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## Accumulation Dynamics of the Brain Total Free Fatty Acids and Free Arachidonic Acid in the Models of Cerebral Hypoxia and Epilepsy in Rats

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The aim of this study was to determine the dynamics of the brain free fatty acid (FFA) pool in rats exposed to: (a) controlled hypoxia, and (b) experimental epilepsy. Animals from group (a) were subjected to hypoxia procedure. Immediately, 5, 15 or 60 minutes after losing the righting reflex, the animals were decapitated and brains were quickly removed. Animals from group (b) received penicillin (5000 U/5  $\mu$ l) into the left lateral ventricle and were decapitated immediately, 5 or 15 minutes after the appearance of epileptic seizures. The FFA were quantified by gas chromatography. An increase in the total brain FFA content with the maximum level at 60 minutes after losing the righting reflex was detected in hypoxia-exposed rats. The highest level of the brain free arachidonic acid (FAA) was detected 15 minutes after hypoxia. Seizures also produced an increase in the total brain FFA and brain FAA, with a statistically significant level 5 minutes after the appearance of epileptic seizures.

### INTRODUCTION

Preserved morphological and functional cell membrane integrity is fundamental for a normal functioning of brain cells. Numerous pathological conditions, including hypoxia and epilepsy, are accompanied by an increase in intracellular  $\text{Ca}^{2+}$  concentration,<sup>1</sup> which causes activation of the membrane phospholipases A<sub>2</sub> and C, degradation of membrane phospholipids and

liberation of cerebral free fatty acids (FFA).<sup>2,3,4</sup> Changes in the membrane phospholipids and FFA metabolism lead to irreversible cell damage. In addition, the permeability of the membrane for  $\text{Ca}^{2+}$  increases due to the disturbed balance between the membrane proteins and phospholipids.<sup>5,6</sup>

The FFA, particularly free arachidonic acid (FAA), are assumed to have detrimental effects on mitochondrial and plasma membrane functions.<sup>7,8</sup> Accumulation of their metabolites causes additional cell damage.<sup>9</sup>

The aim of our study was to determine the dynamics of the brain total FFA and FAA level in rats exposed to controlled hypoxia conditions or experimental epilepsy.

## MATERIALS AND METHODS

### *Animals*

The study was carried out on female Hannover-Wistar rats, weighing 200–250 g. The animals were maintained on a 12hr light-dark cycle at a room temperature of 22–24 °C and allowed free access to food and water. The animals were randomly divided into 8 groups. The control group animals were intact and had no past experimental history.

### *Hypoxia*

Four groups of animals were subjected to controlled hypoxia conditions. They were placed in a hypoxia cage consisting of an airtight plexiglas box into which a mixture of pure nitrogen and oxygen was delivered. The oxygen concentration was gradually reduced by the animal breathing until the level of 3.5V% of oxygen was reached. It was then maintained constant by an automatically regulated release of pure oxygen from separate chamber. The percentage of oxygen was continuously measured and controlled by an oxygen measuring device. The animals were maintained in such conditions until losing the righting reflex. One group of animals was decapitated immediately after losing the mentioned reflex. Other animals were transferred to the normal atmosphere and were decapitated 5 or 15 or 60 minutes after losing the righting reflex. The brains were quickly removed and frozen in liquid nitrogen.

### *Epilepsy*

Three groups of animals were anesthetized with chloralhydrate (400 mg/kg i.p.) and placed in a stereotaxic apparatus. They received a penicillin injection (5000U/5 µl) into the left lateral ventricle. The coordinates for microin-

jection placement were: 0.8 mm posterior to bregma, 1.7 mm lateral to the midline, 4.0 mm below the surface of the skull. The rats were decapitated immediately or 5 or 15 minutes after the appearance of epileptic seizures. The brains were removed and frozen in liquid nitrogen.

### *Biochemical analyses*

The frozen brains were weighed and homogenized in about 25 ml of a chloroform/methanol (2:1 *v/v*) mixture for lipid extraction. Nonadecanoic acid (19:0) was added as an internal standard. The extract was taken to dryness, lipids were dissolved in 1 ml of the chloroform/methanol (2:1 *v/v*) mixture and aliquots were used for analyses. The FFA were separated by preparative thin-layer chromatography (developing solvents: petroleum ether/ether/acetic acid (97:3:1, by vol.) and petroleum ether/ether/acetic acid (80/20/1, by vol.)). Streaks on thin-layer plates were visualized by water spray, scraped off and eluted with chloroform/methanol (2:1 *v/v*).

FFA methyl esters were prepared by methanolysis with methanol/hydrochloric acid (8.3:1.7 *v/v*) for 20 minutes at 120 °C, extracted with petroleum ether and quantified by gas chromatography using an internal standard.

### *Statistical analysis*

Levels of the brain total FFA and FAA are expressed in mg/g tissue. Statistical significance was calculated according to ANOVA, followed by Duncan's multiple range test. The criterion for significance was  $p < 0.05$ .

## RESULTS

Table I shows the brain total FFA and FAA levels in rats of the control group and in hypoxia-exposed rats decapitated at various time intervals after losing the righting reflex. An overall of ANOVA on the brain total FFA levels revealed the significant effect of the time [ $F(4,22) = 18.87$ ;  $p = 0.000001$ ]. Duncan's multiple range test indicated that the brain total FFA content in rats decapitated 60 minutes after losing the righting reflex was significantly higher in relation to the animals of the control group and to the hypoxic animals decapitated immediately, 5 or 15 minutes after cerebral hypoxia had been obtained (Table I).

As ANOVA, [ $F(4,22) = 4.55$ ;  $p = 0.007876$ ], followed by Duncan's multiple range test indicated, the brain FAA content in the hypoxic animals decapitated 15 minutes after losing the righting reflex, was significantly higher relative to the intact animals of the control group (Table I).



TABLE I

The brain total FFA and FAA levels in rats of the control group and hypoxia-exposed rats decapitated at various time intervals after losing the righting reflex

	CONTROL	POSTHYPOXIC TIME INTERVAL (min)			
		0	5	15	60
Total FFA (mg/g)	0.4044±0.127 [8]	0.2007±0.0681** [5]	0.8946±0.1643** [4]	0.5163±0.1061** [6]	1.9151±0.3200* [4]
FAA (mg/g)	0.0034±0.0040 [8]	0.0083±0.0048 [5]	0.0225±0.0080 [4]	0.4153±0.0091* [6]	0.0254±0.0281 [4]

The results are expressed as means ± S.E.M.; [n]: number of rats.

\* $p \leq 0.05$ : Significantly different from the control group.

\*\* $p \leq 0.05$ : Significantly different from the group decapitated 60 minutes after cerebral hypoxia was obtained.

An overall of ANOVA on the brain total FFA level in the control group rats and in rats decapitated at various time intervals after the appearance of penicillin-induced seizures revealed a significant effect of the time [ $F(3,23) = 19.94$ ;  $p = 0.000001$ ]. Duncan's multiple range test indicated that the brain total FFA content in rats decapitated 5 or 15 minutes after the appearance of epileptic seizures were significantly higher than in the intact animals of the control group and the animals with penicillin-induced epilepsy, decapitated immediately after the appearance of the epileptic seizures (Table II).

TABLE II

The brain total FFA and FAA levels in rats of the control group and in rats with penicillin – induced epilepsy decapitated at various time intervals after the appearance of epileptic seizures

	CONTROL	EPILEPSY TIME INTERVAL (min)		
		0	5	15
Total FFA (mg/g)	0.4044±0.1273 [8]	1.1557±0.3310 [6]	3.2703±0.4348** [7]	4.6813±0.1765** [6]
FAA (mg/g)	0.0034±0.0040 [8]	0.0146±0.0057 [6]	0.1530±0.0424** [7]	0.2113±0.0343** [6]

The results are expressed as means ± S.E.M.; [n]: number of rats.

\* $p \leq 0.05$ : Significantly different from the control group.

\*\* $p \leq 0.05$ : Significantly different from the group decapitated immediately after the appearance of the epileptic seizures.

As ANOVA, [ $F(3,23) = 9.83$ ;  $p = 0.00023$ ], followed by Duncan's multiple range test indicated, the brain FAA levels in rats decapitated 5 or 15 minutes after the appearance of the penicillin-induced epileptic seizures were significantly higher than in the animals of the control group. It is also evident that both groups had a significantly higher level of the brain FAA than the group of rats with penicillin-induced epilepsy, decapitated immediately after the appearance of epileptic seizures (Table II).

## DISCUSSION

The results of our experiments demonstrate that an accumulation of the brain FFA, particularly FAA, occurs during the period of posthypoxic normoxic reoxygenation in hypoxia-exposed rats. Concerning the accumulation dynamics of FFA and FAA, our results show that the total FFA level increased gradually, reaching a statistically significant value 60 minutes after hypoxia had been induced. An increase of the brain FAA content at various posthypoxic time intervals was also observed. Maximum level of FAA was detected 15 minutes after losing the righting reflex. After that time period, the level of the brain FAA started to decrease. These findings could be explained by different modes of FFA liberation caused by hypoxia and following normoxic reoxygenation. It seems that, in the early posthypoxic period, a selective degradation of specific membrane phospholipids by phospholipase A occurs with a concomitant significant increase of polyunsaturated FFA, particularly FAA. In the later period of posthypoxic reoxygenation, a degradation of membrane phospholipids by nonspecific phospholipases and slow liberation of various FFA occur. The decrease of the peak brain FAA content is probably due to the conversion of FAA into eicosanoides.<sup>10</sup> The same chemical pathway, which in early phase results in a significant increase of the FAA level and in the later period in an increase of various FFA, occurs during cerebral ischemia.<sup>11,12</sup>

Our results show that penicillin-induced epilepsy is also associated with an increase of the brain FFA and FAA levels. A statistically significant increase of fatty acids was detected 5 and 15 minutes after the appearance of epileptic seizures. Similar results were obtained in bicuculine-induced epilepsy.<sup>13,14</sup> Therefore, we suggest that the nonspecific degradation of membrane phospholipids and the liberation of various FFA, including FAA, occur very early during the penicillin-induced epileptic activity.

In conclusion, we suggest that an increase in cerebral FFA and FAA concentrations could be a common pathological event for both the period of cerebral posthypoxic reoxygenation and epilepsy.

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## SAŽETAK

**Dinamika nakupljanja ukupnih slobodnih masnih kiselina i slobodne arahidonske kiseline mozga u modelima cerebralne hipoksije i epilepsije u štakora**

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Cilj ovog istraživanja bio je utvrditi dinamiku ukupnih slobodnih masnih kiselina (SMK) mozga u: (a) štakora izloženih uvjetima kontrolirane hipoksije, te (b) štakora s penicilinom izazvanim epileptičkim konvulzijama. Životinje iz skupine (a) izlagane su hipoksiji do gubitka »righting» refleksa. Neposredno, 5, 15 ili 60 minuta po hipoksiji štakori su dekapitirani, a njihovi mozgovi izvađeni i zaleđeni. Životinjama iz skupine (b) u lijevu lateralnu komoru aplicirana je injekcija penicilina (5000 U/5  $\mu$ l). Štakori su dekapitirani neposredno, 5 ili 15 minuta po pojavi epileptičkih konvulzija. SMK su određivane metodom plinske kromatografije. U životinja izloženih hipoksiji pronađen je porast razine ukupnih SMK i slobodne arahidonske kiseline (SAK) mozga u različitim vremenskim intervalima po gubitku »righting» refleksa. Maksimalna koncentracija ukupnih SMK mozga zabilježena je 60 minuta, a SAK 15 minuta po hipoksiji. Konvulzije su također uzrokovale porast razine SMK i SAK mozga. Maksimalne vrijednosti koncentracije navedenih tvari zabilježene su 5 minuta po pojavi epileptičkih konvulzija.