Intracellular Redox State Revealed by ^{31}P MR Spectroscopy Measurement of NAD$^+$ and NADH Contents *In Vivo*

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Introduction As an important coenzyme, nicotinamide adenine dinucleotide (NAD) plays key roles in energy metabolism in all living cells (1). The conversion between its reduced form (NADH) and oxidized form (NAD$^+$) occurs in all redox reactions catalyzed by NADH-dependent dehydrogenase, including the major metabolic pathways in glycolysis, TCA cycle and electron transport chain. Intracellular redox state (RX) defined by the NAD$^+$/NADH ratio is considered reflecting the cellular metabolic status and function, and it fluctuates in response to alterations in metabolism, such as aging, diabetes and cancer (2). To date, there have been only two invasive approaches available for assessment of the intracellular RX: the biochemical analysis using tissue/cell extraction and the auto-fluorescence detection of NADH (3). A non-invasive approach for directly measuring RX has been unavailable. In this study, we developed a novel ^{31}P MRS-based method for non-invasive quantification of NAD$^+$ and NADH contents *in vivo*. Our results indicated that the ^{31}P MR signals of NAD$^+$ and NADH could be identified and quantified to determine their absolute contents in cat brains at high magnetic field. NAD$^+$ and NADH have similar T_1 values in normal cat brains at either 16.4T or 9.4T. For the first time, the feasibility of direct measuring NAD$^+$ and NADH contents *in vivo* was revealed in this study. The same approach could readily be applied to study healthy or diseased human brains and other organs.

Theory and Method The molecular structure of NAD$^+$ is identical to that of NADH except for the positive charge on the nitrogen atom in the nicotinamide. However, this positive charge discriminates the nuclear shielding of the two phosphorus atoms in the NAD$^+$. Thus, according to NMR principle, NAD$^+$ is a two-spin system and its ^{31}P spectrum exhibits a quartet resulting from the J coupling effect, whereas NADH presents a singlet. Since the frequency separation between the two phosphorus spins of NAD$^+$ was found to be similar in magnitude to the J_{P-P} coupling constant (see Figure 1), NAD$^+$ yields a second-order coupling spectrum. Based on the NMR theory of second order-effect and chemical shift information extracted from the high-resolution spectrum of NAD$^+$ solution obtained at 11.7T (Figure 1A&B), spectrum prediction of NAD$^+$ can be made at any given field strength via Lorentzian simulations (Figure 1B). Thus, a quantification model for description of resonance signals of NAD$^+$, NADH and α-ATP at a magnetic field strength of interest through linear addition of Lorentzian functions was developed. By least-square fitting of the model outputs to the resonance signals obtained from *in vivo* ^{31}P spectrum, signal intensity and linewidth of each phosphorus peak can be determined. The cerebral contents of NAD$^+$ and NADH were quantified by normalizing their peak integrations to that of α-ATP, which has a stable, known pool size of ~2.8 mM in normal brain. All the *in vivo* ^{31}P MRS measurements were conducted on either a 9.4T/31 cm or a 16.4T/26 cm horizontal bore magnet interfaced to VNMRJ console. Surface-coil localized *in vivo* ^{31}P MR spectra of cat brains were acquired at either 162 MHz (for 9.4T, N=6) or 283 MHz (for 16.4T, N=7) using single-pulse-acquire sequence with 16 s (9.4T) or 3~16 s (16.4T) repetition time, 5.2 kHz (9.4T) or 8 kHz (16.4T) spectral width, 0.058 s (9.4T) or 0.064 s (16.4T) acquisition time, and 64 (9.4T) or 40~64 (16.4T) signal averages. Raw FID signals were converted into frequency domain spectra by Fourier transformation following the application of an exponential filter with 10 Hz line broadening to enhance SNR. *In vivo* T_1 values of NAD$^+$ and NADH were measured in cat brains at fully relaxed conditions using inversion recovery pulse sequence at both field strengths.

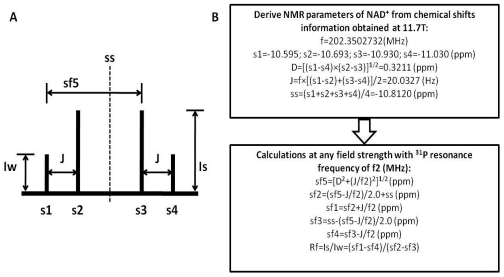

Figure 1. Diagram of two-spin system for the NAD$^+$ quartet (A) and flow chart for spectrum prediction at any given field strength (B).

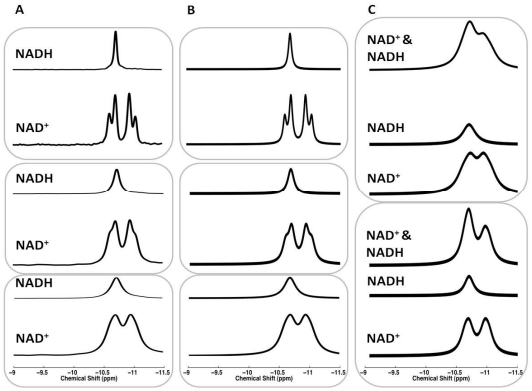

Figure 2. ^{31}P spectra of phantom solutions at 11.7T (A) and model simulations at 11.7T (B), 9.4T and 16.4T (C)

Results As shown in Figure 2A&B, model simulations of NAD$^+$ and NADH resonance signals were highly comparable to the ^{31}P NMR spectra of their phantom solutions at 11.7T. NAD$^+$ signal evolved from quartet to doublet when increasing line broadening (from top to bottom in Figure 2A) or linewidth (from top to bottom in Figure 2B) values were applied. Figure 2C illustrated simulations to mimic the *in vivo* spectra of NAD$^+$ and NADH at both 9.4T (upper) and 16.4T (lower panel). Representative surface-coil localized *in vivo* ^{31}P spectra of normal cat brains were shown in Figure 3. All the resonance signals of NAD$^+$, NADH and α-ATP were satisfactorily fitted at both field strengths, as reflected by the small residues between the original spectra and model fittings. RX (2.6~3.0) and absolute contents of NAD$^+$ (0.38~0.39 mM), NADH (0.14~0.15 mM) and total NAD (0.52~0.54 mM) were obtained. As shown in Figure 4, our results indicated similar T_1 values of NAD$^+$ and NADH in normal cat brains at either magnetic field (1.58 s vs. 1.50 s at 9.4T; 0.82 s vs. 0.87 s at 16.4T).

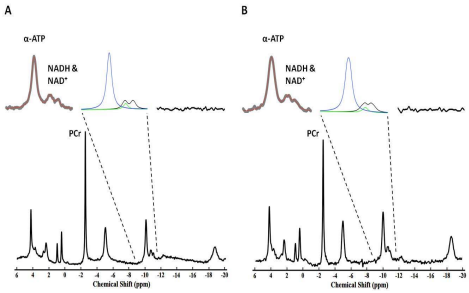

Figure 3. Representative ^{31}P MR spectra of normal cat brains at 16.4T (A) and 9.4T (B).

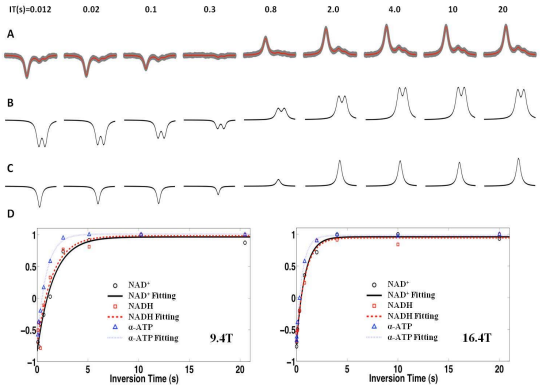

Figure 4. *In vivo* T_1 measurements. (A): Model fittings (red) to the summarized spectra of 5 cat brains (gray) from different inversion time (IT) at 16.4T; (B) and (C): Model decomposed signals of NAD$^+$ and NADH from (A); (D): Exponential model fittings at 9.4T (left) and 16.4T (right).

Conclusion The new ^{31}P MRS approach allows direct and reliable measurements of NAD$^+$ and NADH contents *in vivo*. For the first time, it provides an opportunity to non-invasively investigate the role of RX in brain functions and diseases. Also, it could readily be applied to study healthy or diseased human brains and other organs.

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(1) Berger, F., et al. (2004) *Trends Biochem Sci.* 29, 111-118. (2) Ying, W. H. (2006) *Front Biosci.* 11, 3129-3148. (3) Chance, B., et al. (1962) *Science*. 137, 499-508