Metabolism of sphingolipids by normal and atherosclerotic aorta of squirrel monkeys

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ABSTRACT We studied the synthesis and hydrolysis of sphingomyelin by homogenates of aortic intima plus inner media from normal squirrel monkeys and from monkeys with nutritionally-induced atherosclerosis (6–10 mo on a semipurified diet containing butter and cholesterol).

The concentrations of sphingomyelin in the aortas and plasmas of the atherosclerotic monkeys were higher than those for the normal monkeys. Palmitoyl-1-^{14}C coenzyme A was actively utilized for the synthesis of ceramide (*N*-palmitoyl sphingosine). The addition of sphingosylphosphorylcholine increased the utilization of palmitoyl CoA in sphingomyelin synthesis, and the addition of psychosine (sphingosyl galactoside) increased the incorporation of palmitate into cerebrosides. Rates of sphingomyelin and ceramide synthesis were significantly higher in the atherosclerotic than in the control aortas. Hydrolysis of labeled sphingomyelin to ceramide was also increased in homogenates of the atherosclerotic aortas. Labeled sphingomyelin was taken up from plasma by everted carotid arteries, and this process was also enhanced by atherosclerosis.

Increased rates of synthesis and of uptake from plasma of sphingomyelin may account for the increased concentrations of sphingomyelin in the atherosclerotic arteries, even though the ability to degrade sphingomyelin is also enhanced in the atherosclerotic aorta.

SUPPLEMENTARY KEY WORDS palmitoyl CoA · ceramide · aortic intima plus inner media · sphingosylphosphorylcholine · sphingomyelin synthesis and hydrolysis · semipurified diet · everted carotid artery

ONE OF THE CHANGES in the aortic intima plus inner media consequent to age or atherosclerosis in man is an increased concentration of sphingomyelin (e.g. references 1–5). This change also characterizes aging and early atherosclerosis in monkeys (6–9). The following three aspects of sphingomyelin metabolism are pertinent to an understanding of variations in arterial sphingomyelin concentrations: synthesis, catabolism, and endothelial uptake from, or exchange with, the plasma lipoproteins.

It has been difficult to study the incorporation of phosphate, choline, or fatty acid into sphingomyelins since the rates of such incorporation are so low compared with those for lecithin. In previous studies (10, 11) we reported the high incorporation of palmitate from palmitoyl-1-^{14}C coenzyme A into ceramide (*N*-palmitoyl sphingosine) by cell-free preparations of aortic intima plus inner media. A ceramidase that has been demonstrated in brain (12) may also be active in aorta, just as the sphingomyelinase first studied in liver (13, 14) and in brain (15) has been demonstrated subsequently in the aortas of several species (16).

Observations made in vivo (17) emphasize the position of ceramide as a sphingomyelin precursor, although evidence from in vitro studies shows that particularly in the brain the enzymic reaction of fatty acyl CoA with SPC (18) is important.

In the present paper we have emphasized the synthesis of sphingomyelin from SPC and palmitoyl CoA in aorta because this reaction could be observed in vitro while the reaction of ceramide and cytidine diphosphate choline, as carried out by Fujino, Nakano, Negishi, and Ito (19), could not. The former pathway is not necessarily active in vivo, however. Sribney and Kennedy (20) have observed that only *threo*-sphingosine or ceramide containing *threo*-sphingosine is an effective substrate for sphingomyelin synthesis in vitro, despite the fact that natural sphingomyelin has sphingosine with the erythro configuration. Recently, however, Fujino et al. (19) have shown that the apparent preference for cer-

Abbreviations: SPC, sphingosylphosphorylcholine.

amide containing *threo*-sphingosine in sphingomyelin synthesis may be an artifact resulting from the superiority of the threo form in substrate emulsification. Differences in solubility of substrates may also account for apparent differences in the activity of the SPC and ceramide pathways of sphingomyelin synthesis in vitro.

Aortic sphingomyelin may arise in part from plasma lipoproteins since sphingomyelin levels are relatively high in plasma, being concentrated in the low density lipoproteins (21, 22). Sphingomyelin is concentrated in the plasma membrane (23, 24) of most cells, including the smooth muscle cells of the aorta (7, 9, 25).

This paper describes the formation of sphingomyelin, ceramide, and cerebrosides from palmitoyl CoA by preparations of aortic intima plus inner media. The hydrolysis of sphingomyelin to ceramide was also studied. In some cases, subfractions of brain and liver were included as control tissues with known behavior. Atherosclerosis was also shown to affect some of these arterial activities as well as the uptake of sphingomyelin from plasma by everted carotid arteries.

MATERIALS AND METHODS

Substrates

Palmitoyl-1-^{14}C CoA and unlabeled palmitoyl CoA were prepared by the method of Seubert (26) slightly modified (11). The specific activity of preparations of the former were 10 μc/μmole.

SPC was prepared by the method of Kaller (27). Thin-layer chromatography (chloroform–methanol–water 60:35:8 on Silica Gel H) indicated that at least 90% of this preparation was in the erythro configuration, the threo form being barely detectable. Tentative identification of the erythro and threo forms depended on the R_f values (19). For most of the metabolic studies, the erythro compound was not further purified. The molar proportions of sphingosine (28), choline (29), and phosphorus (30) were 1.06:1.03:1.00.

Palmitoyl-1-^{14}C–labeled sphingomyelin used in hydrolysis studies was prepared biosynthetically by incubation of rat brain mitochondria plus microsomes (1,000–100,000 *g* pellet) with palmitoyl-1-^{14}C CoA and SPC. The contents of 20–40 incubation vessels were pooled for each preparation. Each incubation vessel contained 2–5 mg. of protein, 0.25 μc of palmitoyl-1-^{14}C CoA (10 μc/μmole), and 0.3–1 mmole of SPC in a total volume of 2 ml. Incubations were carried out at 37°C for 3 hr (subsequent studies indicated that nearly full activity was obtained with 15-min incubations).

Reactions were stopped with chloroform–methanol 2:1 and incubated for 1 hr longer to insure complete extraction of lipid. The protein-free filtrate was washed with water and dried under nitrogen, and the residue was subjected to hydrolysis in methanolic NaOH (0.4 N) at 37°C for 1 hr. This treatment was shown to hydrolyze quantitatively all of the glycerophosphatides but not sphingomyelin. We subjected some of these hydrolysates, as well as lipids from incubations of aortas (to be described subsequently), to mild acid hydrolysis in order to hydrolyze any plasmalogens present. Since the levels of radioactivity of the compounds investigated were not influenced by acid hydrolysis, the acid hydrolysis step was eliminated. The hydrolysate was neutralized with HCl, and the mixture was subjected to another equilibration with chloroform–methanol–water 2:1:0.6. The lower layer was dried under nitrogen and applied in a small volume of chloroform across two 20 × 20 cm thin-layer plates coated with specially washed (31) Silica Gel H (400 μ thick) and chromatographed according to the system of Skipski, Peterson, and Barclay (32). The sphingomyelin zone, located by means of reference standards on the plates, was scraped into a column prepared from a 10 ml graduated pipette that contained a packed plug of glass wool. The sphingomyelin was eluted with 20 ml of 95% methanol. We verified the purity of the sphingomyelin by repeating the thin-layer chromatography, by establishing the compound's stability to mild alkali treatment, and by quantitatively converting a sample of it to ceramide with phospholipase C (Sigma Chemical Co., St. Louis, Mo.) obtained from *Clostridium welchii*. The specific activities of the sphingomyelin-^{14}C prepared in this way and subsequently used were 0.49, 0.60, and 1.19 μc/μmole. The molar proportions of sphingosine, choline, and phosphorus were 1.00:1.12:1.00.

To further characterize this product, we dissolved biosynthetic sphingomyelin-^{14}C (see above) in 1 ml of diethyl ether and added 0.5 ml of 0.001 M $CaCl_2$ containing 1 mg of phospholipase C. The incubation mixture was allowed to stand overnight at 24°C. (This two phase system [33] for reaction with phospholipase C was more effective than the single phase system of Macfarlane [34].) The reaction was stopped with 1 ml of ethanol, and the solvents were removed under a stream of nitrogen. The residue was chromatographed on Silica Gel H in chloroform–MeOH–glacial acetic 95:2:5 (15), and the ceramide was located by means of a reference standard (nonhydroxy ceramide; Applied Science Laboratories, Inc., State College, Pa.). Ceramide was eluted from the silica gel with methanol in the manner described for sphingomyelin.

Psychosine (galactosyl sphingosine), bovine cerebrosides, and sulfatide were obtained from Applied Science Laboratories, Inc.

DL-Sphingosine was obtained from the Miles Laboratories, Inc., Elkhart, Ind.

Preparation of Animals

Female squirrel monkeys, each weighing 540–720 g, were used. They had been fed one of two semipurified diets (8, 11) for 6–10 mo. One diet supplied 15% of calories as corn oil and contained no cholesterol; the second supplied 45% of calories as butter and 0.1 g of cholesterol per 100 cal. These diets represent the extremes of numerous diets tested for producing the widest range of plasma cholesterol concentrations and aortic atherosclerosis. Animals fed the first diet had low plasma cholesterol levels (mean = 1.66 mg/ml) and a high incidence of atherosclerosis-free aortas; those fed the butter diet had high cholesterol levels (mean = 3.30 mg/ml) and a high incidence of diffusely atherosclerotic aortas.

Preparation of Tissues and Methods of Incubation

Total homogenates of aortic intima plus inner media and of various subcellular fractions were prepared as previously described (6, 35).

Hydrolyses of sphingomyelin labeled with palmitate-1-^{14}C were measured essentially according to Rachmilewitz, Eisenberg, Stein, and Stein (16). This method includes the use of 0.1 M acetate buffer, pH 5.1, and Triton X-100 (obtained from Sigma Chemical Co., St. Louis, Mo.) at 0.4%. Ceramide hydrolysis was investigated under similar conditions by the method of Gatt (12).

Rates of incorporation of palmitate into different sphingolipids were measured, usually at pH 7.9, with palmitoyl-1-^{14}C CoA (63 μM) as substrate, according to Fujino and Negishi (36). Tetracycline (0.1 mg/0.45 ml) was added. This concentration did not affect the rate of formation of sphingomyelin by active homogenates of aorta, but it did reduce values observed for water or heated tissue blanks in some cases. SPC, psychosine (galactosyl sphingosine), and sphingosine at various concentrations were added for some of the incubations. Palmitoyl CoA, SPC, and psychosine spontaneously form optically clear dispersions in water. Sphingosine micelles were formed in water with the aid of ultrasound.

Measurements of enzymic hydrolysis were made at 1, 2, and 3 hr; measurements of synthesis were made at 10 min and 2 hr. Each determination consisted of the values obtained from two incubations of unheated tissues and one incubation of heated (100°C) tissue. Reactions were stopped by the addition of chloroform–methanol 2:1, kept at 37°C for 1 hr to insure lipid extraction, and filtered. Aliquots of the chloroform–methanol filtrate to which 20 μg of carrier substrate and reaction products had been added were washed and then subjected to thin-layer chromatography. The lipid fractions from the synthesis studies were treated similarly except for the addition of a mild alkaline hydrolysis. The lipids in the hydrolysis studies were separated using only a ceramide (15) system (chloroform–methanol–acetic acid 95:2:5). The thin-layer systems of Skipski et al. (32) for sphingomyelin, of Honegger (37) for cerebrosides, and of Barnholz, Roitman, and Gatt (15) for ceramides were used to separate the lipid extracts from all of the synthesis studies. Compounds were made visible by exposure to I_2 vapor and outlined, and the thin-layer plates were photographed. The iodine was then allowed to sublime, and individual spots were scraped into scintillation vials containing 2 ml of ethanol. Areas between spots were usually also assayed in approximately 1 cm increments. Scintillation fluid (0.5% 2,5-diphenyloxazole and 0.03% -*p*bis 2[5-phenyloxazolyl]-benzene in toluene) was added, and samples were assayed in a Packard liquid scintillation counter. Absolute activity was determined by the channels-ratio method. The radioactivity found at the sphingomyelin locus on the phospholipid plate (32) agreed with that found on the cerebroside plate (37). The ceramide system separated nonhydroxy and hydroxy fatty acid ceramides but did not adequately separate sphingomyelin from cerebrosides. The cerebroside system separated hydroxy and nonhydroxy fatty acid cerebrosides and sulfatides but did not separate ceramides from unidentified labeled products near the front. Palmitoyl CoA was largely or entirely removed in the water washing of chloroform–methanol extracts prior to chromatography. Palmitic acid which was freed by thiolytic activity in the active tissue preparations was adequately separated from other products by thin-layer chromatography.

Determination of Tissue Concentrations of Sphingolipids and Other Lipids

Concentrations of various lipids in the aortas and plasmas of squirrel monkeys were measured according to procedures previously described (6, 11, 38). These consisted essentially of thin-layer chromatography followed by a second development of the thin-layer plates in tanks containing the charring reagent of Ziminski and Borowski (39). The plates were heated and then scanned quantitatively according to Privett, Blank, Codding, and Nickell (40).

RESULTS

Concentration of Sphingomyelin in Aorta and Plasma

Table 1 shows the mean concentrations of sphingomyelin, lecithin, and cholesterol in the aortic intima plus inner media and in the plasma of squirrel monkeys. In both tissues the hypercholesterolemic-atherosclerotic monkeys had higher concentrations of sphingomyelin and cho-

TABLE 1 CONCENTRATION OF SPHINGOMYELIN, LECITHIN, AND TOTAL CHOLESTEROL IN THE AORTIC INTIMA PLUS INNER MEDIA AND IN THE PLASMA OF SQUIRREL MONKEYS FED A BASAL OR AN ATHEROGENIC SEMIPURIFIED DIET FOR 8 MONTHS

	Sphingomyelin		Lecithin		Total Cholesterol
	Concn	% Total PL	Concn	% Total PL	
	mg/g		*mg/g*		*mg/g*
Aorta					
Atherosclerotic	3.28 ± 0.22	26.2	5.83 ± 0.32	46.4	13.64 ± 1.20
Control	2.07 ± 0.28	26.4	2.72 ± 0.20	36.2	5.06 ± 0.74
	mg/ml		*mg/ml*		*mg/ml*
Plasma					
Atherosclerotic	0.23 ± 0.02	7.58	2.46 ± 0.23	81.5	3.30 ± 0.25
Control	0.14 ± 0.006	8.79	1.34 ± 0.095	83.8	1.66 ± 0.11

Values are means ±SEM (n = nine animals per group).

TABLE 2 SYNTHESIS OF SPHINGOLIPIDS BY HOMOGENATES OF SQUIRREL MONKEY AORTIC INTIMA PLUS INNER MEDIA OR OF MID-BRAIN

Tissue	Supplement Added	Radioactive Product Synthesized		
		Sphingomyelin	Ceramide	Cerebroside
		nmoles/mg protein per hr		
Aorta (100°C)	—	0.27	0.13	0.08
Aorta	—	0.42	1.78	0.55
H_2O	100 nmoles SPC	0.35	0.09	0.05
Aorta (100°C)	100 nmoles SPC	0.30	0.19	0.07
Aorta	100 nmoles SPC	3.24	1.45	0.62
Aorta	200 nmoles SPC	2.90	0.53	0.39
Aorta	50 nmoles SPC	2.98	1.72	0.45
H_2O	100 nmoles psychosine	0.12	0.05	0.67
Aorta (100°C)	100 nmoles psychosine	0.18	0.09	0.41
Aorta	100 nmoles psychosine	0.40	0.38	1.46
Aorta	100 nmoles spingosine	0.35	1.54	0.79
Mid-brain	—	2.66	1.06	0.13
Mid-brain	100 nmoles SPC	7.28	1.41	0.10
Mid-brain	100 nmoles psychosine	2.33	0.99	0.40

The incubation media was modified from that of Fujino and Negishi (36) and contained palmitoyl-1-^{14}C CoA (28.3 nmoles), ATP (4 nmoles), $MgCl_2$ (1 μmole), potassium phosphate buffer, pH 7.89 (40 μmoles), tetracycline (0.1 mg), and homogenate containing 0.5 mg of protein, plus indicated supplements in a total volume of 0.45 ml. The incubations were for 10 min.

lesterol than the monkeys with low cholesterol levels and a low incidence of vascular lesions.

Conversion of Palmitate from Palmitoyl-1-^{14}C CoA to Sphingomyelin, Ceramide, and Cerebrosides

Palmitoyl-1-^{14}C CoA (at 63 μM, a concentration at the threshold of detectable substrate inhibition of the formation of various products) was incubated under several conditions with homogenates of apparently normal squirrel monkey aortic intima plus inner media (Table 2). Heating the homogenates in boiling water for 5 min partly prevented the formation of radioactive sphingomyelin, ceramide, and cerebroside. There was little sphingomyelin formed unless sphingosylphosphorylcholine was added. SPC greatly stimulated the incorporation of radioactivity into sphingomyelin, and the addition of psychosine (galactosyl sphingosine) enhanced the apparent formation of cerebrosides. Regardless of which blank was used (tissue homogenate which had been heated to 100°C or water) significant amounts of sphingomyelin or cerebroside were formed when SPC or psychosine was present. Since the quantities formed in these blanks increased with time of incubation, it seemed probable that the products were indeed synthesized during incubations. An alternative explanation would be an alkali catalyzed transesterification of some palmitoyl CoA that escaped the washing procedure prior to hydrolysis of glycerophosphatides. There were several alkali-stable products in the thin-layer chromatograms used to isolate cerebrosides (37). Therefore, in two instances the nonhydroxy cerebroside spot was eluted and rechromatographed; 82% of the radioactivity rechromatographed as a nonhydroxy cerebroside.

Ceramide was the major alkali-stable product from palmitoyl CoA in the absence of added fatty acyl acceptors. The failure of added sphingosine to further

stimulate the incorporation of palmitate into ceramide (see reference 41) may be due to the presence or synthesis of adequate endogenous sphingosine or to an unfavorable physical state of the exogenous sphingosine. Data from comparable experiments with homogenates of pons from the same squirrel monkeys are given for comparison. The failure of psychosine to significantly stimulate the incorporation of palmitate into cerebroside by brain is consistent with the observations of Morell and Radin (42) but not with the report of Brady (43). The identity of the cerebroside radioactivity in aorta is based solely on cochromatography with commercial cerebroside containing nonhydroxy fatty acids. Quantities of the carrier-free cerebroside were much too small for further characterization.

Relation of Concentrations of SPC to Sphingomyelin Formed from Palmitoyl-1-^{14}C CoA

Fig. 1 shows a characteristic substrate concentration–enzymic activity relationship for SPC concentration and apparent activity for sphingomyelin formation. At still higher concentrations of SPC (e.g., 0.5 and 1.0 mM), the reaction was markedly inhibited.

Incubation Times and Incorporation of Palmitate-1-^{14}C into Sphingomyelin and Ceramide from Palmitoyl-1-^{14}C CoA

Fig. 2 shows the time course of incorporation of palmitate-^{14}C into sphingomyelin and ceramide when palmitoyl-1-^{14}C CoA was incubated with homogenates of aortic intima plus inner media. Sphingomyelin synthesis was low in the absence of SPC but was higher when this precursor was added. However, at the relatively low concentrations of palmitoyl CoA (because of the inhibitory effects of palmitoyl CoA at high concentrations), rates of synthesis were not linear for long incubations.

Without SPC, ceramide synthesis from palmitoyl-1-^{14}C CoA continued at a rapid rate for 2 hr. With SPC, ceramide synthesis was initially rapid, but total synthesis after 2 hr was actually lower than without SPC. We assume that the depression of ceramide synthesis in the presence of exogenous SPC results from utilization of palmitoyl CoA by the pathway of Brady, Bradley, Young, and Kaller (18) and by deflection of substrate away from ceramide synthesis. It is possible that ceramide formed during incubations with SPC partly results from the formation of sphingomyelin and subsequent phospholipase C activity.

Distribution of Synthetic Activity in Subcellular Fractions of Aortic Homogenates

Most of the sphingomyelin and ceramide synthetic activity was found in the particulate fractions; 51% of the cell-free activity for sphingomyelin synthesis was in the

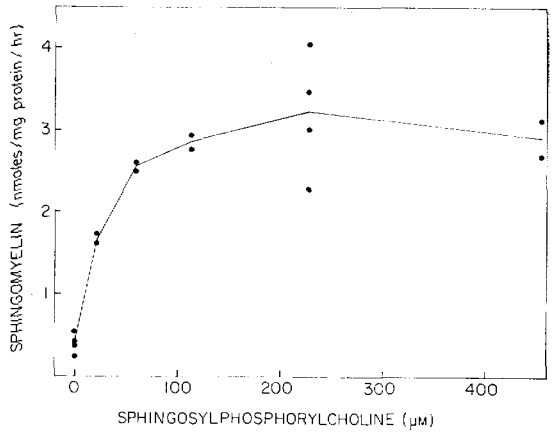

FIG. 1. Effect of concentration of SPC on the activity of homogenates of aortic intima plus inner media to form sphingomyelin-^{14}C from palmitoyl-1-^{14}C CoA (the latter at 63 μM—the highest concentration that did not result in substrate inhibition).

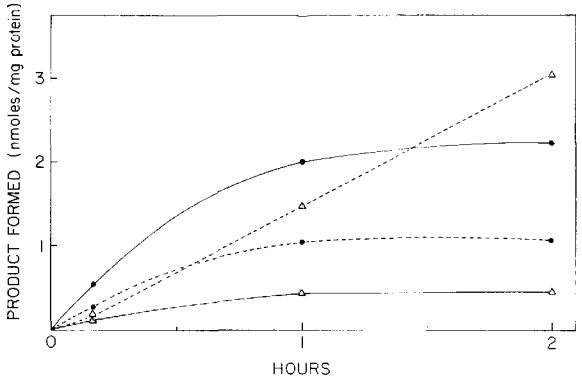

FIG. 2. Effect of incubation time and presence of SPC (222 μM) on formation of sphingolipids from palmitoyl-^{14}C CoA by homogenates of aortic intima plus inner media.
Sphingomyelin formed: ●—● with SPC; △—△ without SPC.
Ceramide formed: ●– – –● with SPC; △– – –△ without SPC.

mitochondria, 45% in the microsomes, and less than 5% in the supernatant fraction. Although the aortic mitochondria we use are heavily contaminated with myofibrils, collagen, and elastin, the high activity of aortic mitochondria is consistent with analogous studies of Fujino and Negishi (36), which showed 80% of the activity in brain to be in mitochondria.

Effects of Nutritionally-Induced Atherosclerosis on Sphingomyelin and Ceramide Synthesis by Aortic Intima Plus Inner Media

In squirrel monkeys the effect of atherosclerosis on sphingomyelin and ceramide synthesis was studied in homogenates of aortic intima plus inner media with and without exogenous SPC. Table 3 (in which some values from Table 2 are included within some of the mean values of the control group) shows increased incorporation of palmitate into sphingomyelin and ceramide in the atherosclerotic group.

TABLE 3 MAXIMUM RATES OF INCORPORATION OF PALMITATE FROM PALMITOYL-1-^{14}C CoA INTO SPHINGOMYELIN AND CERAMIDE BY HOMOGENATES OF AORTIC INTIMA PLUS INNER MEDIA

	Product Formed			
	Sphingomyelin		Ceramide	
SPC Added	+	−	+	−
	nmoles/mg protein per hr		*nmoles/mg protein per hr*	
Atherosclerotic	3.85 ± 0.39	0.39 ± 0.05	1.41 ± 0.18	2.30 ± 0.59
Control	2.61 ± 0.31	0.40 ± 0.05	1.10 ± 0.13	1.38 ± 0.15

Measurements were made with and without added SPC (222 μM). Rates were usually calculated from the first 10 min of incubation, although ceramide synthesis without added SPC was linear for 2 hr. Values are means ±SEM (n = seven animals per group).

Sphingomyelin Hydrolysis by Homogenates of Aortic Intima Plus Inner Media

We studied sphingomyelin hydrolysis by incubating sphingomyelin labeled enzymatically with palmitate-1-^{14}C with homogenates of aorta under conditions similar to those described by Rachmilewitz et al. (16). We confirmed the observations of these workers in that Triton is necessary for activity, that the pH maximum is around 5.0, that at 37°C activity is almost constant for at least 3 hr, and that the effects of tissue and exogenous substrate on the labeled product indicate randomization of the endogenous and exogenous substrate. We did not prepare our tissue homogenates with Triton, as did Rachmilewitz et al. (16). We added the Triton just before the incubations. Under our conditions, most of the hydrolytic activity was in the 3×10^6 g-min precipitate.

The effect of nutritionally induced atherosclerosis was to increase the rate of conversion of sphingomyelin to ceramide, but this increased sphingomyelinase activity (1.34 ± 0.20 vs. 0.95 ± 0.06 [SEM] nmoles/mg protein per hr, n = 10 per group) was statistically significant only at the $P = 0.05$ level.

Uptake or Exchange of Sphingomyelin-^{14}C by Everted Carotid Arteries

Methods for incorporating large amounts of labeled sphingomyelin into incubation media are not available. Nevertheless, the results of labeled sphingomyelin uptake from micelles by everted carotid arteries may be informative. After incubation in a medium containing 40 μM sphingomyelin in micellar form (with plasma added in a few instances), more radiosphingomyelin was in the everted carotid arteries from the atherosclerosis group than in those from the control group (2.44 ± 0.08 vs. 1.89 ± 0.10 [SEM, n = 7 per group] nmoles per carotid per 2 hr, $P = 0.001$). The uptake of sphingomyelin was also dependent on sphingomyelin concentrations in the incubation media (results not shown).

DISCUSSION

Sphingomyelin is a major component of the aortic intima plus inner media. We have observed that the absolute concentration of sphingomyelin and its percentage of the total tissue phospholipid increases during the life span even of nonatherosclerotic monkeys (6, 7, 9). One of the first changes seen in the aortas of squirrel monkeys on an atherosclerosis-inducing diet is an increase in sphingomyelin content (8, 9).

This study indicates that the increase in aortic sphingomyelin concentration with nutritionally-induced hyperlipidemia is associated with increased concentrations of sphingomyelin in the plasma. Everted carotid arteries from the atherosclerotic group took up more sphingomyelin from the incubation media than those from the normal group. The quantity of sphingomyelin taken up also depended on the concentration in the medium. Thus net uptake from the blood could cause increased arterial concentrations of sphingomyelin.

It appears, however, that the aorta may also be able to synthesize sphingomyelin, as well as ceramide and cerebroside, from palmitoyl CoA. Cerebrosides have frequently been demonstrated in aortic tissue (44–46), but there is some confusion about the prevalence of glucose and galactose. Foote and Coles (46) found that only one of 11 aorta samples showed a predominance of galactosyl-cerebrosides, but Hausheer and Bernhard (45) found more galactose than glucose in aorta cerebrosides. We did not use sodium tetraborate thin-layer chromatography to identify the type of cerebroside formed by our aorta homogenates. If glucosyl cerebrosides are the natural aortic cerebrosides, stimulation of cerebroside formation by psychosine may not be relevant in vivo.

When an acceptor molecule such as SPC or psychosine is not added, ceramide is the principal labeled product from palmitoyl-1-^{14}C CoA incubated with aorta homogenates. The efficient incorporation of palmitate from palmitoyl-1-^{14}C CoA into ceramide was also noticed in our previous studies (10, 11), but as far as we know,

ceramide concentrations in arteries have not been reported. Preliminary measurements gave us aortic concentrations of 0.020 mg ceramide per g for normal squirrel monkeys and 0.042 mg/g for the atherosclerotic monkeys (cf. values for sphingomyelin in Table 1). Since ceramide is apparently not present in high concentrations in aorta, it is difficult to appraise the significance of the incorporation of radiopalmitate into ceramide. In our experiments ceramide-^{14}C formed by aorta homogenates was not very actively converted to sphingomyelin or cerebroside. We were also unable in limited experiments to demonstrate the hydrolysis of ceramide-^{14}C (16), or the reaction of ceramide with CDP-choline (21) or UDP-glucose. We have not studied these reactions in brain tissue, and as illustrated by Morell and Radin (42), the reactivity of ceramide is dependent on the way it is presented as a substrate.

Homogenates of monkey aorta can also hydrolyze sphingomyelin to ceramide and phosphoryl choline. Since ceramide is formed by both synthetic and degradative pathways in vitro and yet does not accumulate in the aorta in vivo, ceramidase activity is presumably present, or that compound must be transferred from the aorta. Samuelsson (47) has established the presence of ceramide in human plasma.

It is, of course, impossible from in vitro studies to estimate the relative activities of the arterial wall in vivo in taking up sphingomyelin from the blood and in synthesizing or degrading sphingomyelin. It is difficult to assign one of these mechanisms as a "cause" of the increased sphingomyelin in the aortic intima plus inner media that occurs when nutritional hypercholesterolemia and atherosclerosis is induced in squirrel monkeys. Our evidence indicates that all three processes are enhanced.

We have shown that sphingomyelin in arterial tissues is most prevalent in the microsomal fraction (7, 9). Furthermore, we have observed that the microsomal subfraction richest in sphingomyelin is derived from the plasmalemma (9, 25). It is possible that an accumulation of sphingomyelin in the atherosclerotic aortas is secondary to increased net uptake by the plasma membrane of the smooth muscle cells, the predominant cell type in the aortic intima plus inner media (e.g., references 48–50), including the aorta of squirrel monkeys (51).

Addendum. Since the submission of this manuscript we have seen a paper of Eisenberg, Stein, and Stein (52) which described increased incorporation of labeled choline into sphingomyelin of whole aortas of rats and rabbits with age. They also observed that sphingomyelinase activity did not rise appreciably, and therefore, they attributed the increase in aorta sphingomyelin concentrations with age to this imbalance between synthetic and hydrolytic activities.

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