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EFFECTS OF CDP-CHOLINE ON PHOSPHOLIPASE A_2 AND CHOLINEPHOSPHOTRANSFERASE ACTIVITIES FOLLOWING A CRYOGENIC BRAIN INJURY IN THE RABBIT

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Abstract—Within the tissue surrounding the necrotic lesion, following a cryogenic injury of the brain, there is a definite activation of phospholipase A_2 (at 2 and 4 hr post lesion) that accounts, at least in part, for the phospholipid breakdown. There is also an activation of cholinephosphotransferase (at 2 hr post lesion) that may correspond to an early process of phospholipid resynthesis.

Oral CDP-choline in this model is able to completely inhibit the activation of phospholipase A_2, but has no detectable effect on cholinephosphotransferase activity. The beneficial effect of CDP-choline might be explained by a prevention of destruction rather than by an enhancement of reconstruction of phospholipids.

A severe breakdown of membrane phospholipids is a common finding in a number of brain insults observed in animal models. In hypoxic and ischemic conditions of various types and degrees, a rapid degradation of phosphatidylcholine and phosphatidylethanolamine with release of free fatty acids and diacylglycerols has been repeatedly observed [1–8]. Basically the same alterations are also found following cryogenic lesions [9–12]. In this last model we were able to demonstrate a major impairment of Na^+–K^+-ATPase activity, likely due to the disorganization of membrane structures [13, 14]. These membrane alterations directly entail major disorders in brain function and result in both cytotoxic and vasogenic brain edema which may in turn cause further tissular damage by its mechanical interference with blood supply.

CDP-choline has been used in an attempt to prevent or reduce these disorders and the resultant brain edema. In hypoxic and ischemic conditions, treatment with this drug was able to strongly inhibit the release of free fatty acids. It was postulated that CDP-choline activates the cholinephosphotransferase reaction towards phosphatidylcholine synthesis, thus acting as a reconstructive agent [3, 5, 6, 8, 15]. A beneficial effect of intravenous CDP-choline was also shown in cryogenic lesions with a recovery of ATPase activities and a reduction of edema obtained after 24 hr post injury [13, 16].

In an effort to further understand the mechanisms of CDP-choline effects in brain trauma, this paper will consider a possible interference of the drug with phospholipase A_2 and cholinephosphotransferase activities. Basically our results lead to the conclusion that CDP-choline could inhibit phospholipid degradation rather than enhance phospholipid reconstruction.

EXPERIMENTAL PROCEDURES

Induction of lesion. "Fauve de Bourgogne" female rabbits (weighing 2.0–2.5 kg), selected according to randomized experimental procedures, were divided into two groups. The first group (N = 26) was taken as a control; the second group (N = 36) was submitted to a cryogenic lesion as previously described [17]. The animals were killed by decapitation at 2, 4, and 8 hr after the cold lesion.

The tissue water content was measured by drying to a constant weight at 120° [13].

The water/dry weight ratio is given by:

$$W/Dw = \frac{\text{wet tissue weight} - \text{dry tissue weight}}{\text{dry tissue weight}}$$

Pharmacological treatment. Eighteen injured animals were treated *in vivo* by per os administration of cytidine diphosphate choline (CDP-choline, 200 mg/kg body weight)‡ 5 hr before the cold injury, except for the 8 hr-injured animals which were treated twice, 5 hr before (100 mg/kg) and 1 hr after (100 mg/kg) the cold injury. Eighteen control animals were treated following the same procedure.

Preparation of subcellular particles. Immediately after the sacrifice the injured hemisphere was rapidly removed, the superficial necrosis eliminated and the perifocal area (0.5 cm dia., 800–900 mg) chopped finely and homogenized in ice-cold isolation buffer containing 0.32 M sucrose, 1 mM EDTA and 10 mM

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Tris–HCl, pH 7.4, with 6 up-and-down strokes in a Teflon–glass homogenizer driven at 750 rpm.

The homogenate was centrifuged at 4° for 3 min at 1300 *g* and the supernatant was then centrifuged at 4° for 10 min at 17,000 *g* to sediment the crude mitochondrial fraction.

The supernatant remaining after the sedimentation of the crude mitochondrial fraction was used to obtain the microsomal membranes, according to the method of Woelk and Porcellati [18].

Both particulate fractions were either used at once or frozen at −20° for a few weeks before utilization without loss of activity.

Protein concentration was determined by the method of Lowry *et al.* [19].

Phospholipase A_2 (phosphatide 2-acylhydrolase EC 3.1.1.4). Phospholipase A_2 activity was assayed according to the method of Alberghina *et al.* [20] with slight modifications. The labelled substrate, 1-palmitoyl-2-[1-^{14}C]linoleoyl-3-phosphatidylethanolamine (0.1 μCi, 57 mCi/mmol), diluted with carrier phosphatidylethanolamine (approximately 50 nmol), was incubated in 900 μl of 0.1 M Tris–HCl, pH 8.4 [18], containing 2 mM $CaCl_2$ and 50 μg of sodium deoxycholate to emulsify the substrate. Incubation was started by adding 150 μg of mitochondrial protein and performed at 37° for 30 min in a thermoregulated water-bath, shaking at about 140 strokes/min. The reaction was stopped with 3 ml of chloroform:methanol (2:1, v/v). Control tubes without enzyme were incubated in each experiment. The lipids were extracted from the incubation mixture by the technique of Bligh and Dyer [21] and dried under nitrogen. Fatty acids were separated from phospholipids, together with standard, by thin-layer chromatography in the solvent system hexane/diethyl ether/acetic acid (45:30:0.5, by vol.). The fatty acid-containing regions were revealed by I_2 vapour and scraped into scintillation vials to which were added 0.5 ml of water and 4.5 ml of Instagel as scintillator. The specific activity was expressed as nmoles of fatty acid released per minute per milligram of protein.

Cholinephosphotransferase (1,2-diacylglycerol: CDP-choline cholinephosphotransferase, EC 2.7.8.2.). Cholinephosphotransferase activity was assayed according to the method of Van Golde *et al.* [22]. The standard incubation medium contained: 20 μl of glutathione (0.1 M), 40 μl of CDP-choline (2 mM), 40 μl of CDP-[Me-^{14}C]choline (2.5 μCi/ml), 50 μl of $MgCl_2$ (0.1 M), 170 μl of Tris–HCl (0.1 M), pH 8.4 [23], 0.03% Tween 20, 300 μl of 1,2-diacylglycerol (1,2-dioleoyl- and 1,2-dipalmitoyl-glycerol) sonicated emulsion in Tris–HCl buffer (5 mg/ml). Diacylglycerol suspension was prepared immediately before the experiments. Incubation was started by adding 250 μg of microsomal protein and performed at 37° for 30 min in a thermoregulated water-bath, shaking at about 140 strokes/min. The reaction was stopped with 3 ml of chloroform:methanol (1:2, v/v). Cholinephosphotransferase activity was also assayed without exogenous diacylglycerols as the acceptor substrate. Control tubes without enzyme were included in each experiment.

Labelled phosphatidylcholine was then extracted from the incubation mixture by the method of Bligh and Dyer [21]. The washed chloroform layer was dried under nitrogen and radioactivity measured in a Packard Tricarb automated scintillation spectrometer using 5 ml of Instagel solution as scintillator. An external standard was used for quenching corrections.

The specific activity was expressed as nmoles of substrate incorporated per minute per milligram of protein.

RESULTS

Phospholipase A_2

The time-course of the specific activity of mitochondrial phospholipase A_2 after cryogenic lesion and/or CDP-choline treatment *in vivo* is shown in Table 1. A significant increase of the enzyme activity is observed at an early stage (2 and 4 hr post injury). The activity returns to normal values 8 hr after the lesion. In injured animals, the CDP-choline administration statistically significantly prevents the phospholipase A_2 stimulation, as compared to the untreated group. The drug does not affect phospholipase A_2 activity in control treated animals.

Cholinephosphotransferase

Table 2 shows the specific activity of microsomal cholinephosphotransferase—assayed with or without addition of exogenous 1,2-diacylglycerols—as a func-

Table 1. Effect of CDP-choline on phospholipase A_2 in control and cold-injured rabbit brain

Control	0.164 ± 0.029 N = 8		
	Time of sacrifice (hr post-cryogenic lesion)		
	2	4	8
Treated control	0.153 ± 0.031 N = 6	0.166 ± 0.037 N = 6	0.157 ± 0.017 N = 6
Injured	0.235 ± 0.049 (b) N = 5	0.216 ± 0.034 (b) N = 5	0.174 ± 0.014 N = 6
Treated injured	0.166 ± 0.016 (b') N = 6	0.167 ± 0.028 (a') N = 6	0.190 ± 0.016 N = 6

Specific activity is expressed as nmoles·min^{-1}·mg^{-1} protein (mean ±SD). Significant differences (Student's *t*-test: injured versus control, (a) $P < 0.05$; (b) $P < 0.02$; treated injured versus injured, (a') $P < 0.05$; (b') $P < 0.02$. N = number of animals.

Table 2. Effect of CDP-choline on cholinephosphotransferase activity in control and cold-injured rabbit brain

Control N = 8	—	0.105 ± 0.021	
	1,2-diacylglycerols	3.20 ± 0.65	
		Time of sacrifice (hr post-cryogenic lesion) 2	8
Treated control	—	0.102 ± 0.013	0.091 ± 0.006
	1,2-diacylglycerols	3.62 ± 0.40	3.51 ± 0.16
		N = 6	N = 6
Injured	—	0.128 ± 0.016	0.131 ± 0.025
	1,2-diacylglycerols	3.98 ± 0.34 (a)	3.88 ± 0.57
		N = 6	N = 6
Treated injured	—	0.129 ± 0.018	0.126 ± 0.016
	1,2-diacylglycerols	3.78 ± 0.46	3.62 ± 0.32
		N = 6	N = 6

Specific activity is assayed with or without addition of exogenous 1,2-diacylglycerols and is expressed as nmoles · min^{-1} · mg^{-1} protein (mean ± SD).

Significant differences (Student's *t*-test): as in Table 1.

N = number of animals.

The data at 4 hr post-injury are lost for technical reasons.

tion of time after cryogenic lesion and/or CDP-choline treatment *in vivo*. When 1,2-diacylglycerols are added to the incubation medium a significant increase of the enzyme activity is observed in the injured animals at 2 hr post lesion. CDP-choline treatment does not affect cholinephosphotransferase activity in any experimental conditions.

DISCUSSION

Following a cryogenic injury we find a definite activation of phospholipase A_2 and, to a lesser degree, of cholinephosphotransferase. These results are in keeping with a number of previous data in the literature.

Direct assessment of phospholipase A_2 activity in this model has not been published but indirect evidence is given by the considerable rise of free arachidonic acid repeatedly found following the same lesion [9–12], as well as in ischemic and hypoxic models [1, 2, 7]. The mechanism of this activation may be different in ischemic and in cryogenic lesions. During ischemia the sudden break of energy production allows intracellular Ca^{2+} accumulation which directly triggers phospholipase A_2 [24–27]. We have no evidence of a shortage of energy in the perilesional area surrounding cold lesion: energy charge measured 24 hr post lesion has always been found normal [28–30]. However, comparable accumulation of intracellular Ca^{2+} could be produced by other mechanisms: glutamate is liberated in large amount around cryolesions [31] and may be responsible for an intracellular Ca^{2+} increase [32–34]. Additionally, phospholipase A_2 could also be activated by free radical peroxidations [35–37] which are considered a major cause of tissular damage in this and other types of brain insult (review in ref. 38).

At 2 hr post lesion a significant activation of cholinephosphotransferase was observed. Cholinephosphotransferase catalyses the reaction:

Diacylglycerols + CDP-choline → phosphatidylcholine + CMP

Diacylglycerols are liberated in large amounts following cold lesion [10–12], presumably through an activation of phospholipase C again mediated by free radical reactions [39]. This increase in diacylglycerol concentration yields a straightforward explanation of the cholinephosphotransferase activation. This would mean that from the onset of the lesion, the degradation products of phospholipid breakdown are reused for phospholipid synthesis.

At variance with the assumption of various authors [3, 5, 6, 8, 15] our study is unable to demonstrate an effect of CDP-choline on cholinephosphotransferase activity. Conversely oral pretreatment with CDP-choline completely prevents phospholipase A_2 activation. This important finding is consistent with *in vitro* observations of Kitazaki *et al.* [40] who found a competitive inhibition of phospholipase A_2 activity by CDP-choline. In the literature, CDP-choline as a drug is supposed to help phospholipid synthesis since CDP-choline, as a natural substrate in classical views of phospholipid metabolism [41], does indeed activate cholinephosphotransferase. In fact, experimental evidence is not so clear: we know only that the drug, in a number of models of brain insult in animals, reduces the total amount of free fatty acids and diacylglycerols released. Such findings could be explained by an inhibition of destructive processes, as evidenced in our experiments, rather than by an enhancement of reconstructive processes.

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