Chemical analysis of atherosclerotic plaque cholesterol combined with histology of the same tissue

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Abstract **Sensitive method for chemical analysis of free cholesterol (FC) and cholesterol esters (CE) was developed. Mouse arteries were dissected and placed in chloroform-methanol without tissue grinding. Extracts underwent hydrolysis of cholesteryl esters and derivatization of cholesterol followed by liquid chromatography/mass spectrometry (LC/MS/MS) analysis. We demonstrated that FC and CE could be quantitatively extracted without tissue grinding and that lipid extraction simultaneously worked for tissue fixation. Delipidated tissues can be embedded in paraffin, sectioned, and stained. Microscopic images obtained from delipidated arteries have not revealed any structural alterations. Delipidation was associated with excellent antigen preservation compatible with traditional immunohistochemical procedures. In ApoE$^{-/-}$ mice, LC/MS/MS revealed early antiatherosclerotic effects of dual PPARα,γ agonist LY465606 in brachiocephalic arteries of mice treated for 4 weeks and in ligated carotid arteries of animals treated for 2 weeks. Reduction in CE and FC accumulation in atherosclerotic lesions was associated with the reduction of lesion size. Thus, a combination of LC/MS/MS measurements of CE and FC followed by histology and immunohistochemistry of the same tissue provides novel methodology for sensitive and comprehensive analysis of experimental atherosclerotic lesions.**—Kuo, M-S., J. M. Kalbfleisch, P. Rutherford, D. Giffor-Moore, X-d. Huang, R. Christie, K. Hui, K. Gould, and M. Rekhter. **Chemical analysis of atherosclerotic plaque cholesterol combined with histology of the same tissue.** ***J. Lipid Res.*** **2008.** 49: **1353–1363.**

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Atherosclerosis is a primary cause of heart attack and hence represents the major area of basic research as well as a drug-discovery focus (1). Wide application of genetically modified mice (2) demands development of quantitative analytical endpoints.

Conventionally, atherosclerotic burden in mouse models is evaluated by aortic planimetry (3–6). Aortic lesions, however, take several months to develop, which significantly hampers utility of this analysis, especially in drug discovery. Early atherosclerosis can be detected in specific locations (e.g., aortic root and brachiocephalic arteries) (3, 7). However, quantification of early lesions is largely limited to histology (8, 9), because, due to their small size, the amount of available tissue is often insufficient for biochemical analyses. Similar analytical challenges are typical to various models of accelerated atherosclerosis, where disease process is stimulated by local hemodynamic or mechanical factors in the carotid or femoral arteries (10–12). Development of highly sensitive and robust methodology is needed for fast and accurate analysis of these small lesions.

Chromatography coupled with mass spectrometry is a powerful methodology for analysis of biological samples. Combined liquid chromatography/mass spectrometry (LC/MS) has been increasingly found to be method of choice over GC/MS for many reasons. Chief among them are the ease of method development and higher throughput of the former method. However, analysis of free cholesterol (FC) and cholesterol esters (CE) in biological matrix is a major exception. To date, there are far fewer LC/MS methods than GC/MS methods for analysis of FC and CE. One major contributing factor is that the ionization of cholesterol under normal LC elution conditions is much less favorable than the GC/MS method, which utilizes electron impact. Recently, there are a few reports of measuring cholesterol and its oxides by LC-atmosphere pressure chemical ionization (APCI)-MS method (13, 14, 15). However, these techniques are not directly applicable to analysis of mouse atherosclerosis due to limited sensitivity and inability to measure CE.

In this paper, we describe a sensitive LC/MS-based technique facilitating simultaneous, independent quantification of FC and CE in the small arteries.

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Another major challenge in quantification of mouse atherosclerosis is that biochemical and morphological data are usually derived from different vascular beds within the same animal or from separate mice. We sought to develop a methodology that would facilitate gathering chemical (FC and CE) and morphological data from the same sample. We demonstrated that CE and FC could be quantitatively extracted from mouse arteries without tissue grinding and that lipid extraction simultaneously works for tissue fixation. Microscopic images obtained from delipidated arteries have not revealed any structural alterations. Delipidation was associated with excellent antigen preservation compatible with traditional immunohistochemical procedures. In this paper, we provide detailed description of a technique and demonstrate its utility for drug discovery.

MATERIAL AND METHODS

Materials

Cholesterol, cholesterol-2,2,3,4,4,6-$^{2}H_6$, pentafluorophenyl isocyanate (PFPI), and stearoyl chloride were purchased from Aldrich (Milwaukee, WI). Cholesterol-3,4-$^{13}C_2$ was purchased from Medical Isotopes Inc. (Pelham, NH). Sodium methoxide in methanol was also purchased form Aldrich. Solvents used for LC/MS were HPLC grade. Solvents used for extraction and sample preparation were either HPLC or reagent grade. All reagents were used as received.

Synthesis of 3,4-$^{13}C_2$-cholesteryl stearate

3,4-$^{13}C_2$-cholesteryl stearate was synthesized as described by Swell (16). In a 2-ml vial, 32.7 mg (0.084 mmol) of cholesterol-3,4-$^{13}C_2$ were weighed. A quantity of hexane just sufficient to wet the crystals was added followed by 40 μl (0.118 mmol) of stearoyl chloride. The mixture was warmed on a hot plate for a few minutes, and 7.0 μl (0.087 mmol) of dry pyridine were added. The mixture was heated at 80°C for 30 min. After 30 min the product was dissolved in a minimum amount of hot acetone and isolated by recrystallization.

Animal models

Experimental procedures using animals were approved by the Institutional Animal Care and Use Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Eight-week-old ApoE$^{-/-}$ mice were obtained from Taconic (Hudson, NY). The animals (n = 10–12 per group) were fed with Western diet containing 0.21% cholesterol, and 21% fat. Plasma lipids were analyzed on a Roche/Hitachi automatic analyzer 912 (Roche Diagnostics, Indianapolis, IN), and total cholesterol was used for animal randomization. To analyze early spontaneous atherosclerosis, the mice were euthanized 28 days after the beginning of Western diet feeding, and brachiocephalic arteries were dissected.

To accelerate lesion formation, ApoE$^{-/-}$ mice were prefed with the Western diet for 14 days, and then their left common carotid arteries were ligated under isofluorane anesthesia as described previously (11). Mice were kept on the same diet following the ligation surgery and were euthanized 14 days after ligation. Ligated and unligated (contralateral) common carotid arteries were dissected.

Drug treatment

To assess the utility of our new analytical approach to drug discovery, the animals were treated with the dual PPARα,γ agonist LY465606 at the dose 10 mg/kg/d by oral gavage as described previously (17). Control mice received vehicle solution (1% CMC, 0.25% Tween 80, Fisher Scientific, Fair Lawn, NJ). Drug effects were tested in early spontaneous lesions (brachiocephalic artery) and in accelerated lesions (carotid ligation).

Tissue extraction

Two methods of extracting cholesterol and its esters from arterial tissue were employed.

In the first method, cholesterol and cholesterol ester were extracted from the arterial tissue by the method of Folch (18). Tissue samples were transferred to Potter-Elvehjem type tissue grinders containing 0.5 ml of 2:1 chloroform/methanol and appropriate amounts of cholesterol- 2,2,3,4,4,6-d_6 and 3,4-$^{13}C_2$-cholesteryl stearate. Tissue samples were macerated manually until complete emulsification was observed. After maceration the pestle was rinsed with 0.5 ml of 2:1 chloroform/methanol, the 1.0 ml extract was transferred to a screw cap vial, and the tissue grinder was rinsed with an additional 0.5 ml of 2:1 chloroform/methanol. The rinsings were added to the vial to give a total of 1.5 ml of extract. Then 0.3 ml of water were added to the vial, and after vigorous shaking, the layers were allowed to separate on standing. After separation, the top layer was removed, along with any interfacial solid material, by siphoning with a pipet. The lower layer was then divided into two aliquots of equal volume, one aliquot to be used for FC determination and the second for total cholesterol determination after hydrolysis of cholesteryl esters. Each aliquot was placed in a 2-ml

TABLE 1. HPLC gradient conditions

Time, min	Flow Rate, ml/min	Composition
0.0	0.25	100% A 0% B
2.0	0.25	100% A 0% B
2.5	0.60	10% A 90% B
5.0	0.60	10% A 90% B
5.5	0.60	100% A 0% B
9.5	0.60	100% A 0% B
10.0	0.25	100% A 0% B

Ionization was effected by atmosphere pressure chemical ionization (APCI) in the negative mode using the following source parameters: discharge current 55 μA, vaporizer temperature 200°C, sheath gas 80, ion sweep gas 0, auxillary gas 30, ion transfer capillary 225°C. Collisonally activated decomposition was accomplished using argon @ 1.5 and a collision energy of 30 V.

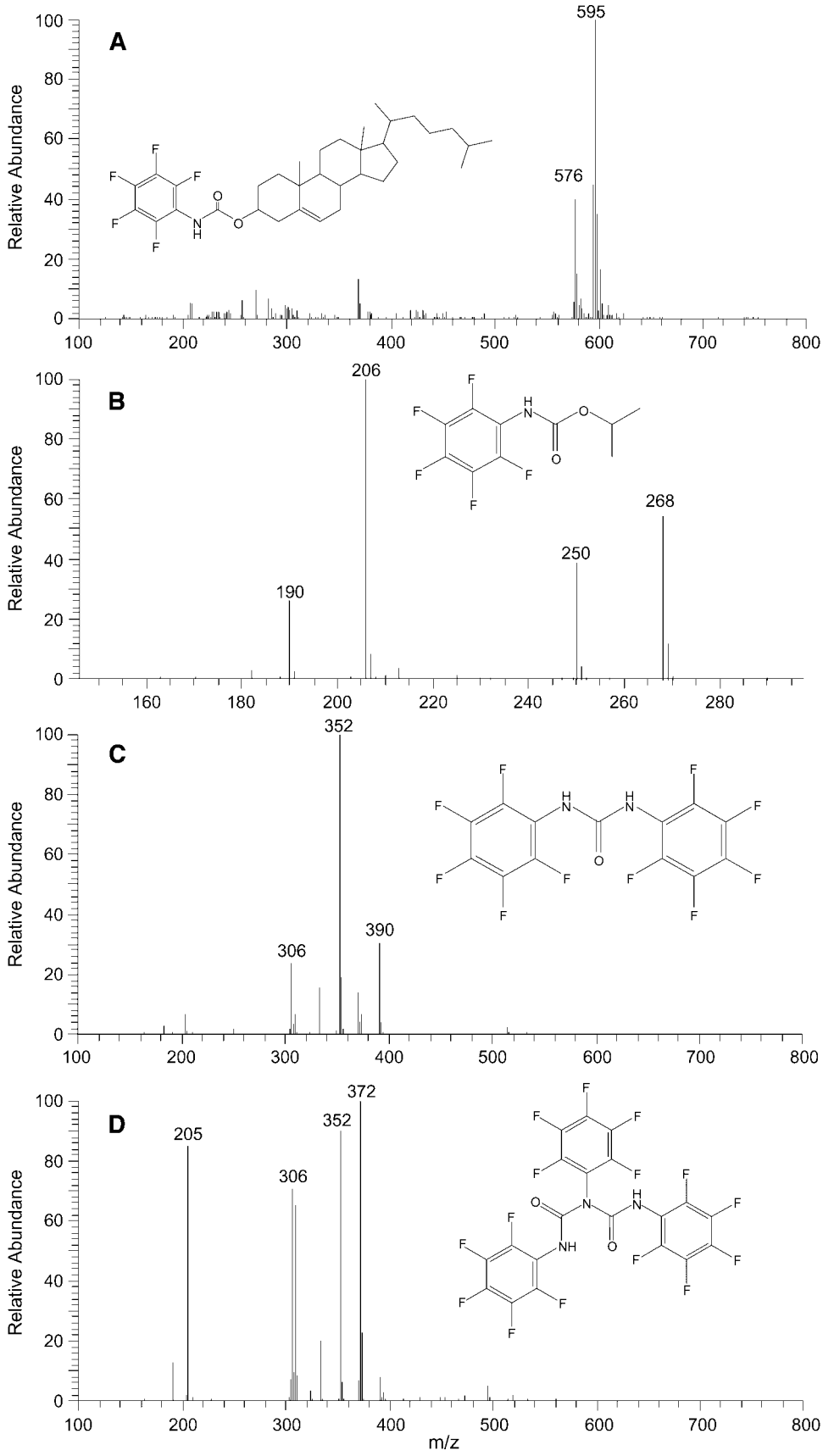

Fig. 1. Negative atmosphere pressure chemical ionization (APCI) mass spectra corresponding to peaks 1-4. A: cholesteryl pentafluorophenyl carbamate. B: isopropyl pentafluorophenyl carbamate. C: pentafluorophenyl urea. D: The biuret of pentafluorophenyl isocyanate (PFPI).

autosampler vial and then evaporated to dryness in preparation for hydrolysis and/or derivatization.

In the second method, tissue samples were immersed in 1 to 3 ml of 2:1 chloroform/methanol. After standing >16 h the tissue sample was removed from the extraction solvent. Appropriate amounts of cholesterol- 2,2,3,4,4,6-d_6 and 3,4-$^{13}C_2$-cholesteryl stearate internal standards were added and two 100-μl aliqouts were taken. Each aliquot was placed in a 2-ml autosampler vial and then evaporated to dryness in preparation for hydrolysis and/or derivatization.

To test the completeness of lipid extraction from the whole artery, we measured cholesterol and CE in the extracts obtained after incubation of the intact artery in chloroform-methanol (previously referred to as a second method), then reextracted already delipidated arteries using classical Folch technique (previously referred to as a the first method).

Hydrolysis of cholesteryl esters

Three hundred μl of dry tetrahydrofuran and 30 μl of a 25 weight % solution of sodium methoxide in methanol were added to the dried aliquot designated for hydrolysis. The vial was capped and heated at 60°C for 30 min. After 30 min, 10 μl of glacial acetic acid were added, and the sample was evaporated to dryness. One ml of hexane and 0.2 ml water were added. The mixture was shaken vigorously and the layers were allowed to separate. Then 0.9 ml of the hexane layer were removed and derivatized.

Derivatization of cholesterol

One ml of hexane was added to the dried extract, which was not hydrolyzed. Derivatization of the hexane solutions was effected by addition of 3 μl pyridine and 5 μl of PFPI. The reaction was allowed to proceed at room temperature for 10 minutes, during which time a white precipitate formed. After 10 minutes 100 μl of isopropanol was added to each vial, which redissolved the precipitate.

Standards

The cholesterol-d_6 and cholesterol-$^{13}C_2$ internal standard solutions were made up in toluene at 0.2 mg/ml and 0.3 mg/ml respectively. A 1 to 10 dilution of the cholesterol-$^2H_6$ solution was prepared for spiking calibration standards.

For the purpose of calibration, a stock solution of cholesterol was made up in toluene at 0.2 mg/ml. Calibration standards were prepared by diluting this solution and adding appropriate amounts of internal standard. The solution was then derivatized.

LC/MS/MS analysis

Derivatized extracts prepared by the first method were run on a Quattro LC (Waters Corporation, Milford, MA; Micromass, Waters Corporation, Milford, MA) triple quadrupole mass spectrometer, equipped with a Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD) comprising two LC-10ADvp pumps and an SCL-10Avp controller. Sample separation was effected by a normal phase isocratic method using an YMC-pack SIL column (150 × 4.6 mm, 5 μm, Waters). The mobile phase was 90/10 hexane/isopropanol at 1.0 ml/min. Ionization was effected by APCI in the negative mode using the following source parameters: corona pin, 3.0kV; cone, 40V; extractor, 0V; source block, 150C; probe, 350C; desolvation gas, 457L/hr. Detection was by multiple reaction monitoring (MRM). The transitions monitored were 595.4→206.0, 597.4→206.0, and 601.5→206.0 corresponding to cholesterol, cholesterol-$^{13}C_2$, and cholesterol-d_6, respectively. The collision energy was 60V and the collision cell pressure was 7.0×10^{-4} mbar.

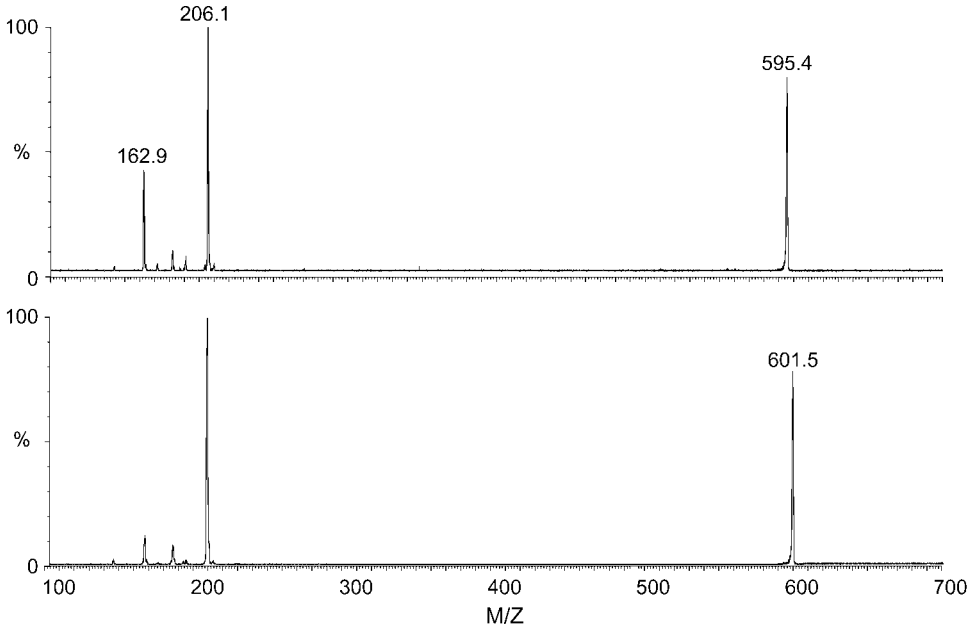

Fig. 2. Product ion spectra obtained from PFPI derivatives of cholesterol (*m/z* 595) and cholesterol-d_6 (mz 601).

Derivatized extracts prepared by the second method were run on a Thermofinnigan Quantum triple quadrupole mass spectrometer (Thermo Fisher Scientific, Inc. Waltham, MA), equipped with an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara CA) comprising a binary pump, a wellplate autosampler, and column heater. Sample separation was effected by a normal phase gradient method using a 2.0 × 150 mm YMC silica column, 3 μm particle size (Waters Corporation, Milford, MA). Eluant A was 95/5 hexane/isopropanol, and eluant B was isopropanol containing 0.1% acetic acid. The gradient is shown in **Table 1**. Ionization was effected by atmosphere pressure chemical ionization (APCI) in the negative mode using the following source parameters: discharge current 55 μA, vaporizer temperature 200°C, sheath gas 80, ion sweep gas 0, auxillary gas 30, ion transfer capillary 225°C. Collisonally activated decomposition was accomplished using argon @ 1.5 and a collision energy of 30 V.

Tissue processing for histology

For conventional histology, the mice were perfused with saline and then with eather 4% formalin or IHC Zink fixative (BD Pharmingen, San Diego, CA) via left ventricle. Brachiocephalic or common carotid arteries were dissected, immersed in the respective fixative for 24 h and paraffin embedded. To enable novel approach of sequestial lipid and histological analysis, the mice were perfused with saline via the left ventricle, the arteries were dissected and placed into 1 ml of 2:1 chloroform/methanol solution. After standing >16 h the tissue sample was removed from the extraction solvent. The solvent was utilized for cholesterol analysis, while delipidated vessels were paraffin embedded.

To explore potential influence of prolonged delipidation on the tissue antigen preservation, several vessels we kept in the solvent solution up to 7 days and then paraffin embedded.

Histology and immunohistochemistry

For histology, 10 equally spaced (200 μm) paraffin cross-sections were stained using modified Masson's trichrome (Sigma, St. Louis, MO) procedure that included elasin staining. Macrophages were visualized immunohistochemically using MAC-2 antibody (clone M3/38, Cedarlane Laboratories, Burlington, Ontario). Sections

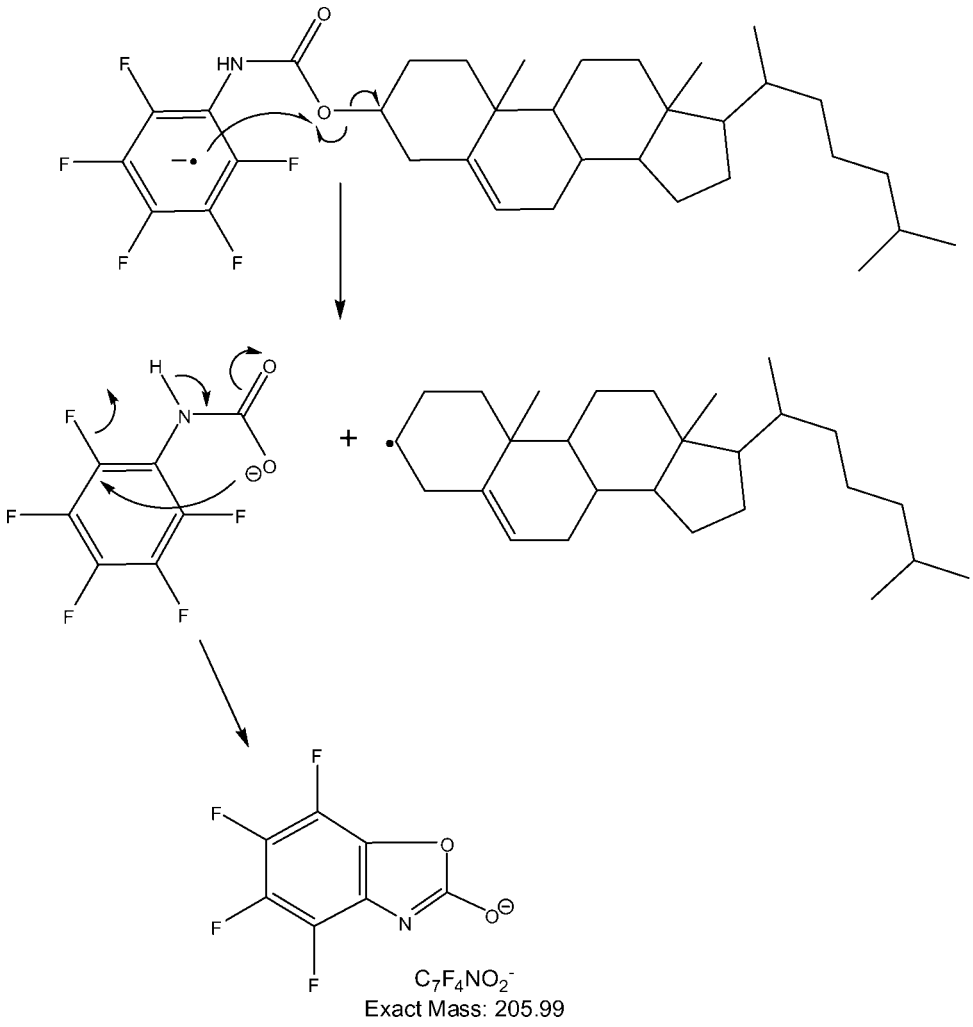

Fig. 3. Mechanism of formation of *m/z* 206 fragment from PFPI derivative of cholesterol.

were incubated for 30 min at room temperature. After primary antibody incubation, sections were incubated with biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA), followed by streptavidan-biotin complex (DAKO, Carpinteria, CA) and development with diaminobenzidine. Rat IgG staining was used as negative control. For smooth muscle cell visualization, α-smooth muscle actin EPOS (DAKO) was utilized. Sections were incubated for 60 min at room temperature followed by development with diaminobenzidine.

The lesion area, defined as an area between the lumen and internal elastic lamina, was calculated using Image-Pro Plus Version 5.0.1 (Media Cybernetics, Inc, Bethesda, MD).

Statistics

The results are shown as the mean ± SEM. Differences between groups were determined using one-way ANOVA with posthoc Dunnett's *t*-test. A value of $P < 0.05$ was regarded as a significant difference.

RESULTS

Characterization of cholesterol derivatives

The reaction product of PFPI and cholesterol was examined by LC/MS using full mass range acquisition (*m/z* 100–800). In addition to the cholesterol derivative, we observed the isopropyl carbamate of PFPI as well as products do due to reaction with water. The negative ion spectrum corresponding to the reaction product is shown in **Fig. 1A**. The molecular radical-anion is observed at *m/z* 595. A peak at *m/z* 594 probably arises from ionization by proton abstraction. The peak at *m/z* 576 corresponds to loss of a fluorine radical from the molecular radical anion, while the peak at *m/z* 367 corresponds to the elimination of pentafluorophenyl carbamic acid from the *m/z* 594 ion. Other observed byproducts from this reaction are isopropyl pentaflourophenyl carbamate, the biuret of PFPI, and the urea of PFPI (Fig. 1A, C, D). Thus, the only reaction product with cholesterol is the expected derivative.

Product ion experiments were performed on the cholesteryl carbamate in order to determine the most appropriate transitions for MRM experiments. Samples of cholesterol and cholesterol-d_6 were derivatized and infused into the APCI source of the Quattro LC triple quadupole MS. Product ion spectra derived from the molecular radical anions at *m/z* 595 and 601 are shown in **Fig. 2**. These compounds produce few fragments with the largest at *m/z* 206 in each case. Although not shown, the PFPI derivative of cholesterol-3,4-$^{13}C_2$ also produces this fragment in the same relative abundance. This fragment is dominant at all collision energies tested. Its mass, 206, corresponds to loss of the hydrocarbon portion of the molecule and a molecule of HF from the molecular radical anion (595– 69–20 = 206), suggesting that the fragment's elemental formula is $C_7F_4NO_2$. A structure having this formula and a plausible mechanism for its formation are shown in **Figure 3**. Based on this information MRM transitions from $M^{+\cdot} \rightarrow$ *m/z* 206 were chosen for quantitative analysis. In order to confirm

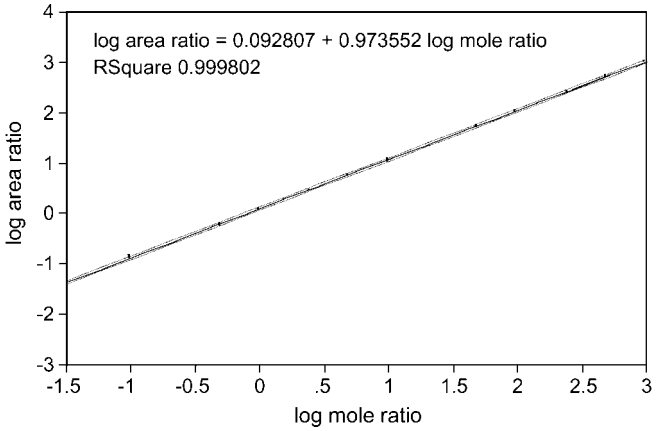

Fig. 4. Calibration curve. Area cholesterol/area cholesterol-d6 vs. moles cholesterol/moles cholesterol-d6.

that the response of cholesterol is equal to the response of cholesterol-d6, calibration solutions were made by diluting a cholesterol stock solution over the range 0.05–450 nmol/ml. An aliquot of 0.510 nmol of cholesterol-d6 were added to each and the solutions were derivatized as previously described. **Fig. 4** shows a log/log plot of the results. The plot is linear over four orders of magnitude, with a slope close to one.

To assess the accuracy of the method, solutions containing known amounts of cholesterol and cholesterol stearate were prepared in extraction solvent. The range of concentrations was chosen based on the range of analytes observed in preliminary experiments. Internal standards were added and the solutions were subjected to the assay as described above. The amount of cholesterol found was calculated by assuming that the response ratios between each analyte and its labeled internal standard were one. The results are shown in **Figures 5 and 6**. Neither slope is significantly different from one, indicating the amount found is equal to the amount taken. The coefficient of variation of the mean response was found to be 3%. From these data we concluded that the method is sufficiently accurate and precise to determine the amounts of FC and CE in mouse brachiocephalic or carotid arteries.

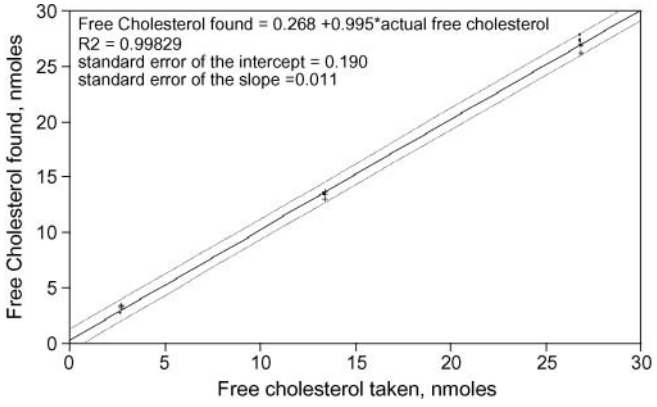

Fig. 5. Method accuracy for free cholesterol (FC).

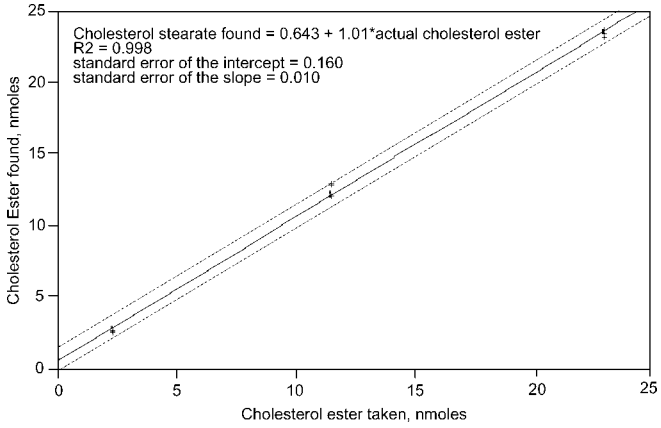

Fig. 6. Method accuracy for cholesterol ester.

The method employs two differently labeled internal standards, $^{2}H_{6}$-cholesterol and $^{13}C_{2}$-cholesterol ester, which allows us to correct for: (*a*) the isotopic enrichment of naturally occurring ^{13}C's, (*b*) the incomplete hydrolysis of the ester under the experimental condition, (*c*) detector response and sample volume variations between injections for each pair of samples, and (*d*) calibration standards for FC and total cholesterol. After the addition of the two ISTDs the samples are divided in half; one half is then subjected to total hydrolysis. The unhydrolyzed sample is used to determine the FC while the hydrolyzed sample represents the total cholesterol. The data analysis of the FC is straightforward as the cholesterol area is calibrated against the cholesterol-$^{2}H_{6}$ internal standard in the unhydrolyzed sample. To calculate the esterified cholesterol, we used data from both sets of samples. The ratio of (M+2)/M ratio of the derivatized cholesterol peak in the unhydrolyzed sample is used to correct for the naturally occurring C_{13} isotope contributions to the C_{13} labeled internal standard ion observed in the hydrolyzed sample. This corrected area represents the response of the $^{13}C_{2}$ labeled IS. The cholesterol/cholesterol-$^{2}H_{6}$ ratio in the hydrolyzed sample is then used to correct the total cholesterol peak area, observed in the hydrolyzed sample for the amount of FC observed in the unhydrolyzed sample. This corrects for the volume loss during hydrolysis and possible detector response variation between injections. The cholesterol ester area is then ratioed against the corrected cholesterol-$^{13}C_{2}$ area to determine the amount of cholesterol ester. This corrects for losses due to incomplete hydrolysis. We have found that this method allows us to estimate the free and total CE in a precise manner.

FC and CE ester analysis in mouse atherosclerotic plaques

Prior to the broad application of described chemical analysis to specific drug discovery projects, we decided to optimize and simplify lipid extraction procedure. Specif-

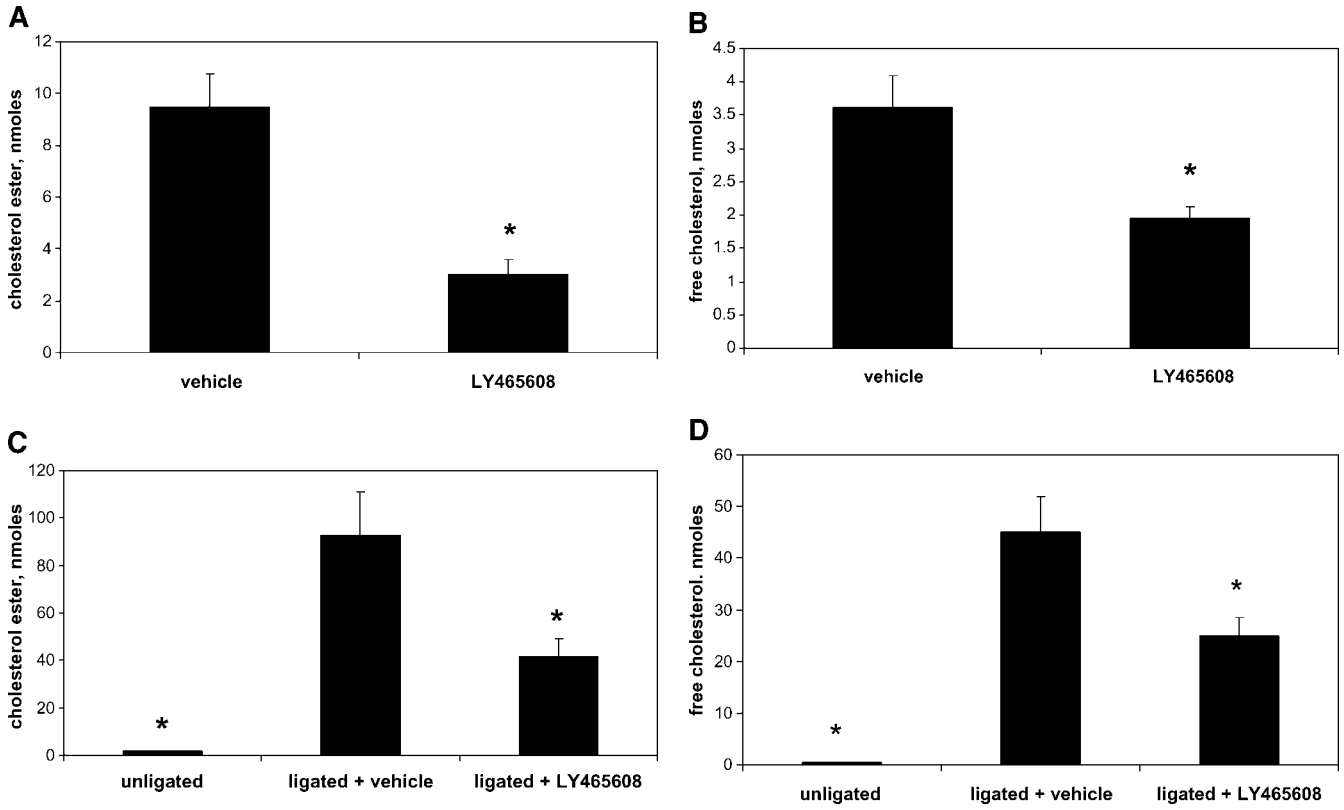

Fig. 7. Effects of LY465608 treatment on lipid accumulation in mouse atherosclerotic plaques. A, B: early atherosclerotic lesions in the BCA. C, D: accelerated atherosclerotic lesions in ligated carotid arteries. A, C: cholesterol ester. B, D: free cholesterol. Results are shown as mean ± SEM. Differences between groups were determined using one-way ANOVA with posthoc Dunnett's t-test. Asterisks indicate a significant difference ($P < 0.05$).

ically, we hypothesized that the lipids can be effectively extracted from the intact whole artery without tissue grinding. FC and CE were measured in the extracts obtained after incubation of the intact carotid artery in chloroform-methanol. Subsequently, already delipidated arteries were reextracted using classical Folch technique that involved grinding of the previously extracted tissues. Examination of 15 samples revealed that only 2.04 ± 1.88% of FC and 1.22 ± 0.98% of CE remained in the tissue after the first extraction thus confirming near complete lipid extraction from the whole artery. Hence, the whole artery extraction was used in the further studies.

FC and CE content was dramatically increased in BCA after 28 days of Western diet feeding that is consistent with the time course of atherosclerotic plaque formation. LY465608 treatment significantly reduced FC and CE accumulation thus revealing its antiatherosclerotic activity in the early vascular lesions (**Fig. 7A and B**). Similar analysis performed on the extracts from the left (ligated) and right (unligated) common carotid arteries in the flow cessation model of accelerated atherosclerosis demonstrated that FC and CE preferentially accumulated in the ligated arteries 14 days after ligation (Fig. 7C and D). It is consistent with the vascular lesion development in association with the blood flow cessation. In this model as well, LY465608 significantly inhibited FC and CE accumulation in lesions. Thus, LC/MS measurements provided required sensitivity to detect early antiatherosclerotic effects of LY465608 in two short-term animal models that are in agreement with the previously described results obtained in 18-week model using aortic en face planimetry (17).

Histology and immunohistochemistry

To test if chloroform-methanol used for lipid extraction would simultaneously work as histological fixative, we

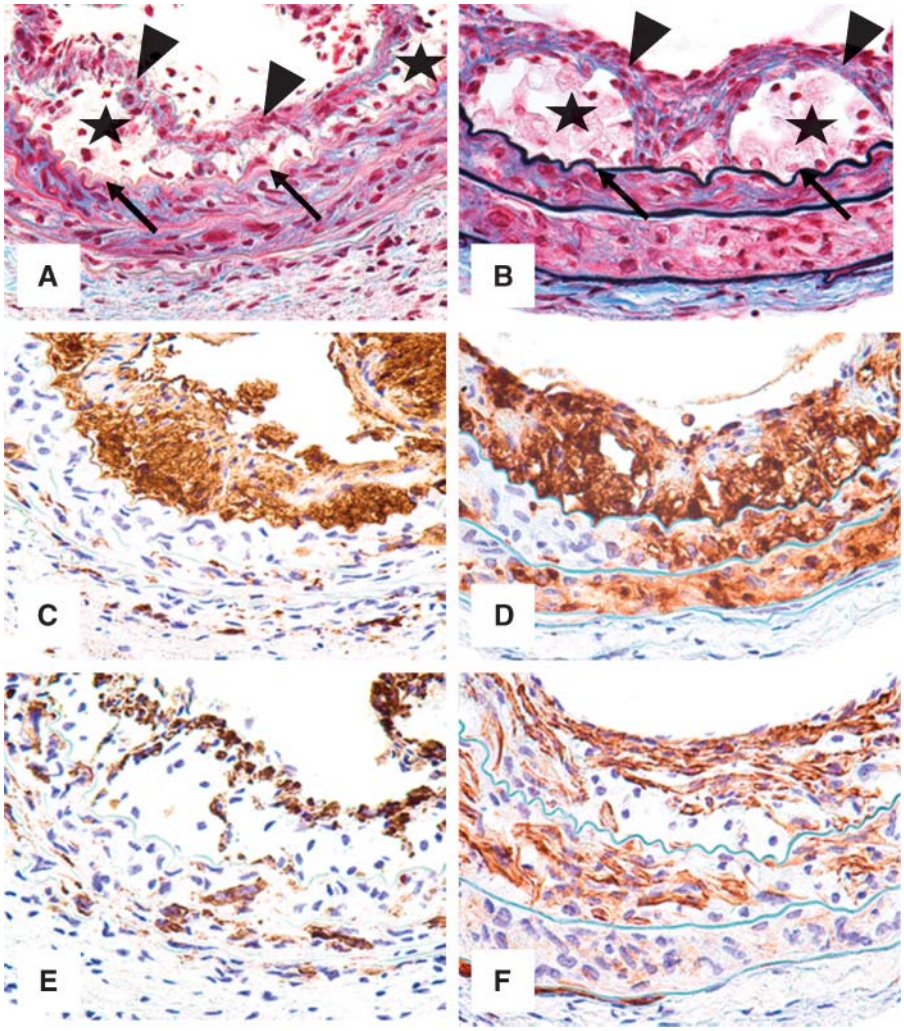

Fig. 8. Histology and immunohistochemistry of mouse carotid lesions. Left panel (A, C, E): after traditional (Zn-Tris) fixation. Right panel (B, D, E): after chloroform-methanol extraction. A, B: Masson trichrome staining (in B, modified by addition of elastin staining). Arrows show internal elastic lamina; arrowheads show fibrous cap; stars exemplify foam cells. C, D: macrophage immunostaining with Mac-2 antibody. Brown staining represents macrophages. E, F: smooth muscle immunostaining with α-actin antibody. Brown staining represents smooth muscle cells.

embedded both traditionally fixed as well as delipidated arteries in paraffin without additional fixation. Parraffin embedding was followed by conventional sectioning and staining. **Fig. 8** exemplifies high quality of routine histological staining (Masson trichrome) and immunostaining using macrophage-specific and smooth muscle-specific antibodies. Left column represents cross-sections of the ligated left common carotid artery obtained from the animals perfused-fixed with Zink-tris fixative and postfixed in the same fixative for 24 h (i.e., fixed and processed in the conventional manner). Right column shows cross-sections of the similar lesions derived from the mice that were perfused with saline only. The arteries were dissected and extracted in chloroform-methanol. While the extracts were analyzed by LC/MS, delipidated tissue samples were paraffin embedded without any additional fixation.

We have not noticed any difference in the affinity of various lesion components to histological dyes or antibodies in the samples that were paraffin embedded after delipidation compare with the conventionally fixed and processed ones. The same staining protocols (i.e., concentration of reagents, incubation time, temperature, etc.) were used in both cases. For immunostaining, no antigen retrieval was necessary. Importantly, when embedding of delipidated arteries was delayed for up to 2 weeks, quality of immunostaining was not impaired (data not shown). That feature alone adds more flexibility to the laboratory routine. Thus, our new approach provides similar qualitative data that are not inferior to the conventional technique.

Moreover, quantitative morphometric aspects of delipidated lesions are comparable with those of traditionally fixed and processed samples. **Fig. 9** demonstrates that mean intimal area of atherosclerotic plaques in BCA were comparable in two separate sets of experiments when the samples were fixed and processed in the traditional manner or delipidated without additional fixation. Noticeably, anti-atherosclerotic effects of LY465608 can be detected regardless of the sample preparation technique. It is currently unclear whether the minor difference in intimal area in the plaques processed by different techniques reflects higher level of tissue shrinkage in the methanol-chloroform extracted samples or represents mere experiment-to-experiment biological variability. Further studies are needed to elucidate this issue. It is also worth mentioning that morphometirc data shown in Fig. 9 were obtained from the same samples that represented in Fig. 7 A and B. It is evident that LY465608 effects on the plaque size are in agreement with its effects on CE accumulation. Thus, lipid extraction did not drastically influence quantitative results of microscopic image analysis. Altogether, it appears that proposed approach offers the benefit of LC/MS/MS-based lipid measurements without impairing either the quality of tissue immunostaining or the accuracy of morphometric analysis.

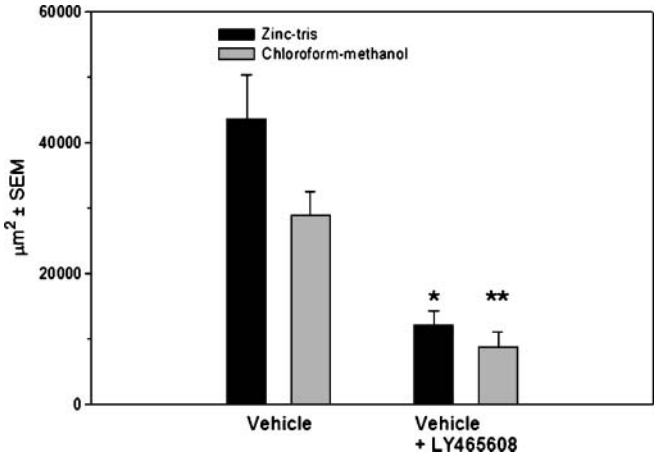

Fig. 9. Effects of LY465608 treatment on the mouse BCA atherosclerotic plaque area. In the first experiment (represented by the black bars), the samples were conventionally fixed (Zn-Tris) and processed. In the second experiment (gray bars), the samples were delipidated (chloroform-methanol extraction) without additional fixation before paraffin embedding. Note that these morphometric data were obtained from the same samples that are represented in Figure 7A and B. Single asterisk denotes statistically significant differences between the drug-treated and vehicle-treated samples that have been fixed in Zn-Tris (represented by the black bars). Double asterisk denotes statistically significant differences between the drug-treated and vehicle-treated samples that have been extracted in chloroform-methanol (represented by the gray bars).

DISCUSSION

We have developed a protocol facilitating simultaneous, independent analysis of FC and CE with high precision, sensitivity, and accuracy. It has been previously demonstrated that the alcohol functional group can be derivatized with electron capture reagents to take advantage of the low-energy ionization of negative APCI source for LC-MS (19). We found that that PFPI provides efficient and clean derivatization reaction for cholesterol over other reagents. More importantly, we found the extra nitrogen atom between the pentafluorobenzene and the benzoyl group provided better precision (data not shown). Poor precision of the derivatives such as pentafluorobenzoyl cholesterol can be attributed to the proton extraction of the cholesterol ring by the ring F atom.

We have demonstrated that early and accelerated atherosclerosis in transgenic mice can be accurately quantified using sensitive LC-MS/MS method. After lipid extraction, the same tissue samples can be used for histological and immunohistochemical analysis.

Conventionally, effects of various compounds or genes on atherosclerosis are studied in ApoE- or LDL receptor-deficient mice (2). Traditional endpoints include atherosclerotic coverage of aortas or cross-sectional histology. Atherosclerotic coverage is quantified by planimetry of en face preparations stained by Sudan IV or Oil Red O, the dies that preferentially bind neutral lipids (3–6). In essence, they demonstrate localization of CE in aortic intima. Although informative, this endpoint is not ap-

plicable for analysis of early atherosclerosis because aortic lesions take several months to develop. Another major limitation is that planimetry is based on the surface area measurements that ignore the lesion thickness.

Histological and immunohistochemical analyses are more sensitive and are widely used at the early stages of atherosclerosis. However, characterization of lipid accumulation, usually done by Oil Red O staining (20), has to be performed on frozen sections. Paraffin embedding is not compatible with this technique because lipids are extracted from the tissue during dehydration process. Although optimal for lipid staining and immunostaining, frozen sections are not ideal for quantitative morphological analysis (e.g., measurements of plaque area on serial sections), because of tissue distortions. Paraffin embedding offers substantially better preservation of tissue morphology. Also, planimetric and histological analyses are conventionally performed using different tissues (i.e., aortas for en face staining and aortic valve area [aortic root] or brachiocephalic artery for histology). If any biochemical analysis is involved, it demands different groups of mice.

Our approach provides opportunity to use the same piece of tissue for chemical analysis of FC and CE and histology. FC and CE can be nearly completely extracted without grinding the tissue. Therefore, tissue structure could be preserved and hence analyzed after lipid extraction. We demonstrated that chloroform-methanol mixture, conventionally used for lipid extraction, could simultaneously work as histological tissue fixative. Similar solutions (e.g., Methanol-Carnoy's [methanol, chloroform and acetic acid]) are used for tissue fixation due to their ability to precipitate proteins (21, 22) and have been utilized for immunohistochemical analysis of atherosclerotic lesions (23, 24). Methanol alone has been also successfully used for tissue fixation (25). Precipitating fixatives, including chloroform-methanol, provide additional experimental benefits because they do not bear the danger of tissue "over-fixation" characteristic to aldehyde-based cross-linking fixatives (26, 27). This feature provides significant experimental and logistics convenience.

High sensitivity and precision of cholesterol measurements facilitate chemical analysis in the small segments of arteries thereby enabling analysis of early and/or locally accelerated atherosclerosis. In the current study, LC/MS measurements provided required sensitivity to detect early antiatherosclerotic effects of LY465608 in two short-term animal models that are in agreement with the previously described results obtained in an 18-week model using aortic en face planimetry (17). Ability to follow-up with morphological analysis of the same sample can improve overall quality of analysis and reduce the number of animal experiments. This feature is important from both humane and economic perspectives.

Thus, LC/MS/MS of FC and CE followed by histology of the same sample provides a novel approach to quantitative, comprehensive analysis of mouse atherosclerotic plaques that can be utilized in basic science research and drug discovery applications.

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