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Effect of Oral CDP-Choline on Plasma Choline and Uridine Levels in Humans

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ABSTRACT. Twelve mildly hypertensive but otherwise normal fasting subjects received each of four treatments in random order: CDP-choline (citicoline; 500, 2000, and 4000 mg) or a placebo orally at 8:00 a.m. on four different treatment days. Eleven plasma samples from each subject, obtained just prior to treatment (8:00 a.m.) and 1–12 hr thereafter, were assayed for choline, cytidine, and uridine. Fasting terminated at noon with consumption of a light lunch that contained about 100 mg choline. Plasma choline exhibited dose-related increases in peak values and areas under the curves (AUCs), remaining significantly elevated, after each of the three doses, for 5, 8, and 10 hr, respectively. Plasma uridine was elevated significantly for 5–6 hr after all three doses, increasing by as much as 70–90% after the 500 mg dose, and by 100–120% after the 2000 mg dose. No further increase was noted when the dose was raised from 2000 to 4000 mg. Plasma cytidine was not reliably detectable, since it was less than twice blank, or less than 100 nM, at all of the doses. Uridine is known to enter the brain and to be converted to UTP; moreover, we found that uridine was converted directly to CTP in neuron-derived PC-12 cells. Hence, it seems likely that the circulating substrates through which oral citicoline increases membrane phosphatide synthesis in the brains of humans involve uridine and choline, and not cytidine and choline as in rats. BIOCHEM PHARMACOL **60**;7:989–992, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. citicoline; cytidylyl diphosphocholine; choline; cytidine; uridine; phosphatidylcholine

CDP-choline† is both an endogenous intermediate in the biosynthesis of PC [1] and a drug used in Europe and Japan to treat stroke and brain injury [2, 3]. When administered orally or by injection to rats, exogenous CDP-choline is metabolized completely to form choline and cytidine [4]. These intermediates enter the circulation, cross the blood–brain barrier [5, 6], and are converted in part to acetylcholine [7], phosphocholine [8], and CTP [9]. The latter two compounds then combine to form endogenous CDP-choline, which reacts with DAG to form PC [1]. Previous reports suggested that, in humans as in rats, the metabolism of exogenous CDP-choline elevates plasma levels of cytidine as well as choline [4, 10]. However, some of the supporting data were based on a cytidine assay that was not fully specific. Using a modification of this assay that increases its specificity, we observed that the principal circulating metabolites of exogenous CDP-choline in humans are uridine and choline.

MATERIALS AND METHODS

Clinical Study

Twelve overnight-fasted subjects (7 males; 5 females) aged 60–80 received each of four different treatments orally, at random, at 8:00 a.m., after a 1-week washout period: placebo; CDP-choline, 500 mg; CDP-choline, 2000 mg; and CDP-choline, 4000 mg. Patients were mildly hypertensive but not on medications. Fasting was terminated at noon by consumption of a standardized lunch, containing about 100 mg choline; the subjects also ate a standardized dinner at 6:00 p.m. Blood samples (10 mL or less) were collected into heparinized tubes prior to treatment and at 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 hr thereafter; plasmas were separated and kept on dry ice pending the performance of assays for choline, uridine, and cytidine.

Assays

CHOLINE. Plasma samples were deproteinized with methanol, and fatty components were extracted into chloroform: methanol. A portion of the aqueous layer was lyophilized for subsequent HPLC analysis. Then the residue was redissolved in water, filtered (Uniflo-25, 0.2 μm, Schleicher & Schuell), and injected into a BAS 200A HPLC-EC (Bio-Analytical Systems), as described by Farber *et al.* [11].

CYTIDINE AND URIDINE. Samples were assayed for cytidine and uridine by a modification of the method described by Savci and Wurtman [9]. An internal standard (fluorouridine, 1 μg) was added to each 1-mL sample of heparinized plasma, which then was deproteinized by adding methanol (5 mL). After centrifugation, the supernatant fluids were lyophilized, and then reconstituted in 5 mL of 0.25 N ammonium acetate (pH 8.8) just prior to purifica-

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† *Abbreviations*: AUC, area under the curve; CDP-choline, citicoline, cytidylyl diphosphocholine; DAG, diacylglycerol; and PC, phosphatidylcholine.

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tion over boronate affinity columns (Affigel-601, Bio-Rad). Samples were run in a cold room: columns were primed first with two 5-mL ammonium acetate washes, the samples then were applied, and the columns were washed again with ammonium acetate, after which the nucleosides were eluted with 0.1 N formic acid (7 mL). The eluates were lyophilized and then reconstituted in 100 μL water for analysis on a Beckman system gold (Beckman Instruments), equipped with a Rainin Dynamax Microsorb C18 column (3 μm packing; 4.6 × 100 mm) at room temperature. In our revised method, we used an isocratic 0.004 N potassium phosphate (pH 5.8) system containing 0.1% methanol, flowing at 1 mL/min and heated to 35°. This sharpened peaks and permitted a faster flow rate. Levels of cytidine in the samples were less than twice blank, or less than 100 nM; basal levels of uridine were 40–60 times blank, or 4–6 μM.

Statistical Analyses

The following analyses were conducted for the plasma concentrations of choline and uridine: Baseline concentrations were compared across the four dose levels using mixed-models ANOVA; if the effect of dose was significant, then contrasts were used to examine pairwise differences between doses.

The effects of dose on plasma concentrations over time were modeled using mixed-models ANOVA. The models included a factor for whether each time point was before or after lunch to allow for a possible effect of the choline-containing lunch on plasma concentrations. From the best-fitting model, the times at which plasma concentrations were increased significantly from the baseline concentration were determined from contrasts of the differences, and the maximum difference from baseline was considered as the peak plasma level. We determined whether lunch influenced plasma concentrations over time by examining whether there was a significant increase in plasma concentrations at either of the two post-lunch measurements (hr 5 and 6) as compared with the last pre-lunch measurement (hr 4).

The effects of dose on AUC were examined using mixed-models ANOVA. If lunch did not affect plasma concentrations, then AUC would be calculated over the 12-hr period. If, however, lunch did matter, then AUC would also be calculated over the pre-lunch period.

In Vitro *Studies*

PC-12 cells were allowed to grow [in 3 mL of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, for 5–6 days] in 6-well tissue culture plates at +37° and 5% CO_2. The medium was changed on days 1 and 3 following sub-plating of the cells. On days 5 and 6, each medium was replaced with serum-free DMEM, with or without various concentrations of cytidine or uridine. In the concentration–response experiments, the cells were incubated for another 6 hr. In the time–course experiments, the cells were incubated for 1, 3, or 6 hr. After the incubation periods, the medium was aspirated, and the cells were washed twice with 2 mL of the balanced salt solution. The cells then were collected and homogenized in ice-cold methanol (80% in water), using an ultrasonic homogenizer. Aliquots of homogenate (20–50 μL in duplicate) were taken for protein assay. The remaining homogenate was centrifuged for 10 min at +4°, and the supernatant fluid was dried using a vacuum centrifuge. The dried samples were reconstituted in 125 or 150 μL water. A 50-μL aliquot was used for measurement of CTP and UTP by HPLC [12]. CTP and UTP values were corrected for protein contents and given as nanomoles per milligram protein.

RESULTS

Choline

Citicoline administration caused a significant ($P < 0.05$), dose-related increase in the AUC of plasma choline levels, throughout the dosage range tested (Fig. 1). Peak plasma choline levels, obtained 2–3 hr after the 500 mg dose and 5 hr after the other doses, were 23, 32, and 43% greater than baseline levels ($P < 0.001$), and significant elevations persisted for 5, 8, and 10 hr, respectively. The elevation in plasma choline levels after lunch was statistically significant only after the 2000 mg dose. Hence, AUCs were calculated over the entire 12-hr period of the experiment.

Uridine

Citicoline administration significantly ($P < 0.001$) increased the AUC of plasma uridine levels at all doses tested (Fig. 2); however, the increase after the 4000 mg dose was no greater than that after the 2000 mg dose.

Peak plasma uridine levels, observed 1.5 hr after the 500 mg citicoline dose, and 3 hr after the other doses, were 101, 136, and 134% greater than baseline levels (all $P < 0.005$). Significant elevations persisted for 5–6 hr in all experimental groups. Lunch had no discernible effects on plasma uridine.

Cytidine

Cytidine levels failed to attain twice blank values, indicating that actual cytidine concentrations must have been less than 100 nM.

In Vitro *Conversion of Uridine to UTP and CTP*

Incubation of PC-12 cells with uridine caused dose-related increases in cellular UTP and CTP levels (Table 1). The increases in CTP were approximately linear at the three lowest doses tested, and then CTP levels attained a plateau. Incubation of the cells with cytidine caused dose-related increases in CTP, but failed to produce significant changes in UTP (Table 1). The increases in CTP and UTP

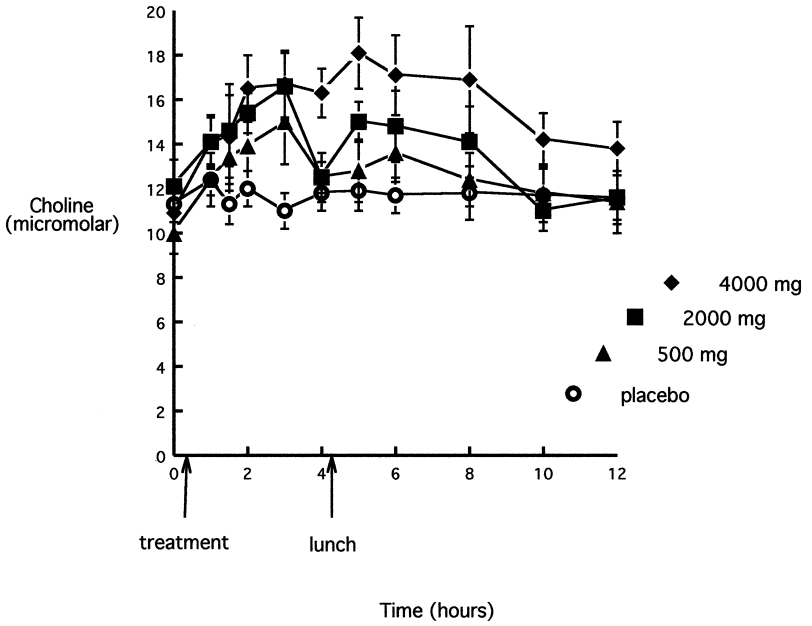

FIG. 1. Effects of oral citicoline on plasma choline levels. Groups of twelve subjects received a placebo or various doses of citicoline (500 mg; 2000 mg; 4000 mg) orally, in random order, while fasting. Blood samples were taken prior to treatment and at 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 12 hr thereafter. Deproteinized, de-fatted plasma samples were assayed for choline, as described in the text. Citicoline caused a significant, dose-related ($P < 0.05$) increase in the AUC throughout the dosage range tested. Peak levels were also dose-related and elevated significantly ($P < 0.001$). Values are means ± SEM.

following incubation with uridine (200 μM) and those in CTP but not UTP after incubation with cytidine persisted for at least 6 hr.

DISCUSSION

These data confirm earlier reports [10] that citicoline consumption increases plasma choline levels (Fig. 1). The peak percent increments obtained after the 2000 or 4000 mg doses were comparable, on a molar basis, to those seen after similar doses of choline chloride [13, 14], although the peaks were delayed by 1 or more hours. Weiss [15] has reviewed the pharmacokinetics of CDP-choline in experimental animals, summarizing cytidine and choline uptake in various organs as well as in plasma. Our data demonstrated for the first time that, in the dosage range in which it is usually administered (e.g. up to 2000 mg daily), CDP-choline increases plasma uridine levels in humans (Fig. 2) and that, in PC-12 cells, uridine can be converted to CTP (Table 1). The increases in choline and uridine persisted for a number of hours, so it might be anticipated that giving the drug twice daily would cause their levels to remain elevated for much of the day.

A previous study from our laboratory [10] was interpreted as showing that citicoline consumption by human subjects increases plasma cytidine levels, just as it does in experimental rodents [4]. However, using a more specific assay, described here, which sharpens the peaks of plasma nucleosides, we failed to detect significant quantities of cytidine in human blood, basally or after citicoline intake. Apparently,

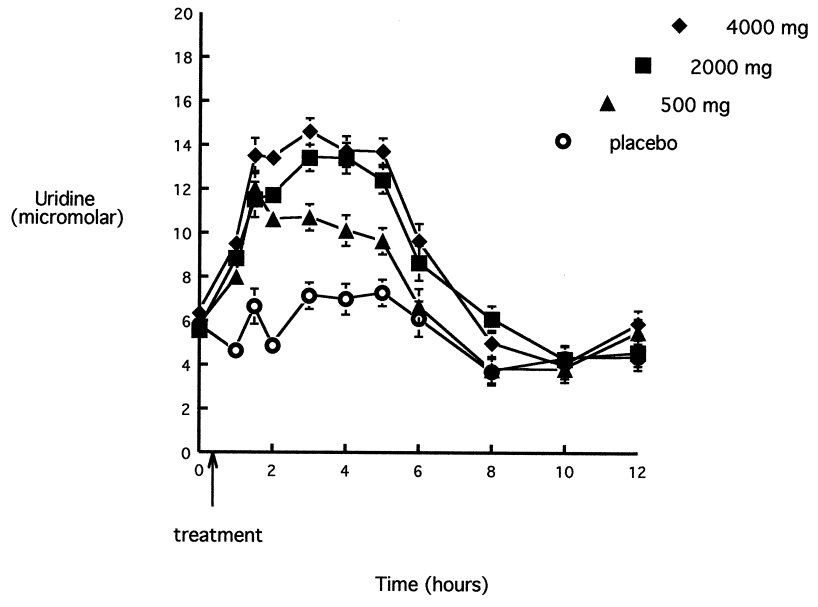

FIG. 2. Effects of oral citicoline on plasma uridine levels. Groups of twelve subjects were treated as described in the legend to Fig. 1, and plasma samples were assayed for uridine, as described in the text. Citicoline significantly elevated ($P < 0.001$) the AUC of plasma uridine levels at all doses tested; however, the increase after the 4000 mg dose was no greater than that after the 2000 mg dose. Peak plasma uridine levels were also elevated significantly ($P < 0.005$), and were greater after receiving 2000 or 4000 mg than after 500 mg of the drug. Values are means ± SEM.

TABLE 1. Effects of cytidine or uridine on CTP and UTP levels in Pc-12 cells

Concentration	Cytidine		Uridine	
(μM)	CTP	UTP	CTP	UTP
0	0.87 ± 0.02	6.32 ± 0.61	0.87 ± 0.02	6.32 ± 0.61
25	2.48 ± 0.20	7.94 ± 0.66	1.24 ± 0.09	11.04 ± 1.45
50	3.35 ± 0.38	7.25 ± 0.65	1.51 ± 0.14	11.72 ± 1.45
100	3.59 ± 0.41	6.11 ± 0.45	2.37 ± 0.24	16.59 ± 1.89
200	5.19 ± 0.36	7.62 ± 0.55	2.54 ± 1.40	14.89 ± 0.96
400	ND	ND	2.15 ± 0.14	14.02 ± 1.20

Cells were incubated with the nucleoside for 6 hr, as described in the text. Data (means ± SEM; N = 4–6) are given as nmol/mg protein. All doses of cytidine or uridine increased CTP levels significantly ($P < 0.01$). Uridine but not cytidine increased UTP levels significantly ($P < 0.05$). ND = no data.

at the citicoline doses tested, the human gastrointestinal tract and liver quantitatively transform the cytidine liberated from the drug to circulating uridine. This compound then enters the brain [6] and other tissues and is phosphorylated to UTP; this nucleoside, in turn, is converted by the enzyme CTP synthetase to form CTP, a rate-limiting cofactor in PC biosynthesis [16]. Conversion of uridine to CTP was demonstrated directly here in PC-12 cells (Table 1).

The proportions of cytidine and uridine in human plasma differ markedly from those in laboratory rodents: in the rat, we and others [17] observed basal cytidine levels of about 5.2 μM and uridine levels of 2.1 μM, yielding a uridine/cytidine ratio of 0.4; in the gerbil, these basal concentrations are 4.5 and 12.8 μM, respectively, yielding a ratio of about 2.8. The present data show that in the human this ratio is at least 58, i.e. if it is assumed that plasma cytidine levels are just below 0.1 μM, the limits of sensitivity of our assay. These differences may reflect less activity of the enzyme cytidine deaminase in the rodents. In such species it might be anticipated that cytidine, and not, as in humans, uridine will be the circulating metabolite through which oral citicoline increases brain CTP levels and enhances phosphatide synthesis.

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