Physical effects of cholesterol on arterial smooth muscle membranes: evidence of immiscible cholesterol domains and alterations in bilayer width during atherogenesis

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Abstract **Small angle X-ray diffraction was used to examine arterial smooth muscle cell (SMC) plasma membranes isolated from control and cholesterol-fed (2%) atherosclerotic rabbits. A microsomal membrane enriched with plasma membrane obtained from animals fed cholesterol for up to 13 weeks showed a progressive elevation in the membrane unesterified (free) cholesterol:phospholipid (C/PL) mole ratio. Beyond 9 weeks of cholesterol feeding, X-ray diffraction patterns demonstrated a lateral immiscible cholesterol domain at 37°C with a unit cell periodicity of 34 Å coexisting within the liquid crystalline lipid bilayer. On warming, the immiscible cholesterol domain disappeared, and on cooling it reappeared, indicating that the immiscible cholesterol domain was fully reversible. These effects were reproduced in a model C/PL binary lipid system. In rabbits fed cholesterol for less than 9 weeks, lesser increases in membrane C/PL mole ratio were observed. X-ray diffraction analysis demonstrated an increase in membrane bilayer width that correlated with the C/PL mole ratio. This effect was also reproduced in a C/PL binary lipid system. Taken together, these findings demonstrate that in vivo, feeding of cholesterol causes cholesterol–phospholipid interactions in the membrane bilayer that alter bilayer structure and organization. This interaction results in an increase in bilayer width peaking at a saturating membrane cholesterol concentration, beyond which lateral phase separation occurs resulting in the formation of separate cholesterol bilayer domains. These alterations in structure and organization in SMC plasma membranes may have significance in phenotypic modulation or aortic SMC during early atherogenesis.—**Tulenko, T. N., M. Chen, P. E. Mason, and R. P. Mason. **Physical effects of cholesterol on arterial smooth muscle membranes: evidence of immiscible cholesterol domains and alterations in bilayer width during atherogenesis.** ***J. Lipid Res.*** **1998.** 39: **947–956.**

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Sterols are obligatory lipids in plasma membranes of nearly all eukaryotic cell lines. In mammals, this sterol requirement is met exclusively by unesterified (free) cholesterol (1). The content of cholesterol in membranes, expressed as the cholesterol to phospholipid (C/PL) mole ratio, is high for plasma membranes compared to intracellular membranes and is relatively fixed for any given cell line, but varies from ≈0.5 to 1.0 between cell lines. The importance of maintaining a constant cholesterol content in the membrane is illustrated by studies which show marked alterations in the activity of transmembrane proteins and cell function following alterations in the C/PL mole ratio (2, 3). Cholesterol-induced changes in membrane protein activity have been suggested to mediate cholesterol-induced changes in cell function in vivo (4). The mechanism for cholesterol's effects on membrane and cell function presumably results, in part, from its ability to modulate the biophysical properties of the membrane bilayer (5). In studies utilizing artificial lipid bilayers, increasing the membrane cholesterol content reduces the order parameter at temperatures below the lipid bilayer phase temperature (Tc) and increases it above Tc (6). Moreover, incorporation of cholesterol into lipid bilayers increases the modulus of compressibility (K) of the liquid-crystalline state (7, 8). These studies demonstrate that changes in membrane cholesterol content alter the physical properties of the lipid bilayer which may, in turn, modulate membrane protein function, and therefore, cell function.

Small angle X-ray diffraction studies have provided direct evidence of physical interactions of cholesterol with membranes, suggesting that cholesterol perturbs the structure of the membrane lipid bilayer (9, 10), including hydrocarbon core width, in a concentration-dependent

Abbreviations: C/P, cholesterol/phospholipid; SMC, smooth muscle cells; BCPC, bovine cardiac phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; HEPES, 4-(2-hydroxethyl)-1-piperazineethane sulfonic acid; APD, alkaline phosphodiesterase; NABGase, N-acetyl-β-glucosaminidase; Na^+/K^+ATPase, sodium/potassium ATPase.

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manner (4). In model membrane bilayers, an increase in the cholesterol content to a level of 50 mole% of total phospholipid was shown to produce an immiscible cholesterol monohydrate phase with a periodicity of 34 Å coexisting with the liquid crystalline lipid bilayer (11). The periodicity of 34 Å corresponds to a tail-to-tail cholesterol bilayer as the long axis of an individual cholesterol molecule is 17 Å in the crystalline state (12). Separate domains of cholesterol in monolayer lipid systems have also been observed using magnetic resonance and fluorescence microscopy as a function of temperature and lateral pressure (13, 14). However, evidence for the formation of cholesterol monohydrate domains in either biological membranes or membranes reconstituted from native phospholipid molecules has not been reported.

Regarding vascular cells, epidemiological studies have provided convincing evidence that an elevation in serum cholesterol represents a primary and independent risk factor for the development of atherosclerotic vascular disease (15, 16). Smooth muscle cells (SMC) in dietary atherosclerosis are characterized by an increase in cytosolic calcium levels which may be related to an alteration in the plasma membrane cholesterol content (4, 17). In the present study, we examined changes in the membrane physical properties of SMC during the genesis of early fatty streak atherosclerotic lesions in the rabbit. A highly enriched SMC plasma membrane fraction was isolated from SMC enzymatically dispersed from aortas of New Zealand rabbits fed a normal or cholesterol-enriched (2%) diet for up to 13 weeks. Changes in the structure and lipid organization of SMC plasma membranes were observed in SMC from cholesterol-fed animals that correlated with an elevated C/PL mole ratio. These observations were reproduced in model C:PL binary lipid bilayers. The results of this study indicate that fundamental changes in SMC plasma membrane structure and lipid organization occur as a direct result of physical interactions of cholesterol with neighboring membrane phospholipid molecules in vivo during the development of dietary atherosclerosis, which may account, in part, for some of the changes in the cell biology of SMC in this disease.

METHODS AND MATERIALS

All chemicals used were reagent grade or better and prepared in ultra-pure deionized water. Bovine cardiac phosphatidylcholine (BCPC), dimyristoyl phosphatidylcholine (DMPC), and cholesterol powders were purchased from Avanti Polar Lipids (Alabaster, AL) and stored at −80°C. Thin-layer chromatography was used to ascertain the presence of any degradative products in the lipid and cholesterol suspensions. The primary fatty acid components of the BCPC lipid molecules were determined by the method of Christie (18) using fatty acid methyl esters. Gas–liquid chromatographic analysis showed the following constituents: 18:2 linoleic acid (29.9%), 16:0 palmitic acid (22.5%), 18:1 oleic acid (13.3%), 20:4 arachidonic acid (11.4%), and 20:3 homogamma linoleic acid (4.6%), with small amounts (<1% each) of palmitoleic acid (16:1), linolenic acid (18:3), 11,14-eicosadienic acid (20:2), and myristic acid (14:0).

Isolation of aortic smooth muscle cells

Freshly dispersed SMC were obtained from the thoracic aorta as previously described (19). Briefly, following surgical excision, the thoracic aorta was cut open longitudinally and pinned intimal site up to a wax substrate submerged in physiological salt solution. The intimal surface was scraped with a scalpel to remove endothelial cells and lesions, and the medial smooth muscle layer was peeled free by stripping. The medial layer was minced and incubated for 1–2 h in minimum essential medium (MEM) containing 275 units/ml collagenase, 0.425 units/ml elastase, and 0.12% soybean trypsin inhibitor. Dispersed cells were washed in MEM and pelleted at 350 × *g* for 10 min and used immediately for isolation of membranes. To confirm cell lineage, an aliquot of cells (≈10^4 cells) was placed in culture medium and incubated overnight to permit cell attachment, followed by exposure to cell-specific monoclonal antibodies and immunostaining performed with an avidin–biotin system conjugated with horseradish peroxidase. Conformation of smooth muscle cell identity was accomplished by immunostaining with the muscle actin-specific HHF-35 monoclonal antibody (Enzo Biochemicals, NY) (20) and macrophage-specific monoclonal antibody RAM-11 (gift from A. Gown) (21). In cells from both normal and atherosclerotic animals, uniform positive staining with the HHF-35 antibody, and absence of staining with the RAM-11 antibody was observed, confirming SMC lineage.

Isolation of plasma membranes from aortic smooth muscle cells

SMC microsomal membranes were isolated using a modification of a procedure previously described (19). Cell dispersions freshly isolated from the aortic medial layer were suspended in cold (4°C) hypoosmolar sucrose (0.25 m) containing 10 mm Tris (pH 7.4) for 6 min, followed by disruption with a tissue homogenizer (Tekmar #60) using 3 homogenization cycles. An equal volume of 0.4 m sucrose solution containing 10 mm Tris, pH 7.4, was then added to the disrupted cells. After removal of an aliquot for chemical and enzymatic analysis, the resultant crude homogenate was centrifuged at 268 *g* for 20 min. The resulting pellet (nuclear/unbroken cells) was resuspended in 0.25 m sucrose, 10 mm Tris, pH 7.4 (as were all subsequent pellets) and the supernatant was centrifuged at 10,950 *g* for 10 min. The resulting pellet (mitochrondrial/lysosomal) was resuspended and the supernatant was centrifuged at 144,000 *g* for 90 min. The final pellet (microsomal) was resuspended after removal of the post microsomal supernatant (soluble fraction).

All fractions were frozen for chemical and enzymatic analysis at a later time with the exception of cytochrome-oxidase activity which was measured on the day of cell fractionation. The following markers were assayed in all subcellular fractions by the indicated procedures: Na^+/K^+ ATPase (22), alkaline phosphodiesterase (23) and free cholesterol (plasma membrane); cytochrome-oxidase (mitochondria) (24) and N-acetyl-β-glucosaminidase (lysosomes) (25); DNA (26) and protein (soluble fraction) (27). Total phospholipid mass (total membrane) was determined in each fraction using a phospholipid phosphorus assay (28).

Dietary atherosclerosis

For cholesterol feeding studies, rabbits were maintained on a cholesterol-rich diet for up to 13 weeks. The diet was a commercially prepared, calibrated rabbit chow (Buckshire Feeds) supplemented with cholesterol (2%). The chow consisted of 30.1% protein, 5.5% fat, 21.1% fiber, and 43.3% carbohydrate (with or without added cholesterol). Control and diet rabbits were housed separately, but in the same room, throughout the feeding period. Control rabbits were fed batch-matched standard chow without added cholesterol.

Preparation of oriented SMC plasma membranes for X-ray diffraction

SMC plasma membrane samples were oriented for small-angle X-ray scattering by stacking using a modification of our previously described technique (29). Briefly, SMC plasma membrane vesicles were spun in an SW-28 rotor (Beckman Instruments, Inc., Fullerton, CA) at 35,000 *g* for 1 h at 5°C in Lucite sedimentation cells, each containing an aluminum foil substrate. On completion of the spin, supernatants were removed and each sample was mounted on a curved glass support. The samples, composed of 250 mg phospholipid, were equilibrated overnight in glass vials by suspending the samples over, but not in contact with, a saturated salt solution that served to define a specific relative humidity of 95% at 5°C ($ZnSO_4$). Oriented membrane samples were then placed in sealed brass canisters containing aluminum foil in which temperature and relative humidity were controlled.

Preparation of reconstituted membrane multilayer samples

The effects of cholesterol content on membrane bilayer structure were directly examined in model membranes using vesicular lipid bilayer preparations. Lipid vesicles for these studies were prepared from dimyristoyl phosphatidylcholine (DMPC) or bovine cardiac phosphatidylcholine (BCPC) and cholesterol reconstituted in chloroform at various cholesterol-to-phospholipid mole ratios (0:1 to 0.6:1). The lipids were dissolved in redistilled chloroform at a concentration of 10.0 mg/ml. Samples containing DMPC or BCPC in the absence and presence of cholesterol were dried down with a stream of nitrogen gas to a thin film on the sides and bottom of 13 × 100 mm glass test tubes while vortexing. Residual solvent was removed by vacuum. A volume of buffer (0.5 mm HEPES, 2.0 mm NaCl, pH 7.3) was added to the other dried lipid preparation, yielding a final phospholipid concentration of 5.0 mg/ml. Multilamellar vesicles were formed by vortexing the buffer and lipids above the thermal phase transition temperature for 3 min. Oriented membrane samples were prepared by centrifugation, as described above for the SMC samples.

X-ray diffraction data collection and reduction

Small-angle X-ray scattering was carried out by aligning the membrane samples at near-grazing incidence with respect to the X-ray beam. The radiation source was a collimated, monochromatic X-ray beam (CuK_α, $\lambda = 1.54$Å) from a Rigaku RU200 (Danvers, MA) rotating anode microfocus generator. The fixed geometry beamline utilized a single Franks mirror providing nickel-filtered radiation ($K_{\alpha 1}$ and $K_{\alpha 2}$ unresolved) at the detection plane. The beam height at the sample was ≈1 mm. Small-angle X-ray diffraction data from the oriented membrane multilayer samples were recorded on a one-dimensional position-sensitive electronic detector (Innovative Technologies, Inc., Newburyport, MA). In addition to direct calibration of the detector system, cholesterol monohydrate was used to verify the calibration.

The unit cell periodicity, or *d* space, of the membrane lipid bilayer is the measured distance from the center between one bilayer to the next, including surface hydration. The *d*-space for the membrane multilayer samples was calculated by Bragg's Law:

$$n\lambda = 2d\sin\Theta$$

in which *n* is the diffraction order number, λ is the wavelength of the X-ray radiation (1.54Å), *d* is the membrane lipid bilayer unit cell periodicity, and θ is the Bragg angle equal to one-half of the angle between the incident beam and scattered beam.

RESULTS

SMC plasma membrane isolation

Microsomal membranes isolated from freshly dispersed SMC cells showed a 13-fold enrichment in the specific plasma membrane marker, alkaline phosphodiesterase (APD). There was only minor contamination of DNA (2.8%), NABGase (5.7%) and cytochrome oxidase (5.5%) in the same fraction. Based on the recovery of the plasma membrane marker APD, the calculated yield of plasma membrane in this fraction was 44.7 ± 7.6% (compared to 41.7 ± 10.0% for the nuclear/unbroken cells pellet, 8.6 ± 1.9% for the mitochondrial/lysosomal pellet and 5.0 ± 1.3% for the soluble fraction). The lipid-to-protein content of these membranes (2.3 mmol phospholipid/mg protein) was similar to that reported for highly enriched cardiac plasma membrane, 1.98 mmol phospholipid/mg protein (30).

Small-angle X-ray diffraction analysis of SMC plasma membrane structure

To characterize the structure of SMC plasma membranes from control and atherosclerotic SMC, small-angle X-ray diffraction was used. In **Fig. 1**, representative X-ray diffraction order patterns from oriented SMC plasma membranes isolated from control and 8-week diet samples are shown. The *d*-space was computed from the diffraction order patterns and revealed a width of 56.3 Å for the control sample (Fig. 1A) and 60.3 Å for the 8-week diet sample (Fig. 1B). In addition, the relative intensity of the diffraction orders also decreased in the diet samples. Thus, the atherosclerotic SMC membrane was 4 Å larger than control. In both samples, there was no evidence for a separate lipid phase.

Between 10 and 13 weeks on the experimental diet, the C:PL mole ratio of the enriched SMC plasma membrane fraction averaged 0.96:1 ± 0.27 (means ± SD, n = 9). Small-angle X-ray diffraction from SMC plasma membranes derived from these animals produced meridonal patterns consistent with two separate lipid phases: a membrane liquid crystalline phase (56 Å) and an immiscible cholesterol monohydrate phase (34 Å-orders 1′ and 2′ at 37°C (**Fig. 2**). The *d*-space of the membrane liquid crystalline phase (56–57 Å) was identical to that reported for control membranes and less than that reported for samples from animals on the cholesterol-enriched diet for less than 9 weeks. These data suggest that the membrane lipid bilayer has an upper limit for cholesterol to remain fully miscible; above this limitation, cholesterol forms a separate, immiscible cholesterol phase.

The presence of the immiscible cholesterol domain was sensitive to the temperature of the sample. When the samples were warmed from 37°C to 45°C or 50°C, a single lamellar lipid bilayer phase was observed and there was no evidence for a cholesterol domain. On cooling back to 37°C, the immiscible cholesterol domain reappeared, indicating its temperature sensitivity. With further cooling to 20°C, the cholesterol domain remained and the lamellar lipid phase demonstrated a further increase in width

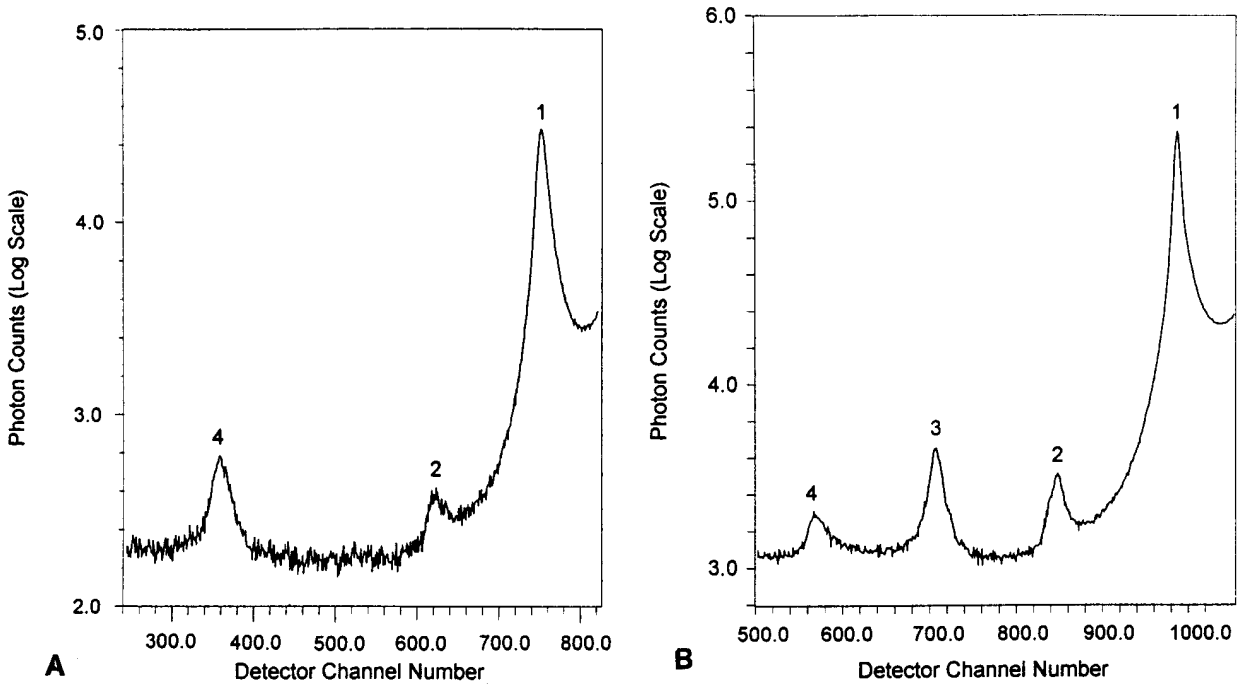

Fig. 1. Typical X-ray diffraction order patterns from oriented SMC plasma membrane samples at 37°C and 93% relative humidity. The diffraction patterns were collected on a one-dimensional position-sensitive electronic detector (see Methods). (A) A typical diffraction order pattern in SMC membranes isolated from normal (control) diet animals that corresponds to a *d*-space value of 56.3 Å (C:PL mole ratio 0.4:1). (B) Under identical conditions, four lamellar diffraction orders were observed in SMC isolated from an animal fed cholesterol for 8 weeks which corresponds to a *d*-space value of 60.3 Å (C:PL mole ratio 0.7:1).

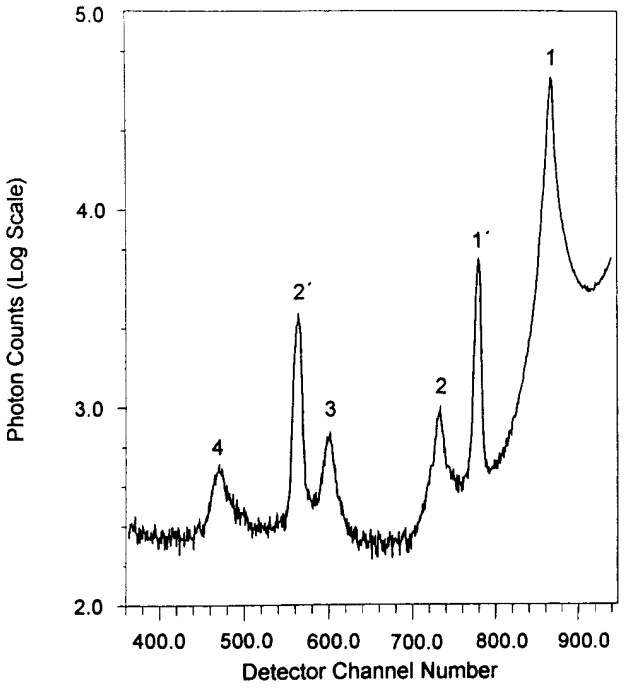

Fig. 2. An X-ray diffraction order pattern from an atherosclerotic animal fed cholesterol for 11 weeks. Under identical conditions as used for data in Fig. 1, two separate lamellar phases were present: a membrane lipid bilayer phase (orders 1–4) corresponding to a *d*-space of 56 Å and an immiscible cholesterol monohydrate phase (orders 1′ and 2′ corresponding to *d*) space of 34 Å at 37°C (C:PL mole ratio 0.9:1). This pattern is typical for membrane preparations from animals fed the cholesterol-enriched diet for 9–13 weeks.

(57.4 to 60.6 Å), consistent with an ordering effect of the membrane bilayer at low temperatures. **Table 1** reviews these changes in membrane structural dimensions.

Cholesterol phase separation reproduced in a reconstituted cardiac lipid bilayer system

Results from the small-angle X-ray diffraction experiments demonstrated that the oriented DMPC/cholesterol and BCPC/cholesterol membrane vesicles produced four strong, reproducible diffraction orders at 37°C and 93%. Membrane width, including surface hydration (i.e., *d*-space or unit cell periodicity), was measured for the samples as a function of cholesterol content. **Figure 3A** illustrates a typical electron density profile demonstrating the effects of adding cholesterol to phospholipid membranes. In the absence of cholesterol, the DMPC *d*-space was 50.9 Å. At the highest cholesterol level measured (0.6:1 choles-

TABLE 1. X-ray diffraction *d*-space measurements for atherosclerotic SMC plasma membranes as a function of temperature

Sample temperature	Membrane Lipid Bilayer *d*-space	Cholesterol Phase *d*-Space
	Å	Å
37°C	57.4	34
45°C	55.4	no
50°C	55.1	no
37°C	57.4	34
20°C	60.6	34

Sample C:PL mole ratio was 1.21:1 (13 weeks on cholesterol diet); no, cholesterol phase not observed.

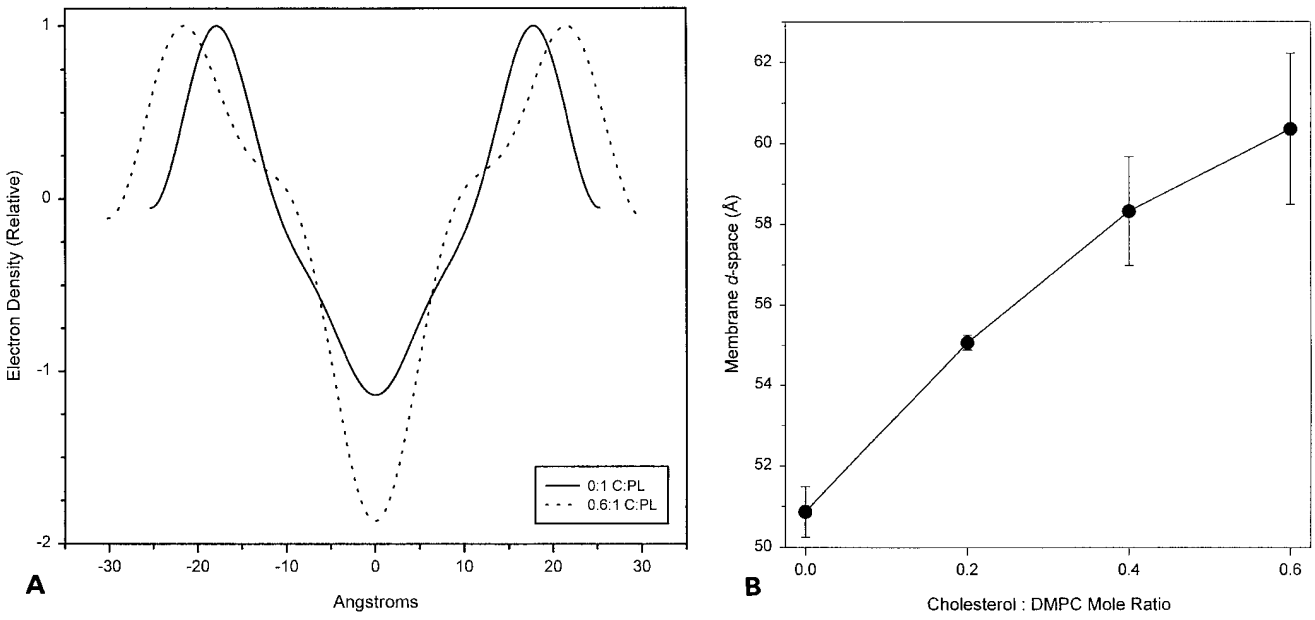

Fig. 3. (A) Electron density profiles generated from diffraction data obtained from DMPC vesicular membranes without cholesterol (0:1 C:PL) and with cholesterol (0.6:1 C:PL) illustrating the increase in intrabilayer phospholipid head group separation and bilayer width (*d*-space). (B) Line graph illustrating the relationship between increased membrane cholesterol content and DMPC membrane bilayer width (*d*-space) (n = 3 different preparations).

terol to phospholipid mole ratio), the membrane width increased by 18.3% to 60.2 Å ($P < 0.01$). In addition, there was a significant, direct relationship between the cholesterol: DMPC mole ratio and membrane width; the correlation coefficient was 0.972 ($P < 0.05$) as determined by the Pearson Product Moment Correlation (Fig. 3B). A similar correlation between cholesterol content and membrane width was observed for BCPC/cholesterol samples (**Fig. 4**). The BCPC membrane *d*-space increased by 10.5% ($P < 0.05$) from 52.5 Å in the absence of cholesterol to 58.0 Å at a 0.6:1 mole ratio (Fig. 4A). As with DMPC, there was a significant, direct relationship between

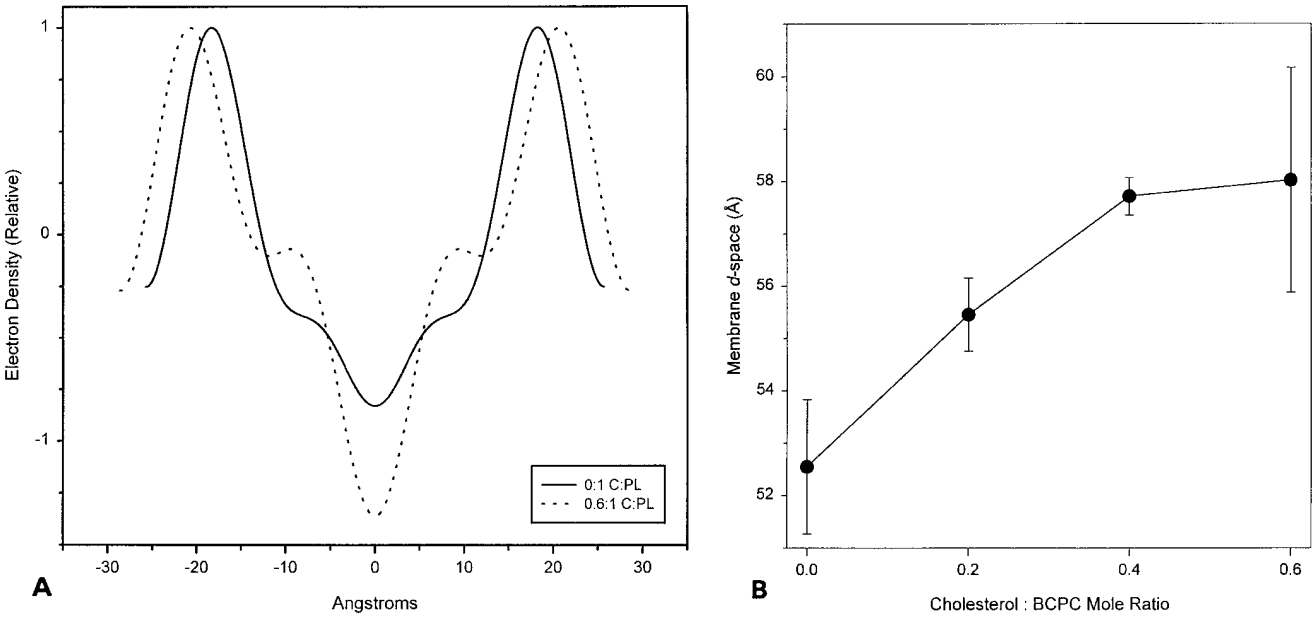

Fig. 4. (A) Electron density profiles generated from diffraction data obtained from BCPC vesicular membranes without cholesterol (0:1 C:PL) and with cholesterol (0.6:1 C:PL) illustrating the increase in intrabilayer phospholipid head group separation and bilayer width (*d*-space). (B) Line graph illustrating the relationship between increased membrane cholesterol content and BCPC membrane bilayer width (*d*-space) (n = 3 different preparations).

the cholesterol:BCPC mole ratio and membrane width, with a correlation coefficient of 0.953 ($P < 0.05$) (Fig. 4B).

In addition to the measurement of membrane *d*-space, the intrabilayer phosphorus headgroup separation (hydrocarbon core width) for these samples was calculated as a function of cholesterol content. The phosphorus headgroup separation was measured directly from one-dimensional electron density profiles calculated from the diffraction data in identical resolution (**Fig. 5**). Similar to that observed for the membrane *d*-space, there was a significant positive correlation between membrane cholesterol content and the measured phospholipid bilayer headgroup separation. The DMPC intrabilayer headgroup separation increased as a function of cholesterol content (Fig. 5A) from 35.6 Å in the absence of cholesterol to 43.0 Å at a 0.6:1 cholesterol:phospholipid mole ratio, an increase of 20.8% ($P < 0.001$). Under identical conditions, the BCPC intrabilayer headgroup separation increased with increasing cholesterol content (Fig. 5B) from 37.2 Å to 42.1 Å, an increase of 13.2% ($P < 0.001$).

To test whether the immiscible cholesterol phase could be reproduced in a reconstituted lipid bilayer system under similar conditions of membrane cholesterol content, binary lipid mixtures were formed composed of native phospholipids derived from cardiac (BCPC) tissue (see Methods and Materials) and cholesterol. The reconstituted membrane lipid bilayers were formed at 0.5:1 and 1:1 C:PL mole ratios to reproduce normal and cholesterol-enriched conditions. **Figure 6** illustrates the results of these experiments conducted at 37°C and 93% relative humidity. At control physiologic levels of cholesterol (0.5:1 C:PL mole ratio), the oriented samples produced a single lamellar phase with a *d*-space of 58Å (Fig. 6A), consistent with the periodicity measured for intact and reconstituted cardiac plasma membranes (31, 32). Under conditions of cholesterol enrichment (1:1 C:PL mole ratio), two separate lamellar phases were observed: a liquid crystalline lipid bilayer phase (56 Å) and an immiscible cholesterol monolayer phase (34 Å) (Fig. 6B). These *d*-space measurements were identical to those reported above for SMC plasma membrane samples under cholesterol-enriched conditions at 37°C. Moreover, in the presence of the cholesterol monohydrate phase, the membrane lipid bilayer phase had a periodicity that was actually less than that measured for cardiac lipid bilayers with a C:PL mole ratio of 0.5:1. These data suggest that the presence of the cholesterol phase actually results in a reduction in cholesterol in the miscible lipid bilayer phase. Only when the cardiac membrane C:PL mole ratio was elevated to 2:1, was the *d*-space of the lipid bilayer component of the two-phase system identical to that reported for control membrane. **Table 2** summarizes the data on cholesterol enrichment inducing an immiscible cholesterol domain (34 Å repeat structure).

Based on the data presented in this paper, we have developed a model to illustrate the effects of cholesterol on membrane bilayer structure that occurs in vivo in the plasma membrane of SMC during early atherogenesis which is illustrated in **Fig. 7**. With increasing cholesterol content of the membrane that occurs during the early period of cholesterol feeding, the cell membrane width increases (swelling), which, if a high cholesterol diet is continued, leads to the appearance of cholesterol monohydrate bilayers in the plane of the cell membrane. As recently demonstrated by Harris et al. in model bilayers (33), the cholesterol molecules appear to align in a tail-to-tail fashion.

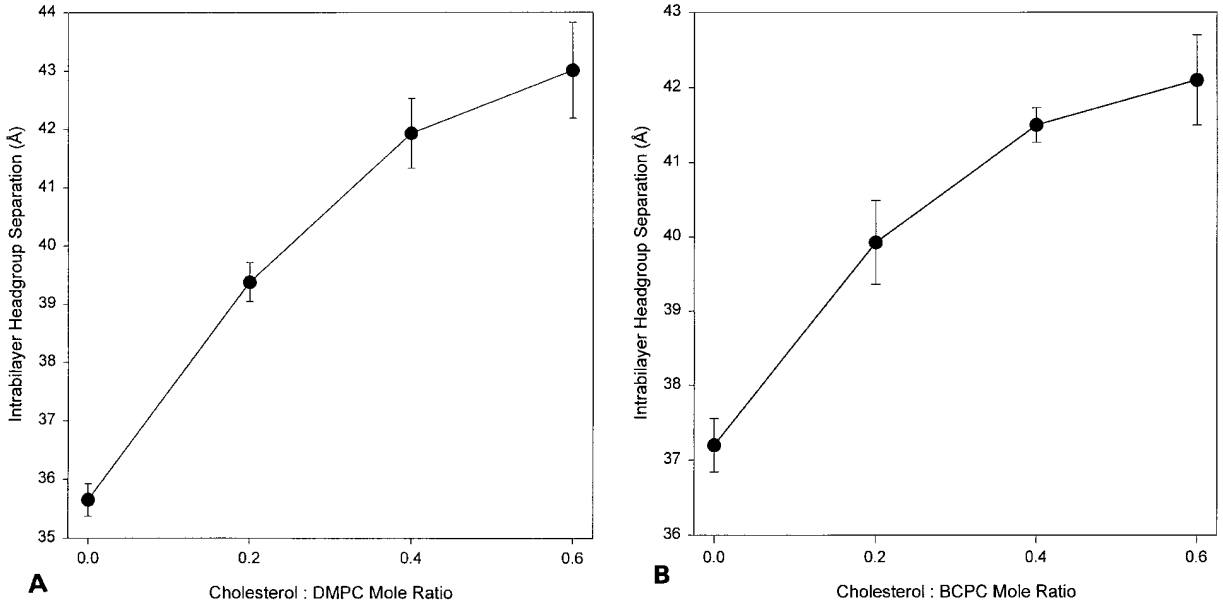

Fig. 5. Relationship between membrane cholesterol content and membrane phospholipid headgroup separation in oriented model membrane bilayers. (A) Line graph illustrating the relationship between increased membrane cholesterol content and membrane phospholipid headgroup separation in DMPC vesicular membranes. (B) Line graph illustrating the relationship between increased membrane cholesterol content and membrane phospholipid headgroup separation in BCPC vesicular membranes (n = 3 different preparations).

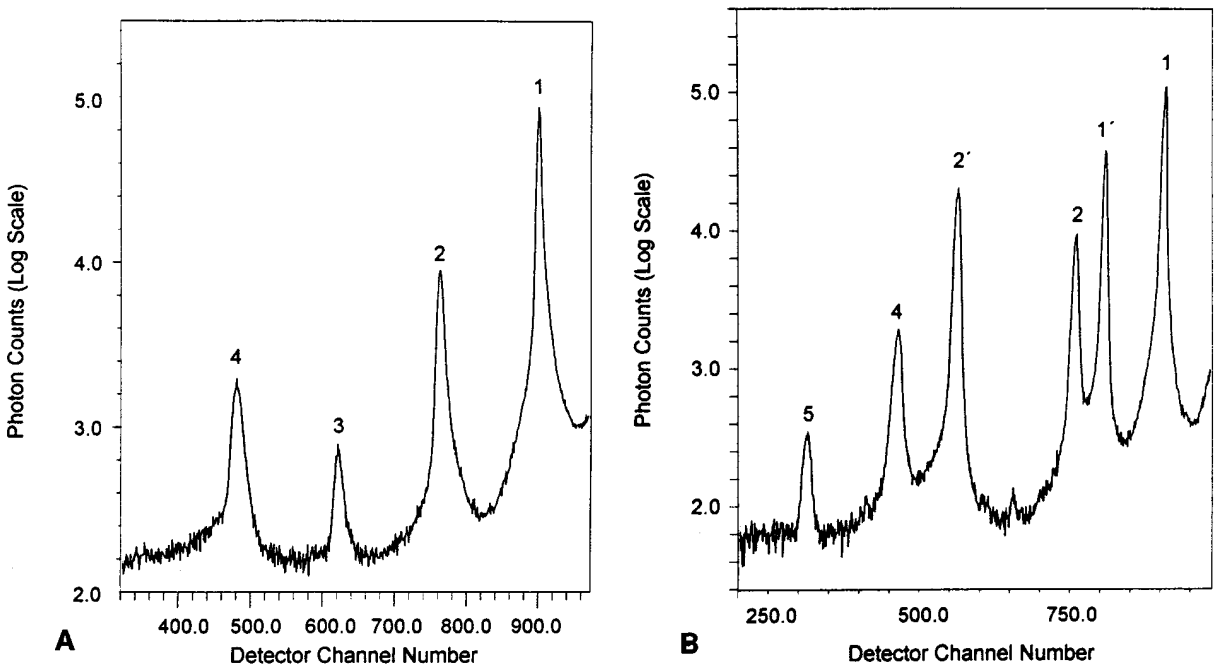

Fig. 6. Typical X-ray diffraction order patterns from oriented BCPC membrane samples at 37°C and 93% relative humidity. The reconstituted membrane lipid bilayers were formed at 0.5:1 (6A left) and 1:1 C:PL (6B right) mole ratio to approximate normal and cholesterol-enriched conditions in vivo. Under normal conditions of cholesterol (0.5:1 C:PL mole ratio), the oriented samples produced a single lamellar phase with a *d*-space of 58 Å (Fig. 6A), consistent with the periodicity measured for intact and reconstituted cardiac plasma membranes (31, 32). Under conditions of cholesterol enrichment (1:1 C:PL; Fig. 6B), two separate lamellar phases were observed: a liquid crystalline lipid bilayer phase (orders 1–4; 56 Å) and an immiscible cholesterol monolayer phase (orders 1′ and 2′; 34 Å).

DISCUSSION

In this study, we examined the lipid organization and structure of SMC plasma membranes derived from rabbits in which atherosclerosis was induced by cholesterol feeding and compared these findings to cholesterol-induced alterations in model lipid bilayers. Using small-angle X-ray diffraction, distinct changes in membrane lipid organization were found, demonstrating, for the first time, the formation of a physical cholesterol domain in vivo. In addition, structural changes were also observed consistent with an increase in membrane bilayer width, confirming and extending our previous report on cholesterol effects on bilayer structure during atherogenesis (4). The observed alterations were associated with a marked increase in the membrane C:PL mole ratio in these same samples and were reproduced in reconstituted lipid bilayer systems.

Within cells, free cholesterol is normally found exclusively in membranes where it is asymmetrically distributed between the intracellular and plasma membranes. The plasma membrane is typically enriched with free cholesterol and is thought to contain up to 90% of the cell's total unesterified cholesterol (34). In SMC, further enrichment of the plasma membrane with cholesterol has been

TABLE 2. Small angle X-ray diffraction *d*-space measurements of atherosclerotic SMC plasma membranes and reconstituted cardiac lipid bilayers

Sample	C:PL Mole Ratio	Membrane Lipid Bilayer *d*-Space	Cholesterol Phase *d*-Space
		Å	Å
Control diet SMC plasma membrane	0.4:1	56	no
Atherosclerotic SMC plasma membrane (10 wk)	0.9:1	56	34
Atherosclerotic SMC plasma membrane (13 wk)	1.2:1	57	34
Reconstituted cardiac lipid bilayer	0.5:1	58	no
Reconstituted cardiac lipid bilayer	1:1	56	34
Reconstituted cardiac lipid bilayer	2:1	58	34

The sample temperatures were 37°C for SMC plasma membrane and 22°C for reconstituted lipid bilayers; no, cholesterol phase not observed.

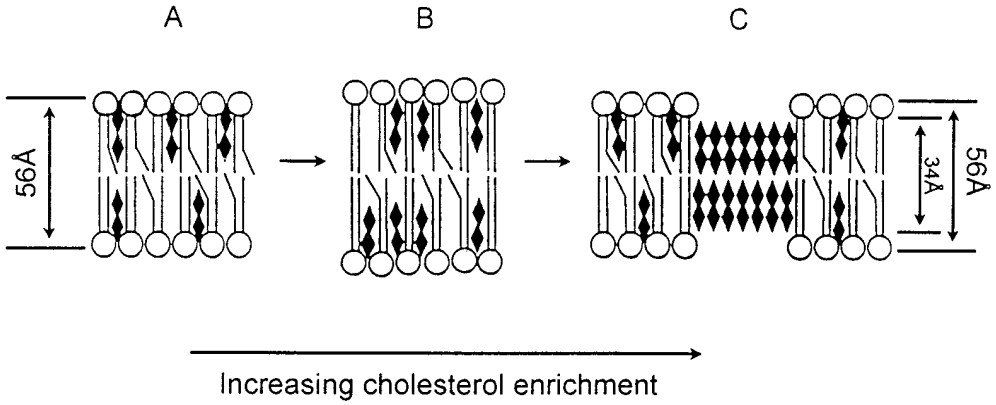

Fig. 7. A schematic model of the effects of cholesterol on plasma membrane structure of arterial smooth muscle cells. The data from this study indicate an initial effect of cholesterol on membrane width. At C:PL mode ratios at or below 0.8:1, bilayer width increases with increasing C:PL. At C:PL mole ratios above 0.9:1, two separate lamellar phases in SMC membranes form, one representing a liquid crystalline lipid bilayer and another representing an immiscible cholesterol phase. The immiscible cholesterol phase forms by phase separation as the membrane "saturates" with cholesterol.

shown to modulate the activity of several transmembrane proteins, including calcium channels (3), potassium channels (35) and the Na^+/K^+ ATPase pump (2). In addition, steady-state intracellular calcium levels have been shown to be significantly elevated in cholesterol-enriched SMC (19). All these alterations have also been demonstrated to occur in SMC in vivo in cholesterol-fed rabbits (4, 17). Cholesterol enrichment of the cell membrane has similar effects on membrane protein activity including impaired NA^+/K^+ ATPase activity in a variety of cells including red cells (36), endothelial cells (37), and renal cells (38). In a recent report, single channel patch electrophysiological recordings in calcium-activated K^+ channels reconstituted into defined lipid bilayers demonstrated an increase in lipid bilayer lateral elastic stress energy by cholesterol enrichment that favored the closed state of the ion channel pore (39). This effect of cholesterol on ion channel pore kinetics appeared to result from structural stress exerted upon the membrane protein. Taken together, these studies indicate that cholesterol levels in the membrane have important effects on membrane protein activity which, in turn, may affect cell function.

The primary finding of the present study is that at membrane C/PL mole ratios above 0.8, seen in SMC obtained from animals fed cholesterol for longer than 9 weeks, two separate lamellar phases coexist: one corresponding to a liquid crystalline lipid bilayer and another related to a cholesterol monohydrate phase (Fig. 2). That this separate lamellar phase represents a cholesterol monohydrate phase is based on earlier studies demonstrating the formation of transbilayer tail-to-tail interactions of cholesterol in model systems (33, 40). The present findings indicate a limitation in the capacity of the biological membrane to maintain cholesterol in a fully miscible state under physiological conditions. Presumably, when the amount of cholesterol exceeds a certain critical concentration (i.e., saturation), an immiscible cholesterol phase is formed. Lateral phase separation of cholesterol into a distinct domain has been shown previously in model bilayers using a variety of techniques (41–45). To our knowledge, however, the present report is the first to demonstrate the formation of physical cholesterol domains in biological membranes in vivo after cholesterol feeding. The relevance of this membrane cholesterol domain likely relates to the fate of cholesterol accumulation in the membrane under conditions of oversupply of this sterol, such as during serum hypercholesterolemia (4).

The formation of immiscible cholesterol domains in the membrane appears to result from a direct physical interaction between cholesterol's highly planar ring structure with adjacent membrane phospholipid fatty acyl chains. This conclusion is drawn from Fig. 6 which demonstrates the formation of immiscible cholesterol domains in model binary lipids containing only cholesterol and phosphatidylcholine. This conclusion is further supported by the observation that warming the samples to 45°C "melted" the cholesterol domains, while cooling to 37°C reversed this effect (Table 1). Likewise, the increase in membrane bilayer width that precedes the formation of these domains with cholesterol enrichment was also reproduced in a model binary lipid system (Figs. 3, 4, and 5), supporting the conclusion that the effect of cholesterol enrichment on membrane bilayer width is a direct result of a physical interaction of cholesterol with the membrane phospholipid fatty acyl chains.

Indirect evidence for distinct cholesterol domains has been previously described from kinetic data obtained in various cells lines, including L-cells, FU5AH hepatoma cells, J774 macrophage cells, and rabbit aortic smooth muscle cells (46–48). In these studies, separate cholesterol kinetic domains were described based on the efflux of labeled unesterified cholesterol from cells maintained in culture. The formation of cholesterol immiscible domains demonstrated in the present study may explain the previous reports of a significant nonexchangeable kinetic cholesterol pool in the cell membrane (49) because immisci-

ble cholesterol domains, being tightly packed, would be expected to be resistant to exchange (48).

The biological relevance of these alterations in SMC membranes is not fully understood at this point in time. One possibility is because these cholesterol domains might provide a nidus for the formation of extracellular cholesterol monohydrate crystals, i.e., cholesterol clefts, structures long associated with atherosclerotic plaques. In addition, increases in membrane width were found preceding the development of early fatty streak lesions in cholesterol-fed rabbits, suggesting this membrane structural "defect" may be involved in the alterations associated with the cellular pathobiology of this disease. Indeed, cholesterol enrichment of the SMC membrane has been shown to augment calcium permeability and suppress Na^+/K^+ ATPase in vitro similar to those occurring in SMC in vivo during atherogenesis (3, 4, 17, 50). In addition, cholesterol enrichment of the SMC membrane also induces proliferation in these cells (51). As atherosclerosis is thought to ultimately result from a cellular injury, it is tempting to speculate that membrane alterations of the type described in this report may be relevant to this injury.

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