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FREE FATTY ACIDS AND EXCITATORY NEUROTRANSMITTER AMINO ACIDS AS DETERMINANTS OF PATHOLOGICAL SWELLING OF ASTROCYTES IN PRIMARY CULTURE

Pak H. Chan, Albert C. H. Yu, and
Robert A. Fishman
Brain Edema Research Center, Department of Neurology, University of California, San Francisco, CA 94143

INTRODUCTION

Brain edema accompanies a wide variety of pathological processes. It contributes to the morbidity and mortality of many neurological diseases, including head injury, stroke, brain tumor, cerebral infections (e.g., brain abscess, encephalitis, and meningitis), lead encephalopathy, hypoxia, hypoosmolality, disequilibrium syndromes associated with dialysis and diabetic keto-acidosis, and some forms of obstructive hydrocephalus. Although many aspects of the pathophysiology of brain edema have been clarified, the molecular mechanisms and biochemical events that underlie the formation of edema are not well understood (Chan and Fishman, 1985). For example, the pathogenesis of early cellular (cytotoxic) edema (characterized by an increase in intracellular water and decrease in extracellular space), and the late development of vasogenic edema (characterized by an expanded extracellular space and increased permeability of brain capillary endothelial cells to plasma components) following ischemia is still poorly understood and believed to be multifactorial (Hossman, 1985). Although cellular edema per se is characterized by swelling of all the brain cells including neurons, glia and endothelial cells, there is ultrastructural evidence that astrocytic swelling is an early and primary event which occurs following ischemia (Kimelberg and Ransom, 1986).

Polyunsaturated Fatty Acids and Cellular Edema in Astrocytes

Numerous factors have been proposed to be involved in the pathogenesis of astrocytic swelling. For example, extracellular K^+ (Moller et al 1974) lactic acid and/or pH_i (Siesjo, 1985; Norenberg et al, 1987), ammonia (Norenberg, 1981) excitatory neurotransmitter amino acids (Chan et al, 1979, 1987), free fatty acids and oxygen radicals (Chan, et al 1986) and factors that affect the sodium pump activity [Na^++K^+-ATPase] and membrane integrity (Siesjo, 1985) have a role in the pathogenesis of swelling of astrocytes. Among these many factors, free fatty acids, especially polyunsaturated fatty acids (PUFAs), have drawn our attention. Our special interest in PUFAS is based on the following reasons: First, PUFAs are rapidly released from membrane phospholipids of brain cells during ischemia and under other pathological insults (Bazan, 1970). Second, PUFAs readily intercalate into fluid domains of membrane and produce significant changes in the packing of the lipid molecules (Klausner et al, 1980). Third, PUFAs are potent uncouplers of mitochondrial respiration (Hillered and Chan, 1987) and inhibitors of Na^++K^+-ATPase (Chan et al, 1983). Fourth, oxygen radicals and other lipid peroxides are formed from PUFAs. These oxygen radicals are known to have a detrimental effect on membrane integrity, protein cross-linking and DNA strand breakage. Fifth, neurotransmitter uptake in brain slices and synaptosomes and in primary cell cultures of cortical neurons and astrocytes are significantly affected by PUFAs (Chan et al, 1983; Yu et al, 1986).

Using rat brain slices as an in vitro system, we have studied the effects of free fatty acids on cellular edema in these slice preparations (Chan and Fishman, 1978) (Table 1).

Table 1

Effects of Fatty Acid on Cellular Edema in Cortical Slices

Fatty acid	Swelling (%)	Inulin Space (%)	Na^+ (mEq/kg dry wt.)	K^+ (mEq/kg dry wt.)
Control	11.3	46.11	509	393
Nonanoic acid	12.6	49.42	519	312
Lauric acid	11.6	49.22	651	315
Palmitic acid	9.4	46.12	838*	387
Oleic acid	11.0	41.07†	694	419
Linoleic acid	32.5*	33.47*	1340*	92*
Linolenic acid	26.0*	42.88*	949*	188*
Arachidonic acid	35.2*	32.6*	1239*	140*
Docosahexaenoic acid	33.5*	41.47*	1215*	77*

Rat brain slices were reconstituted in Krebs-Ringer buffer for 20 minutes followed by incubation with various fatty acids for 90 minutes at 37°. Concentration was 0.5 mM. *$p<0.01$, †$p<0.05$, compared to control group.

These studies have clearly demonstrated that PUFAs including linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4) and docosahexaenoic acid (22:6) cause a 2.5 to 3.5-fold increase in intracellular swelling concomitant with a decrease in extracellular inulin space. Saturated fatty acids: nonanoic acid (9:0), lauric acid (12:0), palmitic acid (16:0), and monounsaturated oleic acid (18:1) were ineffective in inducing edema in cortical slices. Further studies have demonstrated that PUFAs, not saturated fatty acids, cause formation of superoxide radicals and lipid peroxidation in brain slices and in primary cultures of astrocytes of newborn rats (Chan and Fishman, 1980; Chan *et al*, 1986). Table 2 summarizes the effects of PUFAs on superoxide radical ($O_2\cdot-$) formation (measured by the reduction of nitroblue tetrazolium) and lipid peroxidation (measured by the level of thiobarbituric acid reactive malondialdehyde (MDA) formation) in brain slices and astrocytes.

Table 2

Induction of $O_2 \cdot^-$ and MDA by PUFAs in Brain Slices and Astrocytes

Fatty Acid	$O_2 \cdot^-$ (% control)		MDA (%Control)	
	Brain Slices	Astrocytes	Brain Slices	Astrocytes
Control	100	100	100	100
Palmitic acid	104	105	105	110
Oleic acid	93	98	124	95
Linoleic acid	135	138*	211*	108
Linolenic acid	148*	175*	N.D.	170*
Arachidonic acid	134*	200*	256*	200*
Docosahexaenoic acid	N.D.	220*	242*	196*

Brain slice or primary cultures of astrocytes were incubated with fatty acids (0.1 mM) for 30 min (for $O_2 \cdot^-$ assays) and 120 min (for MDA assays) *$P<0.01$, compared to control. N.D. = not determined.

These studies have demonstrated that primary cultures of astrocytes are more sensitive to PUFAs for the induction of $O_2 \cdot^-$ than brain slices. The $O_2 \cdot^-$ formation appears to be intracellular and may be associated with membrane lipid domains since liposome-entrapped CuZn-Superoxide dismutase (SOD) reduced the $O_2 \cdot^-$ level whereas free SOD was ineffective (Chan et al, 1986).

The effects of arachidonic acid on the swelling of the astrocytes were studied by measuring the intracellular volume using 3-O-methyl-[^{14}C]-D-glucose (Kletzien et al, 1975) and by morphological observations. The intracellular volume of the control astrocytes in culture was 3.5 ul per mg protein. Astrocytes treated with 20:4 (0.2 mM) swelled significantly after a 30-minute exposure and continued to swell at 2 hours and thereafter. The degree of swelling corresponds well with the cellular swelling observed morphologically and with the level of intracellular lactate dehydrogenase (LDH) released in the culture medium. The release of LDH from astrocytes increased linearly from control (32 units/L) to a level of 250 units/L at 4 hours following the arachidonic acid

incubation. These data indicate that 20:4 plays a key role in the membrane injury and the subsequent release of LDH. The mode of membrane injury in astrocytes by 20:4 may also involve oxygen free radicals (noted at 30 minutes) followed by a significant increase in lipid peroxidative product malondialdehyde (noted at 2 hours and thereafter) in cultured astrocytes.

Excitotoxins and Cellular Edema in Astrocytes

Brain in situ contains high concentrations of glutamate (GLU). It is well known that excitatory neurotransmitter amino acids, particularly glutamate (GLU) is rapidly released and accumulated in extracellular space. GLU and its agonists N-methyl-D-aspartate (NMDA) have been implicated in selective vulnerability and neuronal death following ischemia, hypoglycemia and epileptic seizures (Meldrum, 1985). NMDA receptor antagonists have been shown to reduce the ischemia or hypoglycemia-induced neuronal death of hippocampus in vivo (Simon et al, 1984; Weiloch, 1985). Furthermore, these NMDA receptor antagonists also reduce the GLU-induced swelling and death in neuronal cell culture in vitro (Choi et al, 1987). Kimelberg et al (1985) have demonstrated recently that, in the presence of ouabain, GLU at 0.1 mM concentration could induce astrocytic swelling in culture. We have reported previously that GLU (1 mM) and its agonists including homocysteic acid, kainic acid, aspartic acid and NMDA caused significant cellular swelling and cation changes in brain slices (Chan et al, 1979). However, the role of GLU and the NMDA antagonists on glial cell swelling and injury was not delineated in those studies. We have studied the excitotoxic mechanisms of glial swelling using intact cerebral cortical astrocytes of newborn rats. The intracellular water space (IWS) (measured by 3-O-methyl [^{14}C]-D-glucose) was increased (control = 3.4 $\pm$ 0.2 ul/mg protein) by GLU (1 mM) by 175%, 213% at 1 hour and 4 hours respectively and was returned to baseline at 24 hours. The GLU-induced IWS changes were dose-dependent. Among the GLU agonists, homocysteic acid at equal molar concentration exhibited similar potency in inducing astrocytic swelling (214%), followed by L-aspartate (ASP) (160%) and quisqualate (152%), whereas NMDA, kainate and quinolinate were not effective. Unlike GLU and ASP, both homocysteic acid and

quisqualate caused a persistent increase in IWS of astrocytes with prolonged incubation time (e.g., 24 hours). DL-2-amino-5-phosphonovaleric acid (APV) and 2-amino-7-phosphonoheptanoic acid (APH), antagonists of NMDA-preferred receptors, and kynurenic acid, a non-specific GLU receptor antagonist at a concentration of 1 mM were not effective in inducing cellular swelling of astrocytes. Furthermore, pretreatment with APV, APH, or kynurenic acid failed to reduce the GLU-induced astrocytic swelling. These data indicate that GLU may exert its (excitotoxic) effects on astrocytic swelling through mechanisms other than those mediated by NMDA receptors.

Does Inhibition of Glutamate Reuptake in Astrocytes and Neurons by PUFAs Contribute to Astrocytic Swelling?

The above section has clearly demonstrated that extracellular accumulation of GLU and its agonists caused swelling of cultured astrocytes. We have demonstrated earlier that PUFAs are potent inhibitors of Na^++K^+-ATPase (Chan et al, 1983). The inhibition of Na^++K^+-ATPase will diminish the ability of astrocytes to maintain the Na+ gradient. Since the uptake of GLU by neurons and astrocytes are dependent on the Na+ gradient, we speculate that the GLU uptake by astrocytes and neurons will be severely affected by the presence of extracellular PUFAs. We now study the effects of arachidonic acid on GLU and gamma-aminobutyric acid (GABA) uptake in primary cultures of astrocytes and neurons prepared from rat cerebral cortex (Yu et al, 1986). The uptake rates of GLU and GABA in astrocytic cultures were 10.4 nmol/mg protein/min and 0.125 nmol/mg protein/min, respectively. The uptake rates of GLU and GABA in neuronal cultures were 3.37 nmol/mg/min and 1.53 nmol/mg protein/min. Arachidonic acid inhibited GLU uptake in both astrocytes and neurons. The inhibitory effect was both dose and time dependent. The effects of arachidonic acid were not as deleterious on GABA uptake as on GLU uptake in both astrocytes and neurons. In astrocytes, GABA uptake was not affected by any of the doses of arachidonic acid studied (0.015-0.6 umol/mg protein). In neuronal cultures, GABA uptake was inhibited, but not to the same degree observed with GLU uptake. Lower doses of arachidonic acid (0.03 and 0.015 umol/mg protein) did not affect neuronal GABA uptake. Other polyunsaturated fatty acids, such as docosahexaenoic

acid, affected amino acid uptake in a manner similar to arachidonic acid in both astrocytes and neurons. However, saturated fatty acids, such as palmitic acid, exerted no such effect. Recently, we further studied the effects of PUFAs on the uptake of GLU in primary cultures of cerebellar granule cells (a glutamatergic neuronal preparation) (Yu et al, 1987). The uptake of GLU was equally sensitive to arachidonic acid in primary cell cultures of cortical neurons (Table 3). On the other hand, the uptake of glutamine was also slightly inhibited by arachidonic acid in both cerebellar granule cells and

Table 3

Changes of Amino Acid Uptake by 0.1 mM 20:4 After 90 Minutes of Exposure in Primary Cultures of Cerebellar Granule Cells, Cerebral Cortical Neurons and Astrocytes

	% of Control Uptake		
	Glutamate	Glutamine	GABA
Cerebellar Granule Cells	13 ± 2.7*	61 ± 7.8*	105 ± 10.6
Cerebral Neurons	23 ± 2.1*	96 ± 6.7	50 ± 3.5*
Cerebral Astrocytes	24 ± 4.6*	65 ± 3.2*	95 ± 7.7

The time for the amino acid uptake was 5 minutes. The control was 100%. * $p<0.01$, compared to control using two-tailed t test.

astrocytes. Our data suggest that 20:4 and its radical metabolites, (which accumulated during ischemia due to the activation of phospholipases, e.g., phosphatidylinositol-dependent phospholipase C, phospholipase A_2) are the key determinants in causing membrane injury in astrocytes. Such injury involves the inhibition of the reuptake of excitatory neurotransmitter glutamate into astrocytes as well as neurons and the inhibition of plasma membrane Na^++K^+-ATPase activity. These alterations in membrane function may contribute to the pathogenesis of astrocytic swelling observed in cerebral ischemia.

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