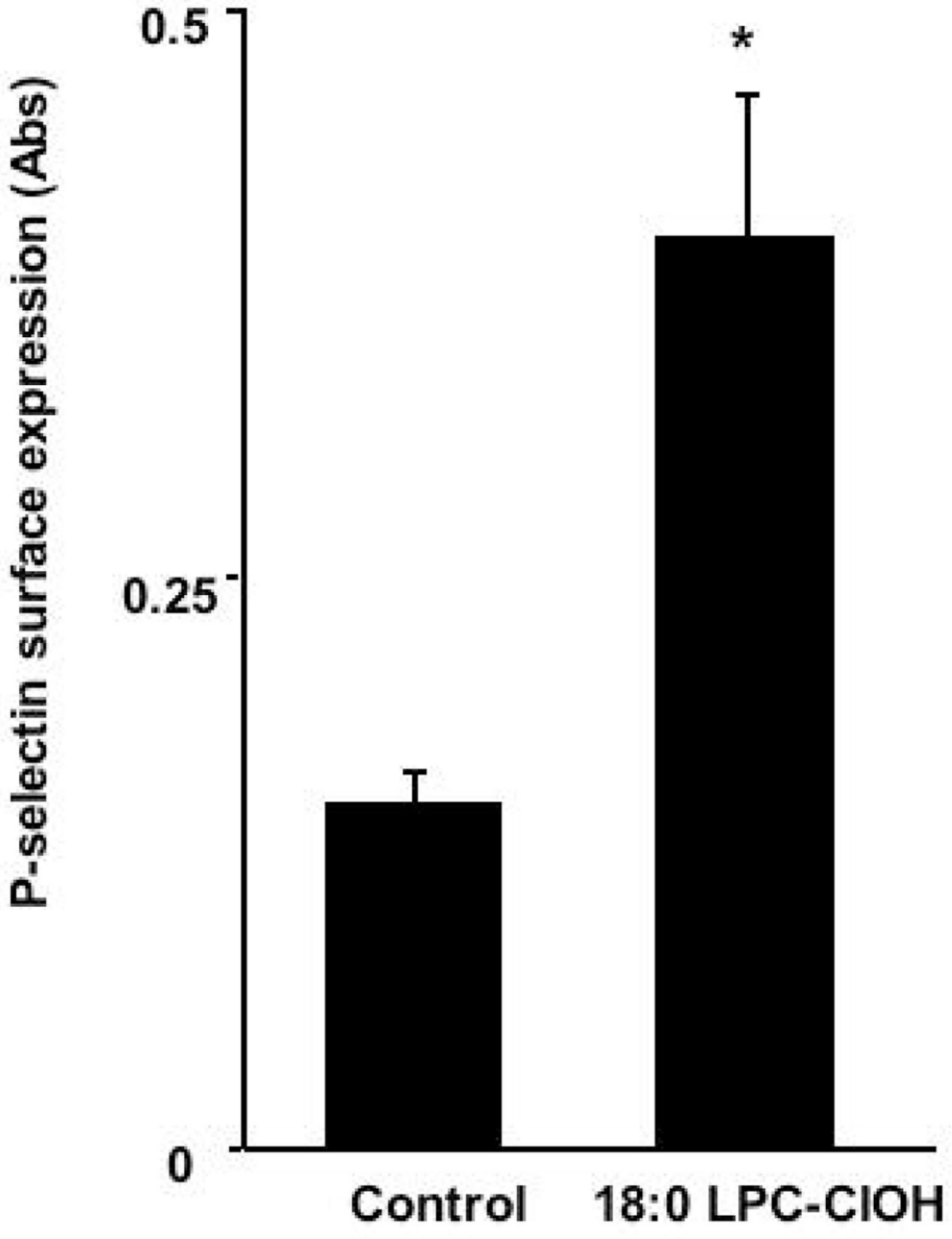


Figure 3. P-selectin is expressed on the surface of HCAEC following 18:0 LPC-ClOH treatment

HCAEC were treated with Hank's buffer alone (control) or buffer containing 10 μM 18:0 LPC-ClOH for 5 minutes. P-selectin surface expression was assayed and expressed as increased absorbance, $p < 0.01$.