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EFFECT OF VALPROATE ON LIPOGENESIS IN NEONATAL RAT BRAIN

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Abstract—The effect of valproate on lipogenesis in brain slices from early neonatal rats was studied. The rate of lipid synthesis from lactate and 3-hydroxybutyrate, but not from glucose, was decreased significantly by 1 mM valproate. Separation by high performance liquid chromatography of brain lipids showed that valproate inhibited the synthesis of major phospholipids (phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine) from lactate and major sterols (desmosterol, cholesterol and lanosterol) from lactate and 3-hydroxybutyrate. Valproate did not affect sterol synthesis but slightly enhanced phospholipid synthesis from glucose. However, the ratio of phosphatidylserine/phosphatidylethanolamine synthesis was decreased from lactate, glucose and 3-hydroxybutyrate, suggesting that valproate changes phospholipid composition of brain structures. These changes may contribute to the pharmacological action of the drug.

Valproic acid is a broad-spectrum antiepileptic drug whose mechanism of action is still unknown. Briefly, valproate inhibits brain α-ketoglutarate dehydrogenase (EC 1.2.4.2) [1], γ-aminobutyric acid aminotransferase (EC 2.6.1.19) [2] and/or stimulates glutamic acid decarboxylase (EC 4.1.1.15) [3]. These effects may increase brain concentrations of the inhibitory neurotransmitter γ-aminobutyric acid, which may explain its pharmacological effect. However, the effects of valproate on the properties of brain membranes also may be relevant in the pharmacological mode of action of the drug. In this sense, it has been reported that valproate inhibits phospholipid synthesis in neuroblastoma cells [4], it decreases the fluidity of brain mitochondrial membranes [5], and it inhibits the monocarboxylate carrier of blood–brain barrier [6] and/or of brain mitochondrial membrane [7]. Thus, the possible changes occurring in brain membrane lipid composition brought about by valproate could be relevant during the postnatal period in which the synthesis of lipids is enhanced temporarily [8]. Prompted by these considerations, we investigated the possible effect caused by valproate on brain lipogenesis during the neonatal period. Since lactate seems to be the best substrate for the brain during this period [9–12], we studied the effect of the drug on lipogenesis from lactate. Lipogenesis from other relevant substrates of the brain during the neonatal period, such as glucose and 3-hydroxybutyrate, was also investigated.

MATERIALS AND METHODS

Chemicals. L-[U-^{14}C]Lactate (specific radioactivity 177 Ci/mol), D-[6-^{14}C]glucose (specific radioactivity 55.8 Ci/mol) and D-3-hydroxy-[3-^{14}C]butyrate (specific radioactivity 44.3 Ci/mol) were purchased from New England Nuclear (Boston, MA, U.S.A.). L-Lactic acid was obtained from Serva Feinbiochemica (Heidelberg, Germany). D-Glucose, DL-3-hydroxybutyrate and sodium valproate were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). High performance liquid chromatography (HPLC)-grade solvents were from Scharlau (Barcelona, Spain). Enzymes and coenzymes were obtained from Boëhringer (Mannheim, Germany).

Animals. Albino Wistar rats fed on stock laboratory diet (by w/v: carbohydrate 58.7%, protein 17.0%, fat 3.0% and added salts and vitamins) and of known gestational age were used for the experiments. Virgin females with a weight of 225–250 g were caged overnight with males. Conception was considered to occur at 1:00 a.m. and was confirmed the next morning by the presence of spermatozoa in vaginal smears. Fetuses weighing 5.2 ± 0. 1 g (mean ± SEM) were delivered on day 21.5 of gestation (21.7 days for full gestation) by rapid hysterectomy after cervical dislocation of the mother. Newborns were carefully wiped and the umbilical cords were tied and cut. Newborns were kept in an incubator at 37° in a continuous stream of water-saturated air without feeding.

Brain incubations. After 1 hr of extrauterine life, the newborns were decapitated and the right hemispheres of the forebrain were removed and immediately sliced in a water-saturated cabin. Brain slices (70 mg of wet weight) were incubated as previously described [9, 11]. The incubation medium was 2 mL of phosphate-buffered saline (11 mM sodium phosphate, 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 1.3 mM $CaCl_2$, pH 7.4) containing 2 μCi of L-[U-^{14}C]lactate (specific radioactivity 0.08 Ci/mol), 2 μCi of D-[6-^{14}C]glucose (specific radioactivity 0.1 Ci/mol) or 1 μCi of D-3-hydroxy-[3-^{14}C]butyrate (specific

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radioactivity 1.0 Ci/mol) and the desired concentration of the unlabeled substrates with or without 1 mM sodium valproate. This concentration has been shown to be close to that reached in blood after the administration of therapeutic doses of valproate, which protects against experimentally induced convulsions in the rat [13]. The atmosphere of the flasks was gassed with O_2; the flasks were then sealed with rubber caps and incubated in a shaken water-bath at 37°. Incubations were stopped after 2 hr by injection of 0.2 mL of 4.75 M $HClO_4$ through the rubber cap into the well.

Extraction of total lipids. At the end of the incubation, the slices were frozen under liquid nitrogen. Total lipids were extracted from the powdered tissue with 2 mL of a mixture of chloroform/methanol (2:1, v/v) by the method of Folch *et al.* [14] for 16 hr at −20°. The extract was washed twice with 0.8 mL of 0.3% (w/v) NaCl saturated with chloroform. The organic phase was divided into two aliquots; 0.1 mL 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° until being subjected to phospholipid separation by HPLC.

Extraction of non-saponifiables. Non-saponifiable material (mainly sterols) was extracted essentially as described by Edmond and Popjäk [15]: brain slices were saponified at 70° with 4 mL of 53.5 M KOH/ethanol (1:1, v/v) for 2 hr; unsaponifiable material was extracted three times with 5 mL of petroleum ether (b.p. 40–60°) and the combined petroleum extracts were concentrated to dryness with a stream of N_2. The extract was kept at −20° until being subjected to separation by HPLC.

Separation of phospholipids by HPLC. Phospholipid species were separated by an HPLC isocratic method, essentially as described by Kaduce *et al.* [16] using a liquid chromatography pump system (114 M-Beckman, Beckman Instruments, Inc., Palo Alto, CA, U.S.A.) on a normal-phase column of silica (4.6 mm × 25 cm, with silica particle diameter of 5 μm, Ultrasphere, Beckman). The eluent was acetonitrile:methanol:9.79 M sulfuric acid (100:3:0.052, by vol.) at a flow rate of 1 mL/min and a pressure of 600 psi. 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 a wavelength of 205 nm (163-Beckman). Signals were channeled 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 sphyngomyelin (21.5 min) were identified by comparison with commercial standards (purity: ≥97%, Sigma Chemical Co.). Eluates (0.5-mL fractions) were collected into scintillation vials using a fraction collector (model 2110, Bio-Rad, Richmond, CA, U.S.A.) coupled to the detector output, and the radioactivity was counted. The recovery of radioactivity was 85–95%. One fraction eluted together with the solvent front contained sterols and sterol esters, which was verified by thin-layer chromatography (Silicagel G-200, Merck, Darmstad, Germany) using a system of chloroform:acetone (95:5, v/v) as the mobile phase (results not shown). The radioactivity incorporated into sphyngomyelin was always undetectable.

Separation of non-saponifiables by HPLC. Non-saponifiable species were separated by an HPLC isocratic 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 and a flow rate of 1 mL/min and a pressure of 700 psi. The dried non-saponifiable extract was redissolved in 30 μL of chloroform and injected into the column. Elution was monitored at a wavelength of 205 nm. Signals were channeled to an integrator and the retention times of desmosterol (30.8 and 34.0 min), lanosterol (42.5 and 46.6 min), cholesterol (53.8 min) and squalene (62.5 min) were identified by comparison with commercial standards (purity: 97–100%, Sigma Chemical Co.). Eluates (0.5 mL fractions) were collected into scintillation vials and the radioactivity was counted. The recovery of radioactivity was 70–80%.

Metabolite determinations and data analysis. D-3-Hydroxybutyrate was determined as described by Williamson and Mellanby [17], D-glucose as described by Bergmeyer *et al.* [18] and L-lactate after Gutmann and Wahlefeld [19]. The specific radioactivity of the substrates found in the blanks was used for the calculations. The rates of substrate utilization by the brain slices were expressed as micromoles (or nanomoles) of L-lactate, D-glucose or D-3-hydroxybutyrate incorporated into total lipids, phospholipids or non-saponifiables per hour per gram of wet weight. Results are presented as means ± SEM. Statistically significant differences were tested by Student's *t*-test.

RESULTS

Effect of valproate on lipid synthesis. Table 1 shows that lactate is the main precursor of lipids in the neonatal rat brain, since the incorporation of this substrate into phospholipids, non-saponifiables and total lipids was higher than the incorporation of glucose or 3-hydroxybutyrate into these lipids. Lactate, glucose and 3-hydroxybutyrate were mainly incorporated into the non-saponifiable fraction, the phospholipid fraction being 28 and 31% of the total amount of lipids synthesized from lactate and glucose, respectively, and 10% of those synthesized from 3-hydroxybutyrate. The presence in the incubation medium of valproate significantly decreased the rate of lipid synthesis from lactate. This inhibition was due to the decrease in the rate of synthesis of both phospholipids and non-saponifiables (Table 1). The synthesis of phospholipids from glucose was enhanced significantly by valproate but the synthesis of non-saponifiables was not modified in these circumstances (Table 1). The rate of total lipid synthesis from 3-hydroxybutyrate was decreased significantly by valproate (Table 1). The ratio of non-saponifiables/phospholipids

*Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; and PS, phosphatidylserine.

Table 1. Effect of valproate on the incorporation of lactate, glucose and 3-hydroxybutyrate into lipids in neonatal rat brain*

Additions	Substrate incorporated (μmol/hr/g wet wt)			N-Sap/Phospholipid ratio
	Total lipids	Phospholipids	N-Sap†	
2 μCi L-[U-^{14}C]Lactate (12 mM)	1.48 ± 0.07 (100)	0.416 ± 0.038 (100)	0.531 ± 0.050 (100)	1.28 ± 0.12
+ valproate (1 mM)	1.09 ± 0.03‡ (74)	0.303 ± 0.023§ (73)	0.426 ± 0.024§ (80)	1.41 ± 0.08
2 μCi D-[6-^{14}C]Glucose (5 mM)	0.83 ± 0.03 (100)	0.261 ± 0.032 (100)	0.375 ± 0.050 (100)	1.44 ± 0.19
+ valproate (1 mM)	0.97 ± 0.05 (117)	0.361 ± 0.024§ (138)	0.372 ± 0.009 (99)	1.03 ± 0.02
1 μCi D-3-hydroxy-[3-^{14}C]Butyrate (1 mM)	0.20 ± 0.01 (100)	0.019 ± 0.002 (100)	0.129 ± 0.011 (100)	6.79 ± 0.58‖
+ valproate (1 mM)	0.15 ± 0.01‡ (75)	0.024 ± 0.004 (126)	0.103 ± 0.009§ (80)	4.29 ± 0.38‖

* Brain slices were incubated in phosphate-buffered saline as described in Materials and Methods. Results are means ± SEM for 4–7 newborns. The percentages are indicated in parentheses.
† N-Sap, non-saponifiables.
‡ $P < 0.01$ vs the values obtained in the absence of valproate.
§ $P < 0.05$ vs the values obtained in the absence of valproate.
‖ $P < 0.001$ vs the values obtained from lactate and glucose.

synthesis was significantly greater from 3-hydroxybutyrate than that from lactate and glucose (Table 1).

Effect of valproate on phospholipid synthesis. Table 2 shows the incorporation of lactate, glucose and 3-hydroxybutyrate into phospholipid species. Brain phospholipids were separated by HPLC and the radioactivity incorporated into each phospholipid was measured by liquid scintillation counting. PC was the main phospholipid synthesized from lactate, glucose and 3-hydroxybutyrate, accounting for about 70–76% of the rate of total phospholipid synthesis (Table 2). The rate of PE synthesis was about 12–17% of the rate of total phospholipid synthesis in these circumstances. The rate of PS synthesis was about 6–11% from all the substrates assayed. The rate of PI synthesis was 1.9 and 4.2% from lactate and glucose, respectively, and 7% from 3-hydroxybutyrate (Table 2). The presence of valproate in the incubation medium decreased the rate of lactate incorporation into PC, PE and PS (Table 2). However, valproate increased the rate of glucose incorporation into PC, PE and PI (Table 2). In the presence of valproate the ratio of PS/PE synthesis was decreased significantly from all the substrates assayed (Table 2).

Effect of valproate on sterol synthesis. Table 3 shows the rates of incorporation of lactate, glucose and 3-hydroxybutyrate into neonatal brain sterols. Squalene accounted for about 1.2–1.7% of the rate of total non-saponifiables synthesis from lactate, glucose and 3-hydroxybutyrate. Lanosterol and cholesterol were synthesized to a similar extent, each accounting for about 18% of the rate of total non-saponifiables synthesis. The rate of desmosterol synthesis from all the substrates assayed was the highest observed (Table 3), accounting for about 62% of the rate of total non-saponifiables synthesis from lactate, glucose and 3-hydroxybutyrate (Table 3). The presence of valproate significantly decreased the synthesis of lanosterol, desmosterol and cholesterol without affecting squalene synthesis from lactate (Table 3). Valproate inhibited the synthesis of squalene, lanosterol, desmosterol and cholesterol from 3-hydroxybutyrate (Table 3). However, the presence of valproate in the incubation medium did not change the rate of the synthesis of sterol species from glucose (Table 3).

DISCUSSION

During the early neonatal period, the very low blood glucose concentration is insufficient to satisfy the energy requirements of the newborn's tissues [20]. Likewise, ketone bodies, which are produced in liver from milk fatty acids, are not available until the onset of suckling, which occurs 2 hr after birth in the rat [21]. Therefore, it has been suggested that lactate, whose blood concentrations are very high immediately after birth [10, 20], plays a relevant role as an alternative energy substrate for the brain during the presuckling period [9–12]. The results obtained in this work reinforce this suggestion since lactate proved to be the main lipid precursor in the neonatal rat brain as compared to glucose and 3-hydroxybutyrate. In addition, lactate was the major

Table 2. Effect of valproate on the incorporation of lactate, glucose and 3-hydroxybutyrate into phospholipids in neonatal rat brain*

Additions	Substrate incorporated (nmol/hr/g wet wt)				PS/PE ratio
	PC†	PE	PS	PI	
2 μCi L-[U-^{14}C]Lactate (12 mM)	310 ± 23 (100)	73 ± 10 (100)	25 ± 3 (100)	8 ± 1.4 (100)	0.34 ± 0.04
+ valproate (1 mM)	232 ± 14‡ (75)	50 ± 4‡ (68)	13 ± 2.6‡ (52)	8 ± 1.9 (100)	0.26 ± 0.05‡
2 μCi D-[6-^{14}C]Glucose (5 mM)	198 ± 21 (100)	35 ± 6 (100)	17 ± 2.5 (100)	11 ± 1.5 (100)	0.49 ± 0.07
+ valproate (1 mM)	269 ± 18‡ (136)	58 ± 4‡ (166)	16 ± 0.6 (94)	18 ± 1.4§ (164)	0.28 ± 0.01‡
1 μCi D-3-hydroxy-[3-^{14}C]Butyrate (1 mM)	13 ± 0.6 (100)	2.2 ± 0.2 (100)	2.1 ± 0.3 (100)	1.3 ± 0.7 (100)	0.95 ± 0.14
+ valproate (1 mM)	15 ± 3 (115)	4.4 ± 0.6§ (200)	3 ± 0.6 (143)	1.2 ± 0.2 (92)	0.68 ± 0.14‡

* Brain slices were incubated in phosphate-buffered saline. The extraction and the analysis by HPLC of the phospholipids were carried out as described in Materials and Methods. Results are means ± SEM for 4–7 newborns. The percentages are indicated in parentheses.

† PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

‡ $P < 0.05$ vs the values obtained in the absence of valproate.

§ $P < 0.01$ vs the values obtained in the absence of valproate.

Table 3. Effect of valproate on the incorporation of lactate, glucose and 3-hydroxybutyrate into non-saponifiables in neonatal rat brain*

Additions	Substrate incorporated (nmol/hr/g wet wt)			
	Squalene	Lanosterol	Desmosterol	Cholesterol
2 μCi L-[U-^{14}C]Lactate (12 mM)	7.2 ± 0.7 (100)	100 ± 8 (100)	333 ± 29 (100)	91 ± 7 (100)
+ valproate (1 mM)	7.4 ± 0.5 (103)	81 ± 3† (81)	257 ± 18† (77)	81 ± 3† (89)
2 μCi D-[6-^{14}C]Glucose (5 mM)	6.5 ± 0.7 (100)	62 ± 5 (100)	230 ± 28 (100)	76 ± 10 (100)
+ valproate (1 mM)	6.1 ± 0.4 (94)	64 ± 3 (103)	227 ± 3 (99)	75 ± 3 (99)
1 μCi D-3-hydroxy-[3-^{14}C]Butyrate (1 mM)	1.5 ± 0.1 (100)	24 ± 1 (100)	81 ± 8 (100)	22 ± 1 (100)
+ valproate (1 mM)	0.8 ± 0.1† (53)	18 ± 2† (75)	68 ± 6† (84)	16 ± 1† (73)

* Brain slices were incubated in phosphate-buffered saline. The extraction and the analysis by HPLC of the non-saponifiables were carried out as described in Materials and Methods. Results are means ± SEM for 6–7 newborns. The percentages are indicated in parentheses.

† $P < 0.05$ vs the values obtained in the absence of valproate.

substrate for the synthesis of brain sterols and phospholipids (Table 1). Under these circumstances, valproate significantly inhibited lipid synthesis from lactate, the effect being observed on the rate of synthesis of the main phospholipid and sterol species (Tables 1–3). These results may be of pharmacological relevance because lactate could be the main substrate during the early postnatal period and hence valproate would interfere with lipid synthesis in these circumstances. In addition, we have reported previously that the administration of valproate to the rat during development decreased the rate of ^3H_2O incorporation into brain sterols *in vivo* [22]. This effect was explained by the fact that valproate inhibits ketogenesis in the liver [23], resulting in a reduced supply of ketone bodies to the brain. However, the results reported in this paper suggest that valproate also inhibits the incorporation of ketone bodies into brain sterols (Table 1). Consequently, valproate may inhibit both ketone body supply and utilization by the neonatal brain, which may result in the inhibition of lipogenesis during the suckling period. Thus, ketone bodies are important substrates for the lactating brain, and meet most of the metabolic requirements of the brain during the suckling period [21]. Moreover, 3-hydroxybutyrate and acetoacetate are precursors for the synthesis of sterols and fatty acids in the central nervous system (Tables 2 and 3) [24]. Nevertheless, conversion to sterols rather than to fatty acids is the main anabolic fate of ketone bodies in the brain (Table 1) [25]. Thus, cytosolic acetoacetate is not necessarily cleaved into acetyl-CoA for cholesterol synthesis in the neonatal brain [25, 26] because of the occurrence of acetoacetyl-CoA synthetase (EC 6.2.1.16) activity in the developing brain [27].

The effect of valproate on the rate of non-saponifiables synthesis from lactate and 3-hydroxybutyrate differed substantially from that from glucose (Table 1). Thus, valproate inhibited sterol synthesis from lactate and 3-hydroxybutyrate but not from glucose (Tables 1 and 3). In addition, valproate decreased the rate of phospholipid synthesis from lactate, did not affect that from 3-hydroxybutyrate, and increased that from glucose (Table 2). These results are not consistent with the suggestion [7] that valproate inhibits the mitochondrial pyruvate carrier because pyruvate from both lactate and glucose obviously uses the same mitochondrial carrier. If valproate inhibits the mitochondrial pyruvate carrier, an inhibition of lipogenesis from glucose should be expected. In fact, α-cyano-4-hydroxycinnamate, a well-characterized inhibitor of the mitochondrial pyruvate carrier [28], inhibited sterol and phospholipid syntheses from glucose together with those from lactate and 3-hydroxybutyrate (results not shown), suggesting that the effect of valproate on lipogenesis (Tables 1–3) is not related to an inhibition of the mitochondrial pyruvate carrier. On the other hand, valproate is transported to the central nervous system by the monocarboxylate carrier [6], which is shared by lactate and 3-hydroxybutyrate [29], but not by glucose, which uses its own transport system [30]. In agreement with this, valproate inhibited to a similar extent sterol synthesis from lactate and 3-hydroxybutyrate but not from glucose, which was unaffected (Table 1). It should be mentioned that phospholipid synthesis from lactate was similarly affected by valproate. In addition, the effect of the drug on phospholipid synthesis from 3-hydroxybutyrate was not observed, possibly because 3-hydroxybutyrate incorporation into phospholipids (Table 1) was below the sensitivity limit of the method. Consequently, valproate may interfere in the transport of lactate and 3-hydroxybutyrate, but not that of glucose, into brain cells by inhibiting the monocarboxylate carrier. In agreement with this, the transport of pyruvate, which also uses the monocarboxylate carrier [28, 29], is competitively inhibited by valproate in brain capillary endothelial cells, although the drug does not affect glucose transport [31]. Consequently, it may be suggested that the inhibition of the plasma membrane monocarboxylate carrier by valproate is responsible for the inhibition of lactate and 3-hydroxybutyrate incorporation into lipids (Table 1).

Under our experimental conditions, the phospholipid most actively synthesized was PC followed by PE, PS and PI. This coincided with the pattern of phospholipids concentrations found in the brain during this period [32]. This is probably a consequence of the low degree of myelination occurring during the early neonatal period [8] when the brain phospholipid composition [32] highly correlates with the rate of newly synthesized phospholipids (Table 2). The inhibition of lactate incorporation into brain phospholipids caused by valproate (Table 2) may be of pharmacological relevance. Roberti *et al.* [4] have reported the ability of valproate to overcome the inhibition of PC and PE synthesis caused by pyridoxal 5′-phosphate. However, no effect of valproate on the rate of choline or ethanolamine incorporation into PC or PE, respectively, was observed under these circumstances [4]. Our results (Table 2) are consistent with the notion of a direct effect of valproate on phospholipid synthesis from lactate rather than on the uptake of phospholipid-borne amines because the effect of valproate on phospholipid synthesis was not observed when glucose was used as a substrate. Likewise, it has been reported [33] that valproate does not change the rate of glycerol incorporation into glycerolipids. In agreement with this, our results suggest that the inhibition of lactate incorporation into PC, PE or PS (Table 2) would not be due to a decrease in the rate of glycerol incorporation into glycerolipids because under our experimental conditions valproate did not inhibit glycerol-borne phospholipid synthesis from glucose (Table 2). Moreover, valproate did not affect the incorporation of [3,4-^{14}C]glucose into brain lipids (results not shown), suggesting that glycerogenesis is not affected by the presence of valproate. Consequently, it may be suggested that valproate inhibits fatty acid-borne phospholipid synthesis from lactate. It should be mentioned that Sklenovsky and Chmela [34] have shown that valproate can reverse the enhanced fatty acid synthesis caused by a convulsive state in the rat, an effect that may be explained by the inhibition of fatty acid synthesis observed in our experiments (Table 2).

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