Chemical basis for deuterium labeling of fat and NADPH

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Materials and methods

Reagents

Glutathione reductase from Baker's yeast, pyruvate dehydrogenase from porcine heart, diaphorase from Baker's yeast (Sigma). NADPH, NADH and deuterium oxide (99.9%) were from Sigma-Aldrich. [3-^{2}H]-glucose (98%) was from Cambridge Isotope Laboratories.

Biochemical experiment

Aqueous buffer contained 2.5 mM Tris and 5 mM NaCl. 8.5 Unit/mL glutathione reductase (or 8.5 Unit/mL pyruvate dehydrogenase or 4 Unit/ml diaphorase) was added in to the buffer with up to 80% D_2O, and then 0.2 mM NADPH (or NADH) was added and the mixture vortexed to start the reaction. The reaction were allowed to sit at room temperature for up to 30 min, and 20 µL of the reaction mixture was added to 80 µL 4°C quenching buffer composed of 40 µL of MeOH and 40 µL of MeCN with 0.625% formic acid. The mixture was then allowed to sit on dry ice for 20 min and the samples were centrifuged for 15 min at 16,200 rpm at 4 °C, and the supernatant placed into LC autosampler vials for subsequent LC-MS analysis. Samples were analyzed directly without drying.

Cell culture

All human cancer cell lines were obtained from American Type Culture Collection and grown in DMEM (Cellgro, 10-017) with 10% FBS (Sigma). The incubator was set at 37 ℃ with 5% CO_2. Isotope-labeled media were prepared from phenol red-, glucose-, glutamine-, sodium bicarbonate-, sodium pyruvate-free DMEM powder (Cellgro) supplemented with 3.7 g/L sodium bicarbonate, 25 mM glucose and 4 mM glutamine and 10% dialyzed FBS (Sigma).

All the labeling experiments were carried out in 6-well tissue culture plates. Cells were seeded at 1 million cells per well. After 24 h, we changed the medium into pre-warmed labeled medium and incubated the cells for 2 h. Then, medium was aspirated using a pasture pipette and metabolism immediately quenched by adding 500 µL 4°C extraction buffer (MeOH : MeCN : H2O 40:40:20 with 0.5% formic acid)[1]. After 5 min incubation on ice, 44 µL 15% NH_4HCO_3 was added to neutralize the acid, the mixture was allowed to sit on dry ice for 20 min. Then the samples were centrifuged for 15 min at 16,200 rpm at 4 °C, and the supernatant placed into LC autosampler vials for subsequent LC-MS analysis. Samples were analyzed directly without drying. Fatty acid extraction was done by adding 1 mL of 0.3 M KOH in 90:10 methanol/water to the cells after aspirating media and washing with 1 mL PBS. Cells were scraped and transferred along with extraction buffer to a 4 mL glass vial. Samples were saponified at 80 °C for 1 h, cooled on ice, and acidified by adding 100 µL formic acid. Samples were then subjected to extraction with 1 mL hexane. The saponified fatty acid in hexane was transferred to a glass sample loading vial, dried under nitrogen, resuspended in 1 mL 1:1 methanol:isopropanol for

subsequent LC-MS analysis.

LC-MS analysis

LC−MS analysis of NAD(P)H was achieved on the Q Exactive PLUS hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific) coupled to hydrophilic interaction chromatography (HILIC). The LC separation was performed on a XBridge BEH Amide column (150 mm × 2.1 mm, 2.5 µM particle size, Waters, Milford, MA) using a gradient of solvent A (95%:5% H2O : acetonitrile with 20 mM ammonium acetate, 20 mM ammonium hydroxide, pH 9.4), and solvent B (100% acetonitrile). The gradient was 0 min, 85% B; 2 min, 85% B; 3 min, 80% B; 5 min, 80% B; 6 min, 75% B; 7 min, 75% B; 8 min, 70% B; 9 min, 70% B; 10 min, 50% B; 12 min, 50% B; 13 min, 25% B; 16 min, 25% B; 18 min, 0% B; 23 min, 0% B; 24 min, 85% B; 30 min, 85% B. The flow rate was 150 µL min^{-1}. Injection volume was 5 µL and column temperature 25 °C. The MS scans were in negative ion mode with a resolution of 140,000 at m/z 200. The automatic gain control (AGC) target was 5×10^5 and the scan range was 75−1000.

Fatty acids were analyzed by reversed-phase chromatography coupled with negative-mode ESI high-resolution MS on the Q Exactive PLUS hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific) with solvent A (1 mM NH_4OAc + 0.2% acetic acid in 90:10 H_2O : MeOH) and solvent B (1mM NH_4OAc + 0.2% acetic acid in 90:10 MeOH : isopropanol). The gradient was 0 min, 25% B; 2 min, 25% B; 4 min, 65% B; 16 min, 100% B; 20 min, 100% B; 21min, 25% B; 22 min, 25% B; 25 min, 25% B. The flow rate was 150 µL min^{-1}. Injection volume was 5 µL and column temperature 25 °C. The MS scans were in negative ion mode with a resolution of 140,000 at m/z 200. The automatic gain control (AGC) target was 5×10^5 and the scan range was 200-600.

Data from labelling experiments were adjusted for natural ^{13}C, ^{15}N, ^{2}H and ^{18}O abundance and impurity of labeled substrate using in-house correction code[2] running in R.

Data analysis

The mass difference between $^{13}C_1$ and $^{2}H_1$ NADPH and $NADP^+$ cannot be resolved using the Q Exactive PLUS. Therefore, the natural ^{13}C abundance was corrected from the raw data. Over 2 h labeling duration, NADPH became M+1 and M+2 labeled, while $NADP^+$ was only M+1 labeled. The labeling of the redox-active hydrogen of NADPH ([Active-H]) was determined using the following equation:

$$[NADP^+] \times [\text{Active-H}] = [NADPH]$$

$$\begin{bmatrix} M+0 & 0 \\ M+1 & M+0 \\ 0 & M+1 \end{bmatrix} \times \begin{bmatrix} x \\ 1-x \end{bmatrix} = \begin{bmatrix} M+0 \\ M+1 \\ M+2 \end{bmatrix}$$

The solvent exchange fraction is the active-H labeling fraction normalized by D_2O percentage.

$$Solvent\ Exchange\ Fraction = \frac{L_{NAD(P)H}}{L_{D_2O}} = \frac{NAD(P)H\ Active\text{-}H\ Labeling\ Fration}{D_2O\ Percentage\ in\ Medium}$$

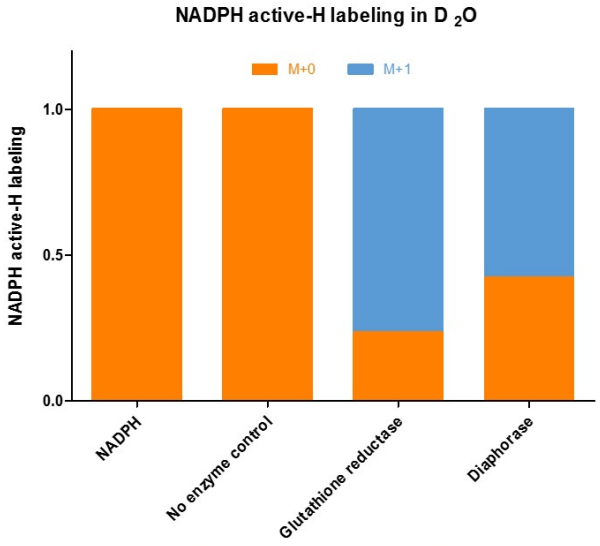
It should be noted that, in cells that mainly use malic enzyme to make NADPH, the solvent exchange correction may overestimate the fractional contribution of the oxPPP, because D_2O not only labels NADPH directly through Flavin-dependent proton exchange, but also labels malate and thereby indirectly labels NADPH.

Throughout the manuscript, we assume for simplicity that the cellular D_2O enrichment is equivalent to the media D_2O fraction. D_2O enrichment in cells can be calculated by matrix deconvolution of the ^2H labeling of 2 closely related metabolites, where one has an additional C-H bond with the hydrogen from ambient water. Calculation is same as the active-H calculation. Appropriate metabolite pairs include malate/fumarate and glutamate/ketoglutarate (Supplementary Fig. 5).

1. Lu, W.; Wang, L.; Chen, L.; Hui, S.; Rabinowitz, J. D., *Antioxid Redox Signal* **2017** [Online early access]. DOI: 10.1089/ars.2017.7014 Published Online: May 11, 2017 http://online.liebertpub.com/doi/10.1089/ars.2017.7014
2. Su, X.; Lu, W.; Rabinowitz, J. D., *Anal Chem* **2017,** *89* (11), 5940-5948 .

Supplementary Figure 1.

a

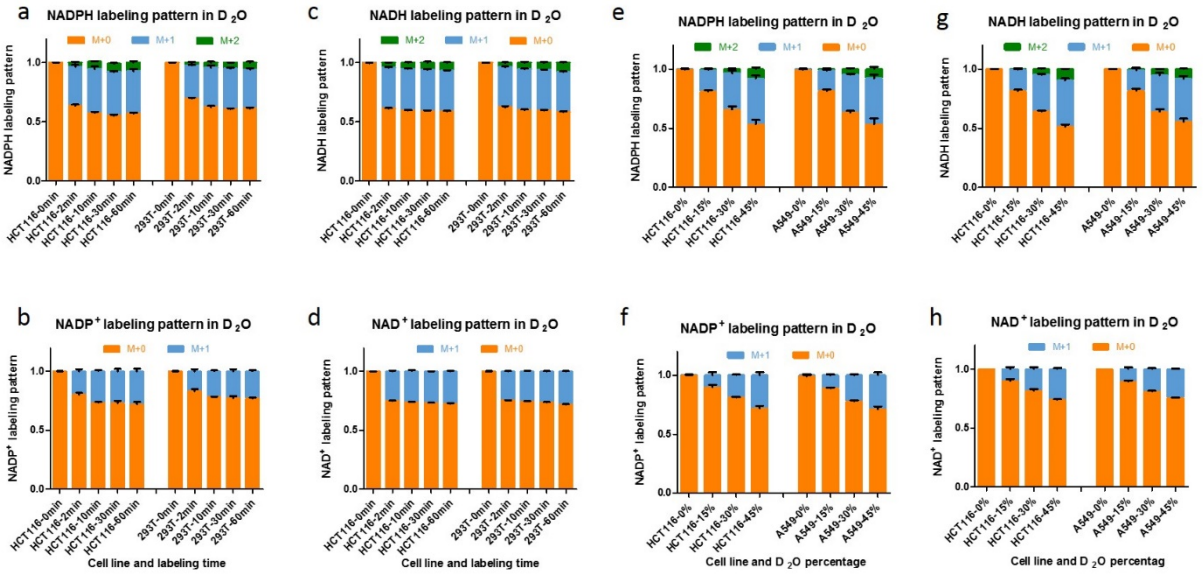


b

	NADPH	No enzyme control	Glutathione reductase	Diaphorase
NADPH	0.2 mM	0.2 mM	0.2 mM	0.2 mM
Enzyme	NO	NO	8.5U/ml	4U/ml
D_2O	NO	85%	85%	85%
Tris	NO	2.5mM	2.5mM	2.5mM
NaCl	NO	5mM	5mM	5mM

Supplementary Figure 1. In addition to glutathione reductase, another FAD enzyme, diaphorase, catalyzes NADPH H-D exchange with water.
a) Steady-state NADPH redox-active hydrogen (active-H) labeling. Labeling duration was 30 minutes.
b) *In vitro* experimental conditions.

Supplementary Figure 2.



Supplementary Figure 2. D_2O labeling of NAD(P)H and $NAD(P)^+$ in different time and D_2O percentage. Data are mass isotope distributions corrected for natural isotope abundance, and were used to calculate the active-H labeling fractions reported in the other figures.

a) NADPH, 45% D_2O, at different time points, used to calculate active-H fraction in Fig. 2a.

b) $NADP^+$, 45% D_2O, at different time points, used to calculate active-H fraction in Fig. 2a.

c) NADH, 45% D_2O, at different time points, used to calculate active-H fraction in Supplementary Fig. 3a.

d) NAD^+, 45% D_2O, at different time points, used to calculate active-H fraction in Supplementary Fig. 3a.

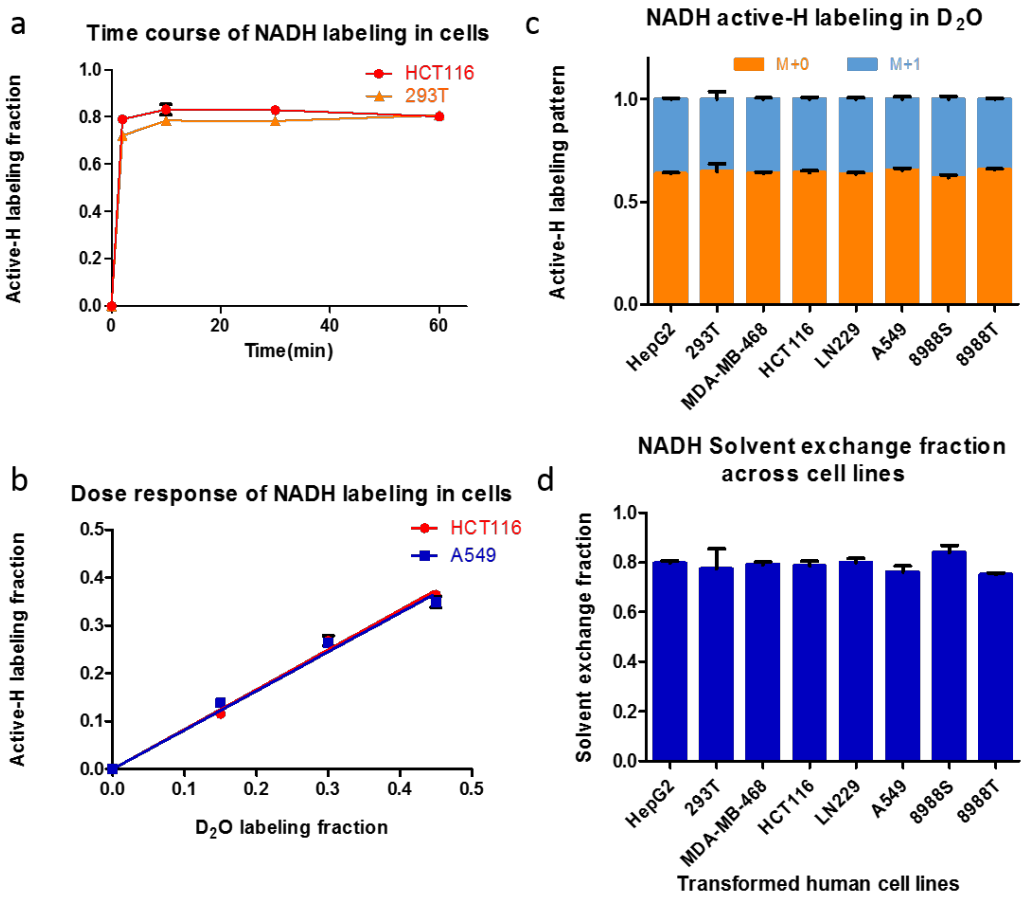
e) NADPH, 2 hours labeling in different D_2O percentage, used to calculate active-H fraction in Fig. 2b.

f) $NADP^+$, 2 hours labeling in different D_2O percentage, used to calculate active-H fraction in Fig. 2b.

g) NADH, 2 hours labeling in different D_2O percentage, used to calculate active-H fraction in Supplementary Fig. 3b.

h) NAD^+, 2 hours labeling in different D_2O percentage, used to calculate active-H fraction in Supplementary Fig. 3b.

Supplementary Figure 3.



Supplementary Figure 3. Rapid H-D exchange occurs between water and NADH in cultured human cells.

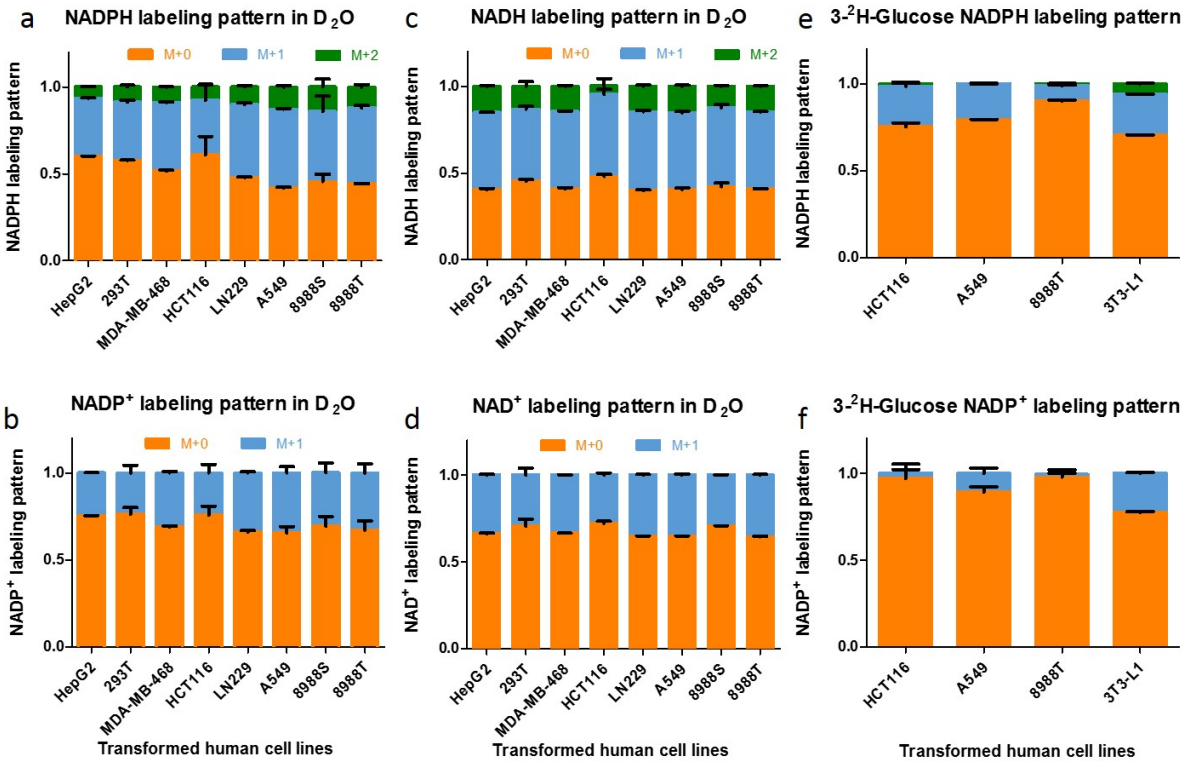
a) Cellular NADH is rapidly labeled on its active-H. Cells were switched from unlabeled media to 45% D_2O medium at t = 0 (Mean ± SD, N = 3).

b) NADH labeling in cells depends linearly on solvent D_2O percentage, cells were labeled in D_2O containing medium for 2 hours (Mean ± SD, N = 3).

c) D_2O (45%) extensively labels NADH's active-H across eight transformed human cell lines (Mean ± SD, N = 3). Active-H labeling is calculated by comparing the mass isotope distribution for NADH and NAD^+.

d) Fraction of NADH's active-H derived from H-D exchange with water. The solvent exchange percentage is calculated by dividing the active-H labeling fraction by the fraction D_2O in the cell culture medium.

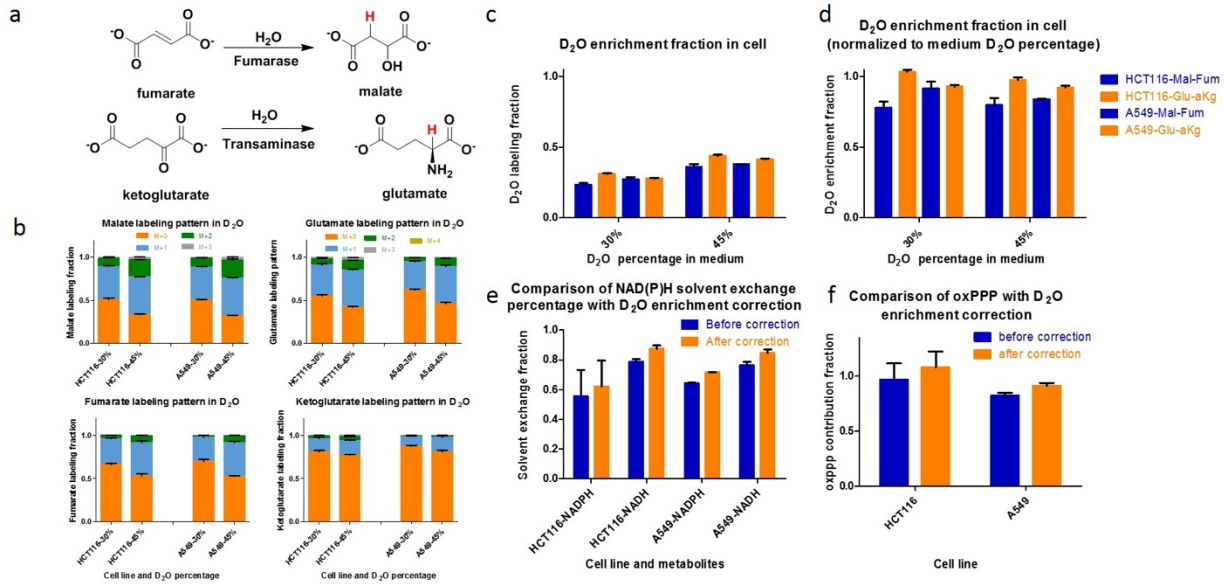
Supplementary Figure 4.



Supplementary Figure 3. D_2O and [3-^{2}H]-glucose labeling of NADPH and $NADP^+$. Data are mass isotope distributions corrected for natural isotope abundance, and were used to calculate the active-H labeling fractions reported in the other figures.

a) NADPH, 45% D_2O, used to calculate active-H fraction in Fig. 2c.
b) $NADP^+$, 45% D_2O, used to calculate active-H fraction in Fig. 2c.
c) NADH, 45% D_2O, used to calculate active-H fraction in Supplementary Fig. 3c.
d) NAD^+, 45% D_2O, used to calculate active-H fraction in Supplementary Fig. 3c.
e) NADPH, 100% [3-^{2}H]-Glucose, used to calculate active-H fraction in Fig. 3d.
f) $NADP^+$, 100% [3-^{2}H]-Glucose, used to calculate active-H fraction in Fig. 3d.

Supplementary Figure 5.



Supplementary Figure 5. D_2O enrichment in cell and the correction for NAD(P)H Active-H labeling in different tracers.

a) Reaction scheme of Fumarase and Transaminase. The products (relative to substrates) contain one additional C-H bond with the hydrogen from ambient water.

b) Malate, fumarate, glutamate and ketoglutarate labeling patterns in HCT116 and A549 cells in 30% and 45% D_2O medium. Labeling duration is 2 h.

c) Cellular D_2O enrichment was calculated by matrix deconvolution of the mass isotope distribution vectors for malate versus fumarate and glutamate versus ketoglutarate.

d) D_2O enrichment in cells relative to added D_2O fraction in media.

e) NAD(P)H solvent exchange percentage corrected by D_2O enrichment percentage.

f) oxPPP contribution corrected by D_2O enrichment percentage.