Available at www.sciencedirect.com

Metabolism

www.metabolismjournal.com

Sources of hepatic glycogen synthesis following a milk-containing breakfast meal in healthy subjects

Cristina Barosa[c], *Claudia Silva*[a], *Ana Fagulha*[b], *Luísa Barros*[b], *M. Madalena Caldeira*[a,c], *Manuela Carvalheiro*[b], *John G. Jones*[c,*]

[a] *Department of Chemistry, University of Coimbra, 3001-401 Coimbra, Portugal*
[b] *Department of Endocrinology, University Hospital of Coimbra, 3001-401 Coimbra, Portugal*
[c] *Biophysics and Biomedical NMR, Center for Neurosciences and Cell Biology, University of Coimbra, 3001-401 Coimbra, Portugal*

ARTICLE INFO

Article history:
Received 4 March 2011
Accepted 26 June 2011

ABSTRACT

During feeding, dietary galactose is a potential source of hepatic glycogen synthesis; but its contribution has not been measured to date. In the presence of deuterated water (^2H_2O), uridine diphosphate (UDP)–glucose derived from galactose is not enriched, whereas the remainder derived from glucose-6-phosphate (G6P) is enriched in position 2 to the same level as body water, assuming complete G6P–fructose-6-phosphate (F6P) exchange. Hence, the difference between UDP-glucose position 2 and body water enrichments reflects the contribution of galactose to glycogen synthesis relative to all other sources. In study 1, G6P-F6P exchange in 6 healthy subjects was quantified by supplementing a milk-containing breakfast meal with 10 g of [U-$^2H_7$]glucose and quantifying the depletion of position 2 enrichment in urinary menthol glucuronide. In study 2, another 6 subjects ingested ^2H_2O and acetaminophen followed by an identical breakfast meal with 10 g of [1-^{13}C]glucose to resolve direct/indirect pathways and galactose contributions to glycogen synthesis. Metabolite enrichments were determined by ^2H and ^{13}C nuclear magnetic resonance. In study 1, G6P-F6P exchange approached completion; therefore, the difference between position 2 and body water enrichments in study 2 (0.20% ± 0.03% vs 0.27% ± 0.03%, $P < .005$) was attributed to galactose glycogenesis. Dietary galactose contributed 19% ± 3% to glycogen synthesis. Of the remainder, 58% ± 5% was derived from the direct pathway and 22% ± 4% via the indirect pathway. The contribution of galactose to hepatic glycogen synthesis was resolved from that of direct and indirect pathways using a combination of ^2H_2O and [1-^{13}C]glucose tracers.

1. Introduction

In the fed state, the liver is capable of synthesizing glycogen from glucose and from 3-carbon gluconeogenic precursors by the direct and indirect pathways [1-2]. In humans, direct and indirect pathway contributions have been noninvasively measured by inferring hepatic uridine diphosphate (UDP)–glucose specific activity or enrichment from glucose tracers [3-4] or

Author contributions: CB researched data and wrote the manuscript; CS researched data; AF, LB, and MC researched the data and reviewed/edited the manuscript; MMC contributed to the discussion and reviewed and edited manuscript; and J.J. wrote/reviewed/edited manuscript and contributed to the discussion.

* *Corresponding author*. Center for Neurosciences and Cell Biology, Faculty of Sciences and Technology, University of Coimbra, 3001-401 Coimbra, Portugal.
E-mail address: jones@cnc.cj.uc.pt (J.G.Jones).

0026-0495/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2011.06.022

deuterated water (^2H_2O) from urinary glucuronide [5]. To date, these approaches have not accounted for contributions from dietary galactose that may be present in the meal. A glass of skimmed milk that is typically included in test meals contains sufficient galactose to contribute significantly to net hepatic glycogen synthesis [5]. In liver, galactose is converted directly to UDP-glucose; and this dilutes the enrichment or specific activity of UDP-glucose from labeled glucose tracers, resulting in underestimation of the direct pathway contribution [6]. With ^2H_2O, hepatic glucose-6-phosphate (G6P) and UDP-glucose are highly enriched at position 2 because of extensive exchange between G6P and fructose-6-phosphate (F6P). If exchange between G6P and F6P is complete, then the position 2 enrichment of UDP-glucose (and that of glucuronide) is equivalent to that of body water. Galactose metabolism dilutes the enrichment of glucuronide position 2; hence, the contribution of galactose to hepatic glycogen synthesis may be estimated from the difference between glucuronide position 2 and body water ^2H-enrichments. In this study, we verified that hepatic G6P-F6P exchange is essentially complete, allowing the contributions of galactose and G6P sources to glycogen synthesis to be measured with ^2H_2O. Glucose-6-phosphate sources were also resolved into direct and indirect pathway contributions by supplementing the ^2H_2O tracer with [1-^{13}C]glucose. Enrichment of glucuronide from both tracers was resolved by multinuclear nuclear magnetic resonance (NMR) spectroscopy.

2. Materials and methods

2.1. Human studies

Study protocols were approved by the Ethics Committee of the University Hospital of Coimbra and performed after informed consent from the subjects. Six overnight-fasted healthy subjects (4 men and 2 women; 21-24 years old; body mass index, 21.8-24.8 kg/m^2) took breakfast (540 kcal, 60% carbohydrate/20% fat/20% protein) containing 200 mL skimmed milk and 1000 mg acetaminophen at 8:00 am. The carbohydrate portion included 10 g of 50% [1-^{13}C]glucose prepared from 99% [1-$_{13}$C]glucose (Cambridge Isotopes, Andover, MA) and unlabeled glucose. Deuterated water (1.5 g/kg body water) was given at 1:00 am and 3:00 am, and drinking water with 0.3% ^2H_2O was provided thereafter. Blood (10 mL) was drawn at 10:00, and urine was collected between 10:00 am and 12:00 pm. Analysis of G6P-F6P exchange was performed in a second group of healthy subjects (1 man and 5 women; 23-25 years old; body mass index, 17.9-24.3 kg/m^2) where an identical breakfast meal was supplemented with 10 g of 30%-enriched [U-$^2H_7$]glucose prepared from 97% [U-$_2H_7$]glucose (Isotec, Miamisburg, OH) and unlabeled glucose, and two 200-mg peppermint oil capsules. Urine was collected between 10:00 am and 12:00 pm for analysis of menthol glucuronide as previously described [7].

2.2. Sample processing

Blood glucose and urinary glucuronides were derivatized as previously described [5,8]. Proton-decoupled ^{13}C NMR spectra were obtained with an 11.75-T Varian Unity spectrometer (Varian, Palo Alto, CA) equipped with a 5-mm broadband probe. Spectra were acquired with a 60° pulse angle, a sweep width of 200 ppm, an acquisition time of 1.5 seconds, a pulse delay of 1.5 seconds, and a temperature of 25°C.

Proton-decoupled ^2H NMR spectra were acquired with a 14.1-T Varian Unity spectrometer equipped with a 3-mm broadband probe as previously described. ^1H NMR spectra were obtained with a 3-mm indirect-detection probe using a standard water presaturation pulse sequence as described [9]. All NMR spectra were analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR, Fremont, CA).

2.3. Metabolic model and calculations

The metabolic model is depicted in Fig. 1. In addition to glycogen synthase contributions, the model also includes G6P-F6P exchange catalyzed by glucose-6-phosphate isomerase and G6P–glucose-1-phosphate exchange catalyzed by phosphoglucomutase. The model is bound by the following assumptions: (1) absence of glycogen phosphorylase flux; that is, no simultaneous synthesis and degradation of glycogen or "glycogen cycling"; (2) complete exchange between glyceraldehyde-3-phosphate and dihydroxyacetone phosphate via triose phosphate isomerase; and (3) negligible oxidative pentose phosphate pathway flux.

2.4. Sources of hepatic glycogen synthase flux

Glycogen synthase flux (GS) is derived from net G6P and galactose fluxes into UDP-glucose. Assuming 95% complete G6P-F6P exchange, the fractional contributions of these fluxes (GS_{G6P} and GS_{Gal}) were estimated by the following equations:

$$GS_{G6P}(\%) = 100 \times H2 / 0.95 \times \text{body water} \quad (1)$$

The balance represents the GS contribution from galactose (GS_{Gal})

$$GS_{Gal}(\%) = 100 - GS_{G6P} \quad (2)$$

The direct pathway contribution to G6P ($G6P_{Direct}$) was estimated from the ^{13}C-excess enrichments of glucuronide and glucose from the meal [1-^{13}C]glucose tracer according to Magnusson et al [10] with correction for glycogenic galactose metabolism, which dilutes the ^{13}C-enrichment of UDP-glucose, according to the following equation:

$$G6P_{Direct}(\%) = 100 \times (C1 - C6)glucur / (C1 - C6)gluc \times 100 / GS_{G6P} \quad (3)$$

Where (C1 – C6)*glucur* is the difference between carbon 1 and carbon 6 excess ^{13}C enrichment of glucuronide and (C1 – C6) *gluc* is the difference between carbon 1 and carbon 6 excess ^{13}C enrichment of plasma glucose. The indirect pathway and de novo gluconeogenic contributions cannot be distinguished from each other and are therefore represented as the balance of G6P synthesis ($G6P_{Indirect}$).

$$G6P_{Indirect}(\%) = G6P_{G6P} - G6P_{Direct} \quad (4)$$

3. Results

3.1. Determining G6P-F6P exchange with [U-$^2H_7$]glucose

When [U-$^2H_7$]glucose is metabolized to UDP-glucose via the direct pathway, the position 3 hydrogen is fully retained; and the ratio of position 2 to position 3 enrichments of glucuronide informs the extent of G6P-F6P exchange [11]. Fig. 2 shows glucuronide spectra following ingestion of meals enriched with [U-$^2H_7$]glucose. Enrichment of position 2 was highly depleted, with only 7% ± 3% retained relative to position 3. Thus, hydrogen 2 had been exchanged in 93% ± 3% of G6P molecules derived via the direct pathway. About 70% G6P is derived via the direct pathway, whereas G6P synthesized by the indirect pathway, whose position 2 hydrogen is derived from body water [5], contributes 30%. Based on this, the position 2 enrichment of the glycogenic G6P pool is predicted to be approximately 95% that of body water. Under these conditions, the contributions of G6P and galactose to glycogenesis can be precisely measured by the ^2H_2O method.

3.2. Enrichment of glucose and glucuronide from [1-^{13}C] glucose and ^2H_2O

As shown by Fig. 3, glucose and glucuronide ^{13}C and ^{2}H enrichments were well characterized by ^{13}C and ^{2}H NMR spectroscopy of their monoacetone glucose (MAG) derivatives. Table 1 shows the enrichment of body water and glucose metabolites for the group of subjects that received the breakfast meal accompanied by [1-^{13}C]glucose and ^2H_2O tracers. Enrichment of glucuronide position 2 was significantly lower than that of body water and is attributable to dilution from galactose-derived UDP-glucose.

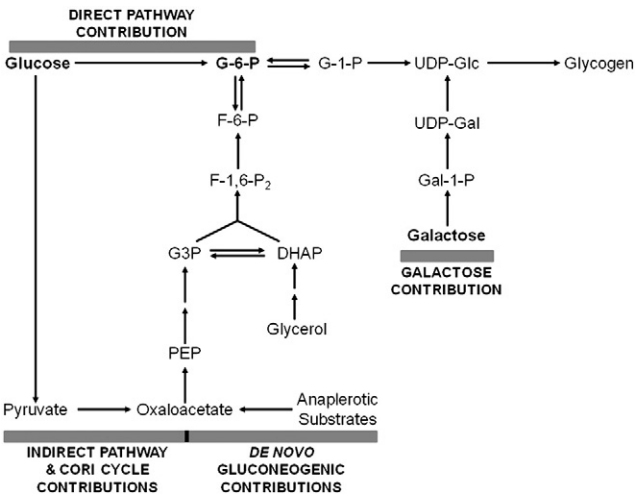

Fig. 1 – Metabolic scheme showing the principal sources and biochemical pathways involved in hepatic glycogen synthesis. Some intermediates have been omitted for clarity. DHAP indicates dihydroxyacetone phosphate; F-6-P, fructose-6-phosphate; F-1,6-P_2, fructose 1,6-bisphosphate; Gal-1-P, galactose-1-phosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; G-3-P, glyceraldehyde-3-phosphate; M-6-P, mannose-6-phosphate; PEP, phosphoenolpyruvate.

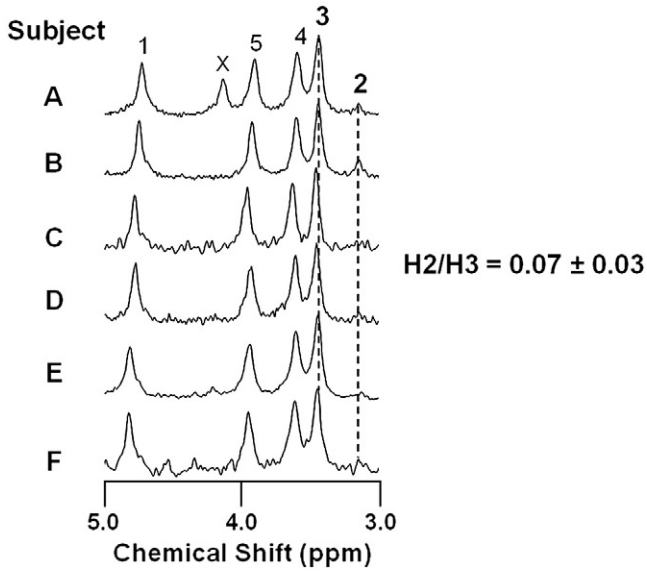

Fig. 2 – ^{2}H NMR spectra of menthol glucuronide obtained from the urine of 6 healthy subjects following ingestion of peppermint oil and a breakfast meal containing 10 g of 30%-enriched [U-$^2H_7$]glucose. The numbers above each signal represent the position of the ^{2}H within the glucuronide hexose skeleton. Signal X is an impurity that is unrelated to menthol glucuronide. Also shown is the mean and standard deviation for the ratio of hydrogen 2 to hydrogen 3 enrichment (H2/H3) for the group.

Enrichment of plasma glucose and glucuronide from [1-^{13}C]glucose by ^{13}C NMR revealed significant excess enrichment in the carbon 1 sites of both metabolites. Significant excess ^{13}C-enrichments of the remaining carbons, including carbon 6, were not detected; hence, subtraction of carbon 6 enrichments from those of carbon 1 as described previously [2-3] gave identical values to direct analysis of carbon 1 enrichment. These data were directly obtained by ^{1}H NMR analysis of glucose and glucuronide-derived MAG, as shown in Fig. 3B. Compared with ^{13}C-direct detection, the ^{1}H NMR measurement has much greater sensitivity; and sampling times are reduced from several hours to a few minutes with standard indirect-detection probes.

3.3. Sources of hepatic glycogen synthesis following a breakfast meal

The principal sources of GS are shown in Table 1. Approximately one fifth was sustained by dietary galactose; and of the remaining proportion, the direct pathway contribution was dominant, accounting for the majority of glycogenic G6P flux, whereas the indirect pathway contributed relatively little. With the exception of subject 4, who presented a remarkably high direct pathway contribution combined with a residual galactose input, the contributions of galactose and direct and indirect pathway G6P fluxes were quite consistent.

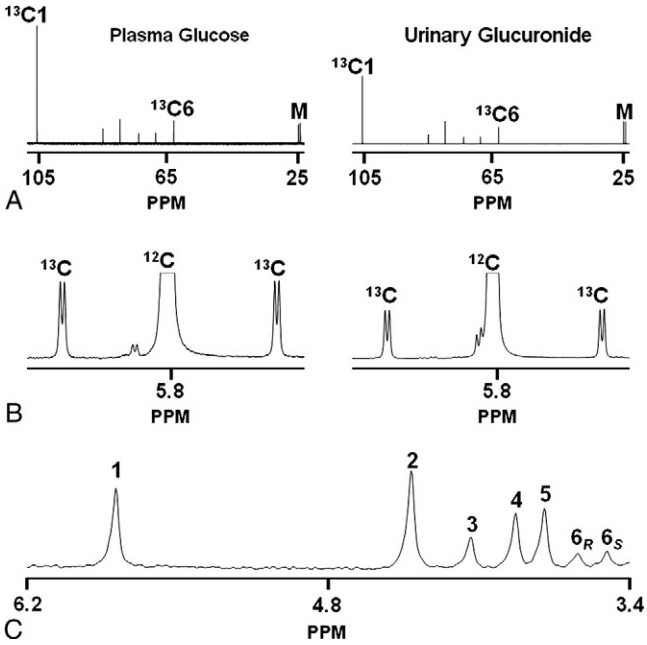

Fig. 3 – ^{13}C NMR spectra of MAG derived from plasma glucose and urinary paracetamol glucuronide (A) showing the carbon 1 and carbon 6 signals ($^{13}C1$ and $^{13}C6$) and scaled to the natural-abundance MAG methyl signals (M); ^{1}H NMR signals of position 1 for the same MAG samples with equivalent scaling and showing the ^{12}C-^{1}H center signal (truncated to fit) and ^{13}C-^{1}H satellites (B) and the ^{2}H NMR spectrum of the MAG sample derived from urinary glucuronide (C). The numbers above each signal represent the position of the ^{2}H isotope within the hexose skeleton.

4. Discussion

The contribution of dietary galactose to glycogen synthesis was based on measurements of glucuronide position 2 and body water enrichments by ^{2}H NMR. The analytical precision is principally limited by the signal-to-noise (SNR) ratio of the glucuronide signal (SNR for body water enrichment measurements being >100:1). The glucuronide position 2 SNRs were 30:1 or higher, translating to an approximately 3% uncertainty in signal quantification due to baseline noise. This error is directly propagated to the glucuronide position 2 to body water enrichment ratio. The principal physiological factor that contributes to the measurement uncertainty is the extent of hepatic G6P-F6P exchange. In this study, G6P-F6P exchange approached completion; but this may not be the case for different human nutritional or pathophysiological states, or for hepatic metabolism in other species. In a study of glycogen synthesis in humans infused with a glucose load and administered with $^{2}H_2O$, G6P-F6P exchange was reported to be only 80% complete based on the values of glucuronide position 2 and body water ^{2}H-enrichments [12]. After an oral glucose tolerance test, direct pathway G6P-F6P exchange measured by the [U-$^{2}H_7$]glucose method was 90% ± 2% [11]. Thus, for confident assessment of glycogenic galactose utilization by the $^{2}H_2O$ method, G6P-F6P exchange needs to be verified for a particular study condition, preferably by a direct method such as the [U-$^{2}H_7$]glucose assay.

Our results indicate that 20% of glycogenic flux was derived from galactose under these conditions. In healthy subjects, cumulative hepatic glycogen synthesis over a 4-hour period after a breakfast meal was estimated to be 28 g [2]. Based on these data, a galactose contribution of 20% corresponds to approximately 6 g of galactose. A 200-mL portion of reduced fat milk contains about 11 g of lactose or 5.5 g of galactose. Therefore, our estimates of absolute galactose contribution to glycogenesis closely match the amount of available galactose from the meal. This suggests that essentially all of the milk galactose was recruited into hepatic glycogen under our study conditions. Galactose uptake and metabolism have been studied in detail with stable-isotope tracers. The fate of galactose is highly dependent on whether it is administered alone or accompanied by glucose. When galactose is administered during

Table 1 – Urinary water and selected urinary glucuronide and plasma glucose excess ^{13}C and ^{2}H enrichments following $^{2}H_2O$ administration and ingestion of a meal enriched with [1-^{13}C]glucose

Subject	Urine water and metabolite excess ^{2}H and ^{13}C enrichments (%)						Sources of hepatic glycogen synthesis flux (%)			
	Urine water ^{2}H	Glucuronide position 2 ^{2}H	Glucose position 1 ^{13}C	Glucose position 6 ^{13}C	Glucuronide position 1 ^{13}C	Glucuronide position 6 ^{13}C	GS_{Gal}	GS_{G6P}	$G6P_{Direct}$	$G6P_{Indirect}$
1	0.24	0.17	2.85	–0.01	1.50	–0.12	25	75	57	18
2	0.30	0.24	3.82	0.09	1.98	–0.02	16	84	54	31
3	0.24	0.20	2.82	–0.08	1.71	–0.05	12	88	61	27
4	0.23	0.17	3.54	0.00	3.22	0.00	8	92	91	1
5	0.29	0.19	3.66	0.08	1.36	–0.05	31	69	39	30
6	0.30	0.22	4.69	–0.02	2.32	–0.01	23	77	49	28
Mean	0.27	0.20*	3.56	0.01	2.02	–0.04	19	81	58	22
SE	0.03	0.03	0.20	0.02	0.21	0.01	3	3	5	4

The contributions of galactose (GS_{Gal}) and glucose-6-phosphate sources (GS_{G6P}) to hepatic GS are also shown. Glucose-6-phosphate sources are resolved into direct ($G6P_{Direct}$) and indirect pathway ($G6P_{Indirect}$) contributions.

* $P < .005$ compared with urinary water enrichment.

fasting, a substantial fraction is converted to glucose via the glycogenolytic pathway [13-15], this fraction being higher with larger galactose loads [16]. When the galactose is coadministered with glucose, its metabolic fate is markedly different. Both absolute and fractional splanchnic extraction rates are significantly enhanced, whereas its appearance in plasma glucose via the hepatic glycogenolytic pathway is sharply reduced [16]. These observations are consistent with the efficient conversion of galactose to hepatic glycogen in the presence of glucose and also support our conclusion that dietary galactose derived from lactose, and therefore accompanied by at least one equivalent of glucose, is also efficiently converted to glycogen.

The sensitivity of hepatic galactose metabolism to coadministered glucose highlights the potential uncertainties in inferring the glycogenic potential of dietary galactose using galactose tracers. By accounting for the true meal-derived galactose inflow into UDP-glucose, our method may provide clearer insights into the contribution of dietary galactose to hepatic glycogen synthesis.

In conclusion, milk or other dairy products can provide a significant amount of dietary galactose for hepatic glycogen synthesis. This contribution is not easily traceable by classic precursor-product methods that rely on ingestion or infusion of labeled galactose. In the presence of $^{2}H_2O$, the dietary galactose contribution can be assessed in a simple manner by comparing glucuronide position 2 and body water enrichments. This analysis indicates that a 200-mL glass of skimmed milk taken as part of a breakfast meal contributes 20% of postabsorptive hepatic glycogen synthesis.

Funding

With support from Fundação para a Ciência e a Tecnologia (FCT) (PTDC/SAU-OSM/65140/2006) and the Juvenile Diabetes Research Foundation International (JDRFI-1-2006-74). The NMR spectrometers are part of the National NMR Network and were purchased in the framework of the Portuguese National Programme for Scientific Re-equipment, contract REDE/1517/RMN/2005, with funds from POCI 2010 (FEDER) and FCT.

Conflict of Interest

No potential conflicts of interest.

REFERENCES

[1] Newgard CB, Hirsch LJ, Foster DW, et al. Studies on the mechanism by which exogenous glucose is converted into liver glycogen in the rat—a direct or an indirect pathway. J Biol Chem 1983;258:8046-52.
[2] Taylor R, Magnusson I, Rothman DL, et al. Direct assessment of liver glycogen storage by ^{13}C nuclear magnetic resonance spectroscopy and regulation of glucose homeostasis after a mixed meal in normal subjects. J Clin Invest 1996;97:126-32.
[3] Petersen KF, Cline GW, Gerard DP, et al. Contribution of net hepatic glycogen synthesis to disposal of an oral glucose load in humans. Metab-Clin Exp 2001;50:598-601.
[4] Fery F, Plat L, Balasse EO. Effects of metformin on the pathways of glucose utilization after oral glucose in non–insulin-dependent diabetes mellitus patients. Metab-Clin Exp 1997;46:227-33.
[5] Jones JG, Fagulha A, Barosa C, et al. Noninvasive analysis of hepatic glycogen kinetics before and after breakfast with deuterated water and acetaminophen. Diabetes 2006;55:2294-300.
[6] Soares AF, Carvalho RA, Veiga FJ, et al. Effects of galactose on direct and indirect pathway estimates of hepatic glycogen synthesis. Metab Eng 2010;12:552-60.
[7] Jones JG, Garcia P, Barosa C, et al. Quantification of hepatic transaldolase exchange activity and its effects on tracer measurements of indirect pathway flux in humans. Magn Reson Med 2008;59:423-9.
[8] Jones JG, Barosa C, Gomes F, et al. NMR derivatives for quantification of ^{2}H and ^{13}C-enrichment of human glucuronide from metabolic tracers. J Carbohydr Chem 2006;25:203-17.
[9] Basu R, Barosa C, Basu A, et al. Transaldolase exchange and its effects on measurements of gluconeogenesis in humans. Am J Physiol 2011;300:E296-303.
[10] Magnusson I, Chandramouli V, Schumann WC, et al. Quantitation of the pathways of hepatic glycogen formation on ingesting a glucose load. J Clin Invest 1987;80:1748-54.
[11] Delgado TC, Silva C, Fernandes I, et al. Sources of hepatic glycogen synthesis during an oral glucose tolerance test: effect of transaldolase exchange on flux estimates. Magn Reson Med 2009;62:1120-8.
[12] Stingl H, Chandramouli V, Schumann WC, et al. Changes in hepatic glycogen cycling during a glucose load in healthy humans. Diabetologia 2006;49:360-8.
[13] Fried R, Beckmann N, Keller U, et al. Early glycogenolysis and late glycogenesis in human liver after intravenous administration of galactose. Am J Physiol 1996;270:G14-9.
[14] Gannon MC, Khan MA, Nuttall FQ. Glucose appearance rate after the ingestion of galactose. Metab-Clin Exp 2001;50:93-8.
[15] Staehr P, Hother-Nielsen O, Beck-Nielsen H, et al. Hepatic autoregulation: response of glucose production and gluconeogenesis to increased glycogenolysis. Am J Physiol 2007;292:E1265-9.
[16] Sunehag AL, Haymond MW. Splanchnic galactose extraction is regulated by coingestion of glucose in humans. Metab-Clin Exp 2002;51:827-32.