hingomyelin and Other Phospholipid Metabolism in the Rabbit Atheromatous and Normal Aorta

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ABSTRACT

Hypercholesterolemia induces atheroma formation, and the concentration of sphingomyelin is increased compared with that in the normal aortic intima-media. The aortic intima-media sphingomyelin concentration appears to increase exponentially with time on the cholesterol diet. Moreover, the entry rate of serum sphingomyelin into the aortic wall also appears to increase exponentially with time on the cholesterol diet and with the extent of aortic exposure to hypercholesterolemia. $^{32}$P-Phosphate incorporation into sphingomyelin and other phospholipids in the perfused rabbit aorta does not increase with atheromatosis, but the rate of sphingomyelin entry increases 27-fold during approximately the same period of atheroma formation and can account for all of the increase in sphingomyelin concentration in the intima-media.

KEY WORDS

perfused aorta cholesterol diet sphingomyelin influx
phosphatidyl ethanolamine phospholipid synthesis
phosphatidyl inositol phosphatidyl choline
lysophosphatidyl choline

An increased concentration of sphingomyelin in arterial wall represents an important feature of atherogenesis (1), but such increases have also been demonstrated with aging in human aortas (2). Positive correlation exists between the cholesterol content and the phospholipid content of atherosclerotic aortic lesions in both rabbits (3) and man (4). Aortic phospholipids in cholesterol-fed rabbits are synthesized in situ (6, 7), but it has not been unequivocally established that this process is the sole mechanism responsible for phospholipid deposition in rabbit atherosclerotic lesions. Sphingomyelin deposition from the plasma compartment constitutes another route of accumulation. To study this process, we injected lipoprotein-bound tritiated sphingomyelin into rabbits, followed its incorporation into aortic lesions, and compared the entry determined by this method with that of sphingomyelin synthesis in perfused rabbit atheromas.

Methods

Tritiated sphingomyelin was biosynthesized in vivo by the method of Rachmilewitz and co-workers (8). Pregnant mice were maintained on a choline-deficient diet for the last 5 days of pregnancy and the first 5 days postpartum. On the second, third, and fourth days postpartum, 0.5 mc of [methyl-3H]choline chloride (New England Nuclear Corp.) at a specific radioactivity of 7.194 mc/mg was injected intraperitoneally into each mouse. During this time each mouse was allowed to nurse its litter. One day after the last injection of labeled choline, the postpartum mothers were killed with ethyl ether, and their liver lipids were extracted by finely dispersing the liver in one volume of methanol in a glass Teflon homogenizer. Two volumes of chloroform were added, and the mixture was allowed to stand overnight. The suspension was filtered, and the filtrate was washed three times with a methanol-0.6% NaCl in water-chloroform solution (48:47:3 v/v/v) according to the method of Folch and co-workers (9). The chloroform layer was dried under nitrogen, and the residue was reconstituted in chloroform.

For labeling the serum with the purified sphingomyelin, blood (25 ml) was withdrawn by heart puncture from each rabbit, allowed to retracted, and centrifuged at 600 g for 20 minutes to obtain serum. The disodium salt of ethylenediaminetetraacetic acid (EDTA) (200 µg/ml) and Polymixin B (250 units) (Pfizer) were added to each milliliter of serum to stabilize and preserve the lipoproteins. The serum was filtered through a 0.45µ Millipore Millex with sterile technique. Tritiated sphingomyelin
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was spotted on 1 x 1-cm Whatman no. 1 paper and equilibrated with serum (10-30 ml) at 37°C for 2 hours in a Dubnoff shaker-water bath at 72 rpm. Under these conditions 30-80% of the \(^3\)H-sphingomyelin transferred to the serum. Electrophoresis on agarose before and after equilibration showed no change in the lipoprotein pattern during the equilibration. When possible the donor serum (6-15 ml) was injected into the same rabbit. Blood samples were taken at 0 minutes and 1, 2, 4, and 8 hours, and the serum was stored at 0°C together with a portion of the donor serum for further processing. After the last sample had been withdrawn, the rabbit was killed by injecting sodium pentobarbital (50 mg/kg) in the ear vein, the aorta was removed, and the intima-media was stripped from the adventitia. The intima-media was rinsed with 0.9% NaCl and blotted; after the wet weight had been determined, the tissue lipids were extracted and freed of nonlipid as described previously. The chloroform layer containing the extracted lipids was dried under nitrogen, and the residue was dissolved in XDC scintillation mixture (naphthalene 112 g, 2.5-bis[2-(5-tert-butybenzoxyazolyl)]-thiophene 4 g, 1, 4-dioxane 600 ml, xylene 200 ml, 2-ethoxyethanol 600 ml) for the assay of radioactivity. Quench correction of radioactivity in the samples was by the channel ratios technique.

After assay of radioactivity, the XDC scintillation mixture was evaporated under nitrogen, and the residue was taken up in chloroform. Neutral lipids were separated from phospholipids, and phospholipid classes were separated by thin-layer chromatography as described previously. Phospholipid was determined by the method of Gottfried (12).

One part of the serum was mixed with 20 parts of a chloroform-methanol solution (2:1 v/v) and kept overnight. The serum lipid extract was washed as described earlier. A portion of the unseparated lipid was analyzed for radioactivity; the remainder of the chloroform solution was taken to dryness under nitrogen. The lipid residue was redissolved in a small volume of chloroform, and the dissolved lipid was applied to a silicic acid column for the separation of neutral lipids and phospholipids as described earlier. The eluted phospholipid was further fractionated into classes by thin-layer chromatography as previously described. After extraction of the phospholipid class from the silica gel, portions were taken for radioactivity and phosphorus assays.

To determine aortic exposure to hypercholesterolemia, blood was withdrawn from all cholesterol-fed rabbits that were recipients of \(^3\)H-sphingomyelin. The serum cholesterol was extracted and analyzed by the method of Parekh and Jung (13). The cholesterol concentrations were plotted as a function of days on the cholesterol-containing diet, and the area under the curve (days \(\times\) cholesterol concentration) was defined as cholesterol exposure.

For determination of phospholipid biosynthesis in the perfused aortic intima-media, five rabbits maintained on a cholesterol-containing diet for 5 months and three controls fed Purina rabbit chow were used. Each rabbit was killed by introducing a bolus of air into an ear vein. The aorta was removed immediately, mounted on one end of a cannula, rinsed with Medium 199 tissue culture fluid (Grand Island Biological Co.), and perfused with Medium 199 containing 0.5 mc of \(^32\)P-

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TABLE 1

Concentration, Influx, and Synthesis of Sphingomyelin in Normal and Atheromatous Aortic Intima-Media from Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Control rabbits</th>
<th>Cholesterol-fed rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 month</td>
</tr>
<tr>
<td><strong>Sphingomyelin concentration</strong> (μmoles P/g tissue)</td>
<td>1.18 ± 0.20*</td>
<td>3.20 ± 0.82</td>
</tr>
<tr>
<td><strong>Mean serum cholesterol (mg/dliter)</strong></td>
<td>69.5 ± 2.27</td>
<td>3240 ± 553</td>
</tr>
<tr>
<td><strong>Total cholesterol exposure (g/dliter day⁻¹)</strong></td>
<td>127 ± 20.8</td>
<td>141 ± 7.8</td>
</tr>
<tr>
<td>% Injected dose per gram of aorta (× 10⁶)</td>
<td>17.5 ± 6.26</td>
<td>29.1 ± 9.92</td>
</tr>
<tr>
<td>% Injected dose/mmole P</td>
<td>15.1 ± 4.61</td>
<td>12.9 ± 8.21</td>
</tr>
<tr>
<td><strong>Intima-media uptake of ³H-sphingomyelin from plasma (nmoles P/g hour⁻¹)</strong></td>
<td>6.6 ± 1.9†</td>
<td>20.5 ± 8.73</td>
</tr>
<tr>
<td><strong>Intima-media incorporation of ³²P into sphingomyelin (nmoles P/g hour⁻¹)</strong></td>
<td>2.40 ± 1.38</td>
<td>0.647 ± 0.172†</td>
</tr>
</tbody>
</table>

Values are means ± SE for three rabbits per group.
*Statistically significant (with Student's t-test on log₁₀ transformed data), P < 0.01 control vs. 4 months, P < 0.05 control vs. 1 or 2 months.
†Statistically significant (with Student's t-test on log₁₀ transformed data), P < 0.05 control vs. 1, 2, or 4 months.
Five animals in the group, 5 months on the cholesterol-containing diet; statistically significant (with nonparametric rank-sum test), α < 0.036 aortic intima-media uptake of ³H-sphingomyelin after 4 months of cholesterol feeding vs. ³²P incorporation into aortic intima-media sphingomyelin after 5 months of cholesterol feeding.

Sphingomyelin phosphate concentrations transformed to log₁₀ confirmed that these transformed concentrations were direct functions of time on the diet (F ratio = 13.16, df 1, 10 for P < 0.01, the critical F ratio = 10.00, y = 0.0045x + 0.13) and that aortic sphingomyelin appeared to increase exponentially.

This accumulation of sphingomyelin in the aortic wall could be due to entry from the serum compartment, de novo synthesis in the arterial wall, or some combination of entry and synthesis. To ascertain the contribution from the serum compartment, labeled sphingomyelin was injected into three normal and nine atherosclerotic rabbits exposed to cholesterol for up to 4 months.

The entry rate of serum sphingomyelin was determined as follows. The specific radioactivities of serum sphingomyelin were plotted as a function of time, and the average specific radioactivity, calculated from the area under the curve, and the hours of perfusion were divided into the total radioactivity found in the aorta. Fractional turnover was expressed as a ratio of the aortic intima-media specific radioactivity of sphingomyelin to the average serum specific radioactivity of sphingomyelin.

Entry rates of sphingomyelin into the aortic intima-media of rabbits fed cholesterol for 1–4 months were significantly different from those of control rabbits (Table 1). No transfer of aortic intima-media sphingomyelin ³H-choline to other arterial phospholipids was detected. A simple linear regression of entry rates of serum sphingomyelin into the aortic intima-media after transformation to log₁₀ of the original rates showed that this entry rate was dependent on exposure to elevated serum cholesterol levels (F ratio = 5.40, df 1, 10 for P < 0.05, critical F ratio = 4.96, y = 0.0098x + 0.75) and on the time of cholesterol feeding per se (F ratio = 11.02, df 1, 10 P < 0.01, critical F ratio = 10.00, y = 0.0030x + 0.98).

To determine the contribution of de novo synthesis of aortic sphingomyelin to the net aortic accumulation of this lipid, aortas were perfused with a medium containing ³²P. This design was employed.
TABLE 2
Phosphate Incorporation and Phospholipid Concentrations in Perfused Rabbit Aortic Intima-Media

<table>
<thead>
<tr>
<th>Phospholipid concentration (μmoles phospholipid P/g tissue)</th>
<th>Lysolecithin</th>
<th>Sphingomyelin</th>
<th>Lecithin</th>
<th>Phosphatidyl inositol</th>
<th>Phosphatidyl ethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.56 ± 0.66</td>
<td>4.67 ± 1.22</td>
<td>5.44 ± 0.71</td>
<td>0.80 ± 0.35</td>
<td>0.94 ± 0.20</td>
</tr>
</tbody>
</table>

P incorporation/intima-media (nmol phospholipid P/g tissue hour⁻¹)

|                                                                 | 0.590 ± 0.121 | 0.647 ± 0.172 | 11.0 ± 1.65 | 5.17 ± 1.17          | 1.06 ± 0.382            |

se % of mean

|                                                                 | 20.5         | 26.5          | 15.0        | 22.7                 | 36.0                    |

P incorporation/cholesterol concentration (nmol phospholipid P/mg hour⁻¹)

|                                                                 | 0.0314 ± 0.007 | 0.0399 ± 0.013 | 0.682 ± 0.209 | 0.354 ± 0.125         | 0.0569 ± 0.025           |

se % of mean

|                                                                 | 21.7         | 28.3          | 30.7        | 35.5                 | 43.7                    |

Normal

|                                                                 | 0.754 ± 0.196 | 2.40 ± 1.37   | 6.09 ± 1.92 | 2.45 ± 1.95          | 0.858 ± 0.386            |

se % of mean

|                                                                 | 26.0         | 57.2          | 31.6        | 79.5                 | 45.0                    |

Values are means ± se for five rabbits per group.

so that there would be a good possibility in this closed system of having the precursor inorganic phosphate of the same specific activity as the possible immediate precursor cytidine diphosphocholine or sphingosine phosphorylcholine. Segments of the artery were removed from the perfusion apparatus, and the incorporation of ³²P into various phospholipids was determined at hourly intervals. Table 1 shows that no significant change in the synthetic rate of sphingomyelin was demonstrable due to atheromatosis (t of control vs. atheroma 1.70, critical t = 2.447 at P < 0.05). The ratio of the synthetic rate to the influx rate was 0.0036. To assess the relevance of ³²P incorporation into sphingomyelin, this observation should be studied in the context of total phospholipid synthesis in the arterial wall. A comparison of the amount of radioactivity in the phospholipids from normal and atheromatous aortas (Table 2) showed that there was no significant increase in synthesis in any of the phospholipid classes due to atheromatosis even though the mean synthetic rate was doubled with atheromatosis. In both the normal and the atheromatous intima-media, lecithin had the highest synthetic rate with phosphatidyl inositol next in the atheromatous aorta. Phosphatidyl inositol and sphingomyelin were next highest in ³²P incorporation in the normal aorta. The lowest rate of synthesis was in the lyssolecithin fraction in both the normal and the atheromatous aortic intima-media.

Gross observation of the atheromatous segments of aortas used for the ³²P incorporation-perfusion studies showed large geographic variations in fatty streaks within the same aorta. Determination of cholesterol concentration in the aorta was considered to be a valid measure of this variation in the fatty streaks. Table 3 shows the degree of variation in cholesterol concentration in the aorta. However, calculation of phospholipid synthesis per aortic intima-media cholesterol concentration (Table 2) did not improve the coefficient of variation significantly. Therefore, we conclude that phospholipid biosynthesis is not dependent on aortic intima-media cholesterol concentrations.

Discussion

It has been shown that during atheromatosis, sphingomyelin appears to increase. This accretion of sphingomyelin is comparable to the exponential increase in rabbit aortic phospholipids during atheromatosis found by Newman and Zilversmit (15). A number of investigators have observed increased total phospholipids (3, 6, 16-18) and sphingomyelin (3, 17, 18). The increase in sphingomyelin parallels the apparently exponential increase in both cholesterol and cholesterol ester (15, 19). Aging in humans also gives increases in aortic sphingomyelin, but this increase is coupled with parallel increases in aortic cholesterol in the artery (2). Higher sphingomyelin concentrations in the arterial wall occur concurrently with an apparently exponential elevation in sphingomyelin in-
Concentrations (mg/g Aortic Intima-Media) and Percent of Cholesterol in Segments of Rabbit Aortic Intima-Media

<table>
<thead>
<tr>
<th>Time of perfusion* (hours)</th>
<th>Rabbit 1</th>
<th>Rabbit 2</th>
<th>Rabbit 3</th>
<th>Rabbit 4</th>
<th>Rabbit 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>77.1</td>
<td>11.2</td>
<td>53.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent</td>
<td>18.7</td>
<td>12.4</td>
<td>17.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>295.0</td>
<td>56.8</td>
<td>204.3</td>
<td>104.4</td>
<td>96.1</td>
</tr>
<tr>
<td>Percent</td>
<td>71.6</td>
<td>62.9</td>
<td>68.0</td>
<td>28.6</td>
<td>61.5</td>
</tr>
<tr>
<td>Concentration</td>
<td>20.9</td>
<td>11.0</td>
<td>28.2</td>
<td>81.0</td>
<td>12.9</td>
</tr>
<tr>
<td>Percent</td>
<td>5.1</td>
<td>12.2</td>
<td>9.4</td>
<td>22.2</td>
<td>8.3</td>
</tr>
<tr>
<td>Concentration</td>
<td>19.2</td>
<td>11.3</td>
<td>14.3</td>
<td>88.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Percent</td>
<td>4.7</td>
<td>12.5</td>
<td>4.8</td>
<td>24.1</td>
<td>16.0</td>
</tr>
<tr>
<td>Concentration</td>
<td>91.4</td>
<td>22.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent</td>
<td>25.1</td>
<td>14.2</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*Segments were taken at intervals up to 5 hours.

flux into the aortic intima-media. This increase is dependent on both time of exposure to cholesterol diet and extent of exposure to elevated serum cholesterol concentrations.

To determine the possible source of this increase, synthetic rates for sphingomyelin in the aortic intima-media were compared using tissue from normal and atheromatous rabbits. There was no significant change in the sphingomyelin synthesis rate due to atheromatosis in the aortic intima-media. This observation has also been made by Morin (18) who used incubations with $^{14}$C-choline and $^3$H-acetate as precursors to study phospholipid biosynthesis in normal and atheromatous rabbit aortas. In earlier studies, McCandless and Zilversmit (17), Newman et al. (20), and Bowyer and co-workers (21) have shown enhanced incorporation of precursors into aortic sphingomyelin during atheromatosis.

The discrepancies in the findings from these studies may be attributed to differences in (1) precursors, (2) conditions of label introduction, and (3) the tissue employed for metabolic studies. McCandless and Zilversmit (17) and Bowyer and co-workers (21) used the whole thoracic aorta with potential periarterial tissue contaminants, whereas in the studies of Morin (18) and the present investigation only intima-media portions were used.

The sphingomyelin entry rate into the aortic intima-media was 272 times the synthetic rate measured in our perfusion studies of atheromatous rabbit aortas. The overall synthetic rate for phospholipids in our perfusion system was 18.5 nmoles/hour g$^{-1}$ wet weight of aortic intima-media, which is about one-twentieth of that obtained by Zilversmit and co-workers (6,16), who made their observations on whole aortic tissue in intact rabbits. It is possible that in the perfusion system in the absence of serum there is an overall reduction in phospholipid synthesis in both normal and atherosclerotic rabbits. This overall reduction has also been observed by Bowyer and co-workers (21). If it is assumed that the variation in phospholipid synthesis is proportionate for all phospholipid classes, then 14.3 nmoles of sphingomyelin would be synthesized per hour in vivo per gram of rabbit atheromatous aortic intima-media. This synthetic rate is still only 8% of the serum sphingomyelin entry rate.

Since the studies of Eisenberg and co-workers (22) show no decrease in sphingomyelin degradation during atheromatosis in rabbits, although decreases in human aortas have been observed (2), we assume that entry from the serum accounts for most of the increased content of sphingomyelin in the aorta of the rabbit during atheromatosis. This finding is in accord with the similarity in fatty acid composition of rabbit serum and aortic atheroma sphingomyelin. In contrast are the more significant differences in fatty acid composition of lecithin in serum and rabbit atheroma (23).

The present findings support the concepts of lipid accretion elucidated by Portman (24) and the relationship he demonstrated between net accumulation of sphingomyelin and hypercholesterolemia.
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