Characterization of glucose metabolism in the diabetic rat by ^{13}C and ^{2}H NMR after oral administration of a novel insulin nanosystem

A. F. Soares[1,2],C. Reis[1], , P. Nunes[2,3], J. G. Jones[3], R. A. Carvalho[2,3], and F. Veiga[1,*]

[1]Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal; [2]Faculty of Science and Technology, University of Coimbra, Coimbra, Portugal; [3]Center for Neurosciences, Coimbra, Portugal (*E-mail address*: franciscasoares@ci.uc.pt)

Introduction

Liver plays a major role on maintaining glycemia in the physiological range. This organ is responsible for both glucose uptake and storage during the post-absorptive sate and its production and release to the bloodstream during fasting. Thus, blood glucose levels are the result of the net flux of opposite metabolic pathways that account for hepatic glucose formation (gluconeogenesis and glycogenolysis) and glucose consumption or storage in the liver (glycolysis and glycogenesis). After glucose load, glycogen synthesis is stimulated by glucose, glucose-6-fosfato (G-6-P) and insulin (Ferrer, 2003). Additionally, hepatic glucose production is suppressed by insulin inhibitory effect on glucose-6-phosphatase.

The present study was designed to access glucose metabolism after oral administration of a novel insulin nanosystem that previously demonstrated strong oral hypoglycaemic effect (higher than 75% of basal values after 14 h) on fasted rats. Following a 15-day oral treatment with insulin nanosystem, an oral glucose tolerance test (OGTT) involving administration of non-recyclable glucose ([U-^{13}C]glucose) tracer and deuterated water ($^{2}H_2O$) was performed. Such tracers were selected with the following aims: (1) evaluate the efficacy of glucose uptake; (2) determine endogenous contributions to plasma glucose; (3) determine sources to liver glycogen.

The efficacy of glucose clearance from the blood can be accessed by NMR methodology. Figure 1 shows a representative ^{13}C NMR spectrum of the glucose derivative monoacetoneglucose (MAG). The typical quartet (Q1,2,5) resonance is due to the intact [U-^{13}C]glucose tracer and will be loss as glucose is taken up into the cells by the action of insulin. Meanwhile, other isotopomer contributions increase. In particular, [1,2-^{13}C] and [1,2,3-^{13}C]glucose resulting from metabolization of the exogenous [U-^{13}C]glucose, are responsible for the dublet (D1,2) resonance in C1 (Perdigoto 2003). Thus, relative contribution of both exogenous and metabolized glucose to blood glucose can be determined by the following equations, respectively: Q1,2,5 / C1 resonance; D1,2 / C1 resonance.

As shown in Figure 2, insulin promotes carbon flux into hepatic glycogen synthesis, (full arrows) and suppresses hepatic glucose production from glucose-6-phosphate (G6P) and gluconeogenesis (doted arrows). Hepatic glucose production during a glucose load can be estimated with $^{2}H_2O$. Additionally, gluconeogenesis and glycogenolysis contributions can be distinguished. Following $^{2}H_2O$ administration, steady-state ^{2}H-enrichment of body water is rapidly attained. In the liver, ^{2}H from body water is incorporated into position 2 of G6P via exchange between G6P and fructose-6-phosphate (F6P) (Landau 1996). Since this exchange is very fast, G6P derived from any hepatic source (i.e. pyruvate or glycogen) is enriched at position 2 by this process. Following hydrolysis by G6P-ase, glucose enriched in position 2 (H2) is generated. Meanwhile, glucose derived directly from the load is not enriched in position 2. Therefore, enrichment of plasma glucose position 2 relative to that of body water (H2/body water) is equal to the fraction of plasma glucose derived from hepatic G6P: Plasma glucose derived from hepatic G6P (%) = 100 × H2/body water. Considering the pathways accounting to

hepatic glucose production (gluconeogenesis and glycogenolysis), ^{2}H incorporation in position 5 occurs from body water at the level of glyceraldehyde-3-phosphate in the gluconeogenesis (Landau 1996). Thus, enrichment in H5 relative to H2 indicates the gluconeogenic contribution to the hepatic glucose production: Gluconeogenesis (%) = 100 x H5/H2.

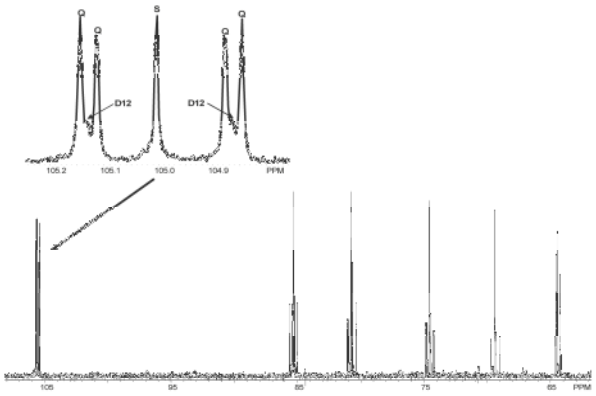
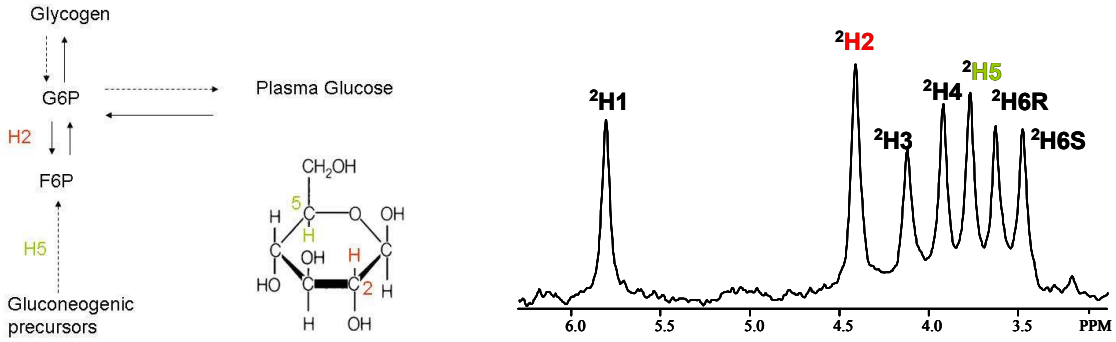


Figure 1. A representative ^{13}C NMR spectrum of MAG from blood glucose.



a **b**

Figure 2. a. Summary of the pathways of hepatic glucose metabolism during a glucose load. b. representative ^{2}H NMR spectrum of MAG from blood glucose

Materials and Methods

Sodium alginate, chitosan and BSA were purchased from Sigma-Aldrich Chimie (France). Setacarb calcium carbonate was obtained from Omya (France). Paraffin oil was supplied by Vaz Pereira (Portugal). The emulsifier, Span 80, dextran sulfate and PEG were purchased from Fluka, Chemie GmbH (Switzerland). Insulin was kindly donated by *Hospitais da Universidade de Coimbra*. Amyloglucosidase from Aspergillus niger, Streptozotocin (STZ) and D-Glucose were supplied from Sigma (Germany). $^{2}H_2O$ (99.9%) and [U-^{13}C]glucose were obtained from Eurisotope (France). All other chemicals were of reagent grade or equivalent.

Nanoparticles were prepared by an emulsification-based method using an internal calcium source. Resultant nanoparticles were recovered using organic solvents and ultracentrifugation (Reis 2007). Chitosan-PEG-coating and then BSA-coating were applied to nanoparticles. Diabetes was induced by STZ ip injection (dose STZ 65 mg/kg in citrate buffer pH 4.5) in male Wistar rats Oral administration of the nanoparticle formulation or placebos was performed id for 15 day period. Animals were randomized in four groups: control group I (water), control group II (non-encapsulated insulin, 50 IU/kg), control group III (empty nanoparticles) and animals with oral insulin-loaded nanoparticles (50 IU/kg). An oral glucose tolerance test (OGTT) was performed to study glucose metabolism on 18 h fasted animals and 10 h after the last oral treatment. Glucose load (2 g/Kg) 5% enriched with [U-^{13}C]glucose was dissolved in 2 mL of $^{2}H_2O$. To achieve proper body water deuterium enrichment, an extra dose of deuterated water (2 mL) was given i.p. Glycemia was monitored every 10 min and when the value started to decreased, animals were anesthetised, blood collected from the hepatic vein and deproteinized with 0.3N $ZnSO_4$ followed by 0.3N $Ba(OH)_2$. Glucose was isolated by solid phase extraction in a cationic resin and amberlite column. Livers were immediately freeze clamped, glycogen extracted with 30% KOH at 70ºC and hydrolyzed to glucose with 16U of amyloglucosidase at pH 4,5 and 50ºC overnight. Both glucose from blood and glycogen were derivatized to MAG as previously described (Perdigoto 2003). NMR spectra of MAG were acquired using a 5 mm broadband probe on a 11.7T Varian Unity spectrometer (Varian Instruments, Palo Alto, CA) at 50ºC. Proton-decoupled ^{2}H NMR spectra were obtained in unlocked mode using a 90° pulse with 1.5 s acquisition time and no interpulse delay. For ^{13}C NMR analysis, samples were dissolved in $^{2}H_2O$ to provide lock signal and spectra obtained using 45° pulse, acquisition time of 1.5 s. The summed free induction decays were processed using Lorentziana function with 0.5 Hz to ^{2}H and 1 Hz to ^{13}C before Fourier transform. Spectra were analysed using the curve-fitting routine supplied with the NUTS PC-based NMR spectral analysis program (Acorn NMR, Fremont, CA). Results are expressed as means ± standard errors of means (S.E.M.). For group comparison, an analysis of variance (ANOVA) with a one-way layout was applied and then Bonferroni multicomparison test. The differences were considered significant when $P < 0.05$.

Results and discussion

Figures 3 and 4 represent the data from the NMR analysis of blood samples. As to liver glycogen samples, deuterium incorporation was detected in a random fashion and thus no trend could be established. Probably the OGTT period was insufficient for all animals to synthesise liver glycogen.

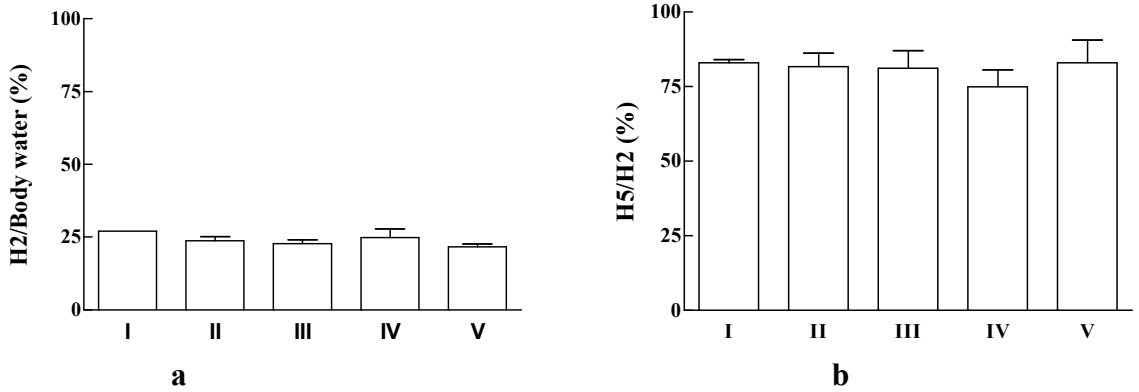


Figure 3. a. Hepatic glucose production; b. gluconeogenesis contribution to blood glucose

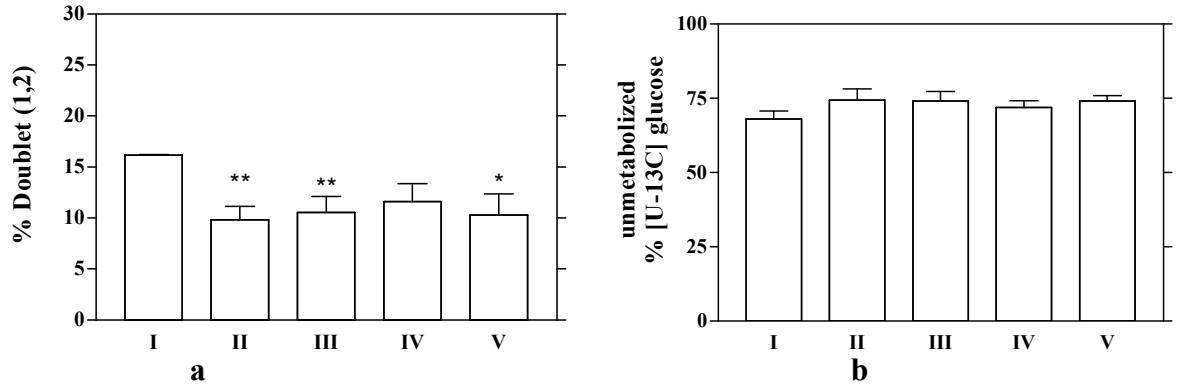


Figure 4. Contributions of a. metabolized exogenous and b. intact exogenous glucose from the oral load to blood glucose.

Conclusions

This study did not demonstrated differences in glucose production after OGTT: 20-25% of plasma glucose was derived from hepatic glucose production and gluconeogenesis was the major contributing pathway. This study could not also conclude the effectiveness of this novel insulin oral nanosystem on the hepatic glycogen synthesis because time period of OGTT revealed to be insufficient and a large inter-variability was as well observed. However, this study could be, in a next future, optimized and be very useful for the glucose metabolism characterization in animal models.

References

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