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Detection of Cerebral NAD⁺ in Humans at 7 T

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Abstract

Purpose—To develop ¹H-based MR detection of nicotinamide adenine dinucleotide (NAD⁺) in the human brain at 7 T. To validate the ¹H results with NAD⁺ detection based on ³¹P MRS.

Methods—¹H MR detection of NAD⁺ was achieved with a 1D double-spin-echo method on a slice parallel to the surface coil transceiver. Perturbation of the water resonance was avoided through the use of frequency-selective excitation. ³¹P MR detection of NAD⁺ was performed with an unlocalized pulse-acquire sequence.

Results—Both ¹H and ³¹P MRS allowed the detection of NAD⁺ signals on every subject in 16 min. Spectral fitting provided a NAD⁺ concentration of 107 ± 28 μM for ¹H MRS and 367 ± 78 μM and 312 ± 65 μM for ³¹P MRS when uridine diphosphate glucose (UDPG) was excluded and included as an overlapping signal, respectively.

Conclusions—NAD⁺ detection by ¹H MRS is a simple method, but comes at the price of reduced NMR visibility. NAD⁺ detection by ³¹P MRS has a near-complete NMR visibility, but is complicated by spectral overlap with NADH and UDPG. Overall, both ¹H and ³¹P MR methods offer exciting opportunities to study NAD⁺ metabolism on human brain *in vivo*.

Keywords

NAD⁺; human brain; ¹H MRS; ³¹P MRS; 7 Tesla

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD⁺) and its reduced form NADH, are cofactors of energy producing pathways present in all tissues, including the brain. In addition to its well-known role in energy metabolism and the maintenance of reduction-oxidation potential

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(NAD⁺/NADH ratio), NAD⁺ also functions as a substrate with roles in gene transcription and repair, homeostasis, cell signaling and the synchronization of metabolic pathway fluxes and metabolite levels via circadian oscillation (1–3).

Despite the importance of NAD⁺ and NADH metabolism the available detection methods were, until recently, limited. Analysis of tissue extracts with various analytical methods, including NMR and MS, provide high sensitivity and specificity, but are incompatible with *in vivo* studies on the human brain (4–6). Autofluorescence of NADH coupled with confocal microscopy allows direct *in vivo* application, but is complicated by limited tissue penetration and the inability to detect NAD⁺ (7). More recently ³¹P MRS (8,9) and ¹H MRS (10) have been proposed to non-invasively detect NAD⁺ and NADH in the human (9) and animal (8,10) brain.

The ³¹P NMR signal of the total NAD pool (i.e. NAD⁺ plus NADH) has been observed since the early days of *in vivo* ³¹P NMR. However, the separation of the heavily overlapping NAD⁺ and NADH ³¹P NMR signals was deemed impossible. The availability of (ultra) high field magnetic fields together with the use of spectral fitting routines makes it possible to disentangle the NAD⁺ and NADH contributions to the observed ³¹P NMR signals. With this novel ³¹P NMR method the concentrations of NAD⁺ and NADH, and thus the redox state, have been established in the normal, aging human brain (9).

The ¹H detection of NAD⁺ has been elusive due to polarization exchange between NAD⁺ and water, whereby destruction of the water signal through standard water suppression techniques indirectly also suppresses the NAD⁺ signal. Recently a ¹H MRS detection method for NAD⁺ was described during which water perturbation is avoided by frequency-selective excitation. In combination with high magnetic field strength and the absence of spectral overlap, ¹H MRS allowed reliable detection of NAD⁺ on the rat brain *in vivo* (10).

Here we describe a modification of the ¹H MRS method to allow detection of NAD⁺ in human brain at 7 T. NAD⁺ signals are quantified with spectral fitting using the *N*-acetyl aspartate (NAA) amide or total creatine (tCr) methyl signals as internal concentration references. NAD⁺ detection and quantification based on ¹H MRS is compared to the ³¹P MRS method. ³¹P MR spectra are acquired with a pulse-acquire method and quantified with spectral fitting using NAD⁺, NADH, ATP, PCr and uridine diphosphate glucose (UDPG). The presence of UDPG is confirmed on rat brain and is demonstrated to be a significant contribution to the combined NAD⁺ plus NADH signal.

METHODS

General

MR measurements were performed on a 7 Tesla actively shielded, 68 cm inner diameter MR magnet interfaced to a Direct Drive spectrometer (Agilent, Santa Clara, CA, USA) and equipped with an asymmetric gradient system (Agilent, Santa Clara, CA, USA) capable of switching 50 mT/m in 512 μ s. The gradient system included a full set of third order shim coils. ¹H MR spectra were acquired with a single 80 mm diameter surface coil tuned to the proton frequency (298.1 MHz), whereas ³¹P MR spectra were acquired with a single 80 mm

diameter surface coil tuned to 120.66 MHz. MR imaging, shimming and ^1H decoupling were performed with a pair of 160 mm diameter surface coils driven in quadrature (11).

Six human subjects (5 men, 1 woman, age 39 ± 12 years) were scanned. All subjects participated in both ^1H and ^{31}P MR studies, in two sessions separated by between 2 and 10 days. All subjects were studied in accordance with Yale Institutional Review Board guidelines for research on human subjects.

***In vivo* ^1H NMR spectroscopy**

The detection of NAD^+ by ^1H MRS requires the use of short echo-time methods to minimize T_2 -related losses and the avoidance of water perturbation to eliminate saturation transfer effects on NAD^+ . The sequence previously employed on rat brain was composed of 3D LASER localization with an echo-time of 14 ms (10). A direct translation to the human brain at 7 T is not feasible as limitations in maximum and average RF amplitudes would lengthen the LASER echo-time to a minimum of 50 ms. As a result the pulse sequence was transformed into a 1D LASER method ($\text{TR}/\text{TE} = 1,500/18$ ms) selecting a 20 mm slice parallel to the surface coil (Fig. 1A) through the occipital cortex. Slice selection was achieved with a pair of 2 ms adiabatic full passage (AFP, hyperbolic secant modulation (12), bandwidth = 5.0 kHz) refocusing pulses. Spatial localization in the X and Z directions are provided by the human head anatomy. The actual brain volume over all volunteers amounted to 96 ± 12 mL as evaluated from multi-slice MR images. The brain volume was not corrected for the coil reception profile. The contribution from muscle was $< 5\%$ on all volunteers. Significant lipid signal from skull and scalp tissue was present, but was inconsequential for NAD^+ detection as lipids do not have NMR resonances beyond 5.5 ppm. Signal excitation was achieved with a minimum-phase 8.0 ms Shinnar-Le Roux pulse (13) of 0.775 kHz bandwidth centered on 8.8 ppm. As water is minimally perturbed, no additional forms of water suppression were required. ^1H MR spectra were acquired as 2,048 complex points over a 12 kHz spectral width with $\text{NA} = 640$ for a total acquisition time of 16 min.

Absolute quantification of NAD^+ requires an internal concentration reference. In addition to the NAA amide singlet at ~ 7.85 ppm, total creatine was also pursued as a concentration reference. Since the lipids signals from skull and scalp overwhelm the spectrum at $\text{TE} = 18$ ms, creatine spectra were acquired at 50, 75, 100, 150 and 200 ms. Following spectral fitting the creatine intensity at $\text{TE} = 0$ ms was obtained via extrapolation.

***In vivo* ^{31}P NMR spectroscopy**

^{31}P NMR experiments were performed with a pulse-acquire sequence ($\text{TR} = 5,000$ ms) in an unlocalized manner. The measured brain volume was estimated at 286 ± 54 mL, not corrected for the coil reception profile. The square excitation pulse angle was adjusted to give the highest phosphocreatine (PCr) signal. ^{31}P MR spectra were acquired as 2,048 complex points over a 12 kHz spectral width with $\text{NA} = 192$ for a total acquisition time of 16 min. On two subjects ^1H decoupling was applied during signal acquisition. Decoupling was achieved by 8 ms AFP pulses (HS4 modulation (14), 1.25 kHz bandwidth, $\gamma B_1 = 0.3$ kHz) incorporated into a 20-step supercycle designed to minimize decoupling sidebands (15).

Animal preparation

To confirm the presence and identity of UDPG a number of *in vivo* and *in vitro* studies were performed on rat brain. Two Sprague–Dawley rats (205 ± 11 g, mean $\pm$ SD) were prepared in accordance to the guidelines established by the Yale Animal Care and Use Committee. The animals were tracheotomized and ventilated with a mixture of 70% nitrous oxide and 28.5% oxygen under 1.5% isoflurane anesthesia. A femoral artery was cannulated for the monitoring of blood gases (pO₂ and pCO₂), pH and blood pressure. Physiological variables were maintained within normal limits by small adjustments in ventilation (pCO₂ = 33–45 mmHg; pO₂ > 120 mmHg; pH = 7.20–7.38; blood pressure = 90–110 mmHg). After all surgery was completed, anesthesia was maintained by 0.3–0.7 % isoflurane. During NMR experiments the animal's core temperature was measured with a rectal thermosensor and was maintained at 37 ± 1 °C by means of a heated water pad. Following the *in vivo* studies, animals were euthanized by focused-beam microwave irradiation (16) after which the entire brain (without the olfactory bulb and cerebellum) was dissected. Rat brains were combined for a total weight of 2,700 mg. Tissue extraction was initialized by adding 7.5 mL 3.0 M perchloric acid, followed by tissue homogenization in a bead-homogenizer (Omni International, Kennesaw, GA, USA) for 3 cycles of 5 s. The sample was centrifuged for 30 min at 15,000 rpm after which the supernatant was removed and neutralized with 10 M KOH. Following centrifugation to remove the precipitated perchlorate salts, the supernatant containing the aqueous soluble NAD⁺, NADH and other metabolites was removed, lyophilized and stored at –80 °C for subsequent NMR analysis.

In vitro NMR spectroscopy

In vitro experiments were performed on a Bruker Avance III HD spectrometer (Bruker Instruments, Billerica, MA, USA) operating at 500.13 MHz for ¹H and equipped with a 5-mm triple resonance probe incorporating triple-axis gradient coils. The magnetic field homogeneity on each sample was optimized with an automated 3D field mapping algorithm capable of adjusting up to fifth order spherical harmonics. Lyophilized brain extracts were resuspended in 700 µL H₂O/D₂O (15/85%) containing 0.5 mM DSS-D6. 2D ³¹P-³¹P COSY data were acquired as a 512 × 512 matrix over a 6.0 kHz bandwidth in both dimensions with TR = 2,000 ms and NA = 128. The ¹H and ³¹P NMR visibility of NAD⁺ was studied on solutions of 1 mM NAD⁺ in 50 mM phosphate buffer (pH 7.2) in the absence or presence of alcohol dehydrogenase (ADH, EC1.1.1.1) at various temperatures. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) and used without further purification.

Data processing

All FIDs were processed in off-line NMR processing software written in Matlab 8.3 (The Mathworks, Natick, MA, USA). Processing included time-domain B₀ correction using a water reference scan ((17) ¹H MRS only), zero-filling, exponential line broadening, Fourier transformation, phase correction and chemical shift referencing (DSS-D6 at 0.00 ppm for ¹H MRS and PCr at 0.00 ppm for ³¹P MRS).

All spectra were quantified with a home-written spectral fitting program in Matlab (18,19) based on the LCModel algorithm (20). For the downfield part of ¹H MR spectra the spectral basis set included NAD⁺, NAA and eight unspecified singlet signals, covering the [7.0 ...

9.5] ppm spectral range. The singlet resonance chemical shifts were initialized at 7.27, 7.51, 7.93, 8.04, 8.16, 8.27, 8.39 and 8.50 ppm and were allowed to change by a maximum of ± 0.03 ppm. Minimum and maximum line widths were constrained to 9.0 and 36 Hz, respectively. The upfield part of ^1H MR spectra was fitted from [2.9 ... 3.1] ppm with a single creatine resonance and a linear baseline. Only spectra with $\text{TE} = 50$ ms were fitted. For ^{31}P MR spectra the spectral basis set included PCr, α - and γ -ATP, NAD^+ , NADH and UDPG, covering the [-12.0 ... 2.0] ppm spectral range. All ^{31}P NMR signals were simulated with the density matrix formalism using homo- and heteronuclear scalar couplings determined *in vitro* at 500 MHz. For all spectra a second-order polynomial baseline was included.

For absolute quantification of ^1H MR spectra, total creatine and NAA are used as internal concentration references assuming concentrations of 9 and 10 mM, respectively. Quantification of ^{31}P MR spectra was achieved with ATP as an internal concentration reference at 2.8 mM (9,21,22).

RESULTS

Fig. 1 shows representative ^1H MR spectra of the [7.0 ... 10.0] ppm spectral range from human brain at 7 T obtained with frequency-selective excitation. Similar to the ^1H MR spectra from rat brain at 11.7 T (10), the three H2, H4 and H6 resonances from NAD^+ can be recognized at 9.33, 8.83 and 9.14 ppm, respectively. Due to the lower magnetic field, the NAD^+ H4 resonance in Fig. 1 is part of the rising edge of signals from adenosine (dominated by ATP) and other, unidentified compounds (23–25). Repeating the measurement with conventional water suppression reduced the NAD^+ signal several fold, in agreement with the studies performed on rat brain. Visual inspection of the spectra on different volunteers suggests a high reproducibility, which is confirmed by the absolute quantification results shown in Fig. 2. The spectral range between 7.0 and 9.5 ppm was adequately approximated with a spectral basis set of NAD^+ , NAA and seven singlet resonances. Using the NAA amide signal at ~ 7.85 ppm as a 10 mM internal concentration reference resulted in a NAD^+ concentration of 107 ± 28 μM . The Cramer-Rao lower bounds for NAD^+ and NAA were $7.3 \pm 1.4\%$ and $1.1 \pm 0.6\%$, respectively. When using the total creatine methyl signal at 3.02 ppm (measured $T_2 = 92 \pm 7$ ms) as a 9 mM internal concentration reference resulted in a NAD^+ concentration of 103 ± 18 μM .

Fig. 3 shows a typical ^{31}P MR spectrum from human brain at 7 T acquired in 16 min. Besides the typical signals from phosphomono- and diesters, inorganic phosphate, phosphocreatine and ATP, the spectrum holds a clear signal from NAD^+ and NADH as an upfield shoulder on α -ATP. In addition, a signal at -9.83 ppm can be consistently seen on all subjects. The signal has previously been observed in rat brain extract (26), rat brain *in vivo* (8) and human brain (9,22) and was tentatively assigned to uridine diphosphate glucose (UDPG). The high-resolution ^{31}P NMR spectrum of UDPG indeed has a resonance at -9.83 ppm (Fig. 3C), but since UDPG contains a diphosphate group (Fig. 3B) it holds another, scalar-coupled resonance at -8.23 ppm ($^3J_{\text{PP}} = 20$ Hz). As the second resonance partially overlaps with both NAD^+ at -8.15 and -8.45 ppm and NADH at -8.10 ppm, it is imperative to confirm the identity of UDPG. A straightforward way to confirm the presence of a

diphosphate group would be to perform a 2D ^{31}P - ^{31}P correlation spectroscopy (COSY) experiment on the human brain *in vivo*. We were unable to obtain a 2D ^{31}P - ^{31}P COSY spectrum that conclusively demonstrates the presence of UDPG, presumably due to the low concentration and sensitivity of UDPG, as well as the short T_2 relaxation times. An attempt on rat brain *in vivo* at 9.4 T also provided inconclusive results. The issue was resolved through the acquisition of a ^{31}P - ^{31}P COSY spectrum on rat brain extract, as shown in Fig. 4. Fig. 4A shows the pulse-acquire ^{31}P MR spectrum from rat brain *in vivo* at 9.4 T, confirming the presence of UDPG at -9.83 ppm. Fig. 4B/C shows the ^{31}P - ^{31}P COSY spectrum from rat brain extract. Besides the expected correlations between β -ATP and α/γ -ATP and between α -ADP and β -ADP, Fig. 4C shows a clear correlation between the signals at -9.83 ppm and -8.23 ppm, thereby conclusively proving that the signals are part of a scalar-coupled diphosphate group.

Fig. 5 shows quantification results for ^{31}P MR spectra from the human brain at 7 T. In Fig. 5A the signal at -9.83 ppm is treated as a singlet signal without a direct link to the NAD^+ and NADH resonances around -8.1 to -8.5 ppm, as done previously (9,22). The spectral fit is excellent, providing reproducible concentrations for PCr (2989 ± 8 μM), NAD^+ (367 ± 78 μM), NADH (159 ± 34 μM) and UDPG (315 ± 67 μM). The Cramer-Rao lower bounds on PCr, NAD^+ , NADH and UDPG were $0.3 \pm 0.1\%$, $9.3 \pm 4.0\%$, $24.0 \pm 6.2\%$ and $5.7 \pm 2.9\%$, respectively. Fig. 5B shows the spectral fitting results when the signal at -9.83 ppm is treated as part of the UDPG doublet-of-doublets multiplet. The overall spectral fit quality is indistinguishable from Fig. 5A, providing concentrations for PCr (3011 ± 9 μM), NAD^+ (312 ± 65 μM), NADH (75 ± 16 μM) and UDPG (339 ± 99 μM). The Cramer-Rao lower bounds on PCr, NAD^+ , NADH and UDPG were $0.2 \pm 0.1\%$, $8.8 \pm 2.8\%$, $33.2 \pm 6.0\%$ and $5.2 \pm 2.6\%$, respectively. Whereas the inclusion of UDPG does not visually affect the overall spectral fitting quality, it does cause a significant decrease in the NADH concentration thereby leading to an increase in the NAD^+/NADH ratio from 2.4 ± 0.6 to 4.2 ± 0.9 (Fig. 5D). The application of proton decoupling led to a 21–46% increase in peak height for the phospho-mono and di-ester resonances (data not shown). However, no significant improvement in spectral resolution could be detected for the spectral region containing the NAD^+ , NADH and UDPG resonances.

Fig. 6 summarizes the central results of the *in vitro* NMR studies on the NMR visibility of NAD^+ . Fig. 6A holds the downfield spectral region of a pulse-acquire ^1H NMR spectrum of 1 mM NAD^+ in buffer solution (298 K) acquired with water presaturation. The NAD^+ H2, H4 and H6 resonances are clearly visible at high spectral resolution. Upon addition of 0.5 mM alcohol dehydrogenase (ADH) the NAD^+ resonances broaden and drop to a 15% NMR visibility as judged from the total integrated area of Fig. 6B relative to that of Fig. 6A. When water perturbation is minimized through frequency-selective excitation of the NAD^+ H2–H6 spectral range, the NAD^+ NMR visibility increases to 49% (Fig. 6C/D). The ^1H NMR visibility increased with increasing sample temperature. Figs. 6E/F show pulse-acquire ^{31}P NMR spectra of NAD^+ in the absence (Fig. 6E) and presence (Fig. 6F) of 0.5 mM ADH. Similar to ^1H NMR, the ^{31}P NMR resonance lines broaden, but the NAD^+ NMR visibility remains high at 86%.

DISCUSSION

Here it has been shown that NAD⁺ can be detected in the human brain *in vivo* at 7 T by ¹H and ³¹P MRS. NAD⁺ detection by ³¹P MRS is a confirmation and extension of the work from Lu et al. (8) and Zhu et al. (9), whereas the ¹H-based NAD⁺ detection is an extrapolation from recent work on rat brain (10). NAD⁺ detection with ¹H MRS is straightforward since (1) frequency-selective excitation eliminates the need for water suppression and (2) NAD⁺ H2 and H6 protons are completely free of spectral overlap. In addition, ¹H-based NAD⁺ detection can take advantage of the high intrinsic ¹H detection sensitivity as well as the multi-element phased-array receivers that are readily available on most MR systems. The ease of detection is offset by the partial NMR invisibility of NAD⁺. NAD⁺ detection with ³¹P MRS has the advantage of providing a broad description of cerebral energy metabolism with the simultaneous detection of NAD⁺, NADH, ATP and PCr. ³¹P-based detection of NAD⁺ is complicated by significant spectral overlap and the need for non-standard (heteronuclear) MR hardware. The relatively low concentration of NAD⁺ requires relatively large volumes for both ¹H and ³¹P NMR methods (90 – 300 mL). In addition, the requirement of avoiding water perturbation and short echo-times further limit the localization options for ¹H MRS on the human brain.

The reduced NMR visibility of NAD⁺ is likely related to the complex interactions between NAD⁺, water and a wide range of NAD⁺ binding enzymes, such as dehydrogenases. The *in vitro* studies shown in Fig. 6 qualitatively reproduce the *in vivo* observations with a combination of NAD⁺, water and alcohol dehydrogenase. Whereas the *in vivo* situation is undeniably more complex, the simplified model system shows the ADH-mediated interaction between water and NAD⁺ (Figs. 6A/B) and the partial NAD⁺ NMR visibility in the absence of water suppression (Fig. 6C/D). The *in vitro* studies also revealed that NAD⁺ detection with ³¹P MRS is much less affected by ADH, yielding a significantly higher NMR visibility (Fig. 6E/F). The ¹H and ³¹P NMR *in vitro* results could be qualitatively explained by the NAD⁺ binding properties to ADH. The structure of horse liver ADH and its interaction with substrates and NAD⁺ have been studied in detail (27,28). The NAD⁺ nicotinamide group is positioned deep within the enzyme, forming strong hydrogen bonds closely interacting with the zinc active site. Even though the NAD⁺ phosphate groups do form several hydrogen bonds with ADH, they are positioned further from the active site with a presumably larger degree of rotational freedom.

The initial report on ¹H-MR-based NAD⁺ detection in the rat brain (10) did not reveal a significant NMR invisibility for NAD⁺ using the water signal as an internal concentration reference. The *in vivo* concentration (296 ± 28 μM) was estimated at 83% of the concentration obtained from brain extract data (355 ± 34 μM). Re-analysis of the rat brain data with spectral fitting using total creatine as a 10 mM internal concentration reference (instead of manual integration with water signal referencing) yields a NAD⁺ concentration of 236 ± 27 μM, representing a 66% NMR visibility.

The differences between human and rat brain may be explained by a number of factors. Firstly, different main magnetic fields B₀ were used for data acquisition (7 T human, 11.7 T rat). The line shape and width of compounds involved in chemical equilibria depend,

among other parameters, on the chemical exchange rate relative to the Larmor frequency. As an increase in magnetic field B_0 shifts an equilibrium to the slow exchange limit, possibly providing less exchange-induced line broadening in the rat brain. Secondly, the pulse sequences used to acquire the signal were different with 3D LASER localization (TR/TE = 2,000/14 ms) for rat brain and 1D LASER localization (TR/TE = 1,500/18 ms) for human brain. Thirdly, the composition of the detected volume was substantially different. Whereas signal on the rat brain *in vivo* was exclusively acquired from cortical gray matter, the slice acquired from human brain *in vivo* included approximately equal amounts of gray and white matter. In addition, a small contribution from muscles in the neck area is possible. Irrespective of the exact mechanisms and contributions, the partial ^1H NMR visibility of NAD^+ does limit the utility of ^1H -MR-based NAD^+ detection.

Anecdotally, using identical acquisition methods as described for the brain, the ^1H -MR-based NAD^+ detection method was unable to detect NAD^+ on rat muscle *in vivo*, rat liver *in situ* and human muscle *in vivo* (data not shown). Since these tissues are known to contain significant amounts of NAD^+ (29), these results imply a near-complete ^1H NMR invisibility in these non-cerebral tissues.

NAD^+ detection with ^{31}P NMR appears to have a near-complete NMR visibility. This may be qualitatively explained by the difference in Larmor frequency between ^1H and ^{31}P and the different chemical position and binding characteristics of the nicotinamide protons and phosphorus atoms. However, unlike the ^1H NMR method, NAD^+ detection by ^{31}P MRS is complicated by spectral overlap. In earlier reports (9) the overlap between NAD^+ , NADH and $\alpha\text{-ATP}$ was recognized and used to obtain separate NAD^+ and NADH levels, as well as the redox potential. Here we have demonstrated that uridine diphosphate glucose (UDPG) also contributes to the spectral overlap with NAD^+ . UDPG has long been tentatively assigned as a single resonance at -9.83 ppm in ^{31}P NMR spectra of brain. Here it has been conclusively shown that the ^{31}P NMR spectrum of rat brain contains two scalar-coupled doublet signals at -8.23 and -9.83 ppm (Fig. 5C). It is well-known that the brain contains a variety of uridine diphosphate sugars (26). Uridine diphosphate glucose and galactose are the most abundant, with smaller contributions from uridine diphosphate mannose. While these different UDP-sugars can be separately detected with ^{31}P NMR on brain extracts (26), the resolution and sensitivity *in vivo* is insufficient to separate the individual contributions. As far as NAD^+ and NADH detection is concerned, the exact nature of the UDP-sugars is less relevant. The diphosphate group of all UDP-sugars generates two signals at roughly -8.23 and -9.83 ppm. While the presence of UDPG further complicates the spectral overlap at the NAD^+/NADH ^{31}P chemical shift position, it is still possible to reliably separate NAD^+ , NADH and UDPG provided that the proper prior knowledge on the spin systems is utilized. Inclusion of the UDPG doublet-of-doublets leads to a lower NADH level without significantly affecting the NAD^+ level (Fig. 5). As a result, the redox potential NAD^+/NADH significantly increased upon inclusion of UDPG.

Since the ^{31}P MRS signal around -8.2 ppm is characterized by significant spectral overlap, any increase in spectral resolution is expected to be directly beneficial for the detection and separation of NAD^+ and NADH . The application of proton decoupling to remove splitting due to heteronuclear ^1H - ^{31}P scalar coupling is a potential method to achieve improved

spectral resolution. Whereas the peak height of the phospho-mono-esters and di-esters increased by 21–46%, in agreement with previous studies, proton decoupling did not lead to an improved spectral resolution for the NAD⁺/NADH/UDPG region. This is likely due to the fact that the spectral line width for NAD⁺ and NADH is dominated by T₂^{*} relaxation with only a minor contribution from heteronuclear scalar coupling. However, at lower magnetic field strengths, proton decoupling may improve the spectral resolution of NAD⁺ and NADH as the ¹H-³¹P scalar couplings become a larger fraction of the observed spectral line width. This was recently demonstrated by Lu et al. (30) for ³¹P MRS of the human brain at 4 T.

In conclusion, NAD⁺ can be detected in the human brain at 7 T with ¹H and ³¹P MRS. Whereas the ¹H MRS method is simple, devoid of spectral overlap and readily implemented on clinical (¹H-only) MR platforms, it is complicated by partial NMR invisibility. The ³¹P MRS method offers the advantage of simultaneous NAD⁺ and NADH detection, but is complicated by spectral overlap. Overall, both ¹H and ³¹P MR methods offer exciting opportunities to study NAD⁺ metabolism on human brain *in vivo*.

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Figure 1.

¹H MR detection of NAD⁺ on human brain at 7 T. (A) MR image displaying the human brain, an 80 mm diameter surface coil transceiver (bottom white line) and a 20 mm thick slice for NAD⁺ detection. The bottom of the slice is typically set immediately above the sagittal sinus, as shown. (B) ¹H MR spectrum acquired from human brain (TR/TE = 1,500/18 ms, NA = 640, approximate volume = 100 mL) displaying the three NAD⁺ H2, H4 and H6 resonances in addition to a NAA amide resonance and various signals from adenosine, ATP, macromolecules (MM) and other, undefined compounds. Besides a minor water resonance at 4.7 ppm (~0.5% of full intensity) the rest of the spectrum is devoid of signal. (C) Representative ¹H MR spectra from four subjects displaying visually good reproducibility. To allow better visualization of the NAD⁺ resonances, the vertical scale has

increased 5 times relative to (B) and the signal ‘hump’ between 7.8 and 8.6 ppm has been truncated.

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Figure 2.

Quantification of NAD $^+$ by ^1H MRS. (A) Spectral fitting result of a ^1H MR spectrum acquired from human brain at 7 T. The top trace shows the experimental spectrum, similar to those in Fig. 1. The second trace shows the total spectral fit using a basis set of NAD $^+$, NAA and seven undefined singlets. The spectral fitting quality is excellent as judged from the residual/difference between the experimental and fitted ^1H MR spectra. The bottom two traces show the NAA and NAD $^+$ contributions to the total spectral fit. (B) Absolute NAD $^+$ concentrations in human brain when using NAA and creatine as internal concentration references.

Figure 3.

(A) ^{31}P MR spectrum acquired from human brain at 7 T (TR = 5,000 ms, NA = 192, approximate volume = 300 mL) without proton decoupling. (B) Chemical structure of uridine diphosphate glucose (UDPG) and (C) ^{31}P MR spectrum acquired at 11.7 T of UDPG. Phosphocreatine was added as an internal chemical shift reference at 0.00 ppm. GPC, glycerolphosphocholine; GPE, glycerolphosphoethanolamine; PC, phosphocholine; PCr, phosphocreatine; PE, phosphoethanolamine.

Figure 4.

(A) ^{31}P MR spectrum acquired from rat brain *in vivo* at 9.4 T ($\text{TR} = 5,000$ ms, $\text{NA} = 384$) without proton decoupling. (B) 2D ^{31}P - ^{31}P COSY spectrum acquired from rat brain extract at 11.74 T. Correlations between β -ATP and α/γ -ATP and between α -ADP and β -ADP are indicated. (C) Zoomed spectral region from the solid line box in (B) clearly showing a correlation between signals at -9.83 ppm and -8.23 ppm.

Figure 5.

Quantification of NAD⁺ and NADH by ³¹P MRS. Spectral fitting results are shown when (A) modeling UDPG as a singlet at -9.83 ppm and (B) modeling UDPG as a doublet signal with contributions at -9.83 and -8.23 ppm. The spectral fit quality in (A) and (B) is excellent and indistinguishable from each other as judged from the residuals and CRLBs (see text for details). (C) The exact spectral signature of UDPG has essentially no effect on the quantification of PCr. However, modeling UDPG as a doublet signal decreases the NAD⁺ and especially the NADH concentrations, which has immediate consequences for (D) the redox potential (NAD⁺/NADH).

Figure 6.

In vitro studies of NAD^+ in the absence and presence of alcohol dehydrogenase (ADH). (A) Water-suppressed ^1H MR spectrum of NAD^+ in water and (B) NAD^+ and ADH in water. The water presaturation reduces the integrated NAD^+ intensity to ~15% of (A). (C, D) Avoiding water perturbation by frequency-selective excitation of NAD^+ increases the NMR visibility of NAD^+ to 49% in the presence of ADH. (E, F) Pulse-acquire ^{31}P MR spectra of (E) NAD^+ and (F) NAD^+ and ADH. While the ^{31}P NAD^+ resonance does broaden in the presence of ADH, the NMR visibility remains high at 86%.