

Detection of an Antioxidant Profile in the Human Brain In Vivo Via Double Editing With MEGA-PRESS

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Vitamin C (ascorbate) and glutathione (GSH) are the two most concentrated non-enzymatic antioxidants in the human brain. Double editing with (DEW) MEGA-PRESS at 4T was designed in this study to measure both antioxidants in the same amount of time previously required to measure one. In the occipital lobe of four human subjects, resolved ascorbate (Asc) and GSH resonances were detected repeatedly and simultaneously using DEW MEGA-PRESS. The Asc and GSH concentrations measured using LCModel analysis of DEW MEGA-PRESS spectra were 0.8 ± 0.1 and 1.0 ± 0.1 $\mu\text{mol/g}$ (mean \pm SD), with average Cramer-Rao lower bounds (CRLB) of 10% and 7%, respectively. Aside from the effects of *J*-modulation at a common echo time (TE), double editing did not compromise sensitivity. To determine the extent to which the oxidized forms of Asc and GSH contribute to DEW MEGA-PRESS spectra in vivo, chemical shifts and coupling constants for dehydroascorbate (DHA) and oxidized glutathione (GSSG) were measured at physiologic pH and temperature. DHA does not contribute to the 3.73 ppm DEW MEGA-PRESS Asc resonance. GSSG contributions to the DEW MEGA-PRESS GSH resonance (3.0 ppm) are negligible under physiologic conditions, and would be evidenced by a distinct GSSG resonance (3.3 ppm) at exceptionally high concentrations. Magn Reson Med 56: 1192–1199, 2006. © 2006 Wiley-Liss, Inc.

Key words: antioxidant profile; ascorbate (vitamin C, Asc); glutathione (GSH); magnetic resonance spectroscopy (MRS)

Vitamin C (ascorbate, Asc) and reduced glutathione (GSH) are the most concentrated non-enzymatic antioxidants in the central nervous system (CNS) (1). Compartmentation of Asc predominantly to neurons and GSH predominantly to glia (1) suggests that sufficient levels of both are important for protection against oxidative damage. On the other hand, the capacity for Asc and GSH to regenerate each other (2), and the presence of transport mechanisms between neurons and glia (3) suggest that the sum of these concentrations may be the best indicator of antioxidant capacity. Because vitamin E, the other important non-

enzymatic antioxidant, is membrane bound and present at micromolar concentrations, it is not detected in vivo. However, the adequacy of vitamin E antioxidant defenses is likely reflected by Asc and GSH levels, since Asc and GSH have been shown to regenerate and spare vitamin E (4). Therefore, the simultaneous quantification of Asc and GSH (i.e., the antioxidant profile) may be a suitable non-invasive assay for determining tissue antioxidant capacity. This assay could be used to study: the role of antioxidants in neuroprotection, factors that impact brain antioxidant levels, and the role of reactive oxygen species in acute and chronic mechanisms of CNS damage.

Homonuclear edited ^1H MRS has been used extensively to separate a single *J*-coupled resonance of interest, such as γ -aminobutyric acid (GABA), reduced GSH, or lactate from overlapping resonances (5,6). Recently, MEGA-PRESS edited spectroscopy was similarly applied to uncover a resolved resonance from ascorbate (Asc) in the human brain in vivo (7). The goal of the present study was to measure resolved resonances of both GSH and Asc in the same amount of time previously required to measure one. Concurrent detection of Asc and GSH was attempted via extension of the difference editing principle. Instead of omitting the frequency-selective editing pulses (“off”) on alternate scans, we encoded additional information by inserting editing pulses selective for a second *J*-coupled spin system. As such, the traditional “on”/ “off” scheme was replaced by a novel “on A”/ “on B” scheme. That is, the refocused spectrum for each of two edited neurochemicals served as a reference spectrum for the other. Our previously described MEGA-PRESS editing schemes for separate detection of GSH and Asc (7,8) were readily combined for double editing with (DEW) MEGA-PRESS.

Although the concentrations of oxidized glutathione (GSSG) and dehydroascorbate (the oxidized forms of GSH and Asc, respectively) are assumed to be below the detection threshold of in vivo ^1H MRS under physiologic conditions, it is of interest to understand the extent to which resonances from these compounds could contribute to DEW MEGA-PRESS spectra under nonphysiologic conditions. GSSG is 50 times less concentrated than GSH under normal conditions (9,10). Dehydroascorbate (DHA) is relatively unstable at physiologic pH and temperature (11). Asc undergoes one-electron oxidation to form ascorbyl radical, and a second one-electron transfer to produce DHA (12). Because it is a free radical, ascorbyl is present at extremely low concentrations (~ 10 – 20 nM) in biological fluids (13) and far below the ^1H MRS detection threshold in living organisms. Biochemical process efficiently con-

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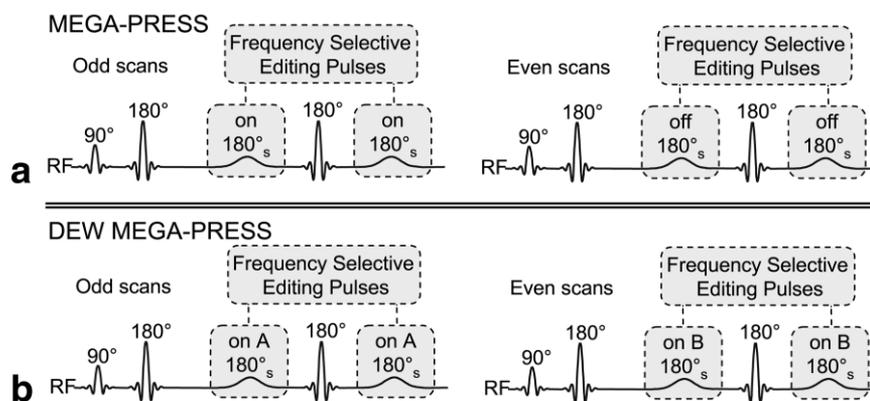
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FIG. 1. **a**: RF portion of the MEGA-PRESS timing diagram (15). The main editing components are frequency-selective inversion pulses. The difference-edited spectrum is obtained by subtracting “on” scans from “off” scans. **b**: RF portion of the DEW MEGA-PRESS timing diagram as designed for difference editing of two J -coupled spin systems. Frequency-selective editing pulses are applied to refocus a second J -coupled system in the case of double editing, as opposed to remaining “off.” The DEW MEGA-PRESS spectrum is obtained by subtracting “on A” scans from “on B” scans.



vert DHA back to Asc before it is rapidly degraded to diketogulonic acid with a half-life of about 6 min (14).

MATERIALS AND METHODS

DEW MEGA-PRESS

Double editing for Asc and GSH was achieved by adapting MEGA-PRESS (15) as illustrated in Fig. 1. The key to double editing was replacement of the editing pulse (180°_s) “on”/“off” pattern typical of difference editing by an “on A”/“on B” scheme (where “A” symbolizes Asc, and “B” symbolizes GSH). In detail, the 3.73 ppm $^6\text{CH}_2$ Asc resonance was refocused during every other scan by applying a 40-ms Gaussian RF pulse at 4.13 ppm such that the chemical shift of the coupled 4.01 ppm ^5CH was well within the bandwidth (39 Hz) of the editing pulse, as previously described (7). The second resonance chosen for observation, the cysteine $\beta\text{-CH}_2$ of GSH (2.95), was refocused on alternate scans by applying a 40-ms Gaussian pulse at 4.56 ppm, the chemical shift of the coupled cysteine $\alpha\text{-CH}$ resonance, as previously described for GSH editing (8) except at the longer TE optimal for Asc detection (TE = 112 ms). The double-edited spectrum was obtained by subtracting the GSH refocused spectrum (“on B”) from the Asc refocused spectrum (“on A”). To enable comparison of double-edited spectra with constituent single-edited resonances, pure reference spectra (180°_s “off”) were also measured *in vitro* and *in vivo*. To estimate the efficiency of Asc and GSH signal recovery, both DEW MEGA-PRESS and pulse-acquire spectra were simulated (16) using MATLAB software (Mathworks, Natick, MA, USA), measured chemical shifts and J -couplings for Asc (see below), and previously reported chemical shifts and J -couplings for GSH (17). Simulated resonances were line-broadened (7 Hz) to mimic *in vivo* line shapes before efficiency was calculated. The optimum TE was determined by measuring MEGA-PRESS spectra from Asc and GSH solutions at TE spanning those previously utilized for MEGA-PRESS editing of GSH (8) and Asc (7). Shorter editing pulses accommodated measurement at shorter TE.

All experiments were performed with a 4 T, 90-cm bore magnet (Oxford Magnet Technology, Oxford, UK) interfaced to a Varian INOVA spectrometer (Varian, Palo Alto, CA, USA) equipped with gradients capable of switching to

40 mT/m in 400 μs (Sonata, Siemens, Erlangen, Germany), and a surface ^1H quadrature transceiver (18).

Quantification

Asc and GSH concentrations were quantified using LCModel analysis (19) of DEW MEGA-PRESS spectra (8) between 1.0 and 3.82 ppm. The basis spectra for Asc, myo-inositol (Ins), lactate (Lac), phosphorylcholine (PC), phosphorylethanolamine (PE), and glycerophosphorylcholine (GPC) were measured previously at 112-ms echo time (TE) for MEGA-PRESS editing of Asc from pure metabolite solutions at physiologic pH and temperature. A MEGA-PRESS metabolite-nulled (20) experiment at 4 T provided a basis spectrum for macromolecules (MMs). The basis spectrum for glycerophosphorylethanolamine (GPE) was estimated by shifting the measured GPC spectrum such that the GPC resonance at 3.67 ppm appeared at 3.29 ppm, a previously reported (21) chemical shift for GPE. The GSH basis spectrum was measured using DEW MEGA-PRESS at physiologic pH and temperature. The N-acetylaspartate (NAA) basis spectrum for DEW MEGA-PRESS was simulated. Global frequency referencing of the *in vivo* edited spectrum was based on GSH and Asc model spectra. The integral intensity of the singlet at 2.01 ppm in the sum of the “on Asc” and “on GSH” scans, corresponding to the acetyl moiety of NAA, was used as an internal reference for quantification, similar to a previously described method (22). The *in vivo* NAA concentration was referenced at 10 $\mu\text{mol/g}$ based on previous findings (17).

Human Subjects and Protocol

Four volunteers (one male and three females, average age = 23 years) gave informed consent for this study, which was conducted according to procedures approved by the Institutional Review Board. The subjects were positioned supine inside the magnet with the RF transceiver subjacent to their occipital lobe. The protocol for each volunteer began with localizer multislice rapid acquisition with relaxation enhancement (RARE) images (TR = 4.0 s, TE = 60 ms, echo train length (ETL) = 8, matrix = 256 \times 128, two averages, slice thickness = 2 mm, and five slices) to select a cubic volume of interest (VOI, 3 \times 3 \times 3 cm^3) centered on the midsagittal plane in the occipital lobe.

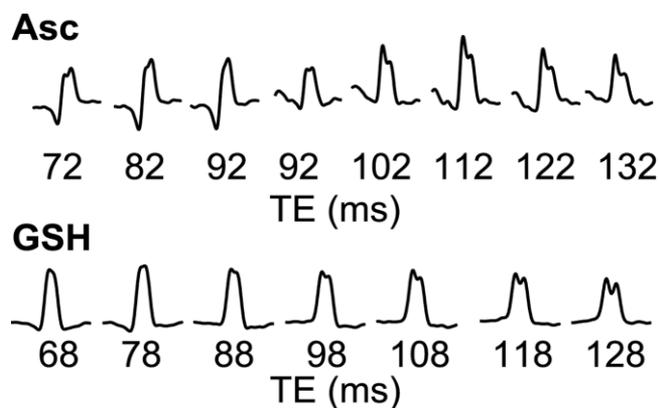


FIG. 2. MEGA-PRESS edited Asc (3.87–3.60 ppm, left to right) and GSH (3.09–2.82 ppm, left to right) resonances measured in respective phantom solutions at the TEs listed. The first three Asc resonances (72–92 ms) were measured with 20-ms editing pulses (Gaussian throughout), and the remainder were measured with 30-ms editing pulses. All GSH resonances were measured with 20-ms editing pulses. Phase was optimized via singlet resonances in the respective reference spectra.

Shimming of all first- and second-order coils was achieved using FASTMAP with EPI readout (23,24). Edited spectra were measured as described above (TE = 112 ms, NEX = 512, TR = 4.5 s) from the 27-mL VOI in 38 min. The entire

scan time was approximately 1 hr per subject. For the editing pulses, RF power (B_1) was optimized by placing the offset of the editing pulse at the water frequency and incrementing B_1 until water signal was minimized (15). Each acquisition (NEX = 1) was stored separately in memory and then frequency- and phase-corrected based on the NAA methyl signal prior to summation (20). Scans during which editing pulses were “off,” “on A,” and “on B” (NEX = 1) were interleaved.

High-Resolution ^1H NMR Spectroscopy

^1H NMR spectra of Asc, GSH, DHA, and GSSG were measured at physiologic pH (~ 7.2) and temperature (37°C) using a high-resolution 600 MHz Varian INOVA spectrometer equipped with a 5-mm probe. ^1H chemical shifts and coupling constants were estimated from measured spectra and then adjusted until simulated spectra visually matched measured spectra. Measured chemical shifts and coupling constants were used in conjunction with spectral simulation to predict which signals (if any) would be contributed by DHA and GSSG to the DEW MEGA-PRESS spectra. GSSG and DHA were purchased from Sigma-Aldrich (catalog numbers G-4376 and 261556). Compounds were dissolved in phosphate-buffered water (600 μL). D_2O containing TSP (100 μL) was added for locking and referencing. Water suppression was used, and baseline correction (based on spline fit) was applied. Compounds were

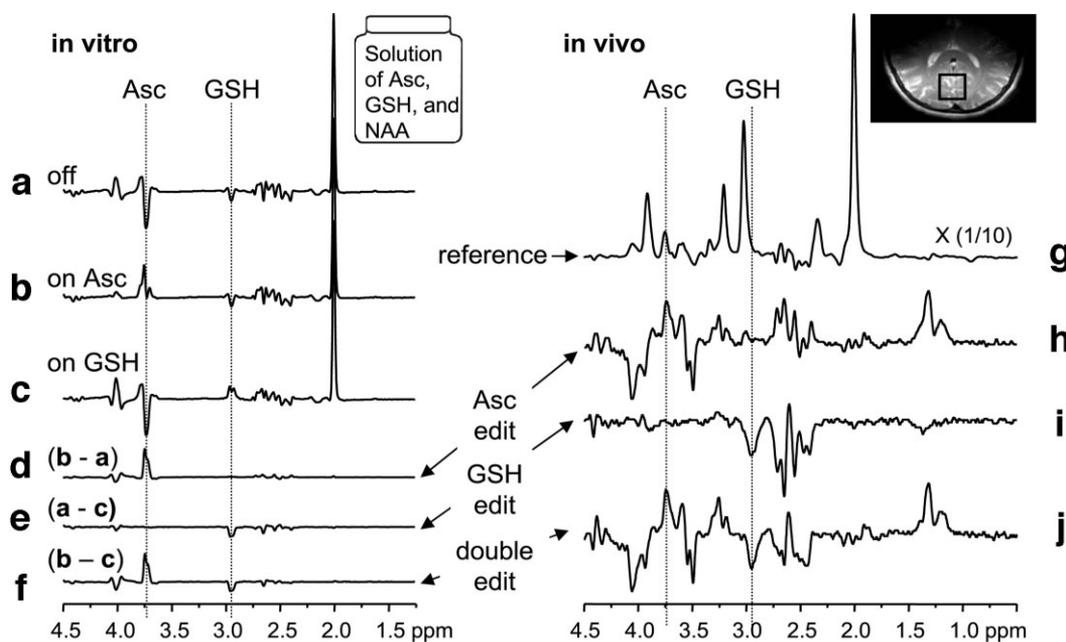


FIG. 3. Illustration of single and double editing via reference (no 180°_s applied), refocused (TE = 112 ms, NEX = 64, TR = 4.5 sec, VOI = 27 cm^3 , 40-ms Gaussian editing pulse bandwidth = 39 Hz FWHM) and edited (subtracted) spectra from a phantom containing 35.5 mM Asc, 13.5 mM GSH, and 35.5 mM NAA and in vivo. **a**: Reference or editing pulse “off” in vitro. **b**: Editing pulse “on Asc,” or applied at 4.13 ppm for editing of Asc in vitro. **c**: Editing pulse “on GSH,” or applied at 4.56 ppm for editing of GSH in vitro. **d**: Standard MEGA-PRESS edit for Asc in vitro (spectrum b minus spectrum a). **e**: Standard MEGA-PRESS edit for GSH in vitro (spectrum c minus spectrum a). **f**: DEW MEGA-PRESS spectrum (spectrum b minus spectrum c) obtained by subtracting “on GSH” (applied at 4.56) from “on Asc” (applied at 4.13 ppm) for simultaneous editing of Asc and GSH in vitro. **g**: In vivo reference spectrum for DEW MEGA-PRESS acquired from the VOI illustrated on the image. **h**: MEGA-PRESS edited Asc in vivo. **i**: MEGA-PRESS edited GSH in vivo. **j**: Asc and GSH DEW MEGA-PRESS spectrum in vivo. For all in vivo spectra, TE = 112 ms, TR = 4.5 s, and NEX = 512, and 40-ms Gaussian editing pulses were applied. In vivo coedited resonances were previously identified (7,8).

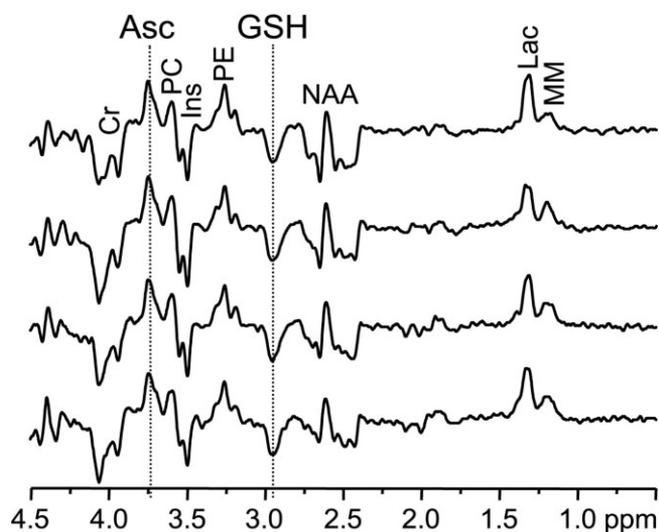


FIG. 4. Asc and GSH DEW MEGA-PRESS spectra measured in all four volunteers studied (TE = 112 ms, TR = 4.5 s, NEX = 512).

measured immediately after preparation to minimize oxidation and degradation. Because DHA decomposes rapidly at physiologic pH and temperature, DHA spectra were measured at a low temperature to substantially reduce the rate of degradation (11). To determine whether DHA chemical shifts and coupling constants varied with temperature and pH, and to separate DHA from degradation products, the DHA sample was prepared and neutralized (pH = 7.25) on ice; scanned at 5°C, 15°C, 26°C, and 37°C in the magnet; and then neutralized (pH = 7.23) at 37°C and remeasured. To achieve a higher signal-to-noise ratio (SNR) for DHA at physiologic pH and temperature, a second fresh DHA sample was quickly prepared at room temperature (pH = 7.11) and then measured at 37°C. To predict the extent to which GSSG would contribute to double-edited MEGA-PRESS spectra at 4T while avoiding expenditure of additional GSSG and scan time, GSSG spectra were simulated under DEW MEGA-PRESS. Accuracy of the simulation was verified using solution spectra.

RESULTS

The dependence of MEGA-PRESS edited Asc and GSH resonance patterns on TE is illustrated in Fig. 2. The optimum edited Asc resonance at 112 ms was a rationale for setting the TE to 112 ms for double editing (7). The intensity of the GSH resonance measured at a TE of 118 ms was approximately 75% as high as that measured at 68 ms, the optimum TE previously reported for GSH editing at 4 T in vivo (8). Appreciable GSH signal was therefore expected at a TE of 112 ms in vivo, provided that the T_2 relaxation was typical of neurochemicals detected in vivo.

Figure 3 illustrates DEW MEGA-PRESS spectra measured from a phantom containing Asc and GSH. Application of an editing pulse at 4.13 ppm refocused the 3.73 ppm Asc resonance (Fig. 3b, “on Asc”) relative to when the editing pulse was “off” (Fig. 3a). Similarly, application of an editing pulse at 4.56 ppm refocused the 2.95 ppm GSH resonance (Fig. 3c, “on GSH”) relative to

when the editing pulse was “off” (Fig. 3a). Figure 3f shows the double-edited spectrum, i.e., the result of subtracting the “on GSH” (Fig. 3c) spectrum from the “on Asc” spectrum (Fig. 3b). The edited Asc ($^6\text{CH}_2$ at 3.73 ppm) and GSH (cysteine $\beta\text{-CH}_2$ at 2.95 ppm) resonances remained in the double-edited spectrum (Fig. 3f), whereas resonances that lacked coupling partners in the bandwidth of the editing pulses (NAA methyl at 2.01 ppm) did not. In this fashion, GSH and Asc were designed to be resolved from overlapping resonances in vivo. For comparison with double editing, Fig. 3d and e show single-edited Asc and GSH spectra measured as previously reported (7,8). The peak intensities and areas of the double-edited Asc and GSH resonances (Fig. 3f) were equivalent to those of the respective single-edited Asc (Fig. 3d) and GSH (Fig. 3e) resonances. The 3.77 ppm GSH singlet was inverted relative to the 2.95 ppm multiplet in the reference spectrum, and appeared as a shoulder to the left of the 3.73 ppm Asc resonance (Fig. 3a). The small 4.01 ppm Asc resonance coedited with single-edited GSH (Fig. 3e) as a result of partial inversion of a coupling partner at 4.50 ppm, within the bandwidth of the editing pulse applied at 4.56 ppm. The efficiency of Asc signal recovery via DEW MEGA-PRESS relative to pulse acquire (TE = 0) without accounting for T_2 relaxation was 52% based on the integrated area, or 69% based on the maximum resonance intensity. Overall efficiency of GSH signal recovery was 54% based on the integral area, and 62% based on intensity.

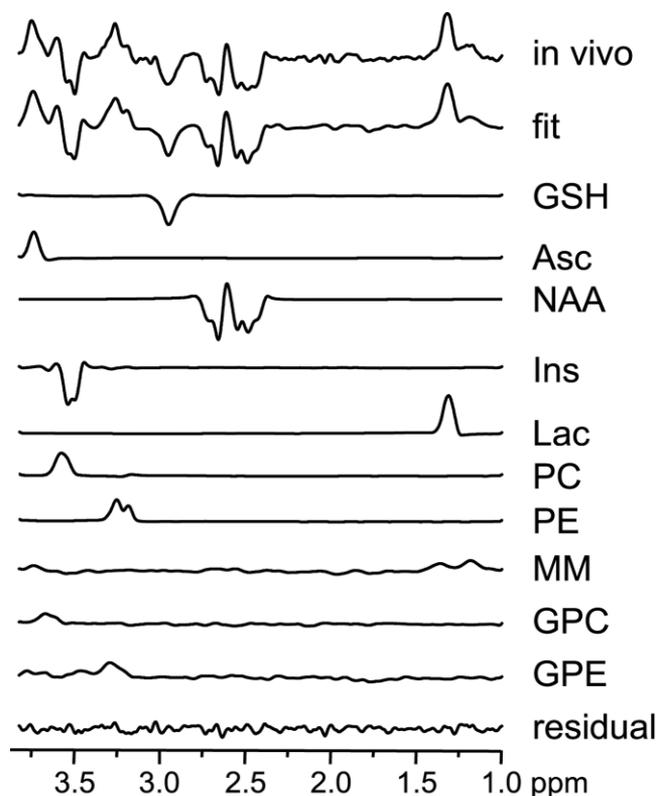


FIG. 5. LCMoel analysis of representative in vivo spectrum. In vivo data, LCMoel fit, and fit residual (bottom). Basis spectra: GSH, Asc, NAA, myo-inositol (Ins), lactate (Lac), phosphorylcholine (PC), phosphorylethanolamine (PE), macromolecules (MM), glycerophosphorylcholine (GPC), and glycerophosphorylethanolamine (GPE).

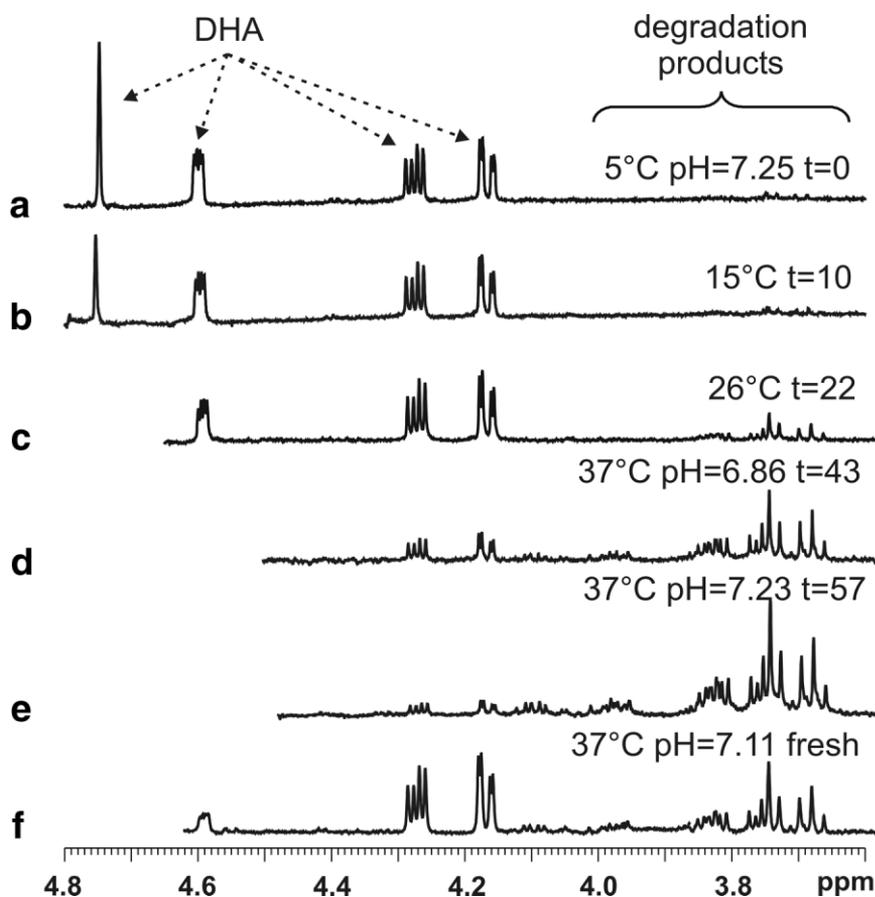


FIG. 6. High-resolution ^1H NMR spectra (5-mm probe, 600 MHz) of DHA dissolved in water showing degradation of DHA and appearance of degradation products with increasing temperature. Spectra were measured sequentially (time, t in min) at (a) 5, (b) 15, (c) 26, and (d) 37°C (four scans, TR = 30 s). e: The sample was neutralized at 37°C and remeasured (four scans, TR = 30 s). f: A fresh DHA sample was prepared at room temperature (pH = 7.11) and measured (16 scans, TR = 36 s).

A representative Asc and GSH double-edited spectrum measured in a human brain *in vivo* is illustrated in Fig. 3j. The strong methyl NAA singlet measured in the reference spectrum *in vivo* (Fig. 3g) was used to phase edited spectra and to set the NAA chemical shift at 2.009 ppm. Single-edited spectra (Fig. 3h and i) were measured *in vivo* for authentication of double-edited resonances. The Asc and GSH double-edited spectrum measured in a human brain *in vivo* (Fig. 3j) retained a resonance from GSH (2.95 ppm) in addition to all coedited resonances expected from Asc editing, as previously described (7) and as illustrated in the single-edited Asc spectrum *in vivo* (Fig. 3h). The single-edited *in vivo* GSH spectrum (Fig. 3i) did not retain coedited resonances with frequencies in the vicinity of the edited Asc resonance, nor did the single-edited *in vivo* Asc spectrum (Fig. 3h) exhibit coedited resonances with frequencies in the vicinity of the edited GSH resonance, which resulted in resolved double editing of these antioxidants *in vivo* (Fig. 3j). The peak intensities and areas of the double-edited Asc and GSH resonances (Fig. 3j) were equivalent to those of the respective single-edited Asc (Fig. 3h) and GSH (Fig. 3i) resonances. The similarity of edited resonance patterns measured *in vitro* (Fig. 3f) and *in vivo* (Fig. 3j) supports assignment of *in vivo* edited resonances to GSH and Asc. The remarkable repeatability of resonances detected using DEW MEGA-PRESS *in vivo* (Fig. 4) indicates that double-edited and coedited resonances were contributed by neurochemicals as opposed to random artifacts.

LCModel analysis of a representative *in vivo* spectrum is shown in Fig. 5. A close match between the LCModel fit and the DEW MEGA-PRESS spectrum was achieved, as evidenced by the noise-predominated fit residual. Asc was quantified at $0.84 \pm 0.11 \mu\text{mol/g}$ (mean \pm SD), with an average Cramer-Rao Lower Bound (CRLB) of 9.75%, corresponding to $0.08 \mu\text{mol/g}$. GSH was quantified at 1.02 ± 0.09 with an average CRLB of 6.5%, corresponding to $0.07 \mu\text{mol/g}$. The simulated NAA basis spectrum closely resembles the corresponding DEW MEGA-PRESS resonance measured in a phantom containing NAA (Fig. 3f) and *in vivo* (Fig. 3j), verifying accuracy of simulation.

Figure 6 illustrates the DHA spectra measured at 600 MHz (5-mm probe). The spectrum measured at 5°C (Fig. 6a) clearly shows DHA resonances from $^6\text{CH}_2$ (4.16 and 4.27 ppm), ^5CH (4.6 ppm), and ^4CH (4.8 ppm). The increase in chemical shifts of DHA resonances relative to Asc is in agreement with deshielding of DHA protons by carbonyl groups and with a previously reported spectrum (25). At low temperature, the residual water peak was shifted to the left of the spectral region shown (Fig. 6a and b), which permitted observation of the ^4CH (4.8 ppm). At higher temperatures ($>25^\circ\text{C}$, Figs. 6c–f), the ^4CH resonance was suppressed along with water (~ 4.7 ppm). Decreasing SNR for DHA resonances with increasing temperature (and time), as shown in Fig. 6a–e, is evidence of DHA degradation. Concurrent buildup of resonances in the region spanning 3.6–4.1 ppm is evidence of accumulation of degradation products. Figure 6e illustrates that

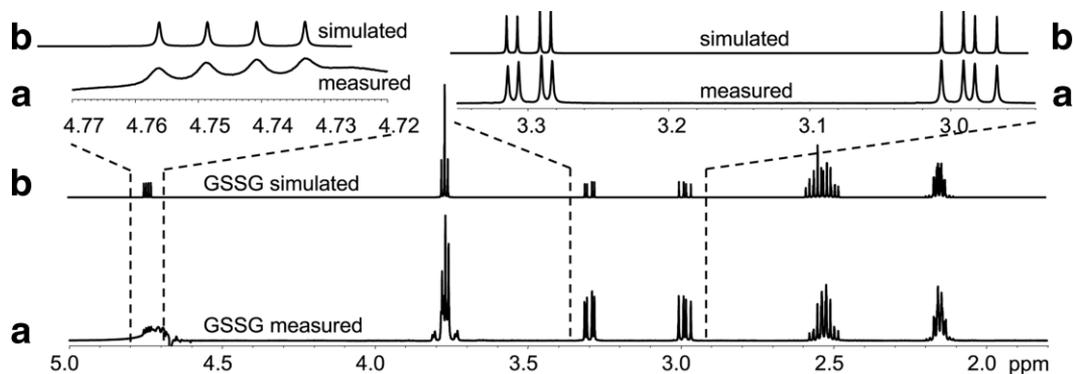


FIG. 7. (a) Measured (5-mm probe, 600 MHz, 16 scans, TR = 36 s, pH = 7.15, 37°C) and (b) simulated GSSG spectra used to determine chemical shifts and coupling constants (see Table 1).

the chemical shifts and coupling constants for DHA do not change substantially between pH 6.7 and 7.2 (Fig. 6d and e). Given this evidence, the resonances at 4.17 (doublet of doublets) and 4.27 (doublet of doublets) in the DHA spectrum measured at physiologic pH and temperature (Fig. 6f) are assigned to $^6\text{CH}_2$ DHA, while the resonances in the 3.6–4.1 ppm range are assigned to degradation products.

Figure 7 illustrates the GSSG spectrum measured (5-mm probe, 600 MHz) at physiologic pH and temperature, as well as the simulated spectrum used to determine the chemical shifts and J -coupling constants. Details are shown for the cysteine protons, since they are located on the moiety detected in vivo using DEW MEGA-PRESS. Asc and GSH chemical shifts and coupling constants determined from spectra simulated analogously (not shown) were in agreement with previous reports (17,26). The chemical shifts and coupling constants determined from the high-resolution GSSG, DHA, Asc, and GSH spectra are reported in Table 1.

Figure 8 illustrates Asc, GSH, and GSSG resonances simulated under DEW MEGA-PRESS (Figs. 8a–c, equimolar) along with analogous resonances measured using DEW MEGA-PRESS in solution (Fig. 8d) and in vivo (Fig. 8e).

Table 1
Chemical Shifts and Coupling Constants Determined by Matching Simulations and High-Resolution ^1H NMR Spectra

Metabolite (moiety)	Proton ID	Chemical shift (ppm)	Coupling constant (Hz)
GSH (cysteine)	1	4.5608	$J_{1,2} = 4.71$
	2	2.9747	$J_{1,3} = 7.09$
	3	2.9264	$J_{2,3} = -14.06$
GSSG (cysteine)	1	4.7408	$J_{1,2} = 4.6$
	2	3.2954	$J_{1,3} = 9.3$
	3	2.9840	$J_{2,3} = -14.2$
Asc	1	4.4965	$J_{1,2} = 2.055$
	2	4.0072	$J_{2,3} = 5.78$
	3	3.7469	$J_{2,4} = 7.373$
	4	3.7194	$J_{3,4} = -11.585$
DHA	1	4.754	
	2	4.598	$J_{2,3} = -5.40$
	3	4.275	$J_{2,4} = 2.40$
	4	4.169	$J_{3,4} = -10.50$

The simulated (Fig. 8a and b) and measured (Fig. 8d) Asc and GSH resonances are in excellent agreement, illustrating accurate simulation. Accurate simulation was also verified using solutions of coediting compounds (not shown). Using the offsets and coupling constants measured above (Table 1) for GSSG, the signal that would be contributed by GSSG is illustrated in Fig. 8c. Simulated spectra (Fig. 8a–c) are shown to scale, so the intensity shown for GSSG would be contributed by approximately 1 mM GSSG (the same as GSH in vivo), which is far more concentrated than expected in a live organism. A concentration of 0.2 $\mu\text{mol/g}$, which is 10 times greater than under physiologic conditions, would only contribute one-fifth of the signal intensity shown.

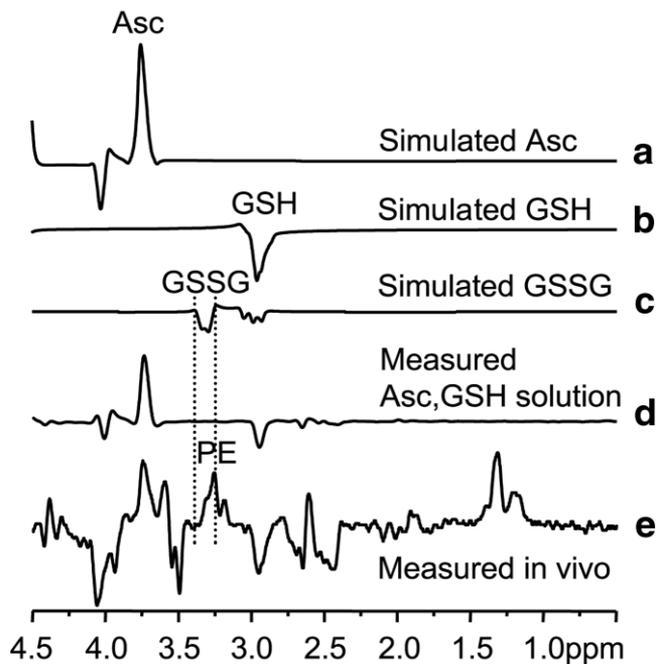


FIG. 8. Simulated equimolar (a) Asc, (b) GSH, and (c) GSSG DEW MEGA-PRESS spectra and measured DEW MEGA-PRESS edited spectra in (d) a solution of 13.5 mM GSH, 35.5 mM Asc and 35.5 mM NAA, and (e) in vivo ($B_0 = 4\text{T}$, TE = 112 ms, TR = 4.5 s, NEX = 512).

DISCUSSION

DEW MEGA-PRESS was achieved for noninvasive quantification of the two most concentrated non-enzymatic antioxidants in the human CNS in the same amount of time previously required to measure one. Double-edited Asc and GSH resonances were as well resolved as respective single-edited resonances measured previously at 4 T (7,8). The sensitivity of double editing at a TE of 112 ms was the same as that observed for editing of each compound separately at the same TE. Sensitivity of GSH detection using double editing was estimated at 75% of that achieved using MEGA-PRESS at an optimal TE for GSH detection (Fig. 2) without accounting for T_2 relaxation. Asc and GSH concentrations and CRLB measured by means of LCModel analysis of DEW MEGA-PRESS spectra were in good agreement with previous findings (7,8).

Because frequency separation between the detected $^6\text{CH}_2$ Asc resonance (3.73 ppm) and the coupled ^5CH (4.01 ppm) increases with increasing field, the editing efficiency for Asc may improve with increasing field and might become difficult at lower field. It is important to note, however, that the feasibility of double editing at different field strengths will depend on the existence of a mutually beneficial TE. Since spectral dispersion varies with field strength while coupling constants (J) do not, coupled resonance patterns manifest differently at different field strengths. These effects are confounded when resonances from more than one non-equivalent proton overlap *in vivo*, as is the case for both Asc and GSH. To retain a practical advantage similar to that provided by difference editing for one compound, the phase and chemical shift offset of DEW MEGA-PRESS spectra can be referenced using intense singlet resonances in either of the subspectra (i.e., “on A” or “on B”).

For double editing, the time previously expended by difference editing methods to measure a reference spectrum was utilized to generate information specific to an additional resonance of interest. As a result, two target resonances were resolved in the same amount of time previously required to resolve one. In contrast to prior applications developed to intentionally retain intense, noncoupled resonances in edited spectra (27–31) or to suppress an undesirable resonance (32), double editing was designed to intentionally resolve two coupled systems from overlapping resonances in one edited spectrum. Double editing was previously applied *in vitro* for simultaneous spectral editing of GABA and taurine using double-banded pulses with double quantum coherence transfer (DQC) (33). As noted therein Ref. 33, several interesting metabolites with proton-proton coupling constants of approximately 7 Hz are suitable candidates for double editing. As also demonstrated by the DQC application therein Ref. 33, double editing can be implemented with schemes other than MEGA-PRESS. To test the versatility of DEW MEGA-PRESS, it was implemented with double-banded pulses (as opposed to the alternating scheme) in the present study. The only change in the double-edited Asc and GSH spectrum was that the resonances from the two compounds were in phase (results not shown). Feasibility of double editing for compounds other than Asc and GSH with editing modalities other than MEGA-PRESS would

depend on editing efficiency at pertinent TEs for the compounds of interest, as well as overlap among edited and coedited resonances. Optimal performance of double editing also depends on sufficient frequency separation between coupled resonances (i.e., the chemical shifts at which the editing pulses are applied). However, coediting in single-edited experiments, while generally considered a nuisance, is common, and may be attempted in cases in which coupled resonances do not have sufficient separation for double editing.

In cases of weak coupling, the TE that gives rise to the optimum edited signal strength can be determined by J , the coupling constant between the resonance targeted for detection and the resonance inverted by the editing pulse (5). The optimum TE was determined experimentally in this study for two reasons. First, the resonance pattern that arose from both compounds targeted for observation (3.73 ppm Asc and 2.95 ppm GSH) arose from nonequivalent protons in CH_2 groups. For example, the signal from GSH at 2.95 ppm was comprised of a resonance at 2.926 ppm and an unresolved resonance at 2.975 ppm. The coupling constants between these protons and the one at 4.56 ppm were not the same (7.09 and 4.71 Hz, respectively) (17). Second, determination of the optimum TE involved a compromise between signal loss due to T_2 decay and signal loss due to incomplete refocusing.

Although the small resonances of Asc and GSH present in short-TE stimulated-echo acquisition mode (STEAM) spectra have been detected using deconvolution software such as LCModel to uncover them, they are not resolved from overlapping neurochemical resonances, and visual affirmation of accurate quantitation as such is absent. Recently we showed that GSH concentrations as quantified using STEAM at 4 T were within error of those quantified using edited spectra measured from the same VOI in the anterior cingulate of 10 human volunteers (34). However, the range of GSH concentrations encountered in that study was not sufficient for establishing a correlation between the two measurement techniques. In a previous study (22) we showed agreement in Asc concentrations measured using ultrashort-TE STEAM and MEGA-PRESS edited spectroscopy in matched regions of interest (ROIs) in the developing rat brain at 9.4 T. In that study the addition of Asc to the LCModel basis set systematically increased or decreased concentrations of some other metabolites quantified from STEAM spectra. This discrepancy reflects the fact that while CRLBs provide a suitable estimate of measurement error, low CRLBs do not guarantee completeness and accuracy of the LCModel basis set. Since the ability to distinguish significant differences in human brain Asc and GSH concentrations using LCModel analysis of STEAM spectra at 4 T has not yet been verified, DEW MEGA-PRESS remains a suitable means of ensuring that these antioxidants are resolved from overlapping contributions.

The GSSG and DHA spectra measured at 600 MHz allowed us to determine the extent to which these compounds coedit with GSH and Asc *in vivo*. Since DHA does not contribute a resonance in the vicinity of 3.73 ppm, the chemical shift of the Asc resonance observed via DEW MEGA-PRESS, DHA does not contribute to the edited Asc signal measured *in vivo*. Given the results of the simulation (Fig. 8), contributions from GSSG to double-edited

(Asc and GSH) MEGA-PRESS spectra are negligible *in vivo*. Furthermore, since the GSSG resonance at 3.3 ppm is larger than that at 2.98 ppm, it would influence the shoulder of the DEW MEGA-PRESS coedited PE resonance *in vivo* (Fig. 8e, 3.25 ppm) if it were present at appreciable levels. Therefore, exceptionally high levels of GSSG would be evidenced by the GSSG resonance at 3.3 ppm *in vivo*.

CONCLUSIONS

In this application, two resonances that did not coedit by coincidence were measured simultaneously without sacrificing resolution and with minor sensitivity loss. Two important antioxidants, Asc and GSH, were resolved and measured simultaneously in the human brain, resulting in highly repeatable detection of an antioxidant profile *in vivo*. Double editing can theoretically be extended to edit for additional compounds and implemented with modalities other than difference editing. *In vivo* Asc and GSH double-edited MEGA-PRESS spectra contained no contamination from DHA and negligible contamination from GSSG. If exceptionally high levels of GSSG were present, they would be evidenced by a resonance distinct from the edited GSH resonance *in vivo*.

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