Lipogenesis from lactate in rat neurons and astrocytes in primary culture

Arantxa TABERNERO, Juan P. BOLAÑOS and José M. MEDINA*

Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad de Salamanca, Aptdo. 449, E37080 Salamanca, Spain

The rate of synthesis of phospholipid and sterol species from L-lactate in neurons and astrocytes in primary culture was studied. Both types of cells actively utilized lactate as lipid precursor, although the rate of lipogenesis was about 2-fold greater in astrocytes than in neurons. The incorporation of lactate into phospholipids was significantly higher than that into sterols in both types of cells, but the ratio of phospholipid/sterol synthesis was much higher in astrocytes than in neurons. Phosphatidylcholine (PC) was the main phospholipid synthesized in both types of cells, followed by phosphatidylethanolamine (PE), phosphatidylserine and phosphatidylinositol. No detectable synthesis of sphingomyelins was observed. The ratio of PC/PE synthesis was about 2-fold higher in astrocytes than in neurons. The main sterol synthesized in neurons was lanosterol, followed by desmosterol. However, the main sterol synthesized in astrocytes was desmosterol, followed by lanosterol and cholesterol. The different ratios of phospholipid/sterol and PC/PE synthesis found in neurons and astrocytes may result in different membrane fluidity being higher in astrocytes than in neurons. This may be associated with differences in the functionality of both types of cells.

INTRODUCTION

During the early neonatal period the rate of liver glycogenolysis is very low (Girard et al., 1973; Cuezva et al., 1980; Fernández et al., 1983) and the gluconeogenic capacity of the liver is negligible (Medina et al., 1980; Fernández et al., 1983), resulting in very low plasma concentrations of glucose immediately after delivery (Girard et al., 1973; Cuezva et al., 1980). Therefore, during the postnatal period the brain utilizes ketone bodies as a source of both energy and carbon skeletons (Williamson, 1982). However, at birth the newborn rat lacks white adipose tissue (Widdowson, 1950), thus preventing active ketogenesis until non-esterified fatty acids from the mother's milk become available. Consequently, during the early neonatal period other metabolic substrates are needed to fulfil brain energy requirements. Lactate accumulates in the blood during late gestation, reaching concentrations higher than 9 mM during the first minutes of extrauterine life (Girard et al., 1973; Cuezva et al., 1980; Juanes et al., 1986). However, most of the lactate accumulated is utilized within the first 2 h of extrauterine life, i.e. before the onset of suckling takes place (Cuezva et al., 1980; Juanes et al., 1986). In addition, a number of observations are consistent with the hypothesis that lactate is an important metabolic substrate for the brain during the early neonatal period in several species, including man (for a review, see Medina et al., 1992). Thus the rate of lactate utilization by neonatal-rat brain slices (Fernández and Medina, 1986) and isolated cells (Vicario et al., 1991; Vicario and Medina, 1992) from early-neonatal rat brain is much higher than that of glucose or 3-hydroxybutyrate, suggesting that immediately after delivery lactate is preferred as a brain fuel to glucose or ketone bodies. In addition, lactate transport into the brain is higher during the perinatal period than in the adult (Cremer et al., 1979; Siesjö, 1988), suggesting that lactate may be used by the brain throughout the perinatal period. In this sense, we have recently shown that fetal rat brain may use lactate as the main metabolic substrate during late gestation

(Bolaños and Medina, 1993). Finally, Dombrowski et al. (1989) have shown that lactate may also be an important substrate for the brain during the early suckling period.

The aim of this work was to investigate the role played by lactate as a lipid precursor in neurons and astrocytes during development. Accordingly, the rate of lactate incorporation into phospholipid and sterol species in perinatal neurons and astrocytes in primary culture was studied.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's Medium (DME), gentamycin, poly-L-lysine and cytosine arabinoside were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal-calf serum (FCS) was obtained from Serva Boehringer Ingelheim (Heidelberg, Germany). Substrates, coenzymes and enzymes were purchased from Boehringer (Mannheim, Germany), Sigma, Merck (Darmstadt, Germany) or Serva Feinbiochemica (Heidelberg, Germany). Standard analytical-grade laboratory reagents were obtained from Merck or Sigma. Fatty-acid-free BSA and silicone (Sigmacote) were purchased from Sigma. Fatty-acid-free BSA was dialysed twice against PBS (11 mM sodium phosphate, 122 mM NaCl, 4.8 mM KCl, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 1.3 mM $CaCl_2$; pH 7.4) for 12 h and filtered through a 0.22 μm filter (Millipore Iberica, Madrid, Spain) before use. L-[U-^{14}C]Lactate was purchased from New England Nuclear (Boston, MA, U.S.A.). Glial fibrillary acidic protein (GFAP) and neurofilament were detected by a specific antibody coupled to peroxidase (Sigma).

Animals

Albino Wistar rats, fed ad libitum on a stock laboratory diet (49.8 % carbohydrates, 23.5 % protein, 3.7 % fat, 5.5 % minerals and added vitamins and amino acids) were used for the experi-

ments. Rats were maintained on a 12 h light/dark cycle. Females with a mean weight of 250 g were caged with males overnight, and conception was considered to occur at 01:00 h; this was verified the following morning by the presence of spermatozoa in the vaginal smears. For preparing neurons in primary culture, fetuses at 17.5 days of gestation were delivered by rapid hysterectomy after cervical dislocation of the mother, wiped, and the umbilical cord was cut. Postnatal 1-day newborn rats were used to prepare astrocytes in culture.

Cell culture

Cell isolation was carried out as described previously (Vicario et al., 1991; Vicario and Medina, 1992) with some modifications. Briefly, animals were decapitated and their brains immediately excised. After removal of the meninges and blood vessels, the forebrains were placed in Earle's Balanced Solution containing 20 μg/ml DNAase and 0.3 % (w/v) BSA. The tissue was minced, washed, centrifuged at 500 g for 2 min and incubated in 0.025 % trypsin (type III) and 60 μg/ml DNAase I for 15 min at 37 °C. Trypsin treatment was terminated by addition of DME containing 10 % FCS. The tissue was then dissociated by gentle trituration, passing it 4–8 times through a silicone-treated Pasteur pipette, and the supernatant cell suspension was recovered. This operation was repeated and the resulting cell suspension was centrifuged at 500 g for 5 min. The cells were resuspended in a known volume of DME supplemented with 10 % FCS and 40 μg/ml gentamycin. For neuron culture the media were also supplemented with 25 mM KCl. Cells were counted, and the test for the exclusion of Trypan Blue dye showed that cell viability was higher than 90 %. The cell suspension was then diluted in DME supplemented with 10 % FCS and 40 μg/ml gentamycin and plated on 10 cm Petri dishes coated with 10 μg/ml poly-L-lysine at a density of 1.5×10^5 cells/cm^2 for neurons and 1.0×10^5 cells/cm^2 for astrocytes. Finally, the cells were incubated at 37 °C in an atmosphere of air/CO$_2$ (19:1) with 90–95 % humidity.

For neuron culture, the medium was replaced by fresh medium containing 10 μM cytosine arabinoside 3 days after plating. After 7 days neuronal cultures reached confluence, as determined by the number of cells and the amount of protein and DNA per dish (results not shown). Antibodies against neurofilament (Dahl and Bignami, 1977) showed that 85 % of the cells counted were immunopositive neurons.

For astrocyte culture, fresh medium was added after 3 days. This medium was replaced by a fresh one containing 10 % FCS twice a week. The number of cells, protein and DNA concentrations increased exponentially until day 10 of culture (results not shown). When confluence was reached, 90–95 % of the cells were astrocytes, as determined by counting immunopositive cells against GFAP (Bignami et al., 1972).

Cell incubation

Quiescent neurons and astrocytes (after 7 and 10 days in culture, respectively) were washed twice with PBS and incubated at 37 °C with 2 ml of PBS containing 10.5 mM L-lactate, 8–13 μCi of L-[U-^{14}C]lactate (600–900 d.p.m./nmol) and 2 % BSA. After 2 h, the incubation medium was removed and the cells were washed three times with ice-cold PBS. The cells were then dissociated with PBS containing 0.05 % (w/v) trypsin and harvested in PBS containing 10 % FCS. The cell suspension was counted and centrifuged at 500 g for 5 min. The pellet was used for lipid extraction.

Under our experimental conditions, neurons and astrocytes

incorporated lactate into lipids linearly with the incubation time over at least 2 h ($r = 0.99$; $P < 0.001$).

Extraction of total lipids

Total lipids were extracted from the pellet with 2 ml of a mixture of chloroform/methanol (2:1, v/v) as described by Folch et al. (1957) for 16 h at −20 °C. The extract was washed with 2×0.8 ml of 0.3 % (w/v) NaCl saturated with chloroform. Then 0.1 ml of the organic phase was used for the measurement of the radioactivity incorporated into total lipids, and 1.0 ml was gently dried under a stream of N$_2$ and was kept at −20 °C until being subjected to phospholipid separation by h.p.l.c.

Extraction of non-saponifiable and saponifiable lipids

Non-saponifiable (mainly sterols) and saponifiable (fatty acids) lipids were extracted essentially as described by Edmond and Popják (1974): the pellet was saponified at 70 °C with 4 ml of 5.35 M KOH/ethanol (1:1, v/v) for 2 h; unsaponifiable material was extracted with 3×5 ml of light petroleum (b.p. 40–60 °C) and the combined petroleum extracts were concentrated to dryness under a stream of N$_2$. The extract was redissolved in 1 ml of chloroform/methanol (2:1, v/v); 0.1 ml was used for determination of radioactivity by scintillation counting (Beckman LS 1800 instrument), and 1.0 ml was dried under a stream of N$_2$ and kept at −20 °C until being subjected to separation by h.p.l.c. The aqueous phase was then acidified by addition of 2 ml of 6 M H$_2$SO$_4$, and the saponifiable material was extracted with 3×5 ml of light petroleum. The combined petroleum extracts were concentrated to dryness under a stream of N$_2$ and used for liquid-scintillation counting.

Separation of phospholipids by h.p.l.c.

Phospholipid species were separated by an isocratic h.p.l.c. method, essentially as described by Kaduce et al. (1983) as modified by Bolaños and Medina (1993), using a liquid-chromatography pump system (114 M; Beckman) on a normal-phase column of silica (4.6 mm × 25 cm, with a silica particle diameter of 5 μm; Beckman Ultrasphere). The eluent was acetonitrile/methanol/9.79 M H$_2$SO$_4$ (100:3:0.052, by vol.) at a flow rate of 1 ml/min and a pressure of 4.14 MPa (600 lb/in^2). The dried lipid extracts were redissolved in 30 μl of chloroform/methanol (2:1, v/v) and injected into the column. Elution was monitored at 205 nm (Beckman 163). Signals were channelled to an electronic integrator (SP 4293; Spectra Physics, San José, CA, U.S.A.) and the retention times of phosphatidylinositol (PI; 5.6 min), phosphatidylserine (PS; 7.8 min), phosphatidylethanolamine (PE; 9.2 min), phosphatidylcholine (PC; 12.6 min) and sphingomyelin (21.5 min) were identified by comparison with commercial standards (Sigma). Eluates (0.5 ml fractions) were collected in scintillation vials by using a fraction collector (model 2110, Bio-Rad) coupled to the detector output, and the radioactivity was counted. The recovery of radioactivity was 80–87 %. One fraction eluted together with the solvent front contained sterols and sterol esters, which was verified by t.l.c. (silica-gel G-200; Merck) using chloroform/acetone (19:1, v/v) as the mobile phase. The radioactivity incorporated into sphingomyelin was always undetectable.

Separation of non-saponifiables by h.p.l.c.

Non-saponifiable species were separated by an isocratic h.p.l.c. method using a reversed-phase column of silica-C$_{18}$ (4.6 mm × 25 cm, 5 μm particle diameter; Ultrasphere-ODS, Beckman) with acetonitrile/methanol (10:1, v/v) as eluent with

a flow rate of 1 ml/min and a pressure of 4.83 MPa (700 lb/in²) (Bolaños and Medina, 1993). The dried non-saponifiable lipid extracts were redissolved in 30 μl of chloroform and injected into the column. Elution was monitored at 205 nm. Signals were channelled to an integrator, and the retention times of desmosterol (30.8 min and 34.0 min), lanosterol (42.5 min and 46.6 min), cholesterol (53.8 min) and squalene (62.5 min) were identified by comparison with commercial standards (Sigma). Eluates (0.5 ml fractions) were collected in scintillation vials and the radioactivity was counted. The recovery of radioactivity was 76–78%.

Analytical procedures

L-Lactate concentrations in the incubation medium were measured by the method of Gutmann and Wahlefeld (1974). GFAP and neurofilament were detected by specific antibodies coupled to peroxidase (Bignami et al., 1972; Dahl and Bignami, 1977). Results are expressed as nmol of lactate incorporated/h per 10⁶ cells and are means±S.E.M. Statistically significant differences were tested by Student's t test.

RESULTS

Lactate incorporation into total lipids, saponifiables, non-saponifiables, phospholipids and sterols in neurons and astrocytes in primary culture is shown in Table 1. The rate of total lipid synthesis from lactate was 2-fold higher in astrocytes than in neurons (Table 1). Alkaline hydrolysis of neuron and astrocyte lipids separated saponifiables (mainly fatty acids from phospholipids) and non-saponifiables (mainly sterols). Accordingly, the rate of total lipid synthesis found in astrocytes was mainly due to fatty acid synthesis, accounting for about 80% of the rate of total lipid synthesis. However, fatty acid synthesis in neurons accounted for about 52% of the total lipids (Table 1). The rate of non-saponifiable synthesis was about 27% of the total in neurons and only about 3.7% in astrocytes (Table 1).

The total lipid fraction was submitted to h.p.l.c. separation, and the radioactivity from L-[U-¹⁴C]lactate incorporated into phospholipid species was counted by liquid scintillation. As shown in Table 1, the main phospholipid synthesized from lactate both in neurons and in astrocytes was PC, followed by PE, PS and PI. However, the distribution of radioactivity among these phospholipid species was not identical in neurons and in astrocytes; PC synthesis in neurons was about 80% of the total phospholipids, but about 88% in astrocytes. In addition, PE synthesis was about 12% of the total of phospholipids in neurons, but about 7% in astrocytes. This resulted in a very different PC/PE ratio in both cell types, being 2-fold greater in astrocytes than in neurons. The rate of total phospholipid synthesis was 3.8-fold higher in astrocytes than in neurons (Table 1).

The non-saponifiable lipid fraction was submitted to h.p.l.c. separation, and the radioactivity from L-[U-¹⁴C]lactate incorporated into the sterol species was counted by liquid scintillation. As shown in Table 1, significant differences in the distribution of radioactivity among sterol species between neurons and astrocytes were observed. Thus lanosterol was found to be the major sterol synthesized in neurons, accounting for about 60% of total sterol synthesis. However, lanosterol synthesis only accounted for about 19% of total sterol synthesis in astrocytes (Table 1). Desmosterol was the major sterol synthesized in astrocytes (58% of total sterols synthesized), followed by cholesterol and lanosterol, which were synthesized at about the same rate (Table 1). In neurons, desmosterol synthesis accounted for about 33% of

Table 1 Lipogenesis from lactate in neurons and astrocytes in primary culture

Neurons and astrocytes in primary culture (7 and 10 days respectively) were incubated in 2 ml of PBS containing 10.5 mM L-lactate, 8–13 μCi of L-[U-¹⁴C]lactate (600–900 d.p.m./nmol) and 2% BSA for 2 h. Lipids were extracted as described in the Materials and methods section. Phospholipids and sterols were separated from the total lipid and the non-saponifiable fraction, respectively, by h.p.l.c. The rate of lactate incorporation into lipids was determined as described in the Materials and methods section. Results are means±S.E.M. for three experiments (N.D., not detectable): *$P < 0.01$; †$P < 0.05$; ‡$P < 0.001$ versus neurons.

	Lipogenesis (nmol of lactate incorporated/h per 10⁶ cells)	
	Neurons	Astrocytes
Total lipids	2.89±0.29	5.94±0.23*
Saponifiables	1.50±0.18	4.77±0.56*
Non-saponifiables	0.79±0.15	0.22±0.04†
Total phospholipids	1.03±0.10	3.86±0.15‡
PC	0.82±0.07	3.38±0.14‡
PE	0.12±0.01	0.28±0.01‡
PS	0.06±0.01	0.16±0.02†
PI	0.03±0.04	0.05±0.01†
PC/PE ratio	6.87	12.3
Total sterols	0.61±0.12	0.17±0.03*
Squalene	< 0.01	N.D.
Lanosterol	0.37±0.04	0.03±0.01*
Desmosterol	0.20±0.06	0.10±0.02
Cholesterol	0.05±0.02	0.04±0.01
Lanosterol/cholesterol ratio	8.16	0.79
Desmosterol/cholesterol ratio	4.49	2.49
Phospholipid/sterol ratio	1.67	23.1

the total sterols, but cholesterol synthesis was only about 7%. Squalene synthesis was very low in neurons and was undetectable in astrocytes (Table 1). Both the lanosterol/cholesterol and desmosterol/cholesterol ratios were much higher in neurons than in astrocytes. The rate of total sterol synthesis was about 3.7-fold higher in neurons than in astrocytes (Table 1). The phospholipid/sterol ratio was about 14-fold higher in astrocytes than in neurons.

DISCUSSION

We have previously reported that in brain slices (Arizmendi and Medina, 1983; Fernández and Medina, 1986; Bolaños and Medina, 1993) and in dissociated brain cells (Vicario et al., 1991; Vicario and Medina, 1992) lactate is the best substrate for brain lipid synthesis during the perinatal period. In the present work our results show that perinatal neurons and astrocytes in primary culture synthesized lipids from lactate at a very high rate (Table 1). Thus, under the same experimental conditions, lipogenesis from [6-¹⁴C]glucose was 1.21±0.48 and 1.40±0.18 nmol of glucose incorporated/h per 10⁶ cells (means±S.E.M.) for neurons and astrocytes, respectively. Accordingly, in terms of carbon atoms incorporated the rate of lipogenesis from lactate (Table 1) was about 1.2- or 2.2-fold higher than the rates from glucose in neurons and astrocytes respectively, suggesting that the two major cell types found in the rat brain during the perinatal period (Miller et al., 1989) use lactate as an important lipid precursor. It should be mentioned that the rate of lipogenesis from lactate may change during electrical activity in both neurons and astrocytes, since our observations were carried out in the resting state.

Lipid synthesis from lactate is significantly higher (about 2-

fold) in astrocytes than in neurons (Table 1), suggesting that astrocytes show a higher capacity for lactate utilization as compared with neurons. In agreement with this, Murthy and Hertz (1988) observed that the rate of pyruvate oxidation was about 2-fold greater in astrocytes than in neurons, probably reflecting an increased rate of the pyruvate dehydrogenase-catalysed reaction in astrocytes. Consequently, the increased activity of pyruvate dehydrogenase in astrocytes may account for the enhanced rate of lipogenesis from lactate found in this type of cell (Table 1). In addition, our results are in agreement with the reported higher total lipid content found in astrocytes as compared with neurons (Norton and Poduslo, 1971); this can be ascribed to the higher surface/volume ratio shown by astrocytes as compared with neurons (Wolff, 1970).

In both types of cells, the rate of lactate incorporation into saponifiables is high (Table 1), suggesting that lactate is mainly incorporated into the fatty acid moiety of phospholipids (Table 1). However, the ratio of phospholipid/sterol synthesis was much higher in astrocytes than in neurons (Table 1), in agreement with the high ratio of phospholipid/sterol concentrations found in astrocytes (Yim et al., 1986). Furthermore, the pattern of lactate incorporation into phospholipid species observed in our experiments (Table 1) correlates highly with the reported phospholipid composition of neurons and astrocytes (Norton and Poduslo, 1971).

The rate of PC synthesis through the sequential methylation of PE is very high in the brain during the neonatal period, PE methyltransferase being the rate-limiting step under these circumstances (Blusztajn et al., 1985). Accordingly, the enhanced ratio of PC/PE synthesis observed in astrocytes as compared with neurons (Table 1) suggests that astrocytes transform PE into PC more actively than do neurons. In agreement with this, it has been reported that the synthesis of PC through the methylation pathway is much higher in astrocytes than that in neurons, suggesting that astrocytes may spare choline for use by neurons (Dainous et al., 1982).

The lower rate of lactate incorporation into sterols observed in astrocytes as compared with neurons (Table 1) may suggest that sterol synthesis in astrocytes is limited by low activity of the 3-hydroxy-3-methylglutaryl-CoA reductase-catalysed reaction. The regulation of this enzyme by phospholipids and sterols has been studied by Volpe and co-workers (Finkel and Volpe, 1979; Volpe and Hennesy, 1977). These authors reported the inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase together with sterol synthesis by the intermediate in PC synthesis, NN'-dimethyl-PE (Finkel and Volpe, 1979). Such a mechanism may be designed to regulate cell membrane fluidity by controlling the phospholipid/sterol ratio. In this sense, our results suggest the occurrence of a high rate of PE conversion into PC through the methylation pathway in astrocytes (Table 1), an observation that may explain an increase in the concentration of the intermediates of this pathway, such as NN'-dimethyl-PE. Moreover, the synthesis of this intermediate might be greater in astrocytes than in neurons, which could be responsible for the lower rate of sterol synthesis observed in astrocytes as compared with neurons (Table 1).

The high rate of desmosterol synthesis from lactate observed in both neurons and astrocytes (Table 1) is consistent with the high concentration of desmosterol found in rodent (Kritchevsky and Holmes, 1962; Bourre et al., 1990) and human (Fumagalli and Paoletti, 1963) brain during the early neonatal period. The enhanced desmosterol synthesis as compared with that of cholesterol observed in neurons and astrocytes (Table 1) could be a

result of the low activity of the sterol Δ^{24}-reductase (desmosterol reductase)-catalysed reaction, which has been reported to be a rate-limiting step in cholesterol synthesis during development (Hinse and Shah, 1971). It should be mentioned that the preservation of the double bond at position 24, which is present in desmosterol, but not in cholesterol, is characteristic of the perinatal brain, suggesting that it may have some relevance for the membrane properties of developing brain cells. However, the high rate of lanosterol synthesis in neurons as compared with astrocytes is intriguing, because to our knowledge this is the first evidence of the occurrence of a preferential synthesis of lanosterol instead of desmosterol or cholesterol in neurons.

The strong differences in the rate of lipid synthesis found between neurons and astrocytes may be addressed to accomplish different physical membrane properties required for the specialized functions of each type of cells.

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