Quantification In Situ of Crystalline Cholesterol and Calcium Phosphate Hydroxyapatite in Human Atherosclerotic Plaques by Solid-State Magic Angle Spinning NMR

Wen Guo, Joel D. Morrisett, Michael E. DeBakey, Gerald M. Lawrie, James A. Hamilton

Abstract—Because of renewed interest in the progression, stabilization, and regression of atherosclerotic plaques, it has become important to develop methods for characterizing structural features of plaques in situ and noninvasively. We present a nondestructive method for ex vivo quantification of 2 solid-phase components of plaques: crystalline cholesterol and calcium phosphate salts. Magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectra of human carotid endarterectomy plaques revealed $^{13}$C resonances of crystalline cholesterol monohydrate and a $^{31}$P resonance of calcium phosphate hydroxyapatite (CPH). The spectra were obtained under conditions in which there was little or no interference from other chemical components and were suitable for quantification in situ of the crystalline cholesterol and CPH. Carotid atherosclerotic plaques showed a wide variation in their crystalline cholesterol content. The calculated molar ratio of liquid-crystalline cholesterol to phospholipid ranged from 1.1 to 1.7, demonstrating different capabilities of the phospholipids to reduce crystallization of cholesterol. The spectral properties of the phosphate groups in CPH in carotid plaques were identical to those of CPH in bone. $^{31}$P MAS NMR is a simple, rapid method for quantification of calcium phosphate salts in tissue without extraction and time-consuming chemical analysis. Crystalline phases in intact atherosclerotic plaques (ex vivo) can be quantified accurately by solid-state $^{13}$C and $^{31}$P MAS NMR spectroscopy. (Arterioscler Thromb Vasc Biol. 2000;20:1630-1636.)

Key Words: magic angle spinning NMR ■ crystalline cholesterol ■ calcium phosphate hydroxyapatite ■ phospholipids ■ atherosclerotic plaques

There is renewed interest in the ultrastructure of atherosclerotic plaques for several reasons. Recent work has shown that plaque stability, which is dependent on composition and ultrastructure, plays an important role in cardiovascular disease. Plaques with a lipid-rich core and a thin, fibrous cap are more likely to rupture, leading to thrombus formation.1-3 Some ultrastructural features underlying plaque stability are now known in sufficient detail to predict plaque stability. The thickness of the fibrous cap and the infiltration of macrophages and T cells are highly correlated with plaque vulnerability and rupture.4 Because there is new evidence that certain diets and drugs may promote regression of plaques,5 it is important to determine the relationship between the physicochemical properties of plaques and regression potential. Lipids present in different phases have different physical properties (eg, diffusion rates), which are directly related to the rate of lipid egress and plaque regression.6 Therefore, quantitative knowledge of the lipid phases in a plaque in situ is crucial.

Cholesterol is 1 of the major lipids found in atherosclerotic plaques.7 At the early stage of plaque development, cholesterol is incorporated mainly into phospholipid bilayers in the liquid-crystalline phase and, to a lesser extent, is partitioned into liquid cholesteryl esters (CEs).7 With progression of the plaque, cholesterol accumulates to higher levels, saturating the liquid and liquid-crystalline phases and forming a separate crystalline phase at body temperature. Methods to date have not succeeded in providing data about the amounts of different lipid phases, particularly the crystalline phase. The total cholesterol in a plaque can be measured by chemical analysis of extracts of homogenized tissue, but information about the physical state and molecular interactions of cholesterol, as well as other lipids, cannot be obtained with this approach. X-ray diffraction and optical microscopy are useful for identification of lipid phases8 but are usually not efficient for quantitative analysis. Raman spectroscopy has been used to quantify lipids in plaques ex vivo,9,10 but this method has not distinguished lipids in different phases.

Calcium phosphate hydroxyapatite (CPH) is another solid component often present in advanced atherosclerotic lesions. Calcification is an active, regulated process and is probably...
involved in the very early development of atherosclerotic lesions.11,12 Potential links between calcification and atherosclerosis have recently been proposed.13 Whereas natural or synthetic calcification can be detected by x-ray diffraction (CT),14 B-mode echography,15 or MRI,16 quantification of calcium salts in biological samples still requires that the tissue sample be delipidated and then digested with concentrated acid before standard chemical assays are performed.17,18 Therefore, introduction of simple and reliable instrumental methods to quantify the plaque composition (ex vivo) will be useful for the interpretation and calibration of the in vivo measurements.

NMR spectroscopy is a nondestructive method that detects the magnetic resonances of nuclei with different chemical environments. NMR spectroscopy studies of human atherosclerotic tissues date back to the early 1970s but were limited mostly to the identification and quantification of the lipids in different liquid phases.19,20 Newer applications of solid-state NMR with magic angle sample spinning (MAS NMR) extend the feasibility of using NMR to study anisotropic biological samples, as documented by reports from our laboratory21–23 and others.24 Our previous study of atherosclerotic plaques focused on establishing protocols to identify different plaque components in a qualitative manner. The current study presents new and detailed protocols to quantify the crystalline cholesterol and calcium phosphate in situ by MAS NMR. The application of this method is demonstrated by using human carotid plaques as the biological sample.

Methods

Plaque Samples

Tissues obtained from endarterectomy specimens, which included atherosclerotic plaques and some adherent intima and media, were dissected, beginning at the common carotid artery and extending to 1 to 2 cm below the bifurcation and ~1 cm into the internal carotid artery. Except as noted below, tissues were fixed in 10% formalin–Tris-buffered saline buffer immediately after excision at the Methodist Hospital, Houston, Tex. Formalin stabilizes the tissue by cross-linking its proteins but does not alter the physical properties of lipids in the plaque.25 Fixed plaque samples were shipped to Boston at ambient temperature. For NMR analysis, samples were cut into a suitable size (~6 × 4 × 2 mm) and then transferred to a 7-mm ZrO2 rotor without buffer. Two fresh tissue samples were shipped in 50% glycerol/PBS with “blue ice.” These specimens were studied by 13C MAS NMR spectroscopy before and after formalin fixation. No significant differences in the 13C spectra of fixed and unfixed tissue were detected.

Chemical Preparation

Cholesterol (>99% pure as checked by thin-layer chromatography) was purchased from Nu-Chek Prep and used without further purification. Cholesterol monohydrate crystals were prepared by recrystallization of anhydrous cholesterol in ethanol/water (70:30, vol/vol).21 Synthetic CPH was purchased from Aldrich Chemical Co and used without further purification. Chicken wings were purchased at a local supermarket (Boston, Mass) and boiled. The cleaned bones were ground into powder for NMR and x-ray diffraction experiments.

NMR Spectroscopy

Experiments were performed on a Bruker AMX-300 spectrometer equipped with solid-state and MAS accessories. A standard cross-polarization (CP) pulse sequence or a dephased CP pulse sequence was used for the study of crystalline cholesterol (2-ns contact time, 45-μs dephasing delay), with a sample spinning rate of 5 kHz. High-power proton decoupling (65 W) was used in all 13C experiments to remove the C-H dipolar couplings. A standard direct-polarization observation pulse sequence with a moderate 1 H decoupling power level (~25 W) was used to study phosphorus in calcified plaques with a sample spinning rate of 4 kHz. Although significant 1H–3P interactions have been described in phospholipid bilayers,26 such interactions are minimal in CPH [3Ca(OH)2Ca(OH)2] because the dipolar interaction decays in proportion to 1/r6 of the internuclear distance. Therefore, a moderate 1 H decoupling was sufficient to remove line-broadening effects by any other nonspecific 1H–3P interactions.

To optimize the sample temperature for the NMR experiments, preliminary tests were performed at ambient temperature (25°C), 37°C, and 50°C. Crystalline cholesterol monohydrate, liquid-crystalline cholesterol, and CPH were thermotropically stable in this temperature range, and the spectral features of these components were essentially unchanged. The results reported in this study were acquired at ambient temperature (~25°C) to maximize sample preservation. It is important to emphasize that calibration of signal intensities must be done for each probe/spectrometer and must be performed at constant-temperature as well as constant-acquisition conditions.

X-Ray Powder Diffraction

X-ray powder diffraction patterns were recorded using nickel-filtered CuKα radiation from an Elliot GX-6 rotating-mode generator (Elliot Automation) equipped with a camera using Franks double-mirror optics.27 The sample (~1.5 mg) was packed into 1.0-mm-diameter Lindeman capillary tubes (Charles Super) and sealed. Diffraction experiments were performed at room temperature. The sample-to-film distance was calibrated by using the data of a standard material (crystalline cholesterol myristate).

Chemical Analysis

After completion of the NMR experiments, the tissue sample was freeze-dried overnight to constant weight. Tissue dry weight was measured by direct weighing on an electric balance. Lipids were then extracted by the Folch protocol.28 The total lipid was measured as the dry weight of the lipid extract, and the quantities of specific lipids were measured by standard methods.28–31 Folch extraction has been widely tested and accepted as 1 of the standard extraction protocols for the analysis of lipids in biological samples.32

Results

Quantification of Cholesterol Monohydrate in Human Carotid Plaques

In atherosclerotic plaques, cholesterol can exist in 3 forms: solubilized in the isotropic CE phase; a liquid-crystalline form within phospholipid bilayers (CholLiqCr); and a crystalline form (cholesterol monohydrate [CholM]). The distribution of cholesterol between the crystalline and liquid-crystalline phases that predominate in the plaque cannot be directly estimated by chemical analysis because this information is lost when the lipids are extracted into the same organic solution. Furthermore, the incorporation of cholesterol into phospholipid bilayers in atherosclerotic plaques depends on their sphingomyelin content,33,34 as well as other (unknown) factors,35 making it difficult to predict the exact amount of liquid-crystalline cholesterol in the bilayer structures.

Previously, we demonstrated that CholM and CholLiqCr can be identified without interference from other plaque components in separate 13C MAS NMR experiments with pulse sequences optimized to highlight each phase separately.23 It would be ideal if both of these phases could also be quantified in situ. However, we were not able to calibrate the signal intensity of CholLiqCr in plaques (see below), probably because of variations in the phospholipids, the more heterogeneous environments that might exist in the lamellar...
phase, and/or the motion characteristics of the CholLiqCr in the plaque. On the other hand, CholM in plaques is relatively inert and essentially the same as chemically pure cholesterol monohydrate. Therefore, we have investigated the feasibility of quantifying CholM in situ and then estimating Chol-LiqCr by using the following equation: CholM + CholLiqCr + cholesterol (iso) = total cholesterol, where cholesterol (iso) represents the amount of cholesterol in the isotropic CE phase. Total cholesterol and CE can be analyzed by chemical analysis after NMR experiments, and cholesterol (iso) can be calculated from its solubility in CE, which has already been established (≈5.2% to 5.5% by weight). Because of its low abundance, cholesterol in this pool has not been detected in natural-abundance NMR spectra of plaques.

As shown in Figure 1, the 13C CP MAS NMR spectrum of a typical carotid plaque contains signals arising from CholM, CholLiqCr, phospholipids, and proteins and CP MAS efficiently filters out most signals from noncrystalline lipids. In the spectrum of Figure 1, the C5 twin peaks are well resolved from other resonances and are also symmetrical as they are for pure CholM, indicating that the signal contribution from the CholLiqCr phase is negligible. Otherwise, the downfield peak would be more intense because its chemical shift is close to that of the C5 peak of cholesterol in a liquid-crystalline phase of a simple model system. (It is not clear why the C5 peak from the CholLiqCr phase in the plaque was not detected, but this is another indication of the problems encountered when attempting to quantify CholLiqCr as discussed above.) Therefore, quantification of CholM in principle can be achieved by integration of the C5 twin peaks without interference from the CholLiqCr phase. A prerequisite for cholesterol quantification in plaque is demonstration of the accurate quantification of CholM.

Figure 1. 13C dephased CP MAS NMR spectrum of human carotid plaque. The spectrum was obtained with 28,000 scans and processed with 10-Hz line broadening. Selected resonances were identified as previously described: protein carbonyl (C=O), fatty acid olefinic (C=C), terminal methyl (CH3), and cholesterol resonances (C, followed by IUPAC carbon numbers).

Figure 2. CP MAS NMR spectra of varying amounts of CholM (C5 region only; left) obtained under the same experimental conditions as in Figure 1. Linear least-squares line fitting of integrated C5 peak intensity as a function of cholesterol mass (middle). Error bars represent experimental uncertainty for the integrated signal intensities (samples with ≈2 mg cholesterol) or for the peak height intensities (samples with ≈2 mg cholesterol) after repeated NMR experiments. Selected spectra (C5 regions; right) of carotid plaques, with compositions corresponding to those of sample Nos. 1, 2, 3, and 4 listed in Tables 1 and 2.
Spectra of pure CholM of varying quantities were obtained under identical conditions. Selected spectra (C5 region) are shown in Figure 2 (left). The integrated intensities of the twin peaks and the corresponding sample quantity (Figure 2, middle) gave a linear relationship. The linear least-squares fit of these data can be used as a calibration curve for the quantification of CholM in plaque samples when the NMR spectra are obtained under the same conditions. As shown in Figure 2, the experimental data and the calculated results agree closely with each other when samples contained an intermediate to high level of CholM. For samples containing less than \( 2 \) mg CholM, the low signal-to-noise ratio (S/N) in spectra of these samples resulted in scatter of the data points.

Spectra of carotid plaques from different patients were acquired under the same experimental conditions to test the feasibility of quantifying CholM in the heterogeneous environment of the plaque. The C5 region of selected spectra (Figure 2, right) exhibited the twin peaks characteristics of CholM.

Table 1 shows the lipid contents of cholesterol (Chol), CE, and phospholipids (PL) obtained from chemical analysis data of plaque samples and tissue dry weight obtained by direct weighing after lyophilization of samples.

Table 2 presents the distribution of cholesterol (Chol) in crystalline (Cr), isotropic (Iso), and liquid-crystalline (LiqCr) phases and the molar ratio of cholesterol to phospholipid (Chol/PL) in the liquid-crystalline phase.

*Some of the results on this sample have been presented elsewhere. All values are in milligrams.

Because carotid plaques generally contain less lipid and more calcium salts than do typical coronary artery or aortic plaques, we studied a large number of tissue samples by MAS NMR to identify the limited number of cholesterol-containing plaques discussed below. The results presented here demonstrate our capability to quantify plaque cholesterol in different phases but are not intended to be representative of plaque features in the majority of carotid plaques.
Quantification of Plaque Calcium

Calcium salt deposits are often found in advanced atherosclerotic plaques, especially in carotid plaques formed near the bifurcation. In atherosclerotic lesions, the predominant chemical form of calcium salts is CPH. The 31P nucleus represents an intrinsic probe for detection of CPH by NMR. The identification of CPH in carotid plaques by 31P and chemical analysis has been described in detail in our previous report. For calibration of the integrated 31P signal intensity in plaques, we first used synthetic CPH purchased from a commercial source as a reference compound. The 31P resonance of this CPH (Figure 3A) had approximately the same chemical shift as that of the plaque (Figure 3B), but the peak for CPH was narrower. CPH prepared from diluted CaCl₂ and NaH₂PO₄ solutions gave a 31P resonance similar to that in the 31P NMR spectrum of freshly prepared CPH (Figure 3A). 31P MAS NMR spectra were obtained with known amounts of CPH to test the validity of using it for the calibration of 31P signals in plaques. The plot of peak intensity of the center band versus phosphorus content showed a strong, linear correlation (Figure 4A). Spectra of plaque samples were then obtained under identical conditions, and the intensities of the 31P resonances were plotted against the phosphorus content of the corresponding samples (Figure 4A). In both cases, the actual sample phosphorus content was determined by chemical analysis after completion of the NMR measurements. The data for intact tissue also showed a linear relationship but with a different slope from that obtained for pure CPH.

These results indicated that synthetic CPH is clearly not an ideal calibration compound for plaque calcification. As an alternative, we tried animal bone powder, which is also composed mainly of CPH. The 31P spectrum of bone powder resembled that of the plaque with respect to both chemical shift and line width (Figure 3C). A plot of the spectral intensities of the bone samples versus mass exhibited a slope similar to that of the plaque samples (Figure 4B). Thus, pulverized bone is an appropriate reference for the phosphate signal in plaques. The total phosphorus contents in selected plaques, as determined by the MAS NMR (referenced to bone) and by chemical analysis, are presented in Table 3. The 8 samples studied contained a wide range of phosphorus, extending from 0.60 to 15 mg, corresponding to 3.2 to 81 mg of CPH. The value of total phosphorus in each plaque sample measured by MAS NMR was within 10% of the value determined by the chemical analysis. The calibration accuracy in the low concentration range was significantly higher for CPH than it was for CholM (Figure 2), mainly because of the higher relative NMR sensitivity of the 31P nucleus. The close correspondence validates the use of 31P MAS NMR as a convenient, nonperturbing method for measuring CPH in intact plaques.

To understand why there were differences in the measured 31P signal intensities of CPH from different sources, we measured the x-ray powder diffraction pattern of the synthetic CPH samples, the bone powder, and an atherosclerotic plaque. Synthetic samples, either freshly prepared or purchased from a commercial source, gave the same diffraction pattern (pattern A, shown schematically in Figure 3A). The plaque sample and the bone sample gave the same pattern: both showed some differences from the synthetic samples (pattern B, shown schematically in Figure 3B). Patterns A and B had the same diffraction bands in the short spacing region (the outer bands), but the long spacing bands (the center bands) were seen only in pattern A. This result agrees with a previous study showing that x-ray diffraction patterns of mineral deposits isolated from human aorta matched the pattern of CPH in the short spacing range only. Synthetic CPH thus has a more ordered crystalline structure than does CPH in biological samples. In the latter, crystallization of CPH is a slow process controlled by many pathophysiological processes and may be limited to very small environments, such as individual cells or cell clusters.

### Table 3. Phosphorus Content in Selected Human Carotid Plaques, as Determined by MAS NMR and Chemical Analysis of Plaques With Different Tissue Dry Weights

<table>
<thead>
<tr>
<th>Sample</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P7</th>
<th>P8</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMR</td>
<td>0.60</td>
<td>2.5</td>
<td>4.8</td>
<td>12.9</td>
<td>1.2</td>
<td>3.0</td>
<td>11.8</td>
<td>15.0</td>
</tr>
<tr>
<td>Chemical analysis</td>
<td>0.60</td>
<td>2.4</td>
<td>5.3</td>
<td>14.8</td>
<td>1.4</td>
<td>3.4</td>
<td>11.8</td>
<td>14.8</td>
</tr>
<tr>
<td>Dry weight, mg</td>
<td>95.5</td>
<td>60.8</td>
<td>108.0</td>
<td>78.4</td>
<td>90.9</td>
<td>107.0</td>
<td>72.9</td>
<td>81.4</td>
</tr>
</tbody>
</table>

*These plaques generally had low amounts of lipids and do not correspond to the samples in Table 1.*
fore, the microcrystals of CPH in plaques are probably small and lack long-range ordering in the lattice. It has been suggested that plaque calcification is controlled by bone-forming proteins such as osteopontin; thus, it is not surprising that the bone powder demonstrates that the bone powder can introduce a calibration factor.

**Discussion**

Until recently, quantitative analysis of plaque components without extensive sample manipulation or destruction has proven to be especially difficult. Katz and Small developed a discontinuous sucrose density gradient centrifugation method to separate homogenized plaque tissue into CE, phospholipids, CholM, and pellet phases. This method is valuable in that each phase can be obtained for physical characterization and chemical analysis. However, it was concluded that the isolated fractions showed significant contamination with other phases. The procedure requires disruption of the tissue and is also very labor-intensive. Other investigators using Raman spectroscopy have quantified the major lipids and calcium salts in plaques. However, Raman studies have not distinguished the different phases of lipids, particularly crystalline from noncrystalline cholesterol, and have been applied mainly to minced or homogenized plaques rather than excised plaques in situ because of technical limitations.

In the present study, we have demonstrated the detection and quantification of 2 major pathological constituents of human carotid plaques, CholM and CPH, by MAS NMR. Our results revealed a wide range of CholM in 9 different samples (Table 2), from 1.0 mg (the lower limit of detection) to 8.1 mg. Based on the NMR measurement and the post-NMR chemical analyses, the amount of cholesterol complexed with phospholipid could be estimated (Table 2).

As cholesterol accumulates in a plaque, it partitions into phospholipid bilayers. This lipid bilayer phase eventually becomes saturated with cholesterol as the disease progresses. The exact saturation ratio depends, in part, on the specific phospholipids present. Thus, our results, showing a range of values between 1.1 and 1.7 (Table 2), reflect varying degrees of supersaturation. MAS NMR provides a reliable method to estimate this ratio in situ and to investigate the factors contributing to its variation in future studies. In any case, the observed variation in the amount of cholesterol complexed with phospholipids demonstrates that the quantity of CholM in the plaque cannot be estimated from the total cholesterol determined by chemical analysis.

We are not aware of any independent quantification of CholM in a plaque, and it is not practical to extract CholM selectively for chemical quantitative analysis. Because the ratios of cholesterol-to-phospholipid in the liquid-crystalline phase obtained for our plaque samples (Table 2) were in the expected range as discussed above, our quantitative measurement of CholM was reasonably accurate. In previous studies of model bilayer systems that were supersaturated with cholesterol, MAS NMR detected CholM reliably, including some cases where it was not visible by light microscopy. Thus, MAS NMR should be able to detect even small crystals in tissue and potentially provide an accurate estimate of CholM in the heterogeneous environment of the plaque. This expectation seems to be verified by the present results.

In our experiments for calibrating the intensity of the 31P resonances from plaques, the intensity data points from synthetic CPH and bone fell on lines with differing slopes; a lower slope (≈0.045) was obtained for CPH compared with that for bone (≈0.125). Therefore, if synthetic CPH is to be used as the standard sample for quantification, then a calibration factor will be needed (biological sample mass = 0.4 x its peak intensity). In a recent report on the determination of human coronary artery composition by Raman spectroscopy, a calibration factor was also required to correctly relate the plaque calcium phosphate and synthetic CPH (biological sample mass = 0.7 x Raman signal intensity). Because animal bone is easy to obtain and prepare for NMR analysis, it serves as a good reference for the quantification of CPH in human plaques without the need to introduce a calibration factor.

In situ characterization of the structures and chemical and physical properties of components in plaques can provide valuable information for understanding the pathogenesis and potential progression, stabilization, or regression of the plaque and for explaining why certain plaques are prone to rupture. The MAS NMR approaches described in this study will make it possible to determine, for example, whether the tendency of a plaque to rupture is related to its content of crystalline (or liquid-crystalline) cholesterol or calcium phosphate. Because MAS NMR is essentially nondestructive, selected samples with desired characteristics can be studied by other methods. Although MAS NMR with its rapid sample spinning is not suitable for in vivo studies, the quantification of solid phases by MAS NMR provides critical information for interpreting magnetic resonance images of the same plaque.

**Acknowledgments**

This work was supported by American Heart Association (AHA) Fellowship 13-434-945 and AHA Grant-In-Aid No. 13519-967 to W.G.; National Institutes of Health grant RO1 HL41904 to J.A.H.; and Welch Grant No. Q-1325 to J.D.M.

**References**


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doi: 10.1161/01.ATV.20.6.1630
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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