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Carbonic Anhydrase I, II, and VI, Blood Plasma, Erythrocyte and Saliva Zinc and Copper Increase After Repetitive Transcranial Magnetic Stimulation

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Abstract

Introduction—Repetitive transcranial magnetic stimulation (rTMS) has been used to treat symptoms from many disorders; biochemical changes occurred with this treatment. Preliminary studies with rTMS in patients with taste and smell dysfunction improved sensory function and increased salivary carbonic anhydrase (CA) VI and erythrocyte CA I, II. To obtain more information about these changes after rTMS, we measured changes in several CA enzymes, proteins, and trace metals in their blood plasma, erythrocytes, and saliva.

Methods—Ninety-three patients with taste and smell dysfunction were studied before and after rTMS in an open clinical trial. Before and after rTMS, we measured erythrocyte CA I, II and salivary CA VI, zinc and copper in parotid saliva, blood plasma, and erythrocytes, and appearance of novel salivary proteins by using mass spectrometry.

Results—After rTMS, CA I, II and CA VI activity and zinc and copper in saliva, plasma, and erythrocytes increased with significant sensory benefit. Novel salivary proteins were induced at an m/z value of 21.5K with a repetitive pattern at intervals of 5K m/z .

Conclusions—rTMS induced biochemical changes in specific enzymatic activities, trace metal concentrations, and induction of novel salivary proteins, with sensory improvement in patients with taste and smell dysfunction. Because patients with several neurologic disorders exhibit taste and smell dysfunction, including Parkinson disease, Alzheimer disease, and multiple sclerosis, and because rTMS improved their clinical symptoms, the biochemical changes we observed may be relevant not only in our patients with taste and smell dysfunction but also in patients with neurologic disorders with these sensory abnormalities.

Key Indexing Terms

Carbonic anhydrase; Zinc; Copper; Sensory changes after transcranial magnetic stimulation; Mass spectrometry

Investigators have used transcranial magnetic stimulation in patients with many disorders, including neurologic disorders such as stroke, epilepsy, facial palsy, dystonia, multiple sclerosis, Alzheimer disease, Parkinsonism,^{1–16} and mood disorders and schizophrenia.^{17,18}

Associated with this treatment have been reports of changes in several neurotransmitters and neuroactive agents, including dopamine,^{19–25} catecholamines and other biogenic amines,^{26–30} serotonin,^{31,32} gamma-aminobutyric acid (GABA),^{33–35} hormones,^{36–38} other chemical moieties,^{39–48} and interactions among these moieties^{27–29,40,46} in the brain, blood plasma, and saliva.

Abnormalities of taste and smell function have been reported in patients with several of these neurologic conditions, including multiple sclerosis,^{49–53} Alzheimer disease,^{54–58} Parkinson disease,^{59–62} and other neurodegenerative disorders.^{63–64} In patients with Parkinson disease, smell loss has been considered the first sign of the disease,⁶⁰ the more severe the disease process the more severe the smell loss.⁶⁵ Phantosmia (the presence of olfactory distortions in the absence of any environmental odor) has also been reported in Parkinson disease.⁶⁶ Changes in several neurotransmitters and neuroactive substances known to play significant roles in taste and smell function have been reported to occur in some of these disorders. These moieties include dopamine,^{67–71} catecholamines,^{72–75} serotonin,^{76,77} GABA,^{78–87} trace metals,^{88–92} adenylyl cyclases,^{93–98} hormones,^{99,100} other chemical moieties,^{101–104} and interactions among these moieties.^{76,83,85,86}

Repetitive transcranial magnetic stimulation (rTMS) has been used to treat clinical manifestations of patients with multiple sclerosis,^{11–13} Alzheimer disease,^{14,15} Parkinson disease,^{17–20} and other neurologic conditions,^{1–10} and changes in several chemical moieties in the brain, blood plasma, and saliva, which play roles in taste and smell function, have been measured with this therapy.^{19–25,35–38,40} Although clinical changes in taste and smell function have not been reported after rTMS in patients with neurodegenerative disorders, other specific therapies have been reported to be successful in improving taste and/or smell dysfunction in patients with Parkinson disease by some^{105,106} but not by other¹⁰⁷ investigators.

Phantogeusia (presence of taste distortions in the absence of food or drink), phantosmia,^{108–110} and loss of taste and smell acuity have been reported in patients without other neurologic or otolaryngologic abnormalities. We previously demonstrated decreased GABA by using magnetic resonance spectroscopy in specific brain regions in some of these patients, including those with phantogeusia and/or phantosmia.^{87,111} On the basis of these results, we used GABAergic drugs to treat these patients^{111,112}; results indicated that this treatment increased their brain GABA^{88,111} and improved their sensory abnormalities.¹¹² In some of these studies, we observed increased secretion of salivary carbonic anhydrase (CA) VI,¹¹³ a zinc-containing metalloglycoprotein,^{114–116} a putative taste bud and olfactory epithelial growth factor^{91,92,117,118} similar to nerve growth factor.^{92,118–121} CA VI secretion was previously considered a marker for both taste^{91,92,118} and smell function¹²² associated with correction of pathologic apoptotic changes in both taste bud⁹⁰ and olfactory epithelial structures.¹⁰⁹ Thus, we hypothesized that increased CA VI was associated with taste bud and olfactory epithelial stem cell stimulation.¹¹⁰

Because correction of these sensory abnormalities occurred with GABAergic drug treatment and because GABA^{33–35} and other neurotransmitters had been altered with rTMS,^{19–29,39–47} we hypothesized that rTMS treatment might also correct these sensory abnormalities. In preliminary studies in a single-blind placebo-controlled fixed sequence clinical trial, rTMS corrected these sensory abnormalities.¹¹² These results suggested that CA VI might also change after rTMS. Thus, before and after rTMS, we measured CA VI activity and observed changes in this enzyme and in other salivary proteins. On the basis of our previous studies of salivary proteins,¹¹⁵ because CA VI is a zinc containing metalloglycoprotein, and to learn more about these phenomena, we also measured salivary zinc and copper concentrations and changes in other salivary proteins. On the basis of our experience with these patients, we

also hypothesized that little or no change would occur in any peripheral enzyme system. Therefore, we also measured changes in erythrocyte CA I, II and concentrations of zinc and copper in both erythrocytes and blood plasma to test this latter hypothesis.

METHODS

Ninety-three patients, aged 18 to 85 years (52 ± 2 years, mean $\pm$ SEM), 49 men, aged 29 to 74 years (51 ± 3 years) and 44 women, aged 20 to 85 years (53 ± 3 years), with phantogeusia and/or phantosmia, hyposmia (loss of smell acuity), and hypogeusia (loss of taste acuity) were studied before and after rTMS in a single-blind placebo-controlled fixed sequence clinical trial. Studies were performed consistent with a protocol approved by the institutional review board of the George Washington University Medical Center, and each patient agreed to participate in this study.

Patient symptoms persisted for 0.4 to 30 years (6.9 ± 1.5 years) before rTMS. Physical examination of the head and neck, including examination of oral and nasal cavities, was within normal limits. Neither neurologic nor psychiatric abnormalities other than taste and/or smell dysfunction was present in any patient. Anatomical magnetic resonance imaging (MRI) of the brain and electroencephalographic studies were within normal limits.

rTMS was performed with a Cadwell magnetic pulse stimulator (Kennewick, WA) monitored with a TECA TD20 (Pleasantville, NY) waveform generator, as previously briefly described.^{113,114} Stimulation was applied in a fixed manner by using a single circular 5 cm (internal diameter) coil to 4 skull locations (left and right temporoparietal, occipital, and frontal). Stimulus frequency was 1 pulse given per 1 to 3 seconds for 30 to 60 seconds, with 20 pulses given at each location. Repeat stimulation was performed in all patients in whom sensory distortions decreased and/or sensory acuity increased; repetition continued (2–6 applications) until no further decrease in sensory distortions and/or increase in sensory acuity occurred.

One hour before and 1 to 2 hours after completion of rTMS, venous blood and parotid saliva were collected. Venous blood was placed into zinc-free tubes containing 100 μ L of zinc-free heparin, on ice, centrifuged at 3000 rpm at 4°C, plasma removed, and stored at -20°C until assayed. Erythrocytes were washed and treated as previously described.¹²³ Parotid saliva was collected using a modified Lashley cup applied to Stensen duct, with maximal stimulation using reconstituted lemon juice applied to the lingual surface as previously described.^{114,115} Five hundred microliters of saliva was placed in dry ice immediately after collection and stored at -60°C for measurements by surface-enhanced laser desorption/ionization time-of-flight mass analysis vide infra.

Zinc and copper were measured by atomic absorption spectrophotometry by methods previously described^{123,124} using a double-beam ThermoJarrell Ash video 22 (Franklin, MA) atomic absorption spectrophotometry modified by the Maxwell Instrument Company (Salisbury, NC). Saliva protein was determined by measurement of total peptide content by using absorbance at A215–A225 and the extinction coefficient, as previously described.¹¹⁴

CA activity was measured by a modification of the method of Richli et al,¹²⁵ as previously described. CA activity is expressed as enzyme concentration per milligram of enzyme protein.

Saliva samples stored at -60°C were thawed, and 1 μ L was directly spotted on an H4 Protein Chip array (prewashed with 0.1% trifluoroacetic acid in 50% aqueous acetonitrile) and their protein profile examined on a Ciphergen (Fremont, CA) PBS IIc mass analyzer. Samples were first incubated in a humid chamber for 5 to 10 minutes at room temperature,

then washed with 5% aqueous acetonitrile, dried, and 1 μ L matrix was added (sinapinic acid in 0.1% trifluoroacetic acid, 50% aqueous acetonitrile). Samples were allowed to dry again and subjected to SELDI-TOF analysis on the PBS IIc. Protein peaks were characterized by their apparent molecular weight [based on their mass/charge ratio (m/z)].

Following initial observation of biochemical changes after rTMS, subsequent measurements in all biological fluids were performed in a blinded manner; all samples were coded and results uncoded only after analyses were completed.

Mean $\pm$ SEM for each parameter was determined before and after rTMS. Differences were calculated for each parameter, and significance of differences was determined by parametric (differences between undifferentiated means, paired t tests, χ^2) and nonparametric (sign test) statistics; $P < 0.05$ was considered significant for all statistical analyses.

Table 2 summarizes changes in each parameter measured in the total patient group of 93 patients. Two sets of numbers are shown. One set reflects the number of patients in whom there was no change from each parameter value measured comparing after with before rTMS. The other set reflects the number of patients (in percent) in whom each parameter value increased after rTMS compared with before rTMS. If there were any increase post-rTMS compared with pre-rTMS value in any parameter, the value for that parameter was considered increased. If there were no change or any decrease in the post-rTMS compared with the pre-rTMS value in any parameter, the value for that parameter was considered decreased.

RESULTS

After rTMS, mean salivary CA VI activity and salivary zinc and copper concentrations increased significantly as did mean erythrocyte CA I, II activity and plasma copper concentrations (Table 1).

Significant increases in both CA I, II and CA VI activity were also measured [paired comparisons ($P < 0.01$, Student t test), sign test ($P < 0.05$, Student t test) (data not directly shown)]. These latter data are reflected in a summary of these results shown in Table 2 in which total changes before and after rTMS are shown with respect to the number of patients (in percent) who experienced either an increase or decrease in the parameters measured in Table 1 (see Methods section for details). Increased CA VI was measured in 90% of patients with changes varying from >0 to $+153\%$ (mean change, $+17\%$) (Table 2); compared with chance, changes of this frequency and magnitude would occur <5 times in 1000 (χ^2), $P < 0.005$. Increased CA I, II was measured in 93% of patients with changes varying from >0 to $+56\%$ (mean change, $+11\%$) (Table 2); compared with chance, changes of this frequency and magnitude would also occur <1 time in 1000, $P < 0.001$ (χ^2). Increased plasma and erythrocyte zinc and copper concentrations were similarly measured in 92% of patients (Table 2); compared with chance, changes of this frequency and magnitude would also occur <1 time in 1000, $P < 0.001$ (χ^2).

SELDI-TOF mass spectrometry revealed a peak at m/z 21.5K in the post-rTMS spectra, which was absent in the pre-rTMS spectra (Figure 1). Also present in the post-rTMS spectra was a repetitive protein pattern separated by intervals of approximately 5K m/z (Figure 1). Similar patterns were observed in about 1/3 of patients studied. Molecular characteristics of these peaks are under investigation.

DISCUSSION

Results indicate that after rTMS, activity of CA VI, CA I, II, and concentrations of zinc and copper in blood plasma, erythrocytes, and saliva increased and induction of novel salivary proteins occurred in patients with phantogeusia and/or phantosmia and loss of taste and smell acuity. These changes accompanied the rTMS-induced sensory improvement in these patients.¹¹²

rTMS in patients with neurologic disorders have been previously observed to improve several aspects of sensory function, including tinnitus inhibition,^{34,126–129} increased visual excitability,¹³⁰ improved picture recognition,¹³¹ increased sensitivity to cutaneous stimuli,¹³² and other aspects of cognition.^{133–136} Other treatment techniques have been reported to improve smell acuity,^{105,106} smell recognition,¹⁰⁶ and phantosmia¹⁰⁵ in patients with Parkinson disease. As noted earlier, rTMS has been used to treat several neurologic disorders, including multiple sclerosis,^{11–13} Alzheimer disease,^{14,15} and Parkinson disease,^{17–20} and changes in several neurotransmitters and neuroactive moieties in the brain, blood serum, and saliva accompanied this procedure.^{19–46} Clinical changes have been measured after a single exposure to rTMS.^{44,137} The current studies extend these observations to patients with taste and smell distortions and loss of taste and smell acuity without other neurologic findings.

Increased enzyme activities and zinc and copper concentrations we observed after rTMS had a rapid onset that persisted for 2 to 10 weeks after treatment (data not shown). However, over time, in some patients, there was a slow, gradual decrease in enzyme activity in saliva and in erythrocytes and in saliva and plasma zinc and copper concentrations associated with return of sensory distortions and loss of sensory acuity. A second rTMS treatment (results not included) increased all CA activities and trace metal concentrations to or above levels measured after initial rTMS with decreased sensory distortions and improvement of sensory acuity; both the biochemical and the sensory changes after the second rTMS were more robust and longer lasting than those measured after the first rTMS.

The rapid biochemical changes after rTMS may relate to increased brain GABA, which we previously observed in patients treated with GABAergic drugs and measured by magnetic resonance spectroscopy,¹¹⁰ consistent with increased GABA function observed by others after rTMS.^{33–35} However, rTMS has increased many neurotransmitters and neuroactive moieties,^{19–46} which suggest that changes in several moieties may contribute to this effect.

Rapid changes in zinc, which we observed after rTMS, may occur because zinc has been associated with GABA and GABA receptors^{138–142} and in other neurotransmitter¹⁴³ pathways. Zinc may act as a modulator of neuronal activity¹⁴³ related to its high concentration in synaptic vessels of zinc-containing neurons in anterior brain associated with its interaction with both GABA and glutamate.^{142–145} Both zinc and copper have been reported to play roles in both physiology and pathology in Parkinson disease,^{146–151} multiple sclerosis,^{152–157} and Alzheimer disease^{158–166} and have induced behavioral changes in Parkinson disease.¹⁶⁷

Increased CA VI after rTMS may relate to direct stimulation of parotid gland CA VI after application of the magnetic field. However, because CA is a well-known constituent of brain parenchyma,^{168,169} primary sensory neural afferents,¹⁷⁰ and interactions with GABAergic events,^{171,172} several factors may influence this CA VI increase.

Increased salivary zinc after rTMS is consistent with increased CA VI because zinc is the major metal in CA VI. Mechanisms by which salivary copper increased are not clear, although copper metalloproteins have been previously implicated in both taste and smell

function.^{173–175} Increased erythrocyte CA I, II after rTMS may have been initially unexpected because there was no a priori reason to assume that rTMS would induce changes in any peripheral tissue. However, CA II has been found in brain parenchyma,¹⁷⁶ and CA II messenger RNA has been found in brain neurons and glia.¹⁷⁷ Thus, some factors secreted from brain may have induced release of CA I, II. Increased erythrocyte zinc after induction of CA I, II is understandable because CA I, II are the major zinc metalloenzymes in erythrocytes. Mechanisms by which plasma zinc and erythrocyte copper increased after rTMS are also unclear. Zinc in plasma is loosely bound to albumin and more tightly bound to α_2 -macroglobulin^{178,179}; perhaps, rTMS induced some factors that initiated release of zinc from its major binding sites^{180–183} in muscle or liver into plasma and its subsequent binding to circulating seroproteins. The majority of plasma copper is bound to ceruloplasmin.¹⁸⁴ Although this protein was not measured directly, increased plasma copper reflects increased ceruloplasmin,¹⁸⁴ suggesting induction of this metalloprotein after rTMS. Induction of heretofore unidentified salivary proteins also occurred. Although we have previously identified the major proteins in saliva,¹¹⁵ the proteins induced by rTMS described in this work have not been previously observed; the multiple peaks of the induced proteins may reflect isomers of the same induced protein (Figure 1).

Mechanisms by which biochemical effects occur after rTMS may be either direct, indirect, or a combination of these effects. Direct effects could include action of magnetic fields, which may induce cooperative signal transduction cascades¹⁸⁵ and thus differentially¹⁸⁶ alter dipole characteristics of neurons,¹⁸⁷ thereby inducing signal amplification¹⁸⁸ and changes in cellular membrane permeability,¹⁸⁸ cellular transport, ion channel activity, or receptor affinity.^{189,190} Direct effects may also include rTMS stimulation of VIIth cranial nerve, which might induce increased salivary gland CA VI secretion but not stimulation of erythrocyte CA I, II. Exposure to magnetic fields has been reported to increase ornithine decarboxylase activity,^{191,192} cAMP,¹⁹³ GFAP levels,¹⁹⁴ catecholamines,²⁸ lipid second messengers,¹⁹⁵ and 5HT_{1B} receptors in brain.¹⁹⁶ Some of these changes could be associated with increased cellular activity¹⁸⁸ or associated with gene activation.¹⁹⁷ Increased synthesis of glycoaminoglycans in cartilaginous bone matrix was also reported after magnetic field exposure¹⁹⁸. Indirect effects could include release of highly charged moieties such as GTP, ATP, Mg²⁺,¹⁸⁹ or substances such as Na⁺ K⁺ ATPases which could, through signal amplification,¹⁸⁶ induce differential dipolochemical activation¹⁷⁸ and cooperative polyelectrolyte organization¹⁸⁶ involving molecular hysteresis phenomena.¹⁹⁹ Another possible indirect effect might initiate release of cytokines, chemokines, or other chemical signal transducers that could act via membrane signal transduction.^{200–203}

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FIGURE 1.

Mass spectroscopic analysis of parotid saliva of patients after repetitive transcranial magnetic stimulation (rTMS). m/z is plotted on the abscissa, relative intensity on the ordinate. Salivary proteins with an m/z value of 21.5K with repeating patterns at intervals of 5K were induced.

TABLE 1

Changes in erythrocyte carbonic anhydrase (CA) I, II, saliva CA VI, and saliva, erythrocyte, and plasma zinc and copper before (pre) and after (post) repetitive transcranial magnetic stimulation (rTMS) in patients with sensory distortions and decreased sensory acuity

Condition	rTMS	
	Pre	Post
Plasma		
Zn ($\mu\text{g/dL}$)	85 ± 2	88 ± 2
Cu ($\mu\text{g/dL}$)	101 ± 2	109 ± 3^a
Erythrocytes		
Zn ($\mu\text{g/g Hb}$)	38.0 ± 0.5	39.7 ± 0.5^b
Cu ($\mu\text{g/g Hb}$)	2.1 ± 0.04	2.2 ± 0.05
CA I, II ($\mu\text{g/mg protein}$)	3.12 ± 0.06	3.45 ± 0.06^d
Saliva		
Zn ($\mu\text{g/L}$)	103 ± 5	121 ± 5^b
Cu ($\mu\text{g/L}$)	13 ± 1	22 ± 3^c
CA VI ($\mu\text{g/mg protein}$)	0.153 ± 0.009	0.197 ± 0.008^d

Data are presented as mean $\pm$ SEM.

Compared with pre-rTCMS:

^a $P < 0.05$

^b $P < 0.025$

^c $P < 0.01$

^d $P < 0.001$.

TABLE 2

Comparison of patient number in whom changes in erythrocyte carbonic anhydrase (CA) I, II, saliva CA VI, and saliva, erythrocyte, and plasma zinc and copper either increased or decreased after repetitive transcranial magnetic stimulation (rTMS) compared with before rTMS in patients with sensory distortions and decreased sensory acuity

Condition	Post- vs. Pre-rTMS	
	Number decreased	Percent increased
Plasma		
Zn ($\mu\text{g/dL}$)	8	91
Cu ($\mu\text{g/dL}$)	7	92
Erythrocytes		
Zn ($\mu\text{g/g Hb}$)	1	99
Cu ($\mu\text{g/g Hb}$)	7	92
CA I, II ($\mu\text{g/mg protein}$)	7	92
Saliva		
Zn ($\mu\text{g/L}$)	25	73
Cu ($\mu\text{g/L}$)	23	75
CA VI ($\mu\text{g/mg protein}$)	9	90