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AN *IN VITRO* STUDY OF HEMICHOLINIUM-3 ON PHOSPHOLIPID METABOLISM OF KREBS II ASCITES CELLS

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Abstract—With [Me-^{14}C]choline as marker and after separation of choline and phosphocholine by ion-exchange column chromatography or thin layer chromatography on alumina, it is shown that 40 μM hemicholinium-3 (HC-3) inhibits the cytosolic choline-kinase of rat liver and Krebs cells. This inhibition is competitive (*Km* different, *Vm* similar) in the first case and mixed in the second (*Km* and *Vm* different). Despite this general inhibition of the phosphocholine formation, the synthesis of phosphatidylcholine (PC) by post-nuclear supernatants of rat liver and Krebs cells is different when tested with HC-3. It is unaffected in rat liver; however, HC-3 induces a PC deficiency in Krebs cells which is time-course dependent between 15 and 120 min and proportional to the drug concentrations in the interval 5–40 μM. Incorporation of AT-[γ^{32}P] or [2-^{14}C]ethanolamine into phospholipids shows that the sequential methylation pathway is not detectable in Krebs cells. These results are discussed in relation to those established concerning HC-3 action on phospholipid metabolism in other tissues.

Drugs affecting lipid metabolism have been reviewed whether in subcellular components, cells and tissue slices [1] or *in vivo* [2, 3]. Among the depressants tested, HC-3§ ([2,2'-(4,4'-biphenylene)-bis-(2-hydroxy-4,4-dimethylmorpholinium)]bromide), with two quaternary ammoniums, is a potent inhibitor of the choline metabolism. Several results have been reported concerning the inhibition by HC-3 of choline acetylation and its effects on the neuronal transmission in the central nervous system and neuromuscular endings [4, 5]. In the same way, studies on the action of HC-3 on another choline pathway have established that it inhibits its phosphorylation by choline-kinase (EC 2.7.1.32) [6]. We have previously reported [7] that chlorpromazine produces an accumulation of PA and PG by a phosphatidate-phosphohydrolase inhibition. But PC remained unchanged, although it represents about 57% of the mammalian cell total phospholipids [8]. For this reason we have investigated the action of HC-3 on PC synthesis in the Krebs II ascites cells treated in previous papers [9, 10]. The present study deals only with subcellular components.

MATERIALS AND METHODS

Chemical products and analysis. Chemical products were obtained as follows: HC-3, aprotinin, ATP and CTP from Sigma (St Louis, MO), silica gel analytical plates from Woelm (Eschwege, F.R.G.), alumina analytical plates from Merck (Darmstadt, F.R.G.), Dowex 1-X2 (500–100 mesh) resin from Biorad Lab. (Richmond, CA). Other products were purchased from Merck or Prolabo (Paris, France). Protein was measured by the method of Lowry [11]; and lipid phosphorus according to Chen [12].

Labelled compounds. [Me-^{14}C]Choline (40–60 mCi/mmole), [Me-$^{3}H_2$]choline (25–50 Ci/mmole), [2-^{14}C]ethanolamine (40–60 mCi/mmole), [9–10, $^{3}H_2$]palmitic acid (250–500 mCi/mmole) were supplied by the Radiochemical Centre (Amersham, U.K.). Phospho-[Me-^{14}C]choline (40–60 mCi/mmole) and AT-[γ^{32}P] were obtained from New-England Nuclear (Dreieich, F.R.G.). Radioactivity determinations were performed using a Kontron-Intertechnique SL 4000 liquid scintillation counter.

Cell suspensions and subcellular fractionation. The Krebs II ascitic tumours were maintained in female Swiss mice, 9–12 weeks old, by i.p. transfer of the ascitic fluid between the eighth and tenth day after their inoculation [7]. Cells harvested were added with 2000 units penicillin and 2000 μg streptomycin per ml. They were separated from the exudate by centrifugation at 700 *g* (10 min) and were washed twice in 3 volumes of PBS. Homogenates (10% w/v in PBS plus antibiotics) were prepared in a steel Dounce homogenizer (10 strokes per 20 ml fraction) under phase-microscopy control. The cytosolic enzyme choline-kinase was measured on a particle-free supernatant obtained at 220,000 *g* (90 min); the biosynthesis of PC was studied on the homogenate previously separated of nuclei and cellular debris at 1000 *g* (15 min). Rat livers were homogenized (10% w/v in PBS plus antibiotics) with a Teflon pestle apparatus. The post-nuclear supernatant and the

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§ Abbreviations used: HC-3, hemicholinium-3; PA, phosphatiolic acid; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PBS, Dulbecco's phosphate buffered saline; PMT I, phospholipid–methyltransferase I; PMT II, phospholipid–methyltransferase II.

cytosol were prepared by the same way as the Krebs cells.

Enzyme assays and separation of products. Incubations were performed at 37° with shaking. Details of the experiments are reported in the figure legends. For measuring the phospho-[Me-^{14}C]choline synthesized, the reaction was stopped by adding trichloroacetic acid at a final concentration of 5% (w/v). The protein precipitate was removed by centrifugation, the supernatant washed 3 times with 2 volumes of diethyl ether and pH adjusted to 9.0 with ammonia. The [Me-^{14}C]choline substrate and the phospho-[Me-^{14}C]choline formed were separated on a Dowex 1-X2 (H^+) column previously standardized by [Me-$^3H_2$]choline and phospho-[Me-^{14}C]-choline eluted respectively by H_2O and 0.1 N HCOOH. The experiments for HC-3 inhibition were performed with [Me-$^3H_2$]choline as internal standard. Assays (A) with HC-3 and controls (C) without HC-3 are expressed as the percentages of total radioactivity loaded on the column eluted as phospho-[Me-^{14}C]choline. The A/C ratio measures the inhibition degree of phosphorylation. For kinetic studies, the reaction products were chromatographed on alumina plates in benzene–methanol–acetone–acetic acid (14:4:1:1, v/v/v/v) after verification of the separation efficiency by autoradiography. Labelled choline and phosphocholine were revealed at 254 nm before measuring their radioactivities. PC synthesized was labelled from [Me-^{14}C]choline, [2-^{14}C]ethanolamine or AT-[γ^{32}P]. Incubations were blocked by 2 volumes of chloroform–methanol (1:1, v/v) and a PC randomly labelled by [9-10,$^3H_2$]palmitic acid ([^{3}H]PC = 4072 dpm/nmole) biosynthesized in our laboratory [13] was added as internal standard in order to rectify the recoveries of the formed ^{14}C-labelled compounds. The organic phase separated by centrifugation was dried under vacuum and the lipid extract washed twice with 18 ml of chloroform–methanol–water (2:1:0.6, v/v/v) according to the method of Folch *et al.* [14], slightly modified. The extracted and washed lipids were separated on silica plates in chloroform–methanol–water–acetic acid (60:30:6:1, v/v/v/v), The biosynthesis of PC or PE is expressed in the assays and controls by ^{14}C/^{3}H or ^{32}P/^{3}H ratios, and the inhibitions by A/C ratios. Statistical significances are computed according to the Student's independent *t*-test.

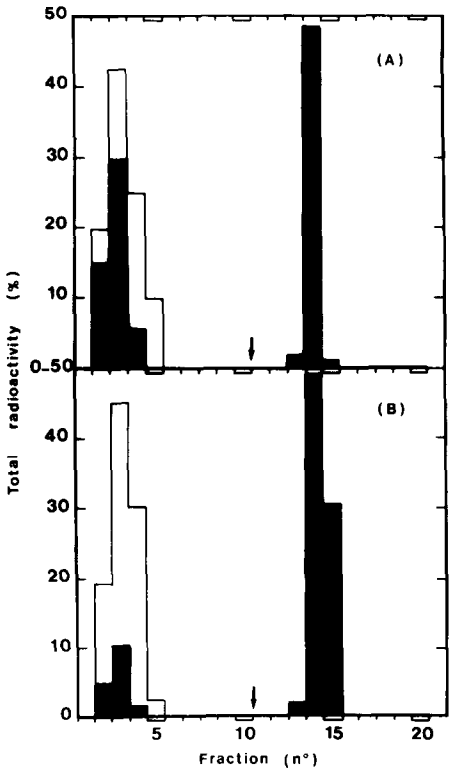

Fig. 1. Choline-kinase activity of Krebs cells cytosol with (A) and without (B) 40 μM HC-3. Medium: protein 10–15 mg, ATP 10 mM, $MgCl_2$ 10 mM, [Me-^{14}C]choline 2 μCi, sodium phosphate –Na buffer 28 mM, pH 7.35, volume 2.5 ml. Incubation 30 min at 37°. Chromatography on Dowex 1-X2 (H^+) of the TCA-soluble products. Column 1 cm × 20, 50 ml/hr, 10 ml each fraction. Elution by H_2O and (↓) 0.1 N HCOOH. □ [Me-$^3H_2$]Choline as internal standard (2 μCi); ■ F_2 to F_4: [Me-^{14}C]choline; ■ F_{13} to F_{15}: phospho-[Me-^{14}C]choline.

Fig. 2. Autoradiography after TLC on alumina plate (see Materials and Methods) of the TCA-soluble products without (1) and with (2) 40 μM HC-3. O = origin; migration: 17 cm; $R_f = 0$: phospho-[Me-^{14}C]choline; $R_f \sim 0.8$: [Me-^{14}C]choline. Incubation conditions as in the legend of Fig. 1.

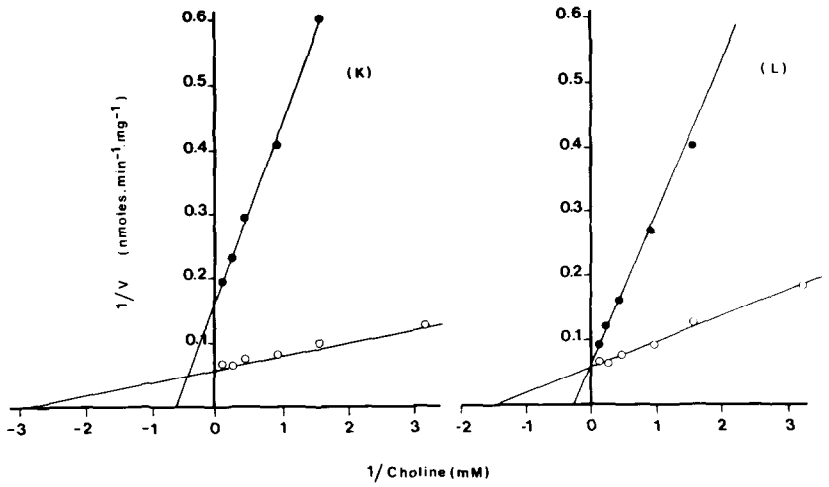

Fig. 3. Cytosolic choline-kinase with (●) and without (○) 40 μM HC-3 of Krebs cells (K) and rat liver (L). Medium: protein 1 mg, ATP 10 mM, $MgCl_2$ 10 mM, sodium phosphate –Na buffer 28 mM, pH 7.35, volume 2.5 ml. Incubation 30 min at 37° ($n = 2$, average values).

RESULTS

Effect of HC-3 on the choline phosphorylation

The action of HC-3 on choline-kinase in the cytosol of Krebs cells is reported in Fig. 1. On Dowex 1-X2 columns the amount of free choline was more important with 40 μM HC-3 than in the controls ($A/C = 0.53 \pm 0.17$, $n = 6$, $P < 0.02$).

After chromatography and autoradiography on alumina plates (Fig. 2) non-phosphorylated choline remained more abundant when HC-3 was added, in agreement with results obtained on the anion exchanger. Determinations of choline-kinase *Km* and *Vm* by the Lineweaver–Burk construction in the cytosols of Krebs cells and rat liver are reported in Fig. 3. In tumourous cells (Fig. 3K), HC-3 produced a mixed inhibition ($Km = 3.5 \times 10^{-4}$ M, $Vm = 18.8$ nmoles/min per mg; $Km_i = 14.3 \times 10^{-4}$ M, $Vm_i = 6.4$ nmoles/min per mg). In rat liver (Fig. 3L), choline-kinase was also inhibited by HC-3 but, whereas the *Km* values were different ($Km = 6.9 \times 10^{-4}$ M, $Km_i = 33.3 \times 10^{-4}$ M), the *Vm* values were similar ($Vm = 16.6$ nmoles/min per mg, $Vm_i = 14.3$ nmoles/min per mg), showing a competitive inhibition. It is noteworthy that the cytosols of Krebs cells and rat liver have nearly identical *Vm* values when tested without HC-3 by the same experimental method.

Effect of HC-3 on biosynthesis

The action of HC-3 was studied simultaneously on Krebs cells and rat liver post-nuclear supernatants in a phosphate buffer (28 mM, pH 7.35; ATP 10 mM, CTP 10 mM, $MgCl_2$ 10 mM, aprotinin 225 μg and antibiotics) with [Me-^{14}C]choline as marker (0.83 μCi) and [^{3}H]PC as internal standard for the PC separation. In 60 min, with and without 40 μM HC-3, [Me-^{14}C]PC synthesized in assays and controls was in the ratio $A/C = 0.56 \pm 0.12$, $n = 6$, $P < 0.02$ in Krebs cells and $A/C = 1.12 \pm 0.15$, $n = 5$, $P > 0.2$ in rat liver. The choline-kinase inhibition by HC-3 induced a decrease in the PC synthesis in the Krebs cells post-nuclear supernatant but not in the same subcellular compartment of rat liver which counterbalanced its choline-kinase inhibition. The time course of this inhibition in Krebs cells is shown in Fig. 4: the action of HC-3 on PC biosynthesis was measurable after 30 min and increased linearly as a function of time until 120 min. This effect was also dependent on the HC-3 concentrations (Fig. 5): it was already obvious at 5 μM and increased to give an inhibition of almost 50% at 40 μM.

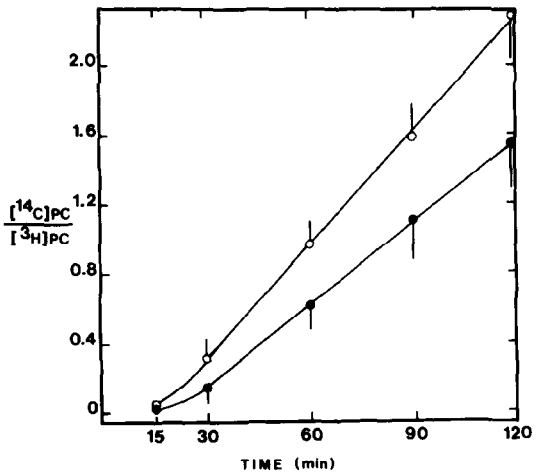

Fig. 4. Time course of PC synthesis with (●) and without (○) 40 μM HC-3 by a Krebs cells post-nuclear supernatant, 10% w/v. [^{14}C]PC = phosphatidyl-[Me-^{14}C]choline; [^{3}H]PC = [$^3H_2$]palmitic acid labelled phosphatidylcholine as internal standard (~100,000 dpm). Medium: protein 15 to 17 mg, [Me-^{14}C]choline 0.83 μCi, ATP 10 mM, CTP 10 mM, $MgCl_2$ 10 mM, aprotinin 225 μg, antibiotics (2000 U penicillin plus 2000 μg streptomycin per ml), sodium phosphate –Na buffer 28 mM, pH 7.35, volume 2.1 ml ($n = 3$). Incubation at 37°.

Incorporation of AT-[γ^{32}P] and [2-^{14}C]ethanolamine

As the *de novo*-synthesized choline was not taken into account in the experiments with [Me-

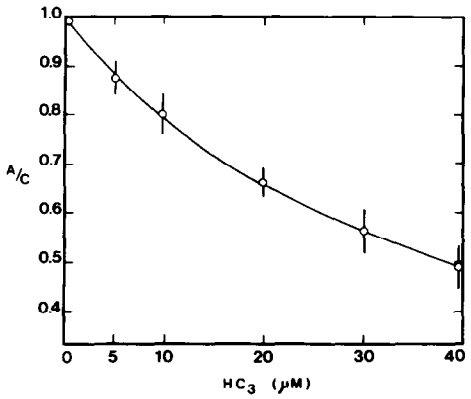

Fig. 5. Action of the HC-3 concentrations on the PC synthesis by a Krebs cells post-nuclear supernatant, 10% w/v. Experimental conditions are the same as in the legend of Fig. 4 except for the HC-3 concentrations and the 60 min incubation. A = [^{14}C]PC/[^{3}H]PC for assays, C = [^{14}C]PC/[^{3}H]PC for controls; [^{14}C]PC and [^{3}H]PC as in the legend of Fig. 4. ($n = 3$).

^{14}C]choline, we used AT-[γ^{32}P] as marker. All the conditions were the same as in the experiments with [Me-^{14}C]choline except that tris buffer was used instead of phosphate buffer. The incorporation of AT-[γ^{32}P] into phospholipids in the presence of 40 μM HC-3 demonstrated that only the labelled phospholipids migrating between PC and PE (mainly PS and PI) were weakly diminished (A/C = 0.82 ± 0.11, $n = 6$, $P < 0.05$). Considering the important isotopic dilution of ^{32}P and the various labelling rates of different phospholipids, it was hard for us to obtain a satisfactory arrangement between efficient product separation and sufficient specific radioactivities. For this reason, we examined a possible compensation of the PC synthesis defect by the PE methylation pathway. Using the protocol described in Fig. 4, and with [Me-^{14}C]choline and [2-^{14}C]ethanolamine (1 μCi each one) as markers, [Me-^{14}C]PC remained lower in assays than in controls after a 2 hr incubation (A/C = 0.63 ± 0.15, $n = 6$, $P < 0.001$), while [2-^{14}C]PE remained unchanged (A/C = 0.91 ± 0.12, $n = 6$, $P > 0.2$). In these experiments, without added *S*-adenosyl-methionine, the PC synthesis by the PMT I and PMT II pathway is not sufficient to compensate the HC-3 inhibition under our assay conditions.

DISCUSSION

These data indicate that HC-3 retards the PC synthesis in a post-nuclear supernatant of Krebs II ascites cells by a choline-kinase inhibition. Although the cytosolic choline-kinase of rat liver is also inhibited by HC-3, the PC synthesis is not diminished. As previously reported [15], the first two enzymes of the cytidine pathway for the PC synthesis, choline-kinase (EC 2.7.1.32) and cytidylyl-transferase (EC 2.7.7.15), catalyse rate-limiting steps. The fact that the studies of the phosphocholine formation were made on a 220,000 *g* (90 min) supernatant exclude membrane mechanisms of choline uptake as they were described in microsomal, mitochondrial or synaptosomal fractions [16–19].

The similarity between the choline-kinase inhibitions of Krebs cells and rat liver is not extended to the PC synthesis. Whereas choline phosphorylation is localized in the cytosol, the formation of CDP-choline and PC are realized in another subcellular compartment, mainly in the microsomal membranes. This fact explains the discrepancy between the Krebs cells and the rat liver metabolic responses against the HC-3 action. Our results relating the action of HC-3 on the PC synthesis in rat liver may be compared to those obtained in the canine caudate nucleus [20, 21], where a direct correlation was established between HC-3 concentrations and the incorporation of radioactive choline into phospholipids. The drug interacted on the initial step of the synthesis but, whereas choline acetylation was diminished, labelling of phosphocholine and cytidinediphosphocholine was increased. In the same way, the intracerebral injection of HC-3 caused inhibition of the phosphocholine synthesis in rat cerebellum and mid brain, but stimulation of PC formation [22]. In order to explain this apparent contradiction relating the HC-3 effect, several mechanisms may be invoked. In liver the drug increases the choline-kinase *Km* but a release of the hepatic choline pool is a possibility for a phosphorylation rate allowing the uptake of phosphocholine by CTP. Further, specific liver detoxication pathways might decrease the HC-3 efficient toxic concentrations and their inhibitory action. Of the four enzymatic pathways of PC synthesis (CDP-choline step, lyso PC cycle, exchange of the base moiety and PE methylations), the first three re-distribute pre-existing choline, and only PMT I and PMT II acting on PE realize the synthesis of new choline molecules. In our experiments on the Krebs cells post-nuclear supernatant, the incorporation of AT-[γ^{32}P] or [2-^{14}C]ethanolamine into phospholipids has not demonstrated a correction to the HC-3 toxic effect. Similar data have been reported in regard to the ^{32}P-labelling of total phospholipids in rat brain cortex [5]. Furthermore, the base exchange mechanism considered in brain in order to compensate the choline-kinase inhibition [22] is unlikely in our experiments carried out in Tris buffer reported as an inhibitor of the choline exchange [23]. As the activity of ethanolamine-kinase was unaffected by HC-3 [24], it was logical to investigate the ability of Krebs cells to assume the sequential methylations of PE so that new PC molecules are generated. Our data, which shows that the methylation pathway is undetectable, are in agreement with other studies concerning tumourous cells such as Hela, KB and Ehrlich [25]. Although PMT I and PMT II were described in several tissues and micro-organisms, they are much more active in liver than in adrenal medulla, erythrocytes or brain, and 15% of the hepatic PC are synthesized *de novo* [26]. This fact contributes towards the choline-kinase inhibition by HC-3 which does not induce a PC deficiency in a liver post-nuclear supernatant.

The action of HC-3 is together an inhibition of the choline penetration in the cell, of its acetylation and phosphorylation. Despite its toxicity, it was tested *in vivo* on mice bearing Ehrlich carcinoma [27]. We are continuing the study of its effects on the Krebs cells *in toto* in order to define its role in the transport

processes of choline and the incidence on the cellular phospholipid metabolism.

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