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Cerebral Microvascular and Parenchymal Phospholipid Composition in the Mouse

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Cerebral microvessels consisting predominantly of capillaries and small arterioles (<30 μm dia.) were isolated from the cerebral cortex and cerebellum of 3-month-old mice. Lipids were extracted from both microvascular and brain parenchymal fractions and the major phospholipid classes (choline phosphoglyceride, ethanolamine phosphoglyceride, inositol phosphoglyceride, serine phosphoglyceride, and sphingomyelin) separated by 2-dimensional TLC. Comparison of mol % determined by phosphate analysis of each phospholipid revealed significant differences in membrane composition of ethanolamine phosphoglyceride, inositol phosphoglyceride, and sphingomyelin between microvascular and parenchymal components of the central nervous system. Moreover, the choline phosphoglyceride/sphingomyelin mol ratio, one of three determinants of membrane fluidity, is significantly lower for microvessel membrane than for membranes of the brain parenchyma.

KEY WORDS: Cerebral microvessels; lipids; phospholipids.

INTRODUCTION

The microcirculation of the mammalian brain consists of a functionally specialized system of capillaries, small arterioles (<30 μm dia.), and venules which together modulate the microenvironment of the brain (neurons and glia). Regulation of molecular and ionic fluxes across the cerebral microvasculature is affected predominantly by anatomical and physiological aspects of the microvessel endothelium, referred to collectively as the blood-brain barrier (BBB).

Recent studies suggest that cell membrane phospholipids are more than static structural components of the cell membrane, and that they influence directly or indirectly many specialized transport functions as well (5, 10, 14). At present only a few studies have reported on the phospholipid composition of endothelial cell membrane of isolated cerebral microvessels (4, 10, 11, 15). In this study, the first reporting on phospholipid composition of mouse cerebral microvessel endothelial membrane, comparisons were made between microvessels and the brain parenchyma (neurons and glia) isolated from the cerebral cortex and cerebellum of 3-month-old mice. The differences found in membrane phospholipid composition are discussed.

EXPERIMENTAL PROCEDURE

The brains from 3 month old, male, C3H mice (Charles River Laboratories, Inc., Wilmington, MA) were used for this study. All animals were maintained at a room temperature of 23 ± 1°C with a normal 12 hr. on-off lighting cycle. Food (Purina 5001 rat pellets) and water were given ad libitum.

Isolation of Cerebral Microvessels. The method used to isolate microvessels was derived from the modified procedures of

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Goldstein, et al. (6), Siakotos, et al. (17), and Selivonchick and Roots (15). Twenty-eight mice/isolation were sacrificed by cervical dislocation and the brains quickly removed and placed in ice cold 0.34 M sucrose containing 20,000 IU sodium heparin/liter (Solution A). Grey matter from the cerebral cortex and cerebellum was suctioned off into cold 0.34 M sucrose containing 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.34 mM MES (pH 6.1) and 1% bovine serum albumin (Fract. V) (Solution B). The tissue was finely minced with a single-edged razor blade and strained through a 125 μm nylon mesh sieve. The residue, consisting of larger pieces of grey matter, was broken down still further by repeated passage through a 590 μm nylon screen, glued to the cut end of a plastic 10 ml syringe tube. The filtrate and particulate fractions were combined and homogenized in a loosely fitting (0.01″ clearance) 30 ml glass Potter-Elvehjem Teflon homogenizer. The brain homogenate was then centrifuged at 2000 *g* for 10 min at 4°C in a Sorvall RC-30 refrigerated centrifuge. The supernatant was drawn off and the pellet resuspended in 130 ml 12% Dextran (Solution C) (60,000–70,000 MW) containing 0.1 mM $CaCl_2$, 0.1 mM ATP and 0.34 mM MES buffer. The microvessel suspension was then centrifuged at 5,000 *g* for 20 min. The use of 12% Dextran allows separation of contaminating myelin and some nonvascular cellular debris. The suspension was agitated vigorously for one minute using a vortex mixer and filtered through a 125 μm nylon mesh filter, and then passed over a 1.0 × 0.5 cm glass bead column under slight vacuum. The semi-purified microvessel fragments were washed off the beads with cold 0.34 M sucrose containing 0.1 mM $CaCl_2$, 0.1 mM ATP, and 0.34 mM MES (Solution D) and resuspended in 25 ml of the same solution. Small aliquots of the suspension were agitated at moderate speed for 2 mins in a 50 ml polyethylene centrifuge tube containing a small amount of glass beads. This procedure proved effective in dislodging most of the glial cells still attached to microvessel segments without significantly damaging the microvessels (Williams, personal observation). Small, well mixed aliquots of microvessel and parenchymal suspension were taken throughout the isolation procedure for light microscopic assessment of the presence or absence of microvessels in the respective suspensions. Finally, microvessels were again passed through a 125 μm nylon mesh filter, washed over a glass bead column and subsequently concentrated by centrifugation at 5000 *g* for 20 minutes. The supernatant was aspirated and the centrifuge tube aerated with argon gas. Microvessels were stored in sealed vials at −20°C.

All supernatants containing brain homogenate were collected at 4°C. The pooled homogenate was concentrated by centrifugation at 2000 *g* for 10 mins, and stored in 1 ml microcentrifuge tubes under argon gas at −20°C.

Lipid Analysis. Total lipids were extracted from microvessel and brain parenchymal membranes and purified by a modified "Folch" wash procedure (19). Pooled samples of isolated microvessels and brain homogenate were placed in a 50 ml glass-on-glass Ten Brock homogenizer and homogenized in chloroform/methanol (2:1). Methanol was added first to facilitate the extraction. The homogenate was then filtered under vacuum through Whatman #2 filter paper which was then washed with 2–3 ml chloroform/methanol (2:1). Phases were separated by the addition of 0.2 vol of 0.1 M KCl and the filtrate shaken vigorously. The filtrate was centrifuged in the cold at 1000 *g* for 10 minutes to facilitate phase separation. After aspirating the top layer the filtrate was washed with 1–2 ml of "Folch Upper Phase" (0.88% KCl/chloroform/methanol, 47:3:48) solution. The chloroform layer was then transferred to a 50 ml pear-shaped flask and evaporated to dryness under vacuum with a Brinkman RE 120 rotary flash evaporator. The lipid extract was redissolved in 0.5 ml chloroform and stored until used in a sealed vial under argon gas at −20°C.

Major phospholipid classes were separated by two dimensional thin-layer chromatography (TLC) using plates pre-coated with silica gel 60 (E. Merck Co.). Lipid extracted from isolated microvessels and brain homogenate was spotted on individual plates which were run simultaneously in unlined chromatographic tanks. Lipids were first developed in chloroform-methanol-H_2O-acetic acid (97:65:4:1), and then in chloroform-methanol-H_2O (90:52:12). Lipid spots were visualized by charring with sulfuric acid-dichromate spray reagent. Phospholipid classes were identified by comparison of sample Rf values with those of authentic standards (Sigma Chem. Co., St. Louis, MO). The relative amounts of each class, expressed as mol %, were determined spectrophotometrically by phosphate analysis of each spot according to the method of Ames and Kielly (1). Absorbance was read at 820 nm.

Protein content of isolated cerebral microvessels and brain homogenate devoid of microvessels was assayed according to the micromethod described by Bradford (3). Determinations were conducted on 50 ul aliquots of microvessel suspension (0.34 M sucrose in distilled water) taken from a known volume of suspension. These samples were pipetted into 13 × 100 mm test tubes and the volume adjusted to 0.1 ml distilled water. One milliliter of the protein reagent Coomassie Brilliant Blue G-250 (Sigma Chem. Co., St. Louis, MO) was added to each test tube and the contents mixed using a vortex mixer. The absorbance of the solution was measured at 595 nm within 5–10 minutes after addition of the protein reagent. Samples were read and values compared against a reagent blank prepared from 0.1 ml of buffer and 1 ml of Coomassie Brilliant Blue G-250. The weight of protein in known amounts was plotted against the corresponding absorbance to generate a standard curve for use in determining protein concentration (μg/ml) in tissue samples.

Assessment of Microvessel Purity. The purity of isolated microvessel preparations was assessed by light and electron microscopy and by the relative presence of sulfatides and cerebrosides which are found in glial membranes, but not those of the microvessel endothelium (17). The presence of glial contamination was also determined by assay for glial fibrillary acidic protein using the unlabelled antibody (peroxidase-antiperoxidase) technique described by Sternberger (18). Contamination by intralumenal red blood cells was not a problem, since these cells were extruded during the course of the isolation procedure as the result of successive centrifugation, and washing over glass beads.

RESULTS

Microvessels isolated from the cerebral cortex and cerebellum consisted predominantly of capillaries, venules and small arterioles (Figure 1). A few larger arterioles (>30 μm) were occasionally observed by light microscopy. The presence of glial fibrillary acidic protein (GFAP) was not detected in microvessel preparations, however, cerebrosides were barely visible on TLC plates indicating pres-

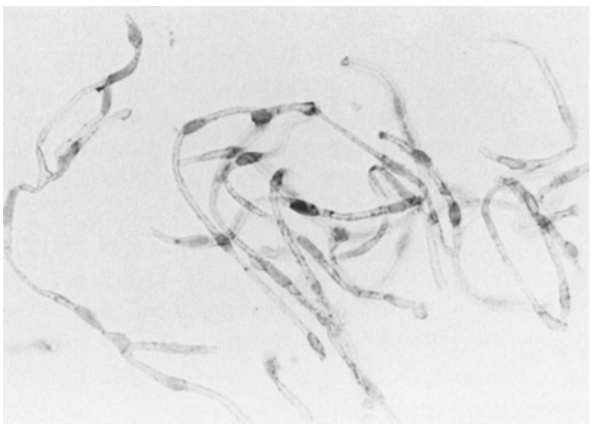

Fig. 1. Light micrograph of isolated microvessels from the grey matter of mouse cerebral cortex and cerebellum. Preparations consist predominantly of capillaries, venules and small arterioles, although minor contamination by larger vessels and glial cells is evident. Note absence of intralumenal red blood cells. (×400, bar = 10 μm).

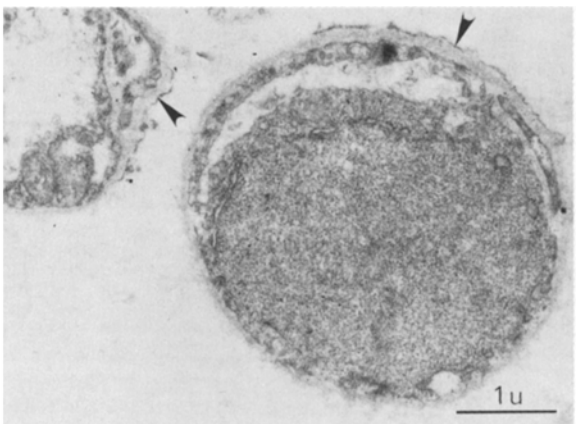

Fig. 3. Electron micrograph of isolated microvessels with small patches of membrane of unknown origin (arrows) attached to the basement membrane. These patches were infrequently observed, but may represent minor contamination by glial endfeet. (×20,000, bar = 0.5 μm).

ence of some glial membrane. The amount of glycolipid present in lipid extracts from microvessels was considerably less than that found in extracts derived from brain homogenate. Electron microscopic examination of randomly selected portions of several microvessel preparations revealed a highly purified concentration of microvessel segments (Figure 2) with occasional membrane fragments of unknown origin (Figure 3). Endothelial cells with prominent nuclei and intermittently attached pericytes were mostly normal in appearance, although an occassional cell showed signs of membrane damage. Such damage most likely resulted from mechanical disruption during isolation. Taken together these observations indicate that the microvessels obtained by the method described herein are highly purified with little contamination by non-vascular elements.

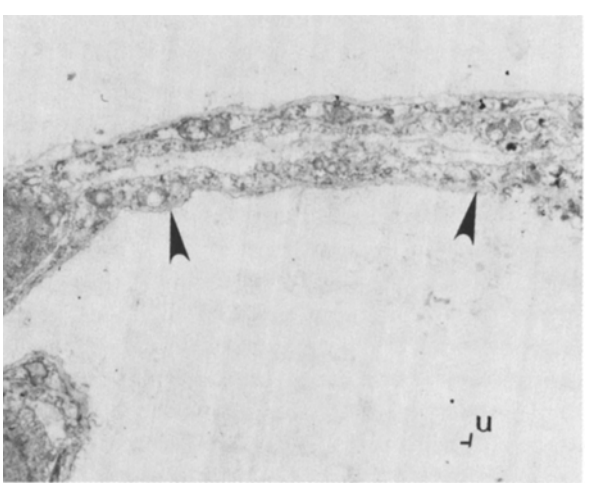

Fig. 2. Electron micrograph of isolated microvessels (arrows) showing intact vessel lumen (L) and organelle ultrastructure. Microvessel membrane shows little or no damage from the isolation procedure. (×6,200, bar = 1.0 μm).

Phospholipid Composition of Microvessel Membranes. The mol % composition for the major phospholipid classes found in microvessel and brain parenchymal membranes is presented in Table I. Comparison of mol % composition between microvessel and brain parenchymal membranes show statistically significant ($P < .05$) differences with respect to ethanolamine phosphoglyceride (EPG), inositol phosphoglyceride (IPG), and sphingomyelin (SM). The presence of choline phosphoglyceride (CPG) was slightly lower in microvessel membrane, but differences were not found to be statistically significant. While the presence of EPG was reduced in microvessel membrane, the levels of IPG and sphingomyelin were significantly increased relative to their levels in membranes of the cerebral and cerebellar parenchyma.

The isolation procedure yielded 0.299 ± 0.08 mg of microvessel protein/g brain.

Table I. Mol Percent of Phospholipids in Isolated Microvessel and Brain Parenchymal Membrane

Phospholipid class	Microvessel	Brain
ethanolamine phosphoglyceride (EPG)	23.4 ± 2.26(5)[a]	40.6 ± 1.57(3)
choline phosphoglyceride (CPG)	36.8 ± 3.87(5)	40.6 ± 1.36(3)
inositol phosphoglyceride (IPG)	8.4 ± 2.52(5)[a]	2.6 ± 0.47(3)
serine phosphoglyceride (SPG)	12.9 ± 1.91(5)	12.0 ± 1.22(3)
sphingomyelin (SM)	18.3 ± 1.84(5)[a]	4.3 ± 1.20(3)
CPG/SM	2.06 ± 0.50(5)[b]	10.21 ± 3.52(3)

Values are means ± SD in mol % of the total lipid phosphorus per sample.
[a] Mol % of microvessel lipid differs significantly from brain homogenate at $P < .05$ (Mann Whitney "U" test).
[b] Ratio for microvessel membrane differs significantly from that of brain parenchymal membrane at $P < .05$ (Mann Whitney "U" test).
Number of parentheses represents the number of determinations.

DISCUSSION

The isolation of metabolically active, cerebral microvessels has become a useful means by which to assess the physiological and pharmacological parameters of the microcirculation. To date, only a few studies have reported on the lipid biochemistry of these vessels with respect to phospholipid composition of the endothelial cell membrane (10, 11, 14, 15). Microvessels isolated from rat brain have been the most widely studied, although lipid analysis of human and bovine cerebral microvessel membrane has also been reported (17). Although species variability in membrane lipid composition between the rat and mouse may not appear likely the ever increasing use of the mouse, as a model for a variety of human diseases, necessitates basic information about membrane lipids in this animal. Our studies, conducted on microvessels isolated from grey matter of the cerebral cortex and cerebellum of 90-day-old mice, indicate that mol% for EPG, CPG, SPG, IPG, and SM are, in fact, similar to that reported for microvessels in the rat (10, 11, 15). The major phospholipids identified also appear in proportions which are similar to those reported for bovine aorta (13).

Unlike previous studies supernatants obtained from the capillary isolation procedure were combined allowing a direct comparison of phospholipid composition to be made between microvascular membrane and membranes of the cerebral and cerebellar parenchyma. Direct comparison such as this is a more valid approach than one where values from separate studies are compared. While mol% for EPG, CPG, IPG, SPG, and SM are essentially identical between glial and neuronal fractions (20) our studies showed significant differences in the relative amounts of EPG, IPG and SM, but not CPG or SPG in microvessel membrane versus brain parenchyma. The significance of these results is difficult to interpret, although conceivably these differences reflect the functional specialization of the microvessel endothelium. Recent studies have confirmed the presence of various membrane receptors, including alpha adrenergic and cholinergic receptors (8), muscarinic receptors (7) and a receptor for insulin (12) on microvessel membrane. At least some of these receptors interact with membrane phospholipids to accomplish signal transduction (9). Moreover, the capillary endothelial membrane is the site for numerous transport mechanisms and enzyme systems which together constitute functional aspects of the blood-brain barrier.

The observed differences in sphingomyelin content between microvessel membrane and parenchymal membrane may reflect differences in membrane fluidity. Three major determinants of membrane fluidity are: (1) the cholesterol to phospholipid mol ratio; (2) the degree of unsaturation of the esterified acyl chains of phospholipids, i.e. the unsaturation to saturation ratio; and (3) the choline phosphoglyceride to sphingomyelin (CPG/SM ratio) (16). Taken together, these three parameters modulate membrane fluidity. Cholesterol levels and acyl unsaturation were not quantified in this study. Levels of sphingomyelin, however, were found to be significantly different between the two fractions with levels being considerably higher in capillary membrane. Since mol % for choline phosphoglyceride was essentially the same for brain parenchymal and capillary membrane, the CPG/SM ratio is significantly lower for the capillary endothelium. The rigidifying nature of sphingomyelin is derived from the highly saturated acyl chains, the trans double bond configuration, and the amide bond in the hydrophobic region of the molecule (16). Taken by itself the lower CPG/SM ratio of capillary membrane would tend to rigidify the membrane structure. However, overall fluidity of the membrane cannot be assessed without the additional knowledge of cholesterol content and degree of acyl saturation.

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