

Citicoline enhances frontal lobe bioenergetics as measured by phosphorus magnetic resonance spectroscopy

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Received 7 September 2007; Revised 24 March 2008; Accepted 24 March 2008

ABSTRACT: Citicoline supplementation has been used to ameliorate memory disturbances in older people and those with Alzheimer's disease. This study used MRS to characterize the effects of citicoline on high-energy phosphate metabolites and constituents of membrane synthesis in the frontal lobe. Phosphorus (³¹P) metabolite data were acquired using a three-dimensional chemical-shift imaging protocol at 4 T from 16 healthy men and women (mean \pm SD age 47.3 \pm 5.4 years) who orally self-administered 500 mg or 2000 mg Cognizin[®] Citicoline (Kyowa Hakko Kogyo Co., Ltd, Ibaraki, Japan) for 6 weeks. Individual ³¹P metabolites were quantified in the frontal lobe (anterior cingulate cortex) and a comparison region (parieto-occipital cortex). Significant increases in phosphocreatine (+7%), β -nucleoside triphosphates (largely ATP in brain, +14%) and the ratio of phosphocreatine to inorganic phosphate (+32%), as well as significant changes in membrane phospholipids, were observed in the anterior cingulate cortex after 6 weeks of citicoline treatment. These treatment-related alterations in phosphorus metabolites were not only regionally specific, but tended to be of greater magnitude in subjects who received the lower dose. These data show that citicoline improves frontal lobe bioenergetics and alters phospholipid membrane turnover. Citicoline supplementation may therefore help to mitigate cognitive declines associated with aging by increasing energy reserves and utilization, as well as increasing the amount of essential phospholipid membrane components needed to synthesize and maintain cell membranes. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: citicoline; ³¹P MRS; anterior cingulate; phospholipids; phosphocreatine; β -NTP

INTRODUCTION

Citicoline (CDP-choline; cytidine 5'-diphosphocholine) is a nucleotide that plays an important role in cellular metabolism (1–3), provides an exogenous source of membrane phospholipid precursors (4,5), serves as a catalyst for acetylcholine synthesis (6,7), and modulates catecholaminergic neurotransmission (1–3). Citicoline also has been shown to reduce memory impairments associated with aging (8,9). Age-related declines in cognitive abilities, particularly related to frontal lobe function (10,11), are thought to be due in part to

decrements in consumption of oxygen and glucose and reductions in cerebral blood flow (12–17). In addition, a 'mitochondrial theory of aging' has been suggested, which asserts that the aging process involves impairment of mitochondrial membrane proteins, declines in electron-transport chain activity, and increases in oxidative stress resulting from mitochondrial respiratory metabolism (18–26).

Orally or intravenously administered citicoline is metabolized to choline and cytidine in the gastrointestinal system of the rat, with cytidine being further metabolized to uridine in the gastrointestinal system and liver of humans (27). Circulating uridine enters the brain via the blood–brain barrier and undergoes phosphorylation to become uridine triphosphate (UTP), which is then converted into cytidine triphosphate (CTP) by CTP synthetase. Free choline undergoes phosphorylation to become phosphocholine, which, in combination with CTP, yields CDP-choline (27,28). Endogenous CDP-choline then reacts with diacylglycerol to form phosphatidylcholine (PtdCho) (29). Thus, the biosynthetic pathway of citicoline provides exogenous sources

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Abbreviations used: 3D-CSI, three-dimensional chemical-shift imaging; ACC, anterior cingulate cortex; ANOVA, analysis of variance; CT, CTP-phosphocholine cytidyltransferase; GPC, glycerolphosphocholine; GPE, glycerophosphoethanolamine; β -NTP, β -nucleoside triphosphate; PC, phosphocholine; PCr, phosphocreatine; PDE, phosphodiester; PE, phosphoethanolamine; Pi, inorganic phosphate; PME, phosphomonoester; POC, parieto-occipital cortex; PtdCho, phosphatidylcholine.

of precursors for the synthesis of acetylcholine and phospholipid membranes, including PtdCho, phosphatidylethanolamine, and sphingomyelin (3,6,30,31). Reduced transport and utilization of choline and a concomitant decrease in an essential structural component of cell membranes, PtdCho, as measured in serum, has been observed in older populations compared with younger cohorts (32–34). Membrane phospholipids provide the structural building blocks of cell membranes, but also play an important role in signal transduction, ion channel and receptor function, regulation of enzymes, and transcriptional activity (5,7,35). The onset of the age-related decline in choline transport has not been well characterized, with most studies comparing young subjects (40 years and younger) with older subjects (60 years and older). It is plausible that declines in active transport of choline may begin to occur before manifestation of memory deficits, but this hypothesis has not been empirically investigated. Regardless, the efficacy of citicoline in reducing memory impairments may be related to an increased dietary source of choline, which is transported into the brain via facilitated diffusion, and also to an increase in cytidine and uridine concentrations, pyrimidines that have been well established to contribute to membrane synthesis (27,28).

Treatment with citicoline has also been shown to modify mitochondrial and synaptosomal proteins and improve brain metabolism in rats (36–39), perhaps related to an increase in the availability of cytidylic nucleotides and content of total adenine nucleotides (24,25,40,41). Thus, citicoline is likely to alter multiple biochemical parameters, in part due to the reciprocal relationship between synthesis and function of phospholipid membranes and efficient energy production and utilization, provided by mitochondria (42,43). Changes in phospholipid membranes and high-energy phosphates may therefore underlie the therapeutic efficacy of citicoline in reducing age- and Alzheimer-related decrements in cognitive functioning, particularly in frontally mediated abilities involving memory.

There is a paucity of *in vivo* human studies, however, conducted to examine the effects of citicoline supplementation on neurochemistry. ^{31}P MRS provides a means of detecting changes in phosphorus-containing metabolites that are associated with levels of high-energy phosphate metabolites and constituents of membrane synthesis, indicating the cellular bioenergetic state and the integrity and function of cell membranes, respectively. Using this method, Babb and colleagues (44) observed a significant increase in phosphodiester (PDEs; phospholipid membrane catabolites) at 1.5 T after 6 weeks of citicoline treatment in older subjects (mean \pm SD age 69.4 \pm 5.6 years). Although the citicoline-related alterations were not regionally specific, because ^{31}P spectra were acquired from a 5 mm axial brain slice prescribed through the frontal and occipital cortices, the findings were consistent with previous cell culture data, which

document increased phospholipid synthesis and turnover (45). The increase in phospholipid catabolites correlated with improved performance on a test of verbal learning and memory (California Verbal Learning Test) (44).

To date, the effects of citicoline on bioenergetic status and synthesis of phospholipid membrane have not been characterized in the frontal lobe or in populations that have not yet reached elderly status. Accordingly, the objective of this study was to collect ^{31}P -MRS data using a three-dimensional chemical-shift imaging (3D-CSI) technique at 4 T in healthy middle-aged subjects. The use of 3D-CSI at high field has several advantages over previous methods: (1) increased spectral dispersion, which allows increased precision of metabolite quantification; (2) post-processing grid shifting which allows 3D placement of voxels of interest; and (3) the ability to co-register voxel placement between baseline and post-treatment follow-up. High-energy metabolite peaks quantified in this study included phosphocreatine (PCr), β -NTP (ATP in brain), and inorganic phosphate (Pi). Phospholipid membrane metabolite peaks quantified included anabolites [phosphomonoesters (PMEs), including phosphoethanolamine (PE) and phosphocholine (PC)] and catabolites [PDEs, including glycerophosphoethanolamine (GPE) and glycerolphosphocholine (GPC)]. A region of interest approach was used to examine phosphorus metabolite concentrations in the anterior cingulate cortex (ACC), given the notable age-related decline in frontal lobe cerebral metabolism and impairment of frontally mediated cognitive functions. A comparison region placed in the parieto-occipital cortex (POC) was also evaluated. It was hypothesized that oral supplementation with citicoline would improve frontal energy metabolism through increased availability of high-energy phosphates, as well as alteration of membrane phospholipid turnover by providing additional membrane precursors.

METHODS AND MATERIALS

Subjects

Subjects were recruited via local advertisement and screened via telephone interview to ensure that they met the basic eligibility criteria for this study. At the baseline visit, information on subject demographics and medical histories was obtained to confirm their inclusion in the study. The resultant group comprised 16 neurologically and psychiatrically healthy adults (mean \pm SD age 47.3 \pm 5.4 years; eight women). Trained research technicians administered a structured clinical psychiatric interview using the Structured Clinical Interview for DSM-IV (46). All subjects were free of Axis I diagnoses, neurological illness, severe medical problems, and psychoactive substance use. Exclusion criteria for all subjects included diagnoses of substance dependence, history of organic mental disorder, head trauma, loss of consciousness,

Table 1. Baseline characteristics of the study group according to dose of citicoline received

	500 mg		2000 mg	
	Female	Male	Female	Male
Age (years)	50.3 ± 7.3	46.5 ± 5.3	45.8 ± 3.1	46.5 ± 6.3
Education (years)	16.8 ± 1.9	16.0 ± 2.8	14.8 ± 1.9	16.3 ± 2.6
Handedness	3R, 1L	4R, 0L	4R, 0L	2R, 2L

Data are mean ± SD. R, right-handedness; L, left-handedness.

seizure disorder or central nervous system disease (e.g. multiple sclerosis or cerebral vascular incident), or contraindications to MR scanning (e.g. metallic implants, pacemaker, aneurysm clips, pregnancy or claustrophobia). All subjects received monetary compensation for their participation. Baseline characteristics of the study sample are presented in Table 1.

Procedure

All aspects of the clinical research protocol were reviewed and approved by the Institutional Review Board of McLean Hospital. After receiving a complete description of the study, all subjects provided written informed consent before participation.

Subjects were randomly assigned to receive a 6-week supply of either 500 mg (four men, four women) or 2000 mg (four men, four women) of Cognizin[®] Citicoline (Kyowa Hakko Kogyo Co., Ltd, Ibaraki, Japan). They were instructed to take one capsule every day (500 mg group) or four capsules every day in a single dose (2000 mg group) for 6 weeks. These two doses were selected, as previous investigations have demonstrated cognitive enhancement and/or alterations in phosphorus metabolites, as well as minimal side effects. MRS was completed on all subjects in two imaging sessions: one before the start of the treatment (baseline) and one after completion of 6 weeks of treatment.

¹H-MRI/³¹P-MRS imaging

All proton imaging was performed using the proton channel of the dual-tuned open-face proton-phosphorus transverse electromagnetic (TEM) whole-head coil from Bioengineering Inc. (Minneapolis, MN, USA) operating at a nominal frequency of 170.3 MHz. A two-dimensional gradient-recalled echo imaging sequence (12 s duration) was used to acquire a single image in all three spatial dimensions (sagittal, coronal, axial) to quickly determine the subject's position and angle. High-contrast, *T*₁-weighted sagittal, coronal images as well as *T*₁- and

*T*₂-weighted transverse images of the entire brain were acquired using a three-dimensional, magnetization-prepared FLASH (fast low angle shot) imaging sequence, allowing clear segmentation between gray matter, white matter and cerebrospinal fluid, as well as clearly delineating between the different anatomical regions of interest. This approach was used to optimize ³¹P-MRS imaging voxel positioning and volumetric correction in the regions of interest.

³¹P-MRS imaging was performed using the phosphorus channel of the dual-tuned proton-phosphorus TEM whole-head coil from Bioengineering Inc. operating at 68.9 MHz. ³¹P-MRS imaging data were recorded using a 3D-CSI sequence (12). Acquisition parameters were: *TR* = 500 ms; tip angle = 32°; *Rx* bandwidth = ± 2 kHz; complex points = 1024; readout duration = 256 ms; prepulses = 10; pre-acquisition delay = 1.905 ms; field of view (*x,y,z*) = 330 mm; nominal volume = 8.8 cm³; maximum phase-encode matrix dimension (*x,y,z*) 14 × 14 × 14 (zero-filled out to 16 × 16 × 16 before reconstruction). This 3D-CSI sequence used a reduced phase-encoding scheme based on prior work (47). This scheme allows the inclusion of spherically bound, reduced-point, weighted *k*-space acquisition, providing ~35% more signal-to-noise for a given scan time and effective voxel volume over conventional methods. The total examination time was ~70 min to complete the series of MRI and MRS imaging scans, including subject positioning and magnetic field homogeneity (shim) adjustments performed for each recording.

Snapshots of the two-dimensional gradient-recalled echo imaging sequence (12 s duration) used to determine the subject's position and angle [in all three spatial dimensions (sagittal, coronal, axial)] were taken and used for co-registration of subject position across study visits (Fig. 1).

Data processing

All offline image processing was conducted on a SunBlade100 UNIX workstation (Sun Microsystems, Mountain View, CA, USA) using both commercial and custom-written software for the purpose of tissue segmentation and partial-volume analysis. The MRS imaging grid was shifted in the *z* dimension, with the center axial image encompassing the genu and splenium of the corpus callosum. The MRS imaging grid was then shifted in the *x* and *y* dimensions so that two 25 cm³ voxels (effective size) were placed in each region of interest, the ACC, and the comparison region (POC) (Fig. 2). Regions of interest were selected, by a trained rater, with reference to an anatomical atlas (48), and placements were made on the basis of gyral boundaries and structural landmarks that were visible on the MR images (49). Images and coordinates from the post-processing grid shifts used to encompass ACC and

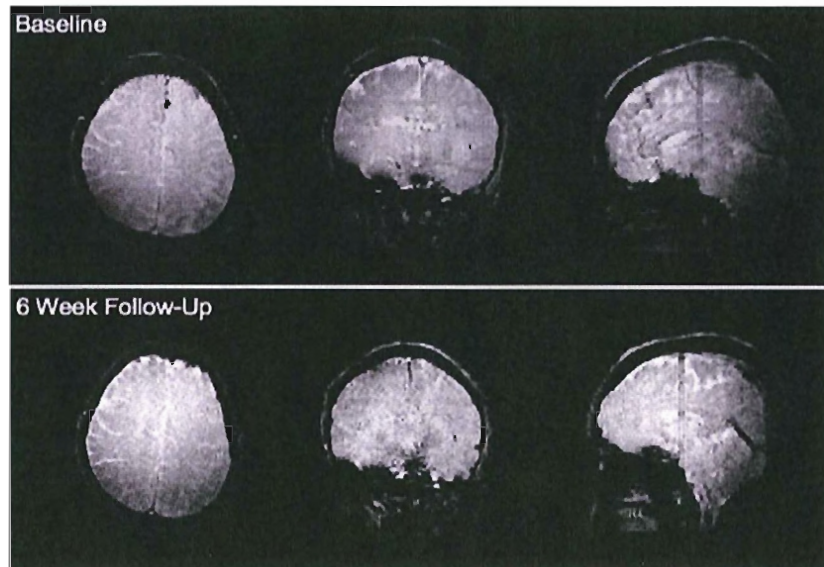


Figure 1. Localizer images used to confirm subject orientation and angle at baseline and 6-week follow-up.

comparison regions from data collected at baseline were used to co-register voxel placement across study visits.

For ^{31}P -MRS imaging spectral analysis, a spectral fitting routine that uses an iterative, non-linear, Marquardt–Levenberg algorithm in combination with prior spectral knowledge was used to fit the acquired spectra precisely. The phosphorus metabolite peaks quantified included individual metabolites within the PME peak (PE and PC) and within the PDE peak (GPE and GPC). The high-energy phosphorus peaks quantified were Pi, PCr, and β -NTP. The

total phosphorus signal (summation of all peaks) was expected to be statistically equivalent between all groups. Thus, each metabolite peak was expressed as percentage metabolite, or the ratio of each peak area divided by the sum total of all peak areas at each visit. The ratio of PCr to Pi was also examined, as this ratio has been used to measure energy at steady-state in isolated mitochondria (50) and in dog brain (51), and is thought to reflect phosphorylation potential (50). Sample *in vivo* ^{31}P brain spectra from 25 cm³ effective voxels in the ACC and POC of a study subject at 4T

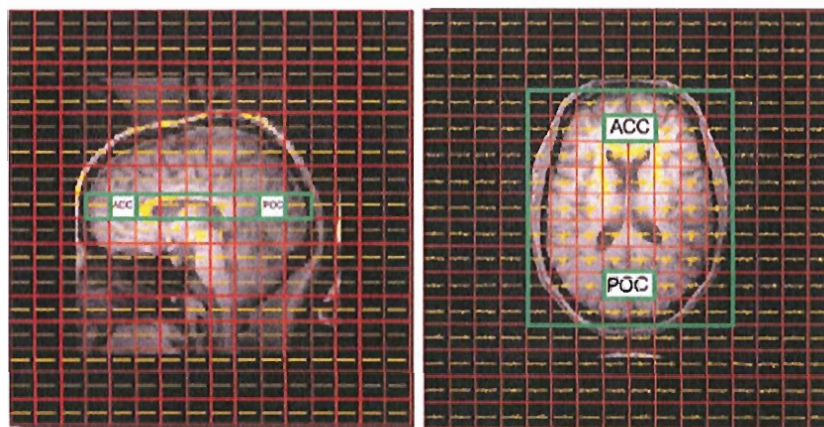


Figure 2. The 3D MRS imaging grid was shifted in the z dimension, using the sagittal image, to align the grid with the top of the corpus callosum. The portion of the MRS imaging grid encompassing the genu and splenium of the corpus callosum (left, sagittal image, outer green box) was then shifted in the x and y dimensions in the axial plane to align the MRS imaging grid (right, axial image, outer green box) with the longitudinal fissure. Two 25 cm³ voxels (effective size) were placed in each of the two regions of interest, the ACC and POC (right, axial image, small green boxes). This figure is available in colour online at www.interscience.wiley.com/journal/nbm

are presented in Fig. 3. Raw data are displayed with modeled fit and residual, with 15 Hz exponential filtering applied for display.

Data analysis

Metabolite data were individually analyzed using 2 (sex) \times 2 (dose: 500 mg/day or 2000 mg/day) \times 2 (visit: baseline and 6 weeks) repeated-measures analysis of

variance (ANOVA). As no significant sex differences, or interactions including sex, were observed, sex was removed as an independent variable in all subsequent analyses. The PCr peak baseline value from one subject (male, high dose) was determined to be an outlier ($>3SD$) and was subsequently removed from the PCr statistical analysis for both regions. The PC baseline value from one subject (male, high dose) could not be fitted and was subsequently not included in the PC statistical analysis for the ACC. All data were tested for violations of sphericity and for *post hoc* testing, and separate repeated-measures ANOVAs were conducted to examine the source of visit \times dose interactions. SPSS V11.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses, with α set at 0.05.

Test-retest reliability

Owing to a lack of a placebo group in this study, spectroscopic data were collected from an additional six healthy subjects (mean \pm SD age 35.7 ± 4.4 years; three female) who did not receive citicoline, on two separate visits. Scan 2 was completed 5.9 ± 0.9 weeks after scan 1. Repeated-measures ANOVAs were conducted, similar to those conducted for subjects who received citicoline supplementation.

RESULTS

Citicoline supplement group

There were no sex or dose differences at baseline for any of the metabolites examined. Total ³¹P area, which served as the denominator for each metabolite ratio, did not differ significantly between visits, between women and men, or between low- and high-dose groups, for either the ACC or POC regions. Mean phosphorus metabolite concentrations and total ³¹P area at baseline and at 6 weeks for the ACC and POC are reported in Table 2.

After 6 weeks of citicoline administration, regardless of dose, significant increases in β -NTP [$F(1,14) = 4.85$, $P = 0.045$] (primarily reflecting concentrations of ATP) and PCr [$F(1,13) = 6.54$, $P = 0.024$] (reflecting the high-energy phosphate buffer stores) were seen in the ACC. There was a significant visit \times dose interaction for change in concentration of Pi in the ACC after 6 weeks of treatment [$F(1,14) = 6.00$, $P = 0.03$]. *Post hoc* analyses revealed that Pi concentrations were significantly reduced in the ACC of subjects who received the 500 mg dose [$F(1,7) = 9.68$, $P = 0.02$; baseline: 0.077 ± 0.012 ; 6 weeks: 0.057 ± 0.023], but not in subjects who received the 2000 mg dose ($P = 0.57$; baseline: 0.069 ± 0.010 ; 6 weeks: 0.073 ± 0.019). No changes in Pi were observed in the POC at either dose. In addition, there was a significant effect of visit [$F(1,13) = 6.34$, $P = 0.03$] and a significant

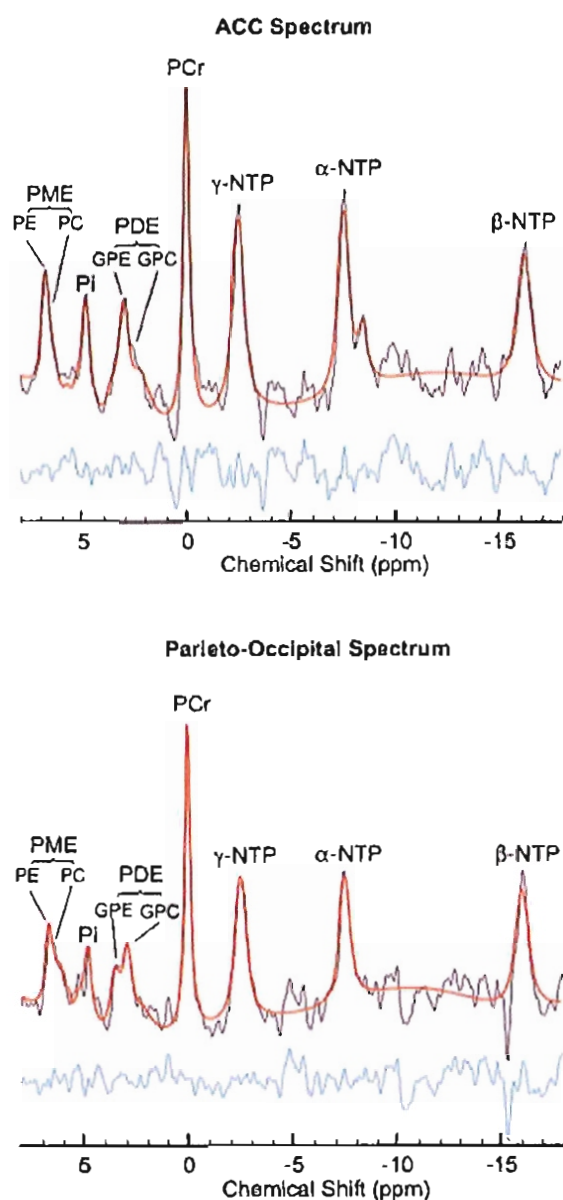


Figure 3. Sample *in vivo* ³¹P brain spectra from 25 cm³ effective voxels in the ACC and POC of a study participant at 4 T. Raw data are displayed with the modeled fit and residual; 15 Hz exponential filtering was applied for display purposes. This figure is available in colour online at www.interscience.wiley.com/journal/nbm

Table 2. Metabolite concentrations at baseline and 6-week follow-up

	ACC			POC		
	Baseline	6 weeks	P value	Baseline	6 weeks	P value
<i>High-energy phosphate metabolites</i>						
PCr	0.154 ± 0.019	0.162 ± 0.015 ^a	0.02	0.164 ± 0.016	0.173 ± 0.015	ns
β-NTP	0.066 ± 0.012	0.075 ± 0.014 ^a	0.05	0.063 ± 0.009	0.066 ± 0.010	ns
Pi	0.073 ± 0.012	0.065 ± 0.022	0.15 ^a	0.072 ± 0.010	0.075 ± 0.013	ns
PCr/Pi	2.17 ± 0.46	2.82 ± 1.04 ^a	0.03 ^b	2.30 ± 0.34	2.38 ± 0.53	ns
<i>Phospholipid membrane anabolites</i>						
PME	0.069 ± 0.016	0.073 ± 0.012	ns	0.088 ± 0.015	0.084 ± 0.011	ns
PC	0.026 ± 0.010	0.019 ± 0.008 ^a	0.02	0.023 ± 0.009	0.026 ± 0.012	ns
PE	0.043 ± 0.015	0.054 ± 0.008 ^a	0.04	0.065 ± 0.009	0.058 ± 0.010	ns
<i>Phospholipid membrane catabolites</i>						
PDE	0.183 ± 0.032	0.154 ± 0.049	0.07	0.165 ± 0.029	0.152 ± 0.030	ns
GPC	0.051 ± 0.015	0.047 ± 0.016	ns	0.047 ± 0.017	0.047 ± 0.010	ns
GPE	0.039 ± 0.010	0.030 ± 0.009 ^a	0.01	0.034 ± 0.011	0.032 ± 0.014	ns
<i>Total phosphorus signal</i>						
	0.366 ± 0.062	0.337 ± 0.061	ns	0.284 ± 0.060	0.295 ± 0.053	ns

^aSignificant change from baseline. Data represent mean ± SD metabolite ratios relative to the total ³¹P signal at each visit.

^bThere was a significant visit × dose interaction ($P = 0.03$). *Post hoc* testing revealed decreased Pi only in the low-dose group: baseline: 0.077 ± 0.012; 6 weeks: 0.057 ± 0.023.

^cThere was a significant visit × dose interaction ($P = 0.04$). *Post hoc* testing revealed increased PCr/Pi only in the low-dose group: baseline: 1.94 ± 0.22; 6 weeks: 3.22 ± 1.22. ns, difference between visits was not significant ($P > 0.05$).

visit × dose interaction [$F(1,13) = 4.98$, $P = 0.04$] for the PCr/Pi ratio in the ACC. *Post hoc* analyses revealed that the PCr/Pi ratio in the ACC had increased significantly in subjects who received the 500 mg dose [$F(1,7) = 10.64$, $P = 0.01$; baseline: 1.94 ± 0.22; 6 weeks: 3.22 ± 1.22], but not in subjects who received the 2000 mg dose ($P = 0.84$; baseline: 2.41 ± 0.52; 6 weeks: 2.33 ± 0.61). No changes in the PCr/Pi ratio were observed in the POC at either dose.

Significant alterations in membrane phospholipids were also observed between baseline and 6 weeks. Although overall concentrations of phospholipid precursors (PMEs) did not differ significantly from baseline in the ACC ($P = 0.53$), there was a significant decrease in concentrations of PC [$F(1,13) = 7.76$, $P = 0.02$] and a significant increase in concentrations of PE [$F(1,14) = 5.23$, $P = 0.04$]. A trend for decreased overall phospholipid catabolites (PDEs) ($P = 0.06$) was observed in the ACC, as well as a significant decrease in GPE from baseline in the ACC [$F(1,14) = 8.41$, $P = 0.01$]. No significant changes in phospholipid membrane metabolites were observed in the POC region of interest.

Effects sizes (f) observed for each of the significant metabolite differences in the ACC were medium to large (52): ↑β-NTP = 0.27, ↑PCr = 0.30, ↓Pi = 0.38, and ↑PCr/Pi = 0.39; ↑PE = 0.28, ↓PC = 0.32, and ↓GPE = 0.32; ↓PDE (trend) = 0.26.

Test-retest group

Mean phosphorus metabolite concentrations and total ³¹P area at scan 1 and scan 2 in test-retest subjects, for the

ACC and POC, are reported in Table 3. Repeated-measures ANOVAs revealed no significant effects of visit ($P > 0.05$) for any of the metabolites examined, in either the ACC or POC, in the test-retest subjects who did not receive citicoline. These findings indicate that the spectroscopic measurements were consistent over a 6-week period, which minimizes the likelihood that the observed metabolite changes after citicoline administration were the sole result of chance or scanner drift.

DISCUSSION

The results of this study are the first to show regionally specific changes in high-energy phosphate and membrane phospholipid metabolites after 6 weeks of citicoline supplementation in healthy middle-aged subjects. The significant citicoline-related metabolite alterations were observed only in the ACC region, some of which were dose dependent, and included increased PCr (↑7%), β-NTP (↑14%), PCr/Pi (↑32%; low dose, ↑66%) and PE (↑26%), and decreased Pi (low dose only, ↓26%), PC (↓29%), and GPE (↓23%). These neurochemical alterations reflect an improvement in brain bioenergetics and synthesis and turnover of phospholipid membranes.

Under steady-state conditions, the rate of ATP synthesis equals the rate of ATP utilization, via suppression of excessive glycolysis and activation of mitochondrial oxidative phosphorylation. In the absence of additional glucose, however, ATP concentrations remain constant, as high-energy PCr serves as a buffer for maintenance of ATP concentrations when turnover is high or synthesis is low (53), as well as a shuttle for

Table 3. Metabolite concentrations in test-retest subjects

	ACC			POC		
	Scan 1	Scan 2	P value	Scan 1	Scan 2	P value
<i>High-energy phosphate metabolites</i>						
PCr	0.156 ± 0.028	0.154 ± 0.026	ns	0.168 ± 0.036	0.165 ± 0.017	ns
β-NTP	0.070 ± 0.011	0.078 ± 0.011	ns	0.066 ± 0.017	0.065 ± 0.015	ns
Pi	0.084 ± 0.012	0.076 ± 0.010	ns	0.069 ± 0.015	0.074 ± 0.024	ns
PCr/Pi	1.89 ± 0.50	1.95 ± 0.16	ns	2.07 ± 0.55	1.97 ± 0.76	ns
<i>Phospholipid membrane anabolites</i>						
PME	0.085 ± 0.017	0.081 ± 0.019	ns	0.084 ± 0.019	0.084 ± 0.018	ns
PC	0.030 ± 0.016	0.024 ± 0.022	ns	0.025 ± 0.018	0.027 ± 0.013	ns
PE	0.055 ± 0.020	0.057 ± 0.018	ns	0.059 ± 0.014	0.057 ± 0.012	ns
<i>Phospholipid membrane catabolites</i>						
PDE	0.170 ± 0.068	0.146 ± 0.057	ns	0.138 ± 0.024	0.168 ± 0.037	ns
GPC	0.045 ± 0.028	0.058 ± 0.013	ns	0.055 ± 0.005	0.051 ± 0.015	ns
GPE	0.038 ± 0.011	0.026 ± 0.020	ns	0.042 ± 0.028	0.040 ± 0.024	ns
Total phosphorus signal	0.337 ± 0.072	0.280 ± 0.072	ns	0.288 ± 0.062	0.281 ± 0.037	ns

Data represent mean ± SD metabolite ratios relative to the total ³¹P signal at each visit. No significant differences were observed between scan 1 and scan 2 (5.9 ± 0.9 weeks after scan 1). ns, difference between visits was not significant ($P > 0.05$).

energy from sites of production to sites of utilization (54,55). Availability of PCr pushes the creatine kinase reaction to generate ATP, through conversion into creatine and high-energy phosphate, at rates that are much faster than oxidative phosphorylation or glycolysis (55). This conversion results in a fall in PCr concentrations while concentrations of ADP and Pi increase to support steady-state concentrations of ATP (50). In this regard, the ratio of PCr relative to Pi has been shown to reflect phosphorylation potential (51). In the present study, citicoline treatment was associated with significant increases in PCr, β-NTP, and the PCr/Pi ratio, as well as decreased Pi (significant at the low dose). The direction of these alterations is consistent with an increase in bioenergetic state, i.e. increased ATP utilization and synthesis (50,56,57). This improvement in bioenergetic metabolism after citicoline treatment may be due, in part, to adaptive modifications of mitochondrial proteins that influence the electron-transport chain, leading to enhancement of cerebral energy transduction (39). Improved energy availability and utilization may also be directly related to increased synthesis and decreased breakdown of phospholipid membranes (43). Reductions in energy metabolism and mitochondrial abnormalities have been shown to be associated with increased phospholipid breakdown, as measured using MRS, in people with Alzheimer's disease (42). Most notably, regionally specific increases in frontal lobe bioenergetics and phospholipid maintenance may contribute to the therapeutic effects of citicoline on memory disturbances by increasing vigilance and working memory capacity, but also by reducing mental fatigue (58). This is consistent with work by Alvarez and colleagues (8), who reported that improvements in the memory performance of older subjects treated with citicoline

were related to facilitation of tissue perfusion and oxygenation, particularly in frontal and temporal regions.

Significant alterations in membrane phospholipids were also observed in this study. Although no overall change in total phospholipid precursors was evident, individual metabolites of the PME peak (PE and PC) changed significantly. Concentrations of PE had increased after treatment, whereas PC concentrations had decreased. This opposite pattern of change was not surprising, given that ethanolamine-containing lipids and choline-containing lipids have unique roles in phospholipid membrane synthesis (35). PE has been shown to be more directly involved in the synthesis of phospholipid membranes, whereas PC contributes more to the synthesis of acetylcholine (35). There was also a trend for a reduction in overall phospholipid catabolites (PDEs, $P = 0.07$) and a significant reduction in the GPE resonance within PDEs, which further supports a citicoline-related change in phospholipid metabolism. These findings are consistent with previous reports that citicoline inhibits phospholipid degradation (7) and enhances synthesis of membrane phospholipids in rat neural tissue and in whole brain (29,59). The present study results differ from those of Babb and colleagues (44), although the data may not be comparable between studies because of differences in the ³¹P-MRS methods used and subject population studied. In the Babb study (44), ³¹P-MRS data were acquired from a large slab of brain tissue using a scanner of lower field strength (1.5 T) from a population of older subjects. In this study, alterations in the building blocks and breakdown products of phospholipid membranes were found to be regionally specific, as citicoline-related changes were observed in the ACC but not in the POC.

There are limitations to this study that merit discussion. First, a placebo control group was not included in the study design, but rather, each subject served as his or her own control, being examined at baseline before supplementation and again after completion of 6 weeks of supplementation. Test-retest reliability data collected from an additional group of healthy subjects, however, demonstrate the stability of the ^4T spectroscopic measurements over a 6-week study period. Thus, it is unlikely that the observed metabolite changes associated with citicoline administration were the sole result of chance or scanner drift. Additional methodological approaches were taken to reduce variability across study visits, including the use of subject placement in the magnet at baseline to guide placement at the follow-up visit and co-registration of regional spectral extractions across study visits using the post-processing grid-shifting capabilities of CSI. There were no significant differences in the total phosphorus signal (summation of all peaks) in either citicoline-treated or test-retest subjects, by region or at either visit. Thus, metabolite concentrations were not confounded by significant differences in the denominator (total phosphorous signal) used to determine metabolite ratios.

A second limitation of this study was the modest sample size. Several significant citicoline-related changes in phosphorus metabolite concentrations were detected, despite a limited number of subjects examined. Furthermore, effect sizes obtained for each of the metabolites that showed significant citicoline-related alterations at the conclusion of the administration period were in the medium to large range (52). Although there was limited power to detect sex or dose effects on phosphorus metabolite concentrations, it is noteworthy that changes in ACC phosphorus metabolites tended to be of greater magnitude in subjects who received the low dose (500 mg) than in those receiving the high dose (2000 mg) (Table 2). Pi concentrations were significantly reduced and PCr/Pi significantly elevated in the ACC at the 6-week follow-up in subjects who received the 500 mg dose, but not in subjects who received the 2000 mg dose. Precursors of endogenous CDP-choline have been shown to increase after exogenous CDP-choline administration. Subsequently, intracellular CDP-choline and PtdCho are synthesized via substrate-dependent activation of unsaturated CTP-phosphocholine cytidyltransferase (CT), the rate-limiting enzyme necessary for the production of endogenous CDP-choline, and choline phosphotransferase enzymes (60). CT reaches a stable level of expression and activity during the initial phase of exogenous CDP-choline administration, followed by enhancement of PtdCho synthesis and activation of CT during prolonged exposure (61). Thus, the low dose may have had a greater influence on phosphorus metabolites than the higher dose, because of a regulatory feedback mechanism that could have inhibited alteration of metabolite concentrations. This mechanism is consistent

with the theory that cellular control systems, which include feedback and feed-forward loops, serve to regulate biological networks (62,63).

Contributions of tissue content to metabolite changes associated with citicoline supplementation were not examined in this study. It is likely that the large voxels (25 cm^3) selected contained both gray and white matter. To this end, previous studies have used linear regression analysis and found higher concentrations of PCr in gray matter and lower concentrations of $\beta\text{-NTP}$ in white matter, in healthy human subjects (64,65). In addition, our interpretation of the observed PME resonance changes should be considered speculative, partly because these metabolites included other prominent phospholipids, e.g. phosphatidylserine, ethanolamine plasmogen, phosphocholine plasmogen, sphingomyelin and rigidly bound PDEs, which cannot be readily quantified *in vivo* (66,67).

In conclusion, this study shows that significant neurometabolic and neurophysiological alterations, i.e. improved frontal lobe bioenergetics and phospholipid membrane turnover, are observed in healthy adults who receive citicoline supplementation for 6 weeks. Furthermore, citicoline-related alterations in brain neurochemistry were regionally specific, targeting a frontal brain region (ACC) implicated in a variety of cognitive functions, including attention and memory. Taken together, these results suggest that citicoline supplementation may mitigate cognitive declines associated with aging by increasing energy reserves and utilization, and by increasing the amount of essential structural components needed to synthesize and maintain cell membranes. Future studies using ^3P MRS are needed to further elucidate the neurobiological mechanisms underlying the therapeutic efficacy of citicoline in reducing cognitive deficits associated with memory loss, dementia, Alzheimer's disease, and other conditions involving cognitive dysfunction, but also to examine the relationships between increases in concentrations of energy metabolites and constituents of membrane synthesis and improvements in cognitive performance.

DISCLOSURES

P.F.R. and D.Y.T. serve as consultants, and T.K. and Y.K. are employees, of Kyowa Hakko Kogyo Co., Ltd, Japan.

Acknowledgements

This study was supported by Kyowa Hakko Kogyo Co., Ltd, Japan.

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