Comparison of Two non-Invasive Approaches for Determination of Gluconeogenesis: *In vitro* ^{2}H versus *In vivo* ^{13}C NMR Spectroscopy.

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Introduction

Previous studies reported a wide range of estimates of gluconeogenesis (GNG) in humans, which may well relate to biochemical limitations of the methods (1,2).

GNG can be also be calculated from the difference between the rates of endogenous glucose production (EGP) and liver glycogen breakdown determined by in vivo ^{13}C nuclear magnetic resonance (NMR) spectroscopy (3). This method quantifies absolute rates of net GNG, but requires patients to lie for hours within a magnet.

Recently, determination of ^{2}H enrichments in hexamethylene tetramine (HMT) derived from blood glucose following $^{2}H_2O$ ingestion was shown to overcome limitations of previous methods in man (4), but may still be subject to losses during extensive chemical preparation.

The aim of the study is to avoid biochemical short-comings of previous methods and invasive or time-consuming techniques of recent methods.A new approach was developed to determine 2H enrichments simultaneously in all individual carbons 1 to 6 of glucose using in vitro ^{2}H NMR spectroscopy. and to compare the results of this method with results acquired by in vivo ^{13}C NMR method.

Methods

Protocol: Seven young healthy male subjects (age: 22-28 years.; BMI: 22.8±0.6 kg/m²) participated in two study protocols spaced ≤ 2 weeks apart. The evening before the study they ingested liquid, carbohydrate rich (60% CHO, 20% protein, 20% fat), meal at 6 p.m. and then fasted overnight. At one day subjects drank 5 g $^{2}H_2O$ p.o. (99% enriched, Cambridge Isotope Lab., Andover, MA) in smaller portions between 6:00 and 7:30 am and blood was sampled at 8:00 am, 10:00 am and 12:00 p.m. (14 h, 16 h and 18 h of fasting). At another day D-[6,6-$^{2}H_2$]glucose (99.9% ^{2}H, Cambridge Isotope Lab., Andover, MA) was infused intravenously between 6:00 am an 12:00 p.m. for the determination of endogenous glucose production (EGP) using GC - MS analysis of blood plasma samples, while hepatic glycogen decline was monitored non invasively using in vivo ^{13}C NMR spectroscopy in three blocks from 12.5-13.5 h, 15-16 h, and 17-18 h of fasting.

^{13}C NMR Spectroscopy was performed in the Medspec 30/80 Spectrometer (Bruker, Germany) using $^{13}C/^{1}H$-surface coil and 1D-ISIS based sequence (pulse length = 225 µs / 135° in the coil plane, TR = 150 ms, acquisition time = 25.6 ms, 5000 scans in 13 min), without ^{1}H-decoupling. Subjects were lying in the supine position with the coil positioned over the lateral aspect of the liver. Magnetic field homogeneity was optimized on the non-localized water signal to a line width of 60-80 Hz. Absolute quantitation of hepatic glycogen concentration was done by comparing the signal intensity of 1-^{13}C-glycogen doublets in the in vivo spectra with that of phantom solution spectra. Corrections for coil loading and sensitive volume of the coil were performed. Liver volumes were measured in a 1.5T Vision MR Imager (Siemens, Germany).

Gas chromatography-mass spectroscopy: For determination of EGP, aliquots of glucose derived from plasma were converted to pentaacetate derivatives. ^{2}H enrichments in carbon 6 of glucose were measured on Hewlett-Packard 5890 gas chromatograph interfaced to Hewlett Packard 5971A mass selective detector operating in electron ionization mode. M+2 enrichments in D-[6,6-$^{2}H_2$]glucose was determinated from the mass-to-charge ratio of 202 to 200 of fragment ion consisting of carbons 2-6.

Derivation of blood glucose and in vitro ^{2}H NMR spectroscopy: After the derivation from blood was glucose converted to its pentaacetate derivative. The product was derivated with a saturated solution of hydrogen bromide in a glacial acetic acid to yield 2,3,4,6-tetra-O-acetyl-a-D-glucopyranosyl bromide which. Glucose was also converted to1,2-5,6-di-O-isopropylidene-a-D-glucofuranose which was dissolved in deuterated acetone. ^{2}H enrichments at carbons 1,2,3,4,5,6 and 6` were obtained from spectra which were acquired on Varian® Unity INOVA spectrometer operating on 400 MHz ^{1}H frequency using 5-mm inverse and switchable probes.

Data Analysis. Data are presented as means±SEM. Linear regression analysis was performed by least-square fitting of data. One-way ANOVA with Bartlett´s test for equal variances and Paired Student`s t-test were used as appropriate.

Results

Spectra of glucopyranosyl bromide gave well resolved signals at positions 1, 2, 3, 4, and 6'. In spectra of glucofuranose, ^{2}H enrichments at positions 6 and 6' were identical (ratio at 6/6': 1.03±0.08) in agreement with studies in rats (5). From the ratios of ^{2}H enrichments at carbon 5 over 2 of glucose, GNG increased ($p<0.05$) from 53.1±6.2% at 14 h to 72.6±5.9% at 16 h and 75.2±5.7% at 18 h of fasting. Mean contribution of GNG to EGP between 14 h and 18 h was 67.0±3.6%.^{2}H enrichments at carbon 3 over 2 and carbon 6 over 2 gave lower ($p<0.05$) estimates.

Hepatic glycogen concentration declined linearly from 249±6 mmol/l liver at 12.75 h to 206±5 mmol/l liver at 17.75 h ($p<0.0001$).Net hepatic glycogenolysis calculated from the decline of liver glycogen concentrations was 3.6±0.4 µmol.(kg body weight)$^{-1}$.min^{-1}. EGP slightly decreased ($p<0.05$). The difference between mean rates of EGP and glycogen breakdown gave a rate of net GNG of 7.3±0.8 µmol·kg body weight-1·min-1, accounting for 66.5±4.4% of EGP.

Estimates of the contributions of GNG to EGP from in vitro ^{2}H NMR spectroscopy and in vivo ^{13}C NMR spectroscopy were linearly correlated ($r = 0.78$, $p < 0.05$, Fig. 1).

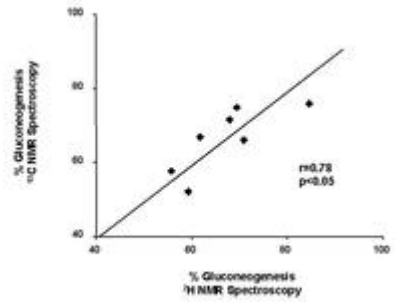

Fig. 1 Correlation between ^{2}H NMR and ^{13}C NMR assessment of gluconeogenesis

Conclusion

Due to the use of safe body water ^{2}H enrichments (~0.5%), the procedures presented here make determination of ^{2}H enrichments at all carbons of glucose feasible in humans.

The linear correlation of estimates of GNG between in vitro ^{2}H NMR spectroscopy and in vivo ^{13}C NMR spectroscopy indicates that the new method offers a valid approach to directly quantify gluconeogenesis in humans which could be of clinical relevance for studying glucose metabolism in man and evaluating new therapeutic strategies in the treatment of diabetes mellitus.

References

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