Fatty acid composition of brain capillary endothelial cells: effect of the coculture with astrocytes

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Abstract We have investigated the fatty acid composition of brain capillary endothelial cells cultured alone or in coculture with astrocytes, using an in vitro model in which endothelial cells and astrocytes were grown from one part of a filter to another. We found that the fatty acid composition of the cocultured cerebral endothelial cells was markedly different from that of non-cocultivated endothelial cells. The most striking difference was the increase of arachidonic acid (20:4n-6) at the expense of its precursor, linoleic acid (18:2n-6). Similar modifications were found for the n-3 family of fatty acids with an increase of docosahexaenoic acid (22:6n-3) at the expense of its precursors, but the differences were less than within the n-6 fatty acids. These changes induced by the coculture were observed only in endothelial cell phospholipids, especially the phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine classes, but were not detected in phosphatidylinositols and in other lipid classes. Only the composition of the n-3 series fatty acids was altered in another capillary endothelial cell type (from adrenal cortex) cocultured with astrocytes under the same conditions. The fatty acid changes observed might be biologically relevant as they tended to make the fatty acid composition of the brain capillary endothelial cells more closely resemble that of brain microvessels.—**Bénistant, C., M-P. Dehouck, J-C. Fruchart, R. Cecchelli, and M. Lagarde.** Fatty acid composition of brain capillary endothelial cells: effect of the coculture with astrocytes. *J. Lipid Res.* 1995. **36:** 2311-2319.

Supplementary key words n-3 fatty acids • n-6 fatty acids • phospholipids • blood-brain barrier

The development of the blood-brain barrier is a complex process that leads to endothelial cells with unique permeability characteristics due to high electrical resistance and with the expression of specific transporters and metabolic pathways (for review see ref. 1). The difficulty in studying properties of brain capillary endothelial cells in vivo, because they represent a small fraction of the total cells in the brain, has led several investigators to use primary cultures of brain capillary endothelial cells as a model system for studying the blood-brain barrier (2, 3). Several lines of evidence suggest that cultured brain endothelial cells rapidly lose the characteristics of the differentiated blood-brain barrier in vivo (1). As the astroglial perivascular sheet is a unique feature of brain capillaries, forming at about the same time as the permeability barrier develops, astrocytes have been implicated in the re-induction of the barrier (4). Several attempts have been made to re-induce blood-brain barrier properties in endothelial cells by coculturing them with astrocytes or glioma cells (5, 6). We have previously described our approach to reconstruct some of the complexity of the cellular environment that exists in vivo by growing endothelial cells on one side of a filter and astrocytes on the other (7). Under these conditions, we have demonstrated a high correlation between the in vivo and in vitro values for the brain extraction for most of the substances tested, showing that the tightness of the monolayer is comparable with the in vivo properties (8).

Although the fatty acid composition of whole brain (9) and brain microvessels (10, 11) is known, no data are available on that of brain endothelial cells in culture. However, membrane lipids and their fatty acyl moieties are important for endothelial cell structure and functions. In particular, numerous studies have pointed out the role of arachidonic acid (20:4n-6) metabolism in the formation of lipid mediators such as prostacyclin (12) and platelet-activating factor (13). In addition, docosahexaenoic acid (22:6n-3) may be relevant as it is found in large quantities in brain microvessels (10, 11). We, therefore, investigated the lipid and fatty acid composition of the brain capillary endothelial cells and the effect

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; bFGF, basic fibroblast growth factor; GFAP, glial fibrillary acidic protein; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; DMA, dimethylacetal.

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of the coculture with astrocytes on composition. We found that the fatty acid composition of brain capillary endothelial cells was markedly different from that of freshly isolated brain microvessels. In addition, we found that astrocytes modified fatty acid composition and moved it closer to that of isolated brain microvessels as the proportions of 20:4n–6 and 22:6n–3 increased at the expense of their precursors in phospholipids of the endothelial cells cocultivated with astrocytes.

MATERIALS AND METHODS

Cell cultures

Brain capillary endothelial cells. Bovine brain capillary endothelial cells were isolated and characterized as described by Méresse et al. (14). In brief, after isolation by mechanical homogenization and sieving the gray matter of bovine brain, microvessels were seeded onto dishes coated with an extracellular matrix secreted by bovine corneal endothelial cells. Five days after seeding, the first endothelial cells migrated out of the capillaries and began to form microcolonies. When the colonies were sufficiently large, the largest islands not contaminated by pericytes were trypsinized and seeded onto gelatin-coated dishes (35 mm diameter) in the presence of Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% calf serum, 2 mM glutamine, 50 μg/ml gentamycin, and 1 ng/ml basic fibroblast growth factor (bFGF) (added every other day). Endothelial cells from one 35-mm diameter dish were harvested at confluence and seeded onto 60-mm diameter gelatin-coated dishes. After 6–8 days, confluent cells were subcultured at the split ratio of 1:20. Cells at this passage (third passage) were stored in liquid nitrogen. For experiments, cells were rapidly thawed at 37°C and used between passage 4 and 7.

Adrenal cortex endothelial cells. Bovine adrenal cortex endothelial cells were isolated and characterized as described by Gospodarowicz et al. (15). These cells (a gift from Dr. S. Saule, Molecular Oncology Laboratory, Institut Pasteur, 59019 Lille, France) were cultured as done with brain capillary endothelial cells.

Rat astrocytes. Primary cultures of mixed astrocytes were made from newborn rat cerebral cortex. After the meninges had been removed, the brain tissue was gently forced through a nylon sieve, as described by Booher and Sensenbrenner (16). Astrocytes were plated on petri dishes (100 mm diameter, Nunclon) at a concentration of 1.2×10^5 cells/ml in 10 ml of DMEM supplemented with 10% fetal calf serum (Hyclon Laboratories, Logan, UT); the medium was changed twice a week. Three weeks after seeding, cultures of astrocytes stabilized and were used for experiments. They were then cultivated using the coculture medium consisting of DMEM supplemented with 15% calf serum, 2 mM glutamine, 50 μg/ml gentamycin, and 1 ng/ml bFGF. The astrocytes were characterized with glial fibrillary acidic protein (GFAP), and more than 95% of the population was GFAP positive (7).

Coculture of endothelial cells and astrocytes

Preparation of filters for coculture. Culture plate inserts (Transwell, 0.4 μm, 75 mm diameter, nucleopore polycarbonate membrane from Costar) were coated on the upper side with rat tail collagen prepared by a modification of the method of Bornstein (17).

Experimental method. These inserts were then set over petri dishes with or without the stabilized astrocyte cultures. The endothelial cells (4×10^5 cells) were then plated on the upper side of the inserts in 8 ml of coculture medium (see above). This medium was changed every other day. Under these conditions, endothelial cells form a confluent monolayer after 7 days. Experiments were performed 5 days after confluence. This arrangement readily permits the use of different cell types, which were separated easily after confluence by removing the insert. The integrity of the endothelial cell monolayers was routinely checked by measuring the electrical resistance of the monolayer using a Millicell-ERS apparatus (Millipore), and the cocultures that had an electrical resistance lower than 500 ohms/cm^2 were not used for experiments. In parallel, stabilized astrocyte cultures with empty precoated filters were prepared. On the eleventh day, all the cultures received 1 μCi/dish of [^{3}H]arachidonic acid (NEN, Boston), which was used to localize the different cell lipid fractions by radioscanning (see below).

Lipid and fatty acid analyses

Lipid extraction. Endothelial cells grown alone or in the presence of astrocytes and astrocytes grown alone or in the presence of endothelial cells were all rinsed twice with phosphate-buffered saline, and scraped into 3 ml ethanol containing 50 μM butylated hydroxytoluene. Several standards (diheptadecanoylphosphatidylcholine, diheptadecanoylphosphatidylethanolamine, triheptadecanoylglycerol, and heptadecanoic acid) were added and cell lipids were extracted from the resulting ethanolic solutions by adding chloroform, water, and the corresponding amount of ethanol to obtain ethanol–chloroform–water 3:6:1 (vol/vol). Freshly prepared brain capillaries were extracted as the cells.

Lipid separations. Lipid separations were performed by thin-layer chromatography (TLC) on silica gel 60 plates. Phospholipids and neutral lipids were separated by using the solvent system hexane–diethyl ether–acetic acid 70:30:1 (vol/vol), and phospholipid classes were

separated using the solvent mixture chloroform–methanol–methylamine 60:20:5 (vol/vol). Bands corresponding to the different lipids were visualized either by radioscanning (Berthold, Silena) or by UV irradiation of dichlorofluoroscein-impregnated plates.

Fatty acid analyses. All the collected lipids were treated with 14% boron fluoride–methanol (18). The resulting fatty acid methyl esters and dimethylacetals were analyzed by gas–liquid chromatography (GLC) as described (19) using a Perkin-Elmer chromatograph model 5830, equipped with a SP 2380 capillary column (60 m × 0.25 mm). The results obtained were expressed in percentage (mol%) or in concentration (nmol/mg prot) by using the calculation mode described by Hill, Husbands, and Lands (20) and a computer program kindly provided by Dr. J. Lecerf.

Statistical analyses

We used the two-tailed paired *t* test to compare the lipid and the fatty acid composition in the matched samples of cells.

TABLE 1. Fatty acid composition of total phospholipids from brain microvessels

Fatty Acid	mol%
16:0	16.0 ± 4.4
16:1n–9	0.5 ± 0.1
16:1n–7	0.3 ± 0.1
18:0	21.5 ± 0.7
18:1n–9	18.7 ± 0.6
18:1n–7	3.8 ± 0.2
18:2n–6	3.2 ± 0.7
18:3n–3	< 0.1
20:3n–6	< 0.1
20:4n–6	9.4 ± 1.6
20:5n–3	< 0.1
22:4n–6	2.9 ± 0.3
22:5n–6	0.8 ± 0.4
22:5n–3	0.7 ± 0.2
22:6n–3	8.6 ± 2.7
16DMA	3.7 ± 0.3
18DMA	4.8 ± 1.0

Brain microvessels were isolated from the brain as reported in the Methods section. Total phospholipids were separated from total lipids by TLC, and fatty acid proportions were determined by GLC. Results expressed as mol% are means ± SD of values from three independent analyses.

RESULTS

Brain microvessels

The microvessels used in the present study were mostly of capillary origin as they typically showed a 40-fold enrichment in γ-glutamyl-transpeptidase compared with brain homogenate (234 ± 25 vs. 5.1 ± 1.6 nmol of product/mg protein per min, respectively). The fatty acid composition of their phospholipids is shown in **Table 1.** Palmitic (16:0), stearic (18:0), and oleic (18:1n–9) acids were the main fatty acids, each accounting for about 20% of total fatty acids. Polyunsaturated fatty acids were found in the form of long chain fatty acids, with 20:4n–6 and 22:6n–3 exceeding their respective fatty acid precursors.

Brain capillary endothelial cells

Figure 1A illustrates the typical phenotype of confluent bovine brain capillary endothelial cells cocultured for 12 days with astrocytes on an insert coated with rat tail collagen. Clear alignments of elongated cells were visible, with neighboring cells tightly packed against each other. This culture is a pure endothelial cell population without contamination by pericytes. The distribution in cultured brain endothelial cells of filamentous actin was determined (Fig. 1B). The actin filament pattern was uniform for each particular culture studied. Bodipy phallacidin staining revealed a delicate fluorescent network of actin filaments throughout the cytoplasm. Under our normal culture conditions, the association between the underlying actin cytoskeleton and the cell membrane was always observed and formed prominent continuous bands at cell borders. These data added to previous published results concerning the high electrical resistance and the low permeability (8) support this model as a legitimate model of the blood–brain barrier.

The fatty acid composition of brain capillary endothelial cell phospholipids is shown in **Fig. 2.** It can be seen that it was markedly different from that of brain microvessels. The proportions of linoleic acid (18:2n–6) and 20:4n–6 were as high as 16:0 and 18:0 in such cells. Moreover, the amount of 22:6n–3 was one-sixth that of 20:4n–6. This was presumably due to the serum fatty acid composition which was found to be poor in n–3 fatty acids (not shown). Astrocytes affected such a composition as the fatty acid profile of cocultured endothelial cells differed from that of the non-cocultivated endothelial cells in several instances. The proportion of 16:0 increased in total phospholipids of cocultured endothelial cells (Fig. 2). Moreover, drastic variations in the proportions of certain polyunsaturated fatty acids were observed (Fig. 2 and **Fig. 3**). In particular, the proportions of 20:4n–6 and of its precursor 18:2n–6 changed reciprocally, the proportion of the former being higher while that of the latter was lower in cocultured endothelial cells (Figs. 2 and 3). The variations could be observed in total phospholipids (Fig. 2) as well as in phospholipid classes, except in phosphatidylinositol (Fig. 3). The increase of longer chain fatty acid at the expense of its precursor, as observed for the n–6 family, could also be found within the n–3 family, although to a lesser extent. As a matter of fact, the proportions of

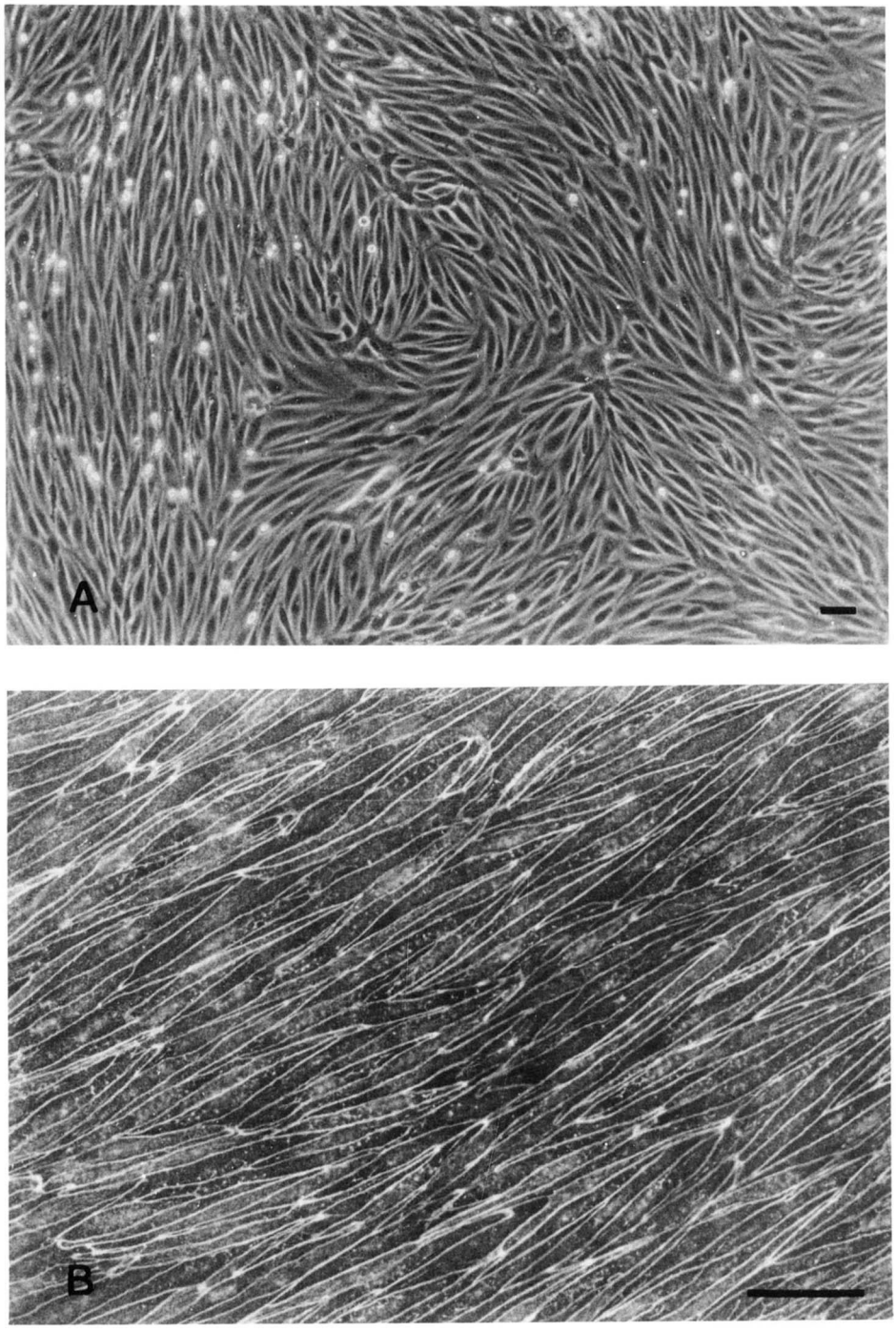

Fig. 1. (A) phase contrast micrograph of confluent brain capillary endothelial cells grown on the upper face of a collagen-coated filter and (B) after F-actin immunolabeling. Bars represent 50 μm.

linolenic (18:3n–3), eicosapentaenoic (20:5n–3), and docosapentaenoic (22:5n–3) acids were significantly lower in total phospholipids from cocultured endothelial cells compared to endothelial cells cultivated alone, and the proportion of 22:6n–3 increased (Fig. 2). These variations were more or less found in phospholipid classes (Fig. 3). Finally, most of the altered proportions that could be seen in Figs. 2 and 3 were also observed when results were expressed in nmol/mg protein which was expected as the concentration of total phospholipids was equal in cocultured endothelial cells and endothelial cells cultured in the absence of astrocytes (not shown).

Astrocytes

We also analyzed the lipid content and fatty acid composition of astrocytes that were cocultured with

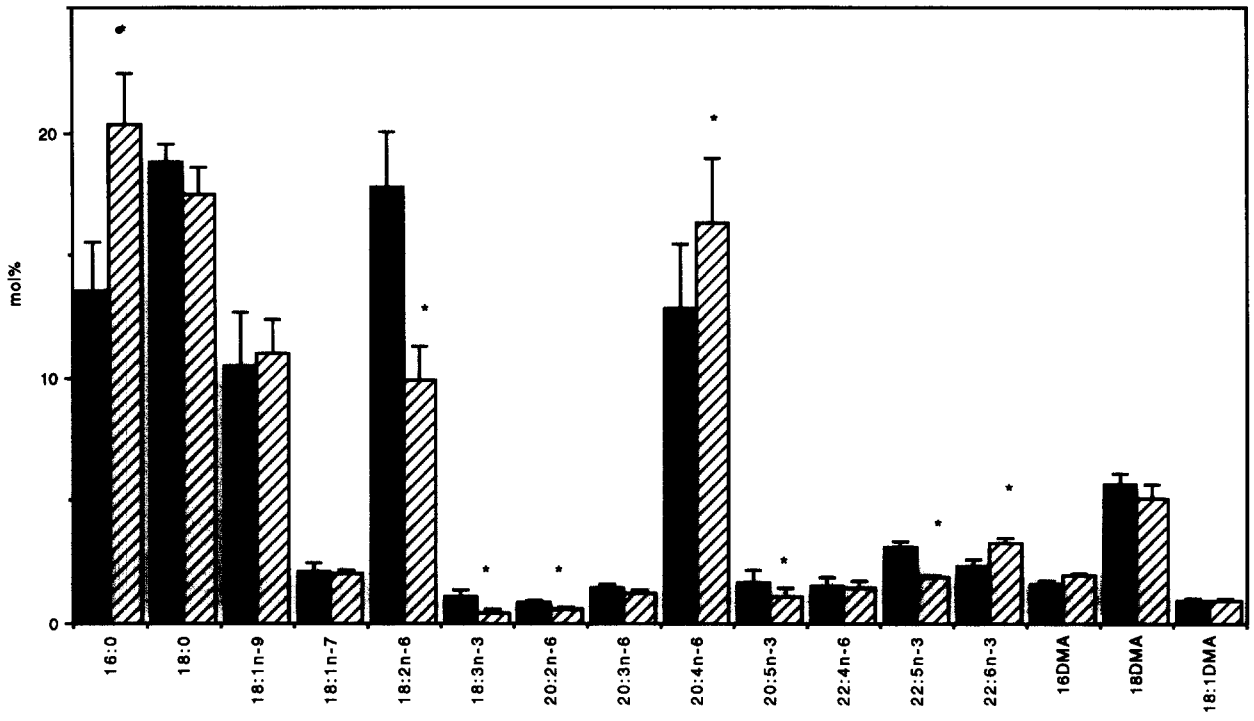

Fig. 2. Fatty acid composition of total phospholipids from brain capillary endothelial cells. Brain capillary endothelial cells were grown on filters either without (closed bars) or with (cross-hatched bars) astrocytes present on the other side. Total phospholipids from endothelial cells were separated from total lipids by TLC, and fatty acid methyl esters were analyzed by GLC. Results are expressed as mol% and are means ± SEM of results from seven separate analyses. *Different from values obtained in the absence of astrocytes at $P < 0.05$.

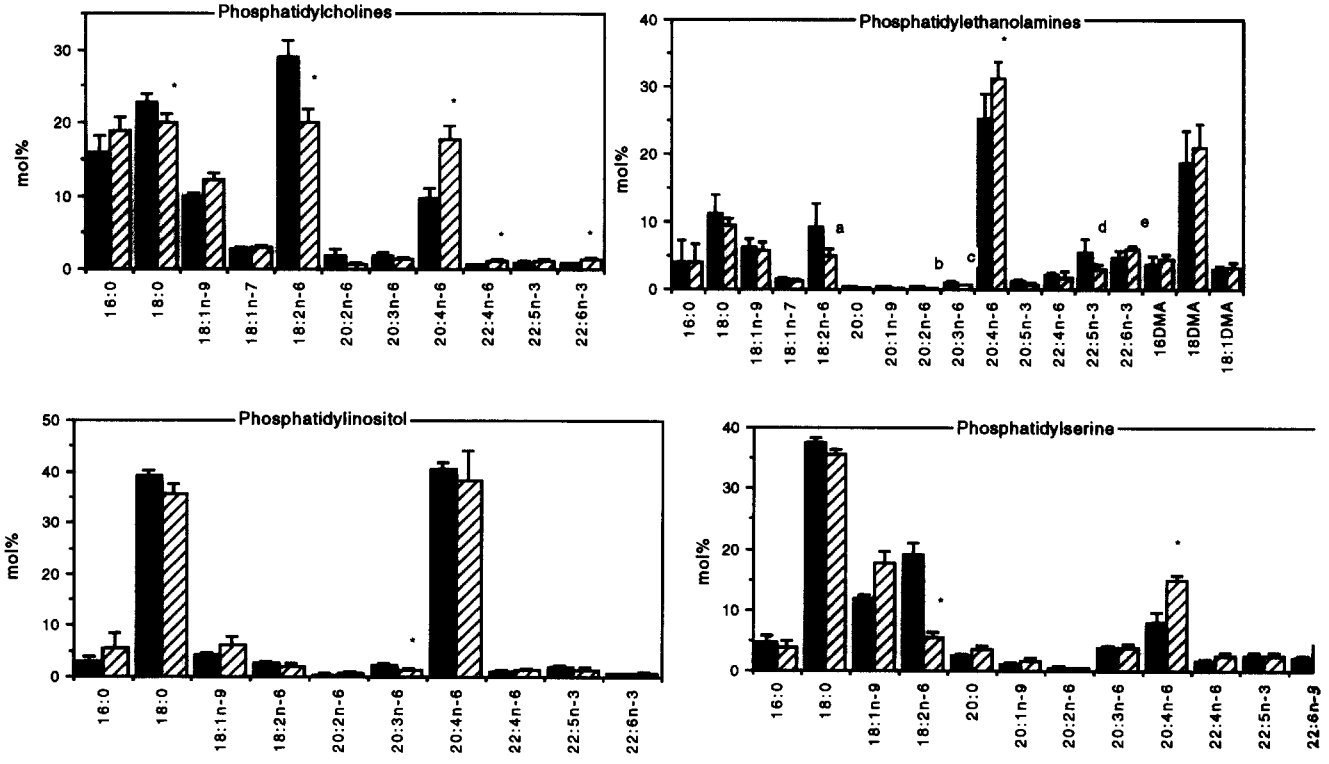

Fig. 3. Fatty acid composition of phospholipid classes from brain capillary endothelial cells. Brain capillary endothelial cells were grown on filters either without (closed bars) or with (cross-hatched bars) astrocytes present on the other side. Endothelial cell phospholipid classes were obtained by TLC and fatty acid methyl esters were analyzed by GLC. Results are expressed as mol% and are means ± SEM of results obtained from four independent analyses. *Different from values obtained in the absence of astrocytes at $P < 0.05$. (a) $P = 0.072$; (b) $P = 0.061$; (c) $P = 0.060$; (d) $P = 0.070$; (e) $P = 0.061$.

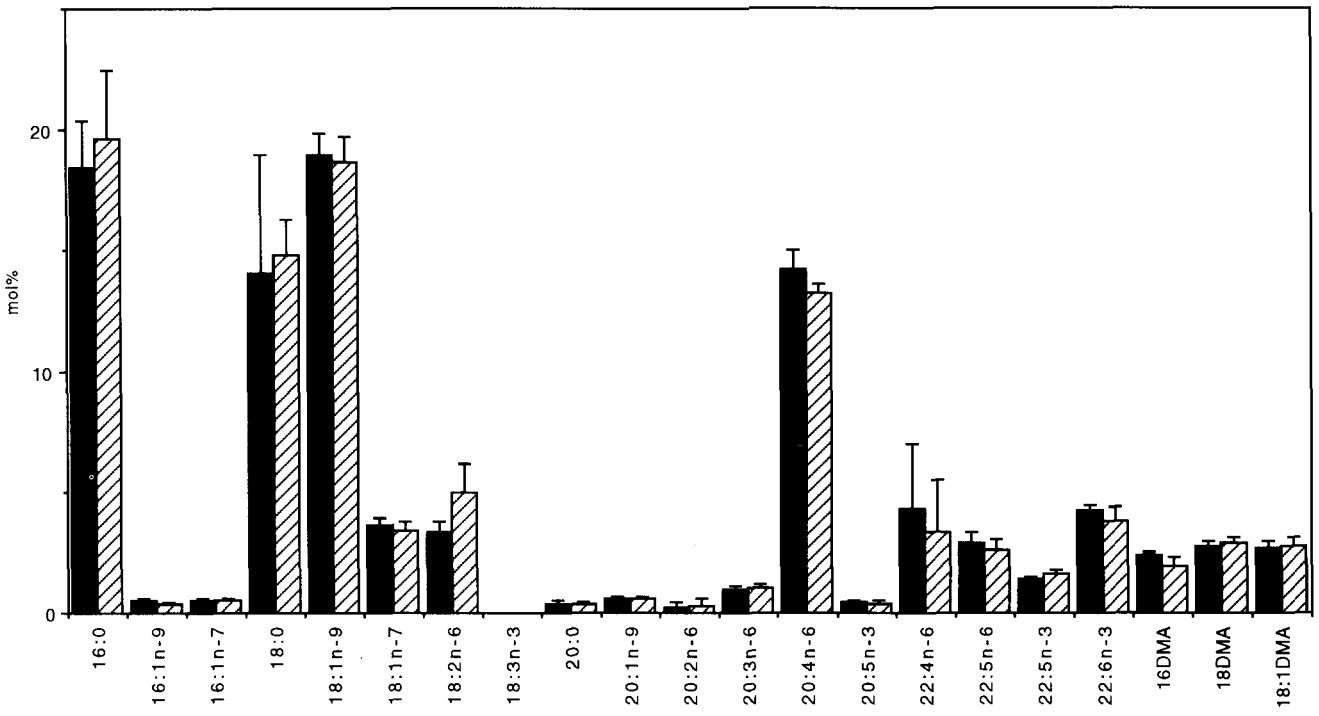

Fig. 4. Fatty acid composition of total phospholipids from astrocytes. Astrocytes were cultured in the absence (closed bars) or presence (cross-hatched bars) of endothelial cells. Total phospholipids from astrocytes were separated from total lipids by TLC and fatty acids contents were determined by GLC. Results presented are expressed as mol% and are means ± SD of results obtained from three independent experiments.

endothelial cells from brain capillaries in comparison with control astrocytes. We found no variations in the fatty acid composition of cocultured versus control astrocytes (**Fig. 4**). However, we found that the cocultured astrocytes contained significantly less phospholipids than the control astrocytes (177.0 ± 22.4 versus 276.3 ± 47.5 nmol/mg prot (n = 3), respectively). The other lipid classes were not altered either in their contents or in their fatty acid compositions (not shown).

Adrenal cortex endothelial cells

In order to determine whether the alteration of the fatty acid composition of brain capillary endothelial cells cocultured with astrocytes could be observed with another cell type, we analyzed the fatty acid composition of total phospholipids of adrenal cortex endothelial cells cultured alone or cocultured with astrocytes using exactly the same conditions as with the brain capillary endothelial cells. These adrenal cortex endothelial cells were chosen as they were also of capillary origin. We found that, as observed with the brain capillary endothelial cells, the adrenal cortex endothelial cells cocultured with astrocytes produced changes in the fatty acid proportion of certain fatty acids (**Table 2**). However, in contrast to what was observed with the brain capillary endothelial cells, only the proportions of the fatty acids of the n–3 series were affected. In particular, the proportions of 22:6n–3 and 20:5n–3 increased whereas that of 18:3n–3 decreased in total phospholipids of the cocultured adrenal cortex endothelial cells (Table 2).

DISCUSSION

We have investigated the fatty acid composition of the brain capillary endothelial cells. These cells constitute the blood–brain barrier and, in this respect, present specific characteristics. Several pieces of evidence, shown here or previously reported (7, 8, 15, 21), argue for the cells we used in the present study as being endothelial cells of the blood–brain barrier type. First, they present all the characteristics of endothelial cells: factor VIII-related antigen, angiotensin-converting enzyme, and nonthrombogenic surface (15). Second, they behave as differentiated blood–brain barrier cells: occurrence of tight junctions, electrical resistance over 500 ohms/cm^2, specific enzyme activities of the blood–brain barrier (γ-glutamyl transpeptidase, monoamine oxidase) and occurrence of carrier-mediated transport (7). In addition, we have previously shown that there was a close correlation between the values of the brain uptake index obtained in vitro on our monolayers and those obtained in vivo with the Oldendorf technique, for a large number of drugs (8), indicating that our model

TABLE 2. Fatty acid composition of total phospholipids of endothelial cells and cocultured endothelial cells from adrenal cortex

Fatty Acid	Endothelial Cells	Cocultured Endothelial Cells
16:0	16.83 ± 3.53	17.62 ± 3.28
18:2n-6	26.30 ± 1.97	24.15 ± 1.76
18:3n-3	0.78 ± 0.05	0.64 ± 0.06[a]
20:2n-6	0.81 ± 0.06	0.86 ± 0.17
20:4n-6	9.18 ± 0.51	9.36 ± 1.41
20:5n-3	0.49 ± 0.04	0.82 ± 0.10[a]
22:5n-3	1.94 ± 0.18	2.12 ± 0.43
22:6n-3	1.60 ± 0.36	3.37 ± 1.00[a]

Endothelial cells from adrenal cortex were grown either in the presence or absence of astrocytes on the other side of a filter. Total lipids of the endothelial cells cultured alone and cocultured endothelial cells were extracted, separated into their different classes by TLC, and the fatty acids of the total phospholipid fraction were analyzed by GLC. Results presented are expressed as mol% and are means ± SD of values obtained from three independent experiments. Only the proportions of fatty acids of interest are shown; others not depicted in the table were unaffected by the coculture.

[a]Different from the value found in endothelial cells at $P < 0.05$.

mimicks the in vivo situation with regard to drug permeability. Moreover, after the 12 days of coculture used in the present study, the paracellular permeability to sucrose of the monolayers was at its lowest (21) and the tight junctions were fully achieved.

Endothelial cells from brain capillaries are unique in that they are in close apposition with astrocytes in situ (1). The barrier properties of these cells are largely dependent on the presence of astrocytes. In particular, it has been shown that the coculture of brain capillary endothelial cells with astrocytes is a determinant for their differentiation (4–8, 14, 21). Owing to the fact that the fatty acid composition of such cells has never been studied and that they interact with astrocytes, we have undertaken the present study. We have determined the fatty acid composition of the brain capillary endothelial cells, the effect of the coculture with astrocytes on such fatty acid composition, and the fatty acid composition of the isolated brain capillaries. We found that astrocytes induced changes in the fatty acid profile of the brain capillary endothelial cells, moving it closer to that of the isolated brain capillaries. The most striking differences in the fatty acid profiles of endothelial cells versus cocultured endothelial cells dealt with the increase of arachidonic acid (20:4n-6) at the expense of its precursor 18:2n-6. This was found in most phospholipid classes, i.e., phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, but not in phosphatidylinositol, in accordance with a recent study showing that the latter is highly resistant to fatty acid modifications (22). Similar modifications appeared for the n-3 family of fatty acids with an increase of 22:6n-3 proportion at the expense of its precursors, but the differences were weaker than among the n-6 fatty acids. As expected, as the total phospholipid mass was not changed by the coculture, the alteration of the fatty acid proportions paralleled that of their concentrations. The shift observed in the presence of astrocytes moved the proportions of 22:6n-3, 22:5n-3, and 20:5n-3 closer to those in brain microvessels, although not equal. This might make relevant the lipid modifications induced by astrocytes during the coculture, although the fatty acid profile of the brain capillary endothelial cells was different from that of the brain microvessels, even when the cells were cocultured. As isolated microvessels do not consist exclusively of capillaries but also of some arterioles and veinules, these differences might be explained by the heterogeneity of the preparation. Furthermore, even if the microvessel preparation could be considered as a pure capillary population, the presence of pericytes within the basement membrane could also interfere with the lipid composition observed (23). Alternatively, it might be that cultured endothelial cells lose part of their lipid specificity, especially when they are cultured in the absence of astrocytes, and that the coculture minimized the effects. It may be pointed out that the only changes induced by astrocytes in the brain capillary endothelial cell lipid composition concerned the n-6 and n-3 families of fatty acids. The one exception was 16:0, the proportion of which in brain capillary endothelial cell total phospholipids increased significantly in the presence of astrocytes. Such an increase is difficult to explain unless one speculates that the molecular species enriched in 20:4n-6 and 22:6n-3 would contain preferentially 16:0 at the *sn*-1 position as has been previously found to occur in platelet phospholipids (24). We may also notice that only endothelial cell phospholipids were concerned by these changes; the fatty acid compositions of the other lipid fractions were not altered by the coculture.

The question of the specificity of these changes to brain capillary endothelial cells was next considered. First, it must be pointed out that only brain capillary endothelial cells would be ever in contact with astrocytes in vivo. However, it has been previously shown that astrocytes can induce changes in non-neural endothelial cell types. For instance, it has been shown that the electric resistance of bovine aortic endothelial cell monolayers was increased after coculture with astrocytes (7). Likewise, the n-3 fatty acid changes observed in brain capillary endothelial cells cocultured with astrocytes were also observed in cocultured adrenal cortex capillary endothelial cells. In contrast, some effects of astrocytes are restricted to brain capillary endothelial cells, suggesting that some kind of connections might exist between these two cell types. As a matter of fact,

astrocytes induce the increased expression of the low density lipoprotein receptor only in brain capillary endothelial cells (21). Similarly, the n-6 fatty acid changes observed in brain capillary endothelial cells cocultured with astrocytes were observed only with this cell population and not with the adrenal cortex capillary endothelial cells.

The lipid modifications induced by astrocytes in brain capillary endothelial cells might arise either from a fatty acid exchange between astrocytes and endothelial cells, as has been proposed to occur between astrocytes and neurons (25), or from an increase of the biosynthetic activities of the enzymes involved in long chain fatty acid synthesis. Indeed, we observed that the n-6 and n-3 fatty acid profiles shifted from the precursor to the longer and more unsaturated homologues. This was particularly obvious for 18:2n-6 which was depressed to the benefit of 20:4n-6. The increased proportion of 22:6n-3 at the expense of its precursors is also in agreement with this idea, especially when we consider that the biosynthesis of 22:6n-3 has been recently recognized as involving the same desaturase activity, namely Δ6 desaturase, as that of the first step in 20:4n-6 biosynthesis, i.e., the conversion of 18:2n-6 into 18:3n-6 (26). However, it may be also pointed out that endothelial cells fail to form 22:6n-3 whereas they can synthesize 20:4n-6 (27). Moreover, we found that the proportion of 20:5n-3 increased as well as that of 22:6n-3 in adrenal cortex endothelial cells cocultured with astrocytes. As previously shown (28), such an increase might result from the retroconversion of 22:6n-3, which is quite active in endothelial cells, leading to a consideration that 22:6n-3 might be taken up rather than formed by endothelial cells.

In contrast to what was observed in endothelial cells, the coculture significantly decreased the phospholipid content of astrocytes, although preserving the other lipid classes. We do not have any explanation for this important alteration but this argues again for profound changes in the lipid composition of both cell populations in coculture, although there was no direct contact between astrocytes and endothelial cells during this coculture.

In conclusion, we found that brain capillary endothelial cells present a fatty acid profile different from that of brain microvessels. However, the coculture of these endothelial cells with astrocytes moved the in vitro situation closer to the in vivo one. These results suggest once more that astrocytes might be vital for setting up a relevant in vitro model of the blood-brain barrier.

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