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THE RELEASE OF BRAIN FREE FATTY ACIDS DURING ISCHAEMIA IN ESSENTIAL FATTY ACID-DEFICIENT RATS

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Abstract—The release of free fatty acids and especially of free arachidonic acid occurring in rat brain during ischaemia has been studied in essential fatty acid-deficient animals. Free fatty acid levels are lower in essential fatty acid-deficient brains before and especially after 5 min of ischaemia. The percentage of arachidonic acid, in respect of total free fatty acids, is similar in both control and essential fatty acid-deficient brains before ischaemia (0 min), but is greatly reduced in deficient brains in respect of controls after ischaemia (5 min). Total levels of free arachidonic acid are thus greatly reduced after ischaemia in the brain of essential fatty acid-deficient rats. Focussed microwave irradiation of the brain, a technique which instantaneously kills the animals, and which was used for the determination of brain basal levels of free fatty acids and free arachidonic acid, does not *per se* modify brain lipid and fatty acid composition.

BRAIN free fatty acid (FFA) levels have been reported to increase during ischaemia (BAZAN, 1970) and after treatment with convulsant drugs or electroshock (BAZAN, 1971). The process is considered to be dependent upon the activation of phospholipase possibly involving cyclic AMP (cAMP) formation (BAZAN *et al.*, 1971). This hypothesis has been substantiated by the recent observation that short-lasting focussed microwave irradiation of the CNS, which inhibits post mortem cAMP accumulation and other enzymic activities in the tissue (GUIDOTTI *et al.*, 1974) prevents FFA increase during ischaemia (CENEDELLA *et al.*, 1975). The levels of FFA in the CNS, after microwave irradiation, are similar to those measured after decapitation and rapid freezing of the tissue (CENEDELLA *et al.*, 1975). FFA production in the ischaemic brain may have a physiological significance in relation to prostaglandin formation since arachidonic acid, a precursor of brain prostaglandins, is preferentially released under these conditions (BAZAN, 1970) and recent experiments have shown, on the other hand, that the prostaglandins $PGF_{2\alpha}$ and PGE_2 are synthesized from endogenous substrates in brain slices (GALLI & NICOSIA, 1975). Arachidonic acid (20 : 4 n-6) levels in tissues are affected by the dietary intake of the essential precursor linoleic acid (18 : 2 n-6). A long-term exposure of growing rats to essential fatty acid (EFA) deficient diets results in reduced levels of arachidonic acid in brain structural lipids (GALLI *et al.*, 1970). It has also been shown that FFA formation in EFA-deficient animals is increased in adipose tissue after stimulation of the hormone-sensitive triglyceride lipase (BIZZI *et al.*, 1967). The aim of the present study was to assess whether or not changes in FFA release during EFA deficiency also occur in the CNS and if the concentrations of free arachidonic acid are particularly affected. Finally, since focussed microwave irradiation of the animal head may result in degradation of complex lipids and/or of their highly unsaturated fatty acids in the CNS, this possibility was tested prior to the study of the effects of EFA deficiency on brain FFA release.

MATERIALS AND METHODS

Animals and diets. Male Sprague–Dawley rats of average age 3 months and weighing between 250 and 350 g were used. The animals were raised on a standard diet or a semisynthetic diet containing 10% saturated fat (EFA-deficient), which were fed previously to the mothers, starting 1 week before delivery, and were subsequently fed to the young rats after weaning as previously described (GALLI *et al.*, 1970). The fatty acid composition of the two diets was analysed by GLC. The animals were killed by exposure of their heads to focussed microwave irradiation (GUIDOTTI *et al.*, 1974) or by decapitation followed by removal of the brain from the skull after being left for 4 min in the head kept at room temperature and homogenization in chloroform–methanol (2 : 1, v/v) for lipid extraction within 5 min.

Brain lipid extraction and FFA determination. Lipids were extracted with chloroform–methanol 2 : 1 (twice). The filtered and water-washed extracts (FOLCH-PI *et al.*, 1957) were fractionated by TLC (CENEDELLA *et al.*, 1975), the band of powder containing FFA was collected, extracted with chloroform and, after reduction to a known volume by evaporation under stream of nitrogen, utilized for quantitative determination by the colorimetric method of DUNCOMBE (1963). The total lipid content of the extracted brains was determined by weighing dried samples of the

Abbreviations used: cAMP, cyclic AMP; EFA, essential fatty acids; FFA, free fatty acids.

extract on a microbalance (ROUSER *et al.*, 1969). The FFA content measured was expressed as μg/100 mg of brain total lipids. In a second group of both control and EFA deficient animals, FFA recovered after TLC separation were analysed by GLC (GALLI *et al.*, 1970). The recovery of FFA from each sample through extraction and chromatography was checked by using [1-^{14}C]stearic acid, added in a total amount which was less than 0·5% of the endogenous FFA pool, as internal standard (CENEDELLA *et al.*, 1975). Recovery was on the average 68·5 ± 2·2%. Finally, in the study of the effects of microwave irradiation on brain phospholipid composition and their fatty acid pattern, control animals were killed by microwave irradiation or decapitation and lipids extracted within 3 min. Phospholipids were analysed by two-dimensional TLC (ROUSER *et al.*, 1970) and the fatty acid composition of ethanolamine phosphoglyceride, purified by TLC, was carried out as previously described (GALLI *et al.*, 1970).

RESULTS

The effects on brain phospholipid composition of microwave irradiation vs decapitation and homogenization of the tissue within 3 min are shown in Table 1. No significant difference between brain phospholipid composition in the two groups of animals is observed. Table 2 presents the fatty acid composition (weight percentage) of ethanolamine phosphoglyceride, the most unsaturated phospholipid in the CNS, in animals killed by microwave irradiation or decapitation. No significant difference is observed between the fatty acid composition in the two groups. The values of FFA levels (μg/100 mg of brain total lipids) in the brains of control and EFA-deficient rats killed either by microwave irradiation or decapitation are presented in Table 3. During ischaemia, brain FFA levels rise both in control and in EFA-deficient rats. However FFA are significantly lower in brains of EFA-deficient animals both after microwave irradiation and after 5 min of ischaemia. It also appears that the increment of brain FFA levels occurring during ischaemia is less pronounced in EFA-deficient animals. Figure 1 shows the composition of brain FFA in control and EFA-deficient rats killed by the two different procedures. In the control group the 5-min period of ischaemia results in a significant decrease of shorter chain saturated fatty acids (14:0 and 16:0) of monoenoic fatty acids (16:1, 18:1 and 20:1) and of docosatetraenoic acid (22:4). On the other hand, stearic acid (18:0) increases 20% and arachidonic acid (20:4) undergoes a 3·5-fold increase. In the EFA-deficient group shorter chain saturated fatty acid levels are not affected by ischaemia, whereas stearic acid increases as in controls. Eicosatrienoic acid (20:3 n-9), present in very low concentrations in controls, tends to rise, whereas arachidonic acid increases 2·1-fold. Docosatetraenoic acid (22:4) decreases as in the case of controls. The comparison between the fatty acid composition in animals killed by microwave irradiation of the control and deficient groups shows the presence of lower percentage values of linoleic acid (18:2) and of higher values of eicosatrienoic acid (20:3) in the deficient group. Basal percentage values of free arachidonic acid are similar in both control and EFA-deficient brains. Finally the comparison between the percentage composition of the control and EFA deficient groups 5 min after decapitation shows that eicosatrienoic acid (20:3) is present in a higher percentage in the deficient animals, being practically negligible in control animals. The percentage composition value of arachidonic acid shows instead a 50% reduction in respect of controls. Table 4 shows the total levels of brain free arachidonic acid (μg/100 mg total lipids) in control and deficient rats before and after ischaemia. Levels are respectively 40 and 70% lower before and after ischaemia in the deficient brains, in respect of controls. The increment of free arachidonic acid during ischaemia is about half in the EFA-deficient brains compared to that observed in control brains.

TABLE 1. COMPOSITION OF BRAIN PHOSPHOLIPIDS OR RATS KILLED BY FOCUSSED MICROWAVE IRRADIATION OR DECAPITATION (3 min)

Phospholipid classes	Microwave (4)	3 min (4)
EPG	38·4 ± 0·4	38·9 ± 0·1
CPG	37·5 ± 0·3	37·3 ± 0·3
SPG	13·0 ± 0·2	12·8 ± 0·1
Sph + IPG	7·7 ± 0·4	7·9 ± 0·1
PA	1·6 ± 0·04	1·3 ± 0·2

Number of animals in brackets.
EPG, ethanolamine phosphoglyceride; CPG, choline phosphoglyceride; SPG, serine phosphoglyceride; Sph, sphingomyelin; IPG, inositol phosphoglyceride; PA, phosphatidic acid.

TABLE 2. FATTY ACID COMPOSITION (WEIGHT PERCENTAGE) OF BRAIN ETHANOLAMINE PHOSPHOGLYCERIDE OF RATS KILLED BY FOCUSSED MICROWAVE IRRADIATION OR DECAPITATION (3 min)

Fatty acid methyl esters	Microwave (4)	3 Min. (4)
16:0	6·9 ± 0·2	6·9 ± 0·5
18:0	21·1 ± 0·6	21·4 ± 0·4
18:1 n-9	24·9 ± 0·9	22·6 ± 0·5
18:2 n-6	0·6 ± 0·04	0·5 ± 0·1
20:1 n-9	5·0 ± 0·1	4·3 ± 0·3
20:4 n-6	13·4 ± 0·4	14·3 ± 0·2
22:4 n-6	7·1 ± 0·1	7·2 ± 0·2
22:5 n-6	2·5 ± 0·1	2·1 ± 0·2
22:6 n-3	15·3 ± 1·7	17·2 ± 1·6

Number of animals in brackets.

DISCUSSION

The data presented in Tables 1 and 2 indicate that focussed microwave irradiation, a procedure for killing small laboratory animals which has been introduced for the correct evaluation of neurotransmitter

TABLE 3. FFA LEVELS (μg/100 mg OF TOTAL LIPIDS) IN THE BRAINS OF CONTROL AND EFA-DEFICIENT RATS, ANALYSED AFTER MICROWAVE IRRADIATION (0 min) OR DECAPITATION AND 5 MIN OF ISCHAEMIA (5 min)

	(a) 0 min	(b) 5 min	b/a
Control	(8) 98 ± 8	(9) 201 ± 21	2·06
Deficient	(8) 73 ± 7*	(6) 120 ± 17†	1·64
Deficient/control	0·75	0·60	

Number of animals in brackets
Statistical significance of difference from controls.
* $P < 0.05$.
† $P < 0.01$.

and cyclic nucleotide levels in the CNS, can also be used to study complex lipids of the brain. In fact, both phospholipid composition and the fatty acid composition of the most unsaturated phospholipid (ethanolamine phosphoglyceride) in the CNS are not modified by microwave irradiation of the brain, in spite of the high temperature developed in this tissue. The analyses carried out in control and EFA-deficient rats after microwave irradiation and 5 min of ischaemia confirm that, during brain ischaemia, release of FFA occurs in this tissue. Considerable differences between control and EFA animals are, however, observed in respect of the levels and composition of EFA released after microwave irradiation or ischaemia. In fact, the basal levels (after microwave irradiation) of brain FFA are lower in EFA-deficient rats than in controls, suggesting reduced hydrolysis of lipid in brain during EFA deficiency. The percentage composition of the brain FFA pool is instead not very different in animals killed by microwave irradiation both in control and EFA-deficient groups. More specifically, the percentage values of free arachidonic acid are not significantly different in control and deficient brains, although their levels in brain total phospholipids (not shown in the figures) are significantly lower in the deficient group (5·8 ± 0·3% in EFA-deficient animals vs 9·0 ± 0·5% in controls). Total basal levels of free arachidonic acid (μg/100 mg total lipids) are, however, lower in the deficient brains.

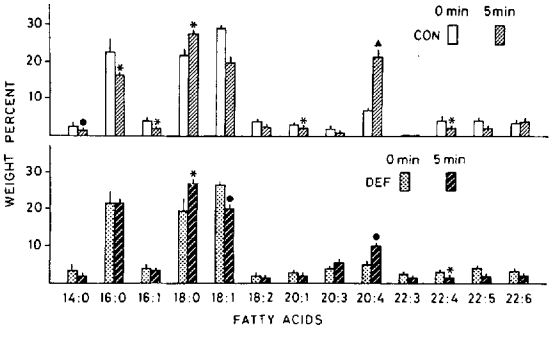

FIG. 1. Weight percentage composition (plus and minus S.E.M.) of brain free fatty acids of control (CON) or EFA-deficient (DEF) rats before (0 min) or after 5 min of ischaemia (5 min). Number of animals used: CON 0 min, 3; CON 5 min, 6; DEF 0 min, 5; DEF 5 min, 6. Difference from controls * $P < 0.05$; ● $P < 0.01$; ▲ $P < 0.005$.

During brain ischaemia, increased FFA release occurs both in control and EFA-deficient brains, but the increase is greater in control animals. Thus in EFA deficiency, reduced stimulation of lipid hydrolysis with respect to controls occurs in the brain under conditions of ischaemia. In contrast to this observation, it has been observed that lipolytic processes in adipose tissue are increased, after hormonal stimulation, in EFA-deficient animals (BIZZI *et al.*, 1967). The increased mobilization of FFA in the stimulated adipose tissue during EFA deficiency has been interpreted as a consequence of a reduced formation in the tissue, under these conditions, of prostaglandins, which are known to inhibit the lipolytic action of several hormones (STEINBERG *et al.*, 1963). A differential release of FFA in brain with respect to adipose tissue during EFA deficiency, may partly depend upon the difference in the enzymes presumably involved in this process (phospholipase A instead of triglyceride lipase). The increased formation of FFA under conditions of ischaemia and the modifications of the FFA composition in the control groups are similar to those already reported by BAZAN (1970).

Arachidonic acid and, to a much lesser extent, stearic acid, are preferentially released both in control and in deficient brains. Polyunsaturated fatty acids with the same degree of unsaturation but longer chain length (22 : 4) than arachidonic acid are released to a lower degree. Eicosahexaenoic acid (22 : 6 n-3) which is present in high concentrations together with arachidonic acid in membranes with greater fluidity is present (SUN & HORROCKS, 1970) in very low concentrations in the brain FFA pool and is not preferentially released during ischaemia. Other factors, besides subcellular location of polyunsaturated fatty acids as suggested by BAZAN (1970), appear thus to be relevant to the phospholipase activity. The reduced release of FFA in deficient brains compared to controls during ischaemia, and the lower percentage values of free arachidonic acid in the released pool, result in considerably lower levels (μg/100 mg total lipids) of free arachidonic acid in the ischaemic, EFA-deficient brains. During ischaemia of deficient brains, a slightly increased release of eicosatrienoic acid (20 : 3 n-9) also occurs. However, the increase is less pronounced than

TABLE 4. LEVELS OF FREE ARACHIDONIC ACID (μg/100 mg TOTAL LIPIDS) IN BRAIN OF CONTROL AND EFA-DEFICIENT RATS BEFORE (0 min) AND AFTER (5 min) ISCHAEMIA

	0 min	5 min	5 min/0 min
Control	6·17*	44·02	7·13
Deficient	3·80	13·32	3·50
Deficient/control	0·61	0·30	

* Values are calculated as follows:
Average levels of brain FFA × average percentage of arachidonic acid.

that of arachidonic acid and, thus, the triene/tetraene ratio decreases. This suggests that, with a given chain length (20 carbon atoms) tetraenes are preferentially released than trienes. In conclusion, it appears that EFA deficiency induces a considerable reduction of the activation of FFA release, normally occurring during ischaemia. A decrease, in comparison to the release measured in controls, is observed for both total FFA and for arachidonic acid levels. The reduced formation of free arachidonic acid, a substrate for prostaglandin formation, and the presence of free eicosatrienoic (20 : 3 n-9) acid, a potent inhibitor of prostaglandin synthetase (ZIBOH *et al.*, 1974), in the brain FFA pool of EFA-deficient rats after stimulation, suggests reduced prostaglandin formation in the CNS during EFA deficiency.

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