REVIEW

Beyond blood sugar: the potential of NMR-based metabonomics for type 2 human diabetes, and the horse as a possible model

Michael S. Hodavance · Sarah L. Ralston · István Pelczer

Abstract Metabonomic analysis is a powerful tool for identifying and characterizing metabolic disorders, for example type 2 diabetes and the metabolic syndrome. Nuclear magnetic resonance (NMR) spectroscopy is an essential tool for such analysis, with special benefits. The review assesses the current status and potential of NMR-based metabonomics of type 2 diabetes. The horse is proposed as a possible model for studying this condition and disease. Some examples are shown of horse blood analyses by NMR.

Keywords Metabonomics · Type 2 diabetes · Horses · Glucose tolerance · Insulin resistance · NMR

Introduction

Type 2 diabetes (non-insulin dependent diabetes mellitus, NIDDM) has been described as an "epidemic" of contemporary society. There will be an estimated 221 million diabetics worldwide in 2010 and that number will expand to 300 million by 2025 [1]. Only recently has the medical profession realized that NIDDM is not an isolated disease entity but is, instead, part of a more general metabolic

M. S. Hodavance · I. Pelczer (✉)
Department of Chemistry, Princeton University,
Princeton, NJ 08544, USA
e-mail: ipelczer@princeton.edu

S. L. Ralston
Department of Animal Science,
School of Environmental and Biological Sciences, Rutgers,
The State University of New Jersey,
New Brunswick, NJ 08981, USA

disorder ("Metabolic Syndrome") [2]. Persons affected with metabolic syndrome usually have glucose intolerance, insulin resistance, central obesity, high blood pressure, and dyslipidemia (abnormally high lipid concentrations in the blood).

In both NIDDM and metabolic syndrome, body tissues are resistant to the actions of insulin on glucose uptake, resulting in prolonged hyperglycemia after intake of carbohydrates. The pancreas tries to compensate by producing more insulin (hyperinsulinemia). Clinical manifestations include heart attack, stroke, and damage to the optic nerve (blindness) and renal tissues (kidney failure) [2, 3]. Both are associated with obesity and inactivity, although a genetic component also has recently been recognized. Although historically an adult-onset disease, NIDDM is becoming increasingly prevalent in adolescents [3].

Insulin has traditionally been regarded as primarily a glucoregulatory hormone which facilitates cellular glucose uptake [4]. It also stimulates glycogen storage, increases fatty acid and protein synthesis, and inhibits fat and protein degradation, however [4]. Because of the diverse functions of insulin, hyperinsulinemia can have multiple metabolic consequences, only a few of which have been identified.

Many different methods, including oral and intravenous glucose-tolerance challenges, insulin-sensitivity tests, and combined glucose and insulin testing [5, 6], are used to screen for NIDDM and metabolic syndrome. All are expensive, generate many false positives, and rely solely on the glucose and/or insulin responses to challenges [5].

Nuclear magnetic resonance (NMR) spectroscopy of biofluids, for example blood, saliva, and urine, can detect an infinitely wider array of metabolites and may enable more accurate detection of "markers" of metabolic disorders [7, 8]. Studies in which the ^{1}H NMR spectra of blood, urine, and saliva of diabetic patients were compared with

those of healthy individuals found consistent differences between the spectra of healthy and diabetic patients [9–11]. Application of metabonomic statistical analysis to such spectra can identify the specific metabolites contributing to the observed differences.

Animal models also play an important role in understanding metabolic disorders. The main animal models for diabetes/metabolic syndrome are currently db/db mice and Zucker diabetic fatty rats [12]. Neither model has "naturally" occurring disorders, however, the animals being highly inbred mutant strains.

Hyperinsulinemia is very common in horses, being most prevalent, as in humans, in adolescents and aged animals [13–15]. Horses have a high incidence of diseases associated with insulin resistance and hyperinsulinemia, including laminitis (disturbance of blood flow to the distal limbs), hyperlipidemia, pituitary adenoma, and osteochondrosis [13, 14]. The purpose of our study is to use metabonomics analyses of NMR spectra of plasma samples to investigate normal and abnormal metabolic responses to a dextrose challenge, using the horse as a model.

Materials and methods

Samples taken from six mixed-breed horses (Table 1) were used in this study. All were approximately 5 months old at the time of the first challenge. The horses were part of a larger herd being used in a long term (7-month) study investigating the effects of standard hay/pelleted concentrate rations compared with an alfalfa-based total mixed ration cube on glucose/insulin metabolism in young horses. A low-dose oral dextrose (LDOD) challenge [13] was administered on October 31, 2005 (one week before dietary changes were made) and again on January 27, 2006. In the LDOD challenge each horse is given an oral dose of dextrose (0.25 g kg^{-1} body weight), mixed with unsweetened apple sauce, by oral dose syringe. During the January challenge Gilbert (#47) was given 38 g of his 83 g dextrose in the form of uniformly labeled ([U-^{13}C]) glucose (Isotec, Stable Isotope Products: A Sigma–Aldrich Company, St Louis, MO, USA). In both trials blood samples were drawn into heparinized vacuum tubes (Vacutainer) from indwell-

ing intravenous catheters before administration of the oral challenge, and then at 30-min intervals for 3 h. Samples were placed immediately on ice and centrifuged at 131 g force at 10 °C in a refrigerated centrifuge (Sorvall Instruments RT6000; Global Medical Instrumentation, Ramsey, MN, USA) within 3 h of collection. Plasma samples were stored at −40 °C pending ^{1}H NMR analysis. Subsamples were stored at −20 °C pending glucose colorimetric (Glucose-SL, Diagnostic Chemicals, Charlottetown, PE, Canada) and insulin ELISA (Mercodia Insulin Elisa, ALPCO Diagnostics, Windham, NH) analysis.

^{1}H NMR analysis was performed with a Varian (Palo Alto, CA, USA) Unity/Inova 600 MHz spectrometer using excitation sculpting (ES) water suppression [16] for all but the diffusion filtered experiments, for which selective flip-back pulses were used [17]. ^{13}C NMR spectra were collected at Bruker–Biospin, (Billerica, MA, USA) using a 600 MHz (^{1}H) NMR instrument equipped with a ^{13}C-detection-optimized 5 mm cryoprobe. The samples were in their native condition; we used capillary inserts with D$_2$O for lock.

Results and discussion

Glucose and insulin responses in the six horses to the initial challenge revealed that Black Magic (#3) and Giselle (#1) were relatively insulin resistant with high ($P<0.05$) plasma glucose and insulin (Figs. 1a and b) compared with the other horses and with previous results for young horses (Ralston, unpublished data, 1999–2005). For both horses there was clinical evidence of abnormal bone development, with epiphysitis and, in Black Magic, flexure deformities in both front legs. Pollyanna (#23) did not receive the full dose of dextrose during the first trial.

Glucose signals are easy to recognize in the ^{1}H NMR spectra. By comparing the integrated areas of selected, well-resolved signals in the sugar region of the NMR spectrum we generated an approximate, relative measure of changing glucose concentration in the samples (Fig. 2). As expected, the spectra revealed a pattern of increasing glucose concentration which reached a maximum after 60 to 90 min, followed by a return to near baseline levels (the normal physiological response to an oral dose of glucose [13, 14]) except for the spectra from Pollyanna (#23), who did not receive the full dose. Relaxation filtered spectra were used for the analysis to isolate the small molecule contribution from large-MW components as much as possible. The regions specified as "glucose" were between 3.32–3.48 and 3.68–3.96 ppm where the α and β-glucose signals appear [20]. Initial analysis (not shown) using the entire sugar region as the range, including peaks such as choline (3.21 ppm), generated similar but less consistent results.

Table 1 Horses used in the experiment

Name	No.	Sex	Breed	Glucose/insulin responses
Giselle	1	Female	Han/TB	Insulin resistant
Magic	3	Male	Perch/TB	Insulin resistant
Poppet	22	Female	QH/Belg	Normal
Polly	23	Female	QH/Belg	Incomplete dose given
Skeeter	31	Male	QH/Belg	Normal
Gilbert	47	Male	QH/Perch	Normal

Fig. 1 Individual horse plasma glucose responses (**a**) and insulin responses (**b**) to LDOD challenge
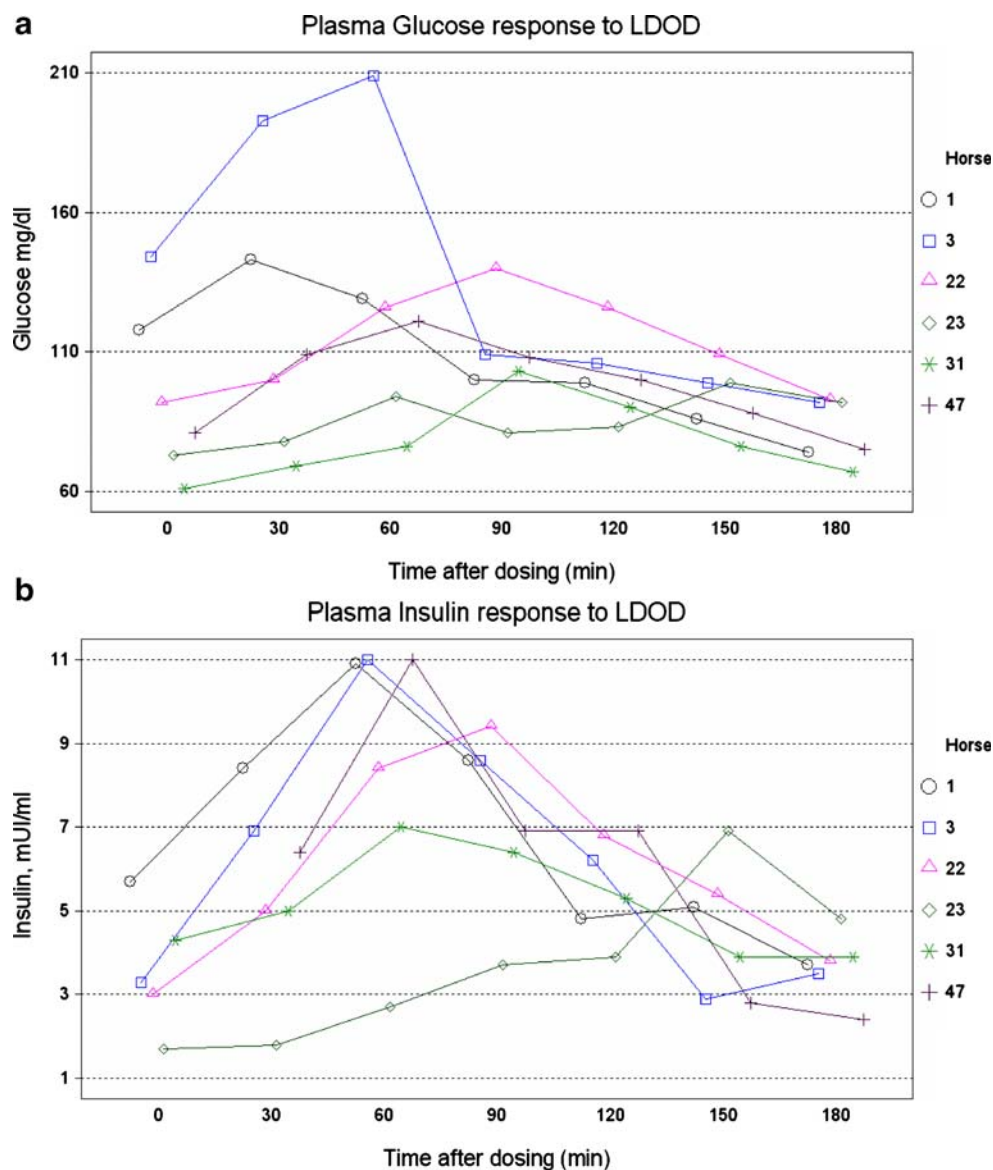

Fig. 2 Plots of the relative spectrum integrals in the regions 3.32–3.48 and 3.68–3.96 ppm normalized to the total integral of the spectrum. The vertical scale is in arbitrary units
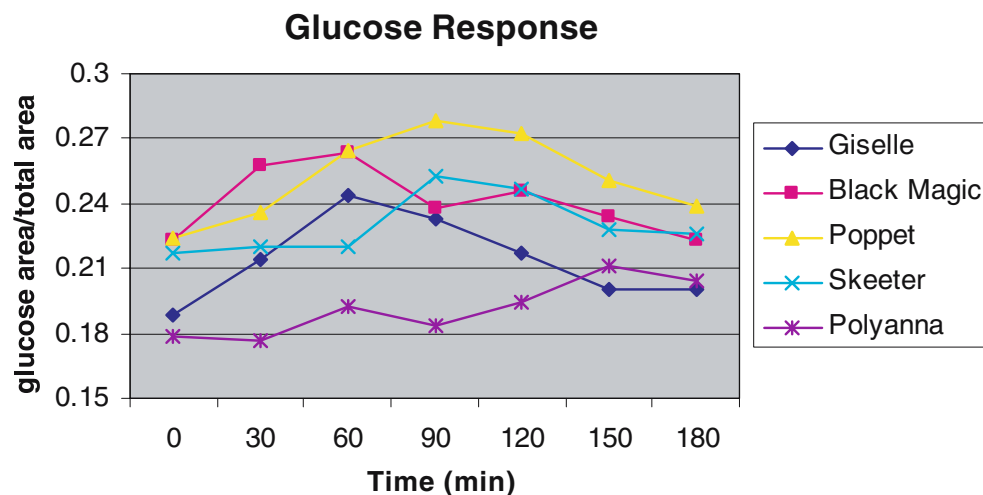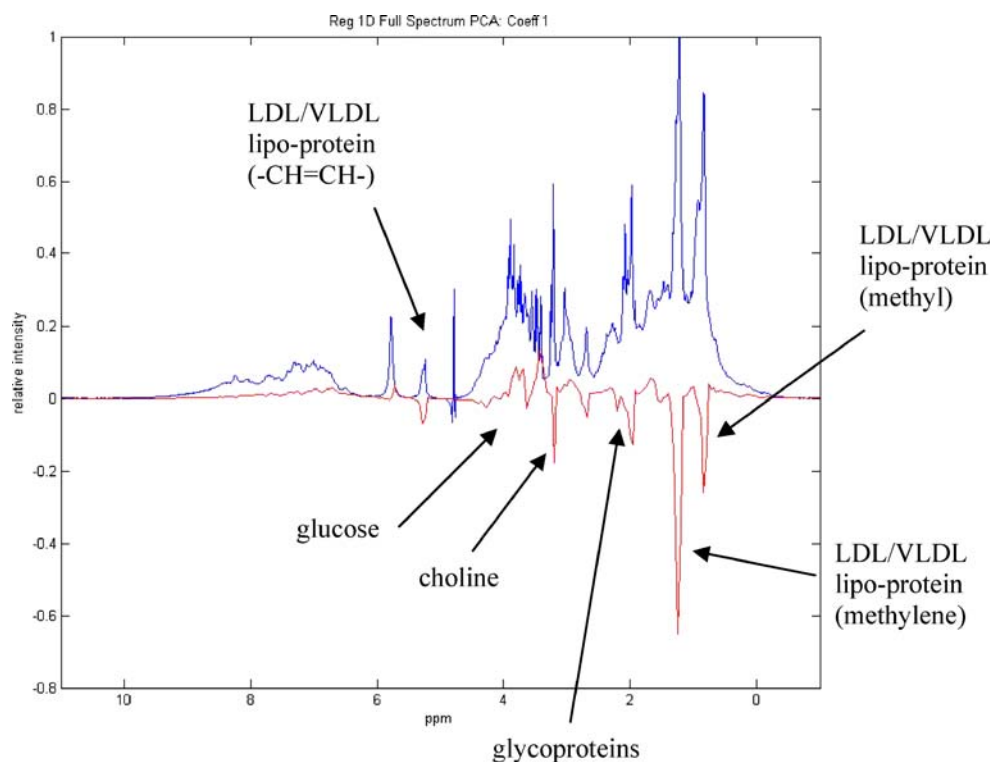
Fig. 3 A representative full ^{1}H NMR spectrum of horse blood plasma (*upper trace, blue*) correlated with the PC1 loadings trace (*lower trace, red*). Selected components are *highlighted* on the plot


Our integrated results compare very well with data from conventional glucose assays [1], even though signals in addition to those of glucose may be contributing to the intensities of the integrals calculated from the spectra. Further refinement of this analysis can include integration using careful curve-fitting and deconvolution.

Identification of contributing components

One-dimensional loadings traces from principal component (PC) analysis were used to identify the most significant components enabling differentiation between samples. The loadings present the relative contribution of each "bucket"

Fig. 4 ^{13}C NMR spectra of horse blood plasma from Giselle. On the *left* (**a**) the full kinetic set is shown. On the *right* (**b**) the control spectrum is compared with the result 150 min after administration of glucose. Two new metabolites are shown by the *arrows*
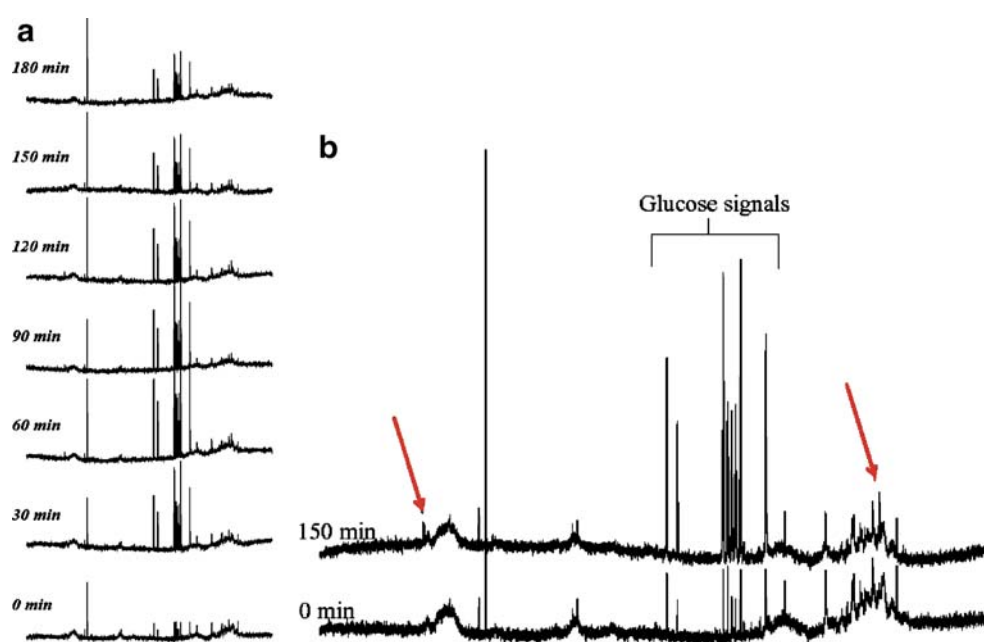
or peak in the spectrum to the variance across the whole dataset; in this presentation, therefore, the (relatively low resolution) traces can be correlated with the original spectrum. In Fig. 3 a representative full spectrum is shown at the top (blue trace) and the PC1 loadings trace is shown inverted (red trace). Individual components most responsible for the variation between spectra in this group are highlighted on the plot. A variety of LDL/VLDL and sugar resonances can easily be recognized, in addition to choline, the importance of which in insulin resistance has not previously been documented. Further investigation of changes in lipo-proteins and choline in normal and insulin-resistant subjects is warranted. It is worth mentioning that this analysis highlights components as a result of blind statistical analysis, without any bias or a priori knowledge.

^{13}C NMR data using labeled glucose

Labeling studies are commonly used for metabolic flux analysis and to identify yet unknown metabolic pathways but, to the best of our knowledge, have never been conducted on a large animal, for example a horse. Use of the ^{13}C-detection-optimized cryoprobe resulted in detection of specific metabolites at natural abundance (0 min spectrum in Fig. 4). Administering ^{13}C-labeled glucose to the horse leads to elevated intensities of the glucose signals, however; these then follow the expected kinetic trend shown already in ^{1}H NMR (Fig. 4a). Even more important are peaks that appear for the first time after administration of ^{13}C-labeled glucose. These are downstream metabolites in glucose metabolism that would not be visible without the specifically labeled material. Some of these peaks are indicated with arrows (Fig. 4b), but remain unidentified. Further analysis of these spectra is in progress.

Conclusion

NMR-based metabonomics is a powerful tool for studying NIDDM and insulin resistance. It enables identification of metabolites previously unrecognized as being part of the metabolic response to standard challenges. The high incidence of naturally occurring insulin resistance in young horses makes this species an attractive model for future investigations of the pathogenesis and metabolic pathways involved in the disorder. As the sensitivity of measurements increases with the introduction of enhanced technology, for example specialized cryoprobes, and more efficient metabonomic data analysis, we can expect significant contributions to further scientific and medical understanding of the underlying biochemistry and system-level biology of this condition and disease.

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