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SYNERGISTIC EFFECT OF CHOLINE AND CARNITINE ON ACETYLCHOLINE SYNTHESIS IN NEUROBLASTOMA NB-2a CELLS

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Summary: An influence of carnitine on acetylcholine synthesis from radiolabeled glucose was monitored in neuroblastoma NB-2a cells. Upon addition of carnitine the distribution of its derivatives was found significantly different than the values published for brain, the level of long-chain acyl derivatives being much higher and reaching 60%. Carnitine itself did not change acetylcholine level. Together with choline (20 μM), carnitine was observed to stimulate (by 36%) acetylcholine synthesis in a synergistic way,which indicated that both substrates could be limiting factors of this process in NB-2a cell line of neuroblastoma.

The synthesis of acetylcholine catalyzed by choline acetyltransferase (acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6) demands two substrates - acetyl-CoA and choline. It was proposed that choline could be produced and taken up by brain. For instance in brain slices the absence of free choline speeded up the choline release from phosphatidylcholine (1). Moreover, choline can be taken up from extracellular fluid into neural cells by the high- and low-affinity carriers (2) and mammmalian neurons were described to be capable of synthesizing choline (3).

Abbreviations: EMEM, minimum essential medium with Earle's balanced salt solution; PBS, phosphate buffered saline; SDS, sodium dodecylsulphate; TCA, trichloroacetic acid.

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As regards acetyl moiety for acetylcholine synthesis it has been established in a number of studies that acetyl-CoA originates mainly from glucose and pyruvate (4, 5). The intramitochondrial acetyl-CoA can pass the inner mitochondrial membrane in a form of citrate, delivering acetyl-CoA to cytosol due to the action of citrate lyase. It was, however, demonstrated, that citrate pathway supplied not more than 30% of acetyl moieties for acetylcholine synthesis in mammalian brain (4, 6, 7). The other possible pathway of acetyl-CoA transfer through the inner mitochondrial membrane would be by the carnitine shuttle, which is involved in transfer of acyl compounds (8). The pathway through carnitine shuttle consists from a central translocation step and several activation and deactivation reactions of fatty acyl chain, catalysed by several enzymes of different specificities towards degree of saturation and the chain length of the acyl moiety. We have recently purified the carnitine/acylcarnitine translocase from rat brain mitochondria (9). This carrier revealed a higher specificity towards short and medium chain acyl derivatives (9) in comparison with other tissues (10), suggesting that this activity in neural cells would deliver acetylcarnitine to cytoplasm, rather than transport long-chain acylcarnitines as the substrates of ß-oxidation which is less pronounced in nervous system. Therefore the metabolism of externally added carnitine was checked in cultured neuroblastoma NB-2a cells and the present study was focused on the possible influence of carnitine on the synthesis of acetylcholine in this cell line.

MATERIALS AND METHODS

Materials: Mouse neuroblastoma NB-2a cell line derived from the C 1300 tumor (11) was kindly supplied by Prof. A.Azzi from the Institute for Biochemistry and Molecular Biology, University of Berne, Switzerland. Tissue culture plastics were from Corning and growth media, serum, antibiotic/antimycotic mixtures were obtained from Gibco. L-[methyl-^{3}H]carnitine was purchased from Amersham, D-[U-^{14}C]glucose was from the Radioisotope Production and Distribution Centre (Prague, Czech Republic). L-Acetylcarnitine, L-propionylcarnitine, L-palmitoylcarnitine were delivered by Serva, L-carnitine was provided by Fluka. Paraoxon (diethyl p-nitrophenylphosphate) and choline were purchased by Sigma. Chromatography paper 3MM was from Whatman, aluminium silica gel 60 sheets were from Merck. Ultima Gold (Packard) was used as a scintillation cocktail. All other reagents were of analytical grade.

Cell culture: Neuroblastoma NB-2a cells were grown in EMEM containing 25 mM bicarbonate, 100 U penicillin/ml, 100 μg streptomycin/ml, 0.25 μg fungizone/ml and 10% foetal calf serum, pH 7.4. Cells were usually seeded into 100 mm plastic culture dishes at a subcultivation ratio 1:3 and grown to confluence at 37°C in a humid atmosphere of 5% CO_2. Culture media were changed every three days. Cells were harvested with a cell lifter, suspended in 90% EMEM, 10% foetal calf serum, spun down at 5,800 x g for 10 min. The pellet was washed once with PBS and finally resuspended in PBS containing 5 mM glucose.

Accumulation of carnitine: Cells were incubated for 1 h at 37°C in PBS supplemented with 5 mM glucose in the presence of 50 μM L-carnitine (15 Ci/mol). The monolayer

was washed 5 times on the Petri dish with PBS, collected with a cell lifter and spun down at 5,800 x g for 10 min. Separation of free carnitine and its acyl derivatives was performed as described in (12). The cellular pellet was treated with 10% TCA and homogenized by passing through a 25G (0.45x16 mm) needle. After keeping for 30 min on ice, the material was spun down for 20 min at 20,000 x g. The pellets containing long-chain acylcarnitines were dissolved in 0.1 M NaOH, 2% Na_2CO_3 and 1% SDS at 40°C and samples were taken for radioactivity counting and protein estimation. Free carnitine and its short- and medium-chain acyl derivatives were quantified in supernatants after extraction of TCA with diethylether and separation by TLC, as described in (12). The position of various carnitine derivatives was defined by comparison with the respective markers run in parallel and visualized by iodine.

Acetylcholine synthesis: Cells were incubated for 1 h at 37°C with [^{14}C]glucose (specific activity of 250 mCi/mol). The incubation medium was supplemented with the additions indicated in the legends. The reaction was terminated by spinning at 5,800 x g for 10 min and washing with PBS. The pellet was suspended in 50 mM NaCl and passed through 25G (0.45x16 mm) needle in order to disrupt cells. Samples were taken for protein estimation and the rest was lyophilized. The lyophilized cell powder was resuspended in water with acetylcholine carrier (1 $\mu g/\mu l$) and subjected to paper chromatography subsequently developed with butan-1-ol/ethanol/acetic acid/water (8:2:1:3, v/v) according to (13). The position of acetylcholine spot, determined in iodine vapour when compared with a marker was cut off and taken for radioactivity counting.

Protein estimation: Protein concentration was determined by the Lowry procedure (14), modified by the addition of 1% SDS (15).

RESULTS AND DISCUSSION

In order to check the influence of carnitine on acetylcholine synthesis, the metabolism of carnitine in neuroblastoma NB-2a cells was analysed. The distribution of carnitine and its acyl derivatives was explored by TCA precipitation and TLC separation. Accumulation of carnitine was observed to increase with time and after 6 h equalled 0.4 nmol/mg protein. Already after short incubation times a significant content of palmitoylcarnitine was detected, reaching 60% of all carnitine derivatives (Fig. 1A). This observation would point to a high activity of carnitine palmitoyltransferase and a low level of ß-oxidation, typical for neural cells. It has to be emphasized, that the observed extremely high content of long-chain acylcarnitines in neuroblastoma represented a reverse situation to the carnitine distribution in brain, where these derivatives did not exceed 20% (16). The total accumulation of carnitine and its derivatives after 24 h were, however, comparable in neuroblastoma and brain, reaching 1.5 nmol/mg protein in NB-2a cells, whilst the values reported for several brain regions were oscillating between 0.8-1.5 nmol/mg protein (excluding hypothalamus where carnitine accumulation was reported to reach almost 4 nmoles/mg protein (16)). The level of free carnitine was always by 50% higher than that of acetylcarnitine, as presented in Fig. 1B for 1 h, moreover, this proportion did not change with time. This

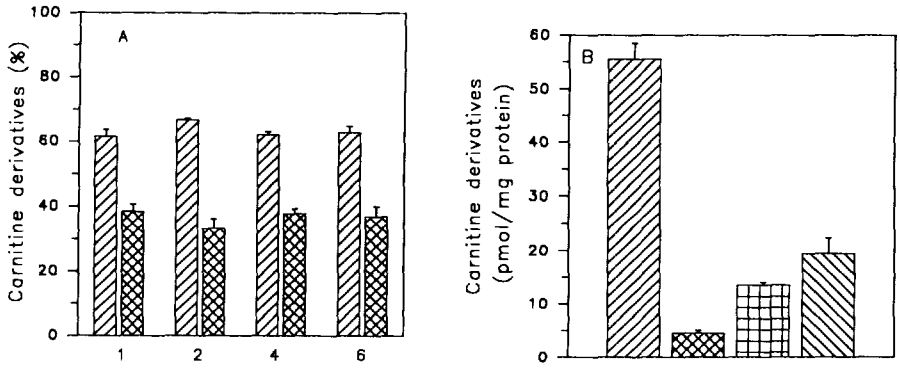

FIGURE 1. Distribution of carnitine and its derivatives in neuroblastoma NB-2a cells. Long-chain acylcarnitines were separated from free carnitine and its medium- and short-chain acyl derivatives by TCA precipitation, as given in Materials and Methods. A. The amount of accumulated palmitoylcarnitine (hatched bars) and acid soluble carnitine derivatives (crossed bars) as a function of time indicated by the amount of hours. The values are expressed as a percentage of total accumulation for each time point. B. The amount accumulated after 1 h free carnitine (rising to left hatched bar) and its derivatives, in particular long-chain acylcarnitines (rising to right hatched bar), medium-chain acylcarnitines (crossed bar) and acetylcarnitine (horizontal crosshatched bar). The results are means ± SEM from 6 separate experiments.

would reflect either a low activity of carnitine acetyltransferase or a quick removal of acetylmoieties by another metabolic pathway, for example acetylcholine synthesis.

In order to measure the ability of NB-2a cells to synthesize acetylcholine, the acetylcholine esterase (EC 3.1.1.7) activity had to be inhibited. Although the early observations presented low activities of this enzyme in cultured neuroblastoma NB-2a (11), it was also reported that in the same cell line acetylcholine esterase is produced in cellular and secreted form (17). Therefore acetylcholine synthesis was measured in the presence of 50 μM paraoxon - a known inhibitor of acetylcholine esterase (18).

Incubation of neuroblastoma NB-2a cells with labelled glucose resulted in a significant synthesis of acetylcholine, reaching 135 ± 8 pmol/mg protein after 1 h (Fig. 2), a value comparable with those reported for slices of rat caudate nuclei (18). Addition of carnitine, a compound postulated to stimulate acetylcholine synthesis (18) revealed, unexpectedly, no statistically significant effect on the acetylcholine synthesis in NB-2a cells. Since also the choline moiety is a necessary substrate for this synthesis, an effect of externally added choline was studied. Due to the fact that carnitine accumulates in neural cells in a Na-dependent way (19), the concentrations of choline transported by a low affinity transporting system (also Na-dependent) were avoided. Choline was added at a concentration saturating the high affinity choline transporting system (2, 20), i.e.

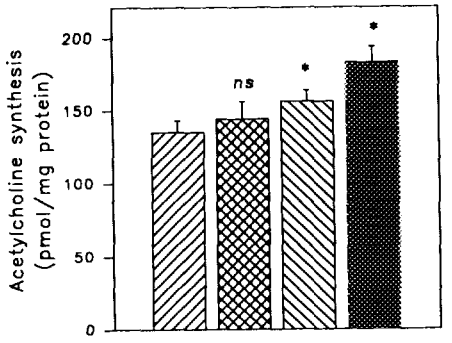

FIGURE 2. Effect of carnitine and choline on acetylcholine synthesis in neuroblastoma NB-2a cells.
Synthesis of acetylcholine was estimated in the presence of 50 μM paraoxon from [^{14}C]glucose, as given in Materials and Methods. The cells were incubated for 1 h either in the absence of additional substrates (rising to right hatched bar) or in presence of following additions: 50 μM L-carnitine (crossed bar), 20 μM choline (rising to left hatched bar), both, 50 μM carnitine + 20 μM choline (filled bar). Statistical analyses of all data were performed using Student's paired t-test against the control without any additions. The results are expressed as means $\pm$ SEM from duplicates obtained in 5 separate experiments (* $p < 0.01$; ns - not significant).

20 μM. As presented in Fig. 2, choline alone revealed a slight stimulatory effect. When, however, both components, carnitine and choline, were added together, a significant increase of acetylcholine synthesis was observed (Fig. 2). This synergistic effect reached 136% of a control value. Such an observation would lead to a conclusion that both the accessibility of choline and acetyl moieties could be limiting factors of acetylcholine synthesis and a stimulatory effect of carnitine on this process can be visualized only in the presence of the excess of choline.

These observations could have physiological implications, since carnitine and its esters have been proven to be useful pharmacological agents for treatment of chronic degenerative diseases in aging human subjects (21), for instance acetylcarnitine has been shown in clinical trials to be effective in slowing down the progression of mental deterioration in Alzheimer's disease (22, 23). The more detailed studies on the effect of carnitine on acetylcholine synthesis in isolated brain cells are now under progress.

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