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Changes in Free Fatty Acids in Rat Brain Subcellular Fractions after Cerebral Ischemia

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Changes in the cerebral free fatty acid pool size and fatty acyl chain composition in different subcellular fractions were examined in a rat model of cerebral ischemia. In control rats, $C_{16:0}$, $C_{18:0}$, $C_{18:2}$ and $C_{20:4}$ were the most prominent components in all brain subfractions. Cerebral ischemia led to progressive increase of FFAs with the largest increase in total FFAs in synaptosomes – 4.8 times the control values. In nuclei, homogenate and mitochondria the increases were 1.7-fold, 1.6-fold and 1.4-fold the normal values, respectively. Microsomes and myelin showed a decrease in FFAs content – 3.7- and 4-fold, respectively. The major components of increased FFAs were $C_{16:0}$, $C_{18:0}$ and $C_{20:4}$ acids. A notable observation was the accumulation of $C_{16:1}$, $C_{18:1}$, $C_{20:2}$ and $C_{22:6}$, which were absent in control rats. Our results showed that FFA accumulation is one of the first detectable changes in brain lipids after cerebral ischemia and it is considered to be a sign of evolution in ischemic brain damage.

Key words: brain, rat, cerebral ischemia, subcellular fractions, free fatty acids.

Introduction

A number of studies on animal brains with experimentally induced ischemia have been reported. Most of these studies have focused on the impaired cerebral metabolism and the reduced cerebral blood flow (CBF). Energy failure, free radical, cytokine, and excitatory aminoacid release, alterations in lipid metabolism are known to contribute to neuronal death [6].

Other investigations report on histologic changes in different ischemic brain areas and ultrastructural changes of neural mitochondria [10]. There is evidence that mitochondria play a key role in neuronal cell death after acute cerebral ischemia [2].

Ischemia causes an increase of the catabolism of membrane phospholipids in brain. Released free fatty acids (FFAs), especially arachidonic acid, impair mitochondrial function. Therefore, FFAs accumulation is considered to be a sign of evolution in ischemic brain damage [8].

Numerous studies have focused on FFAs accumulation in rat brains after various periods of ischemia and recirculation [3]. Levels of total FFAs rise rapidly in the early ischemic period. There are marked differences in the percentage increases of different

FFAs. It is reported that the major components of increased FFAs are palmitic ($C_{16:0}$), stearic ($C_{18:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), arachidonic ($C_{20:4}$) and docosahexaenic ($C_{22:6}$) acids [4].

In our study, we report changes in FFAs levels in different brain subcellular fractions (nuclei, microsomes, myelin, synaptosomes and mitochondria) and in brain homogenate, in a rat model of cerebral ischemia.

Materials and Methods

Ten 3-month-old male Wistar rats were used in the experiment. Five animals (the rest five were used as controls) were subjected to cerebral ischemia according to the model of Smith with minor modifications [9]. Ischemia was caused in the course of three contiguous days for 2 min a day. Anesthesia was induced with diethyl ether in a closed jar, following which the rats were quickly removed. The common carotid arteries were then clamped for 2 min by reversible clasps. A simplified EEG recording assessed ischemia.

Ischemic rats were killed by decapitation 24 hours after the last ischemic insult.

Isolation of Brain Subcellular Fractions

Brains (except cerebellum) were homogenized in ice-cold 0.32 M sucrose in a glass homogenizer with Teflon pestle and 10% homogenate was made. For lipid analysis, 1.2 ml of the homogenate was saved. The remaining homogenate was centrifuged at 3000 g (1500 rev/min) for 10 min. The nuclear fraction (the residue) was collected and the supernatant was centrifuged at 17 500 g (14 500 rev/min) for 1 hour in 8×50 AR. The resulting supernatant (i.e. the microsomal fraction) was collected. The residue was resuspended in 0.32 M sucrose to yield a final volume of 40 cm^3. Then it was layered on a sucrose density gradient, containing 20 ml of 1.2 M sucrose and 20 ml of 0.8 M sucrose, prepared in tubes for 3×65 SW rotor. The tubes were centrifuged at 55 000 g (21000 rev/min) for 2 hours. After centrifugation, top layer, middle layer (at the interface 0.8 M–1.2 M sucrose) and pellets were collected separately and they represented myelin, synaptosomes and mitochondria, respectively.

Lipid Extraction

Lipids were extracted according to the method described by K a t e s [14].
For lipid extraction, 3.75 ml of chloroform:methanol 1:2 (v/v) were added to 1 ml of each subcellular fraction. The mixture was left at room temperature for 1–2 hours, shaken vigorously from time to time. The tube was then centrifuged at 2500 rev/min for 5 min and the chloroform layer was transferred to a new tube. The residue was re-extracted with 4.75 ml of chloroform:methanol:water 1:2:0.8. The second chloroform layer was combined with the first and they were then supplemented with 2.5 ml of chloroform and 2.5 ml of water. The mixture was centrifuged again at 2500 rev/min for 5 min to obtain phase separation. The chloroform phase was dissolved in an equal volume of benzene and evaporated to dryness in a rotary vacuum evaporator. Lipids were weighed in each subcellular fraction.

Gas Chromatographic Determination of FFAs

The fatty acids were converted to fatty acyl methylesters (FAME) as follows: 1.8 ml of methanol and 900 μl of HCl were added to 1.8 ml of the lipid extract and the resulting mixture was left overnight at room temperature. Then the samples were supplemented with 900μl of water to obtain an opaline residue of FAME. The FAME were extracted by the addition of 900 μl of petroleum ether, then concentrated in a rotary vacuum evaporator and subjected to GC analysis. A gas chromatograph with flame ionization detector and connected with Trio Vector computing integrator was used. The analysis was performed by injecting 5 μl of the sample into SE-35 column. The temperature was programmed from 85°C to 205°C (2.5°C/min). Nitrogen was used as carrier gas at a flow-rate of 40 ml/min. We used mix computering program with inner and outer standard to determine the real quantity of FFAs.

Results

In control rats, in the homogenate, microsomes, myelin and mitochondria the percentage of saturated FFAs was higher in comparison with unsaturated FFAs (Table 1).

The same tendency was observed in the composition ratio between the short-chain FFAs and long-chain FFAs (Table 2).

In contrast to this tendency, in synaptosomes and nuclei unsaturated FFAs predominated over saturated FFAs and long-chain FFAs predominated over short-chain FFAs.

Nevertheless, $C_{16:0}$, $C_{18:0}$, $C_{18:2}$ and $C_{20:4}$ were the most prominent components in the control FFA pool in all brain subfractions (Table 3).

In our experimental model of cerebral ischemia we found an increase in total FFAs in four brain fractions.The changes in the amount of total FFAs after ischemia are shown in Fig. 1.

The largest increase was observed in synaptosomes – 4.8 times the control value. In nuclei, homogenate and mitochondria the increases were less pronounced – 1.7-fold, 1.6-fold and 1.4-fold the normal values, respectively.

To our surprise, in microsomes and myelin FFAs content was decreased by 3.7-fold and 4-fold, respectively.

Ischemia led to change in the ratio saturated/unsaturated FFAs and in the ratio short-chain/long-chain FFAs (Table1 and Table 2).

The changes in the amounts of individual FFAs after ischemia are given in Table 3.

Table 1. Saturated/unsaturated FFAs ratio in control and ischemic rats.
Hom = homogenate; Nuc = nuclei; Ret = microsomes; Myel = myelin; Syn = synaptosomes; Mit = mitochondria

	HOM	NUC	RET	MYEL	SYN	MIT
Control	2.03	0.18	1.69	14.83	0.22	2.03
Ischemia	0.03	0.28	–	0.16	0.52	1.22

Table 2. Short-chain/long-chain FFAs ratio in control and ischemic rats

	HOM	NUC	RET	MYEL	SYN	MIT
Control	2.01	0.21	1.92	9.03	0.21	2.07
Ischemia	0.17	0.72	0.2	0.21	0.56	1.24

Table 3. Free fatty acid concentrations in rat brain after cerebral ischemia. Values are expressed in mg/g dry lipid residue; n=5; p<0.05
A dash indicates trace amounts

	HOM		NUC		RET		MYEL		SYN		MIT	
	Control	Ischemia	Control	Ischemia	Control	Ischemia	Control	Ischemia	Control	Ischemia	Control	Ischemia
$C_{14:0}$	–	–	–	–	–	–	–	–	–	–	0.29 ±0.02	–
$C_{14:1}$	–	–	0.27 ±0.02	–	0.6 ±0.02	–	0.02 ±0.001	–	0.26 ±0.01	–	0.32 ±0.09	–
$C_{16:0}$	3.5 ±0.2	0.002 ±0.001	0.32 ±0.01	–	0.06 ±0.01	–	0.29 ±0.03	–	0.51 ±0.02	–	0.83 ±0.01	0.87 ±0.03
$C_{16:1}$	–	2 ±0.03	–	1.73 ±0.03	–	0.96 ±0.02	–	0.15 ±0.02	–	0.6 ±0.02	–	0.31 ±0.02
$C_{18:0}$	4.13 ±0.4	0.59 ±0.02	0.26 ±0.02	1.83 ±0.04	13.29 ±0.9	–	13.54 ±0.8	0.53 ±0.03	0.35 ±0.07	10.51 ±0.9	22.63 ±1.1	26.67 ±1.4
$C_{18:1}$	–	0.41 ±0.02	–	1.5 ±0.1	–	2.32 ±0.2	–	0.53 ±0.02	–	0.84 ±0.04	–	16.62 ±1.1
$C_{18:2}$	0.06 ±0.01	–	0.44 ±0.01	–	0.98 ±0.04	–	0.95 ±0.02	–	0.8 ±0.06	–	–	–
$C_{20:0}$	0.03 ±0.1	–	0.15 ±0.02	–	–	–	0.58 ±0.01	–	0.31 ±0.01	–	0.15 ±0.02	0.03 ±0.001
$C_{20:2}$	–	–	–	–	–	0.11 ±0.01	–	–	–	0.26 ±0.02	–	–
$C_{20:4}$	3.71 ±0.2	8.3 ±0.4	3.47 ±0.3	3.42 ±0.3	6.31 ±0.1	2.41 ±0.2	0.002 ±0.001	2.62 ±0.02	4.22 ±0.7	18.58 ±0.9	11.46 ±0.8	5.33 ±0.09
$C_{22:6}$	–	6.59 ±0.3	–	–	–	–	–	–	–	–	–	0.42 ±0.06
Total	11.43 ±0.9	17.9 ±1.1	4.92 ±0.2	8.5 ±0.6	21.22 ±1.2	5.81 ±0.7	15.38 ±0.8	3.82 ±0.1	6.45 ±0.2	30.78 ±1.5	35.67 ±1.7	50.25 ±1.9

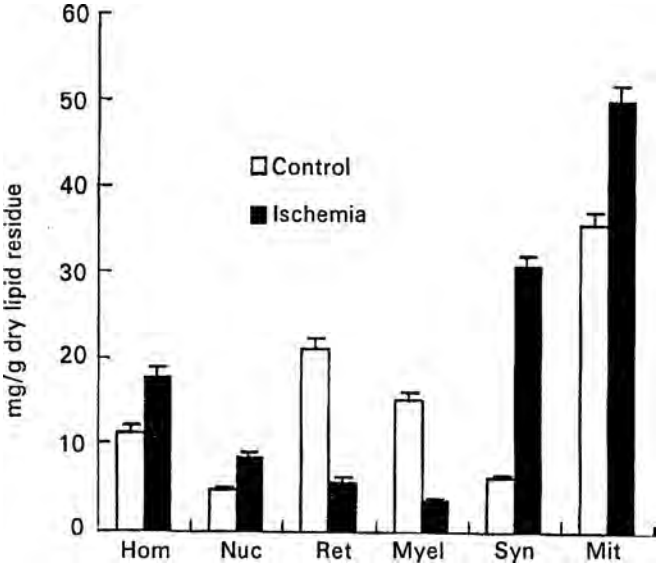

Fig. 1. Changes in total FFAs in rat brain after ischemia. n=5; p<0.05

In the homogenate the values of $C_{20:4}$ were more than 2 times the control values. Except the high content of free $C_{20:4}$ – 8.3 mg/g dry lipid residue (dry l. r.), the homogenate was relatively enriched in $C_{22:6}$, whose concentration was 6.59 mg/g dry l. r.

In the nuclear fraction, there was a significant increase in the amount of $C_{18:0}$ – 7.1 times the control values, from 0.26 mg/g dry l. r. to 1.83 mg/g dry l. r. After ischemia, some monounsaturated FFAs were liberated – palmitoleic ($C_{16:1}$) and $C_{18:1}$ acids. This subfraction contained also $C_{20:4}$ – 3.42 mg/g dry l. r.

In the microsomal FFAs pool the following mono- and polyunsaturated FFAs appeared after ischemia: $C_{16:1}$, $C_{18:1}$ and $C_{20:2}$, with concentrations 0.96 mg/g dry l. r., 2.32 mg/g dry l. r., and 0.11 mg/g dry l.r., respectively. Nevertheless, as a whole the total FFAs level decreased.

A large increase in the concentration of free $C_{20:4}$ was found in the myelin in comparison with control values, from 0.002 mg/g dry l. r. to 2.62 mg/g dry l. r. This fraction was enriched in $C_{20:4}$, but it contained also $C_{16:1}$, $C_{18:0}$ and $C_{18:1}$.

In the mitochondria, the largest increases in concentration were for $C_{16:0}$ and $C_{18:0}$ – 1.07 and 1.18 times the normal levels, respectively. Other mono- and polyunsaturated FFAs released – $C_{18:1}$ and $C_{22:6}$.

The synaptosomal fraction had the highest content of free $C_{20:4}$ in comparison with other subfractions – 18.58 mg/g dry l. r. Stearic acid comprised of 33.5% of the total free fatty acid pool. This subfraction contained also $C_{16:1}$, $C_{18:1}$ and $C_{20:2}$.

Discussion

In the present study we examined the changes in both total FFAs content and individual FFAs in rat brain subcellular fractions in an experimental model of cerebral ischemia.

In controls we found relatively low concentration of the total FFAs – from 4.92 mg/g dry lipid residue to 35.67 mg/g dry l. r. and this is in agreement with previously

published reports [4]. It is well known that FFAs in the brain are normally present in small amounts and the brain FFAs pool is maintained at a low level in a state of dynamic equilibrium with membrane phospholipids.

The lowest concentration of FFAs was found in nuclei and this is probably due to the fact that as a whole the quantity of lipids in the nuclei is very small.

In control rats the analysis showed FFAs with 14 to 20 C-atoms. We found both saturated and unsaturated FFAs. Among them, $C_{16:0}$, $C_{18:0}$, $C_{20:4}$ were the most prominent in all subcellular fractions. These observations are confirmed by other investigators [3].

The analysis of the mitochondrial fraction showed that mitochondria contain the largest quantity of arachidonic acid. This is due to the fact that brain mitochondria are rich in phospholipids containing unsaturated fatty acids. We measured relatively high concentration of free $C_{20:4}$ in the synaptosomes, too – 4.22 mg/g dry l. r. Most probably it is due to the biological role of $C_{20:4}$ as a main precursor of prostaglandins, considered as modulators in synaptic processes.

In myelin as a whole we measured low content of total FFAs –15.38 mg/dry l. r. These results are easy to explain taking into consideration that the main lipid groups in myelin in CNS are cholesterol, phospholipids and glycolipids. Our study indicated that myelin is enriched with saturated fatty acids and this fraction had the highest ratio saturated/unsaturated fatty acids – 14.8. In myelin, $C_{16:0}$, $C_{18:0}$, $C_{18:2}$, and $C_{20:0}$ were the most prominent FFAs and $C_{18:0}$ had the highest percentage – 86% of total FFAs. Arachidonic acid levels were low. The higher percentage of saturated FFAs is probably due to the fact that as a whole the lipids in myelin mostly consist of saturated FFAs, and this is relevant to the thicker packing of myelin membranes.

In our experimental model of cerebral ischemia we found significant changes in FFAs in all subcellular fractions. It is well known that FFAs are the first that undergo changes in different pathological states, including cerebral ischemia.

Brain is very sensitive to impaired blood flow and O_2 delivery. The interrupted cerebral blood flow quickly results in ATP depletion in brain, required in the biosynthesis of acyl-CoA and for phospholipase A_2 inhibition. Glutamate released during ischemia stimulates neuronal receptors, resulting in elevated intracellular Ca^{2+} and activation of phospholipases C and A_2. The activation of both two key enzymes in the catabolism of membrane phospholipids, and of lysophospholipases, diglycerid lipases and other lipases, lead to hydrolysis of the phospholipids and subsequent release of fatty acids. Therefore the main fatty acids, decreasing in the FA composition of the phospholipids, correspond to those, accumulating in the brain after ischemia.

It is known that the biochemical functions of mitochondria strongly depend on membrane lipids. Released $C_{20:4}$ may be metabolized by cyclooxygenases, lypoxygenases to form prostaglandins, leucotriens, and reactive oxygen species (ROS). Oxidative metabolism of $C_{20:4}$ is considered to be a major source of ROS in ischemia, which may generate lipid peroxides and cytotoxic products like 4-hydroxynonenal (4-HNE), acrolein and MDA [7].

Brain mitochondria show an exceptional sensitivity to the process of free radical oxidation. Total reduction of electron transport chain elements (such as pyridine and flavine nucleotides, coenzyme Q, cytochromes) results in the formation of free radicals leading to the initiation of a free radical-mediated peroxidation [11].

Arachidonic acid and all, which are liberated from membrane phospholipids, are known to inhibit the Na^+, K^+-ATPase activity and thus to alter the energy metabolism.

In most subcellular fractions we found the largest increases in the level of $C_{18:0}$ and $C_{20:4}$ – from 1.18 to 29.2 and from 2.23 to over 1000-fold, respectively.

It is considered that $C_{18:0}$ and $C_{20:4}$ are derived from inositol-containing phospholipids by the action of phospholipase C. Numerous studies show that inositol-containing

phospholipids are first degraded in ischemia followed by the release of diacylglycerides and their subsequent degradation to FFAs and glycerol, which is mediated by specific lipases [12].

Considering that synaptic membranes consist of phospholipids with high metabolic activity, including phosphatidylinositols, it is easy to explain the increased content of $C_{18:0}$ and $C_{20:4}$ in synaptosomes – 29.2 and 4.4 times, respectively. The total increase in the level of FFAs in synaptosomal fraction appears to be due to the elevated content of $C_{18:0}$ and $C_{20:4}$.

We found increased concentrations, though in small degree, of $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and $C_{22:6}$ in all subcellular fractions. Probably they are derived from phosphatidylethanolamine and phosphatidylcholine by the action of phospholipase A_2. Numerous studies show that the fatty acid composition of PC plus PE is more abundant in palmitic acid and docosahexaenoic acid than in stearic acid or arachidonic acid [1].

Our studies showed the highest percentage of unsaturated FFAs in microsomes and homogenate after cerebral ischemia. The percentage of $C_{22:6}$ was the highest in homogenate – 36%, but relatively large amount of this acid were measured in mitochondria – 0.42 mg/g dry l. r. Mitochondrial membrane is a basic site for synthesis of $C_{22:6}$, which subsequently is involved in the composition of the membrane phospholipids. The other long-chain FFAs, including $C_{20:4}$ are synthesized mainly in the endoplasmaticum reticulum.

The high concentration of unsaturated FFAs after cerebral ischemia is probably due to their neuroprotective effect. It is reported that polyunsaturated FFAs (especially $C_{20:4}$) block neuronal death by inhibiting glutamatergic transmission [5]. The specific liberation of unsaturated FFAs may be interpreted as a physiological adaptive response to ischemia.

Conclusion

Our results show that cerebral ischemia causes various changes in the FFAs in different subcellular fractions and changes in the ratios saturated/unsaturated FFAs and short-chain/long-chain FFAs. The ischemic process disrupts to a great extent the brain metabolism and the metabolism of its membrane structures. This leads to changes in the functional adaptive possibilities of the brain during crisis situations.

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