

METABOLIC PHENOTYPING GUIDELINES

Assessing glucose homeostasis in rodent models

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Abstract

The pathophysiology of diabetes as a disease is characterised by an inability to maintain normal glucose homeostasis. In type 1 diabetes, this is due to autoimmune destruction of the pancreatic β -cells and subsequent lack of insulin production, and in type 2 diabetes it is due to a combination of both insulin resistance and an inability of the β -cells to compensate adequately with increased insulin release. Animal models, in particular genetically modified mice, are increasingly being used to elucidate the mechanisms underlying both type 1 and type 2 diabetes, and as such the ability to study glucose homeostasis *in vivo* has become an essential tool. Several techniques exist for measuring different aspects of glucose tolerance and each of these methods has distinct advantages and disadvantages. Thus the appropriate methodology may vary from study to study depending on the desired end-points, the animal model, and other practical considerations. This review outlines the most commonly used techniques for assessing glucose tolerance in rodents and details the factors that should be taken into account in their use. Representative scenarios illustrating some of the practical considerations of designing *in vivo* experiments for the measurement of glucose homeostasis are also discussed.

Key Words

- ▶ diabetes
- ▶ insulin secretion
- ▶ insulin resistance
- ▶ glucose metabolism

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Introduction

The incidence of diabetes mellitus, particularly obesity-related type 2 diabetes, is increasing at an alarming rate in the developed world, and this epidemic is driving numerous research programmes into the causes of, and new treatment regimens for, this metabolic disorder. The complex hormonal control of nutrient homeostasis involves numerous tissues and organs, including liver, skeletal muscle, adipose, endocrine pancreas and CNS. While *in vitro* studies can provide cellular mechanistic insights, it is inevitable that *in vivo* models are needed to study the integrated control systems. Many animal models for the study of diabetes already exist, with various

mechanisms for inducing either type 1 or type 2 diabetes (King 2012). Furthermore, genetically modified mouse models in which genes are up- or down-regulated either globally or in a tissue-specific manner are increasingly used to assess the physiological role of a potential target in glucose homeostasis and the development of diabetes. Consequently, techniques for accurately assessing glucose homeostasis *in vivo* in rodents are essential tools in current diabetes research.

Mice and rats are by far the two most commonly used species for experimental studies of glucose homeostasis, and both models have specific advantages and disadvantages.

The primary advantage of using a rat model is a technical consideration in that the larger size of the rat facilitates complex surgical procedures such as catheterisation, and the larger blood volume allows the sampling of more frequent and/or larger blood samples to enable detailed and simultaneous monitoring of multiple plasma hormone levels. Surgical techniques developed in the rat have been successfully miniaturised for use in mouse models, although they are technically challenging, and blood sampling in mice can be a limiting factor in experimental design. However, despite these limitations, the choice of mice for experimental studies allows access to numerous genetically modified models, and knockout and transgenic mice have become powerful tools in elucidating the role of specific genes and pathways. The increasing availability and use of tissue-specific transgenic mice as tools for investigating the (patho) physiology of diabetes has greatly increased the application of *in vivo* studies of glucose homeostasis.

Glucose homeostasis in rodents, as in humans, is primarily determined by two factors – the rate of insulin release from the pancreatic islets of Langerhans in response to circulating glucose, and the sensitivity of the target tissues to insulin. The balance between these two factors determines the overall physiological tolerance of the animal to glucose and its ability to maintain glucose homeostasis within the normal physiological range. Thus, when assessing glucose homeostasis there are multiple end-points that can be measured, and a comprehensive assessment of glucose homeostasis should involve three main elements – quantification of islet hormone secretion in response to changes in plasma glucose; assessment of the sensitivity of target tissues to these hormones, particularly insulin; and an overall determination of glucose tolerance, which reflects the combination of these two factors. However, the most comprehensive methods for analysing glucose homeostasis *in vivo* may not be appropriate for all experimental designs, and choosing the appropriate methodology is dependent on the desired end-points. Studies of islet adaptation and function may focus on hormone release in response to glucose and other stimuli, whilst studies in obesity and type 2 diabetes may focus on insulin resistance, the reduced sensitivity to insulin characteristic of these states. In this review, we will discuss the methods available for measuring the multiple end-points of glucose homeostasis in rodent models, along with their advantages, disadvantages and the considerations that must be taken into account in their use (Fig. 1).

It is beyond the scope of this review to consider in detail the principles of designing experimental protocols

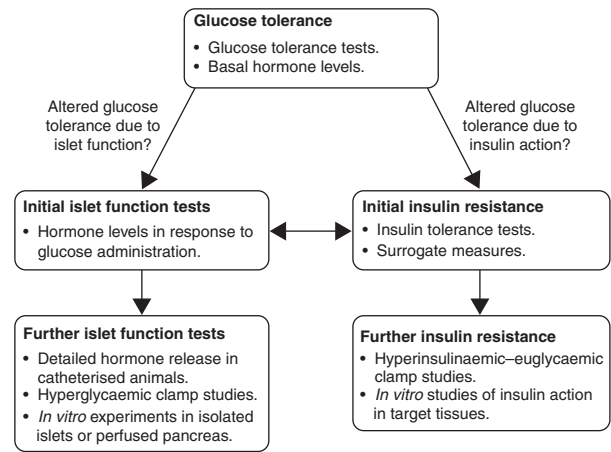


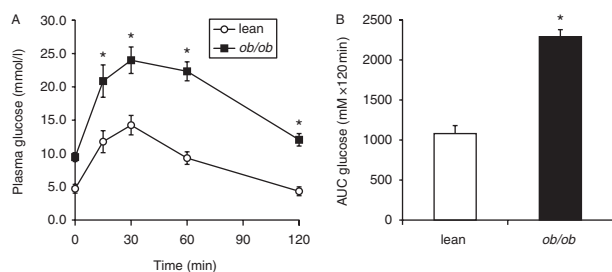
Figure 1

Designing a study of glucose homeostasis in rodents. Schematic diagram summarising the different methodologies available for the study of glucose homeostasis and their place in an overall plan for assessing glucose homeostasis. For a given animal model the first step is to screen for differences in fasting blood glucose and plasma insulin and to carry out a glucose tolerance test. If altered glucose tolerance is observed then further tests will depend on the likely hypothesis. If the changes in glucose tolerance are suspected to be due to effects on islet function more detailed measures of islet hormone release in response to glucose administration would be taken, followed by hyperglycaemic clamp studies or further *in vitro* work if results are promising. If the hypothesis is that changes in glucose tolerance are due to altered insulin sensitivity an initial insulin tolerance test or use of surrogate measures of insulin resistance would be recommended, potentially followed by hyperinsulinaemic-euglycaemic clamp studies and *in vitro* work. Ideally both aspects of glucose homeostasis will be tested, though this will depend on the objectives of the study.

which replace, refine, or reduce (3Rs) the use of animals in research studies, but detailed information is available through the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs – <http://www.nc3rs.org.uk>). The NC3Rs have also been instrumental in developing guidelines for reporting *in vivo* studies using experimental animals (ARRIVE guidelines) as a mechanism to ensure that data from animal experiments can be fully evaluated and utilised (Kilkenny *et al.* 2010a,b). We recommend the 3Rs and ARRIVE guidelines to all researchers using *in vivo* animal models.

Glucose tolerance

The most commonly used method for assessing glucose homeostasis in rodents is the glucose tolerance test (GTT). In brief, mice or rats are routinely fasted before administration of a glucose load, most commonly either through oral gavage or by a single i.p. injection. Baseline (fasting) blood glucose measurements are taken before glucose administration, and further measurements are made at

**Figure 2**

Comparing glucose tolerance in obese and lean mice. Obese *ob/ob* mice had significantly impaired glucose tolerance following i.p. glucose administration (2 g/kg) when compared with lean controls. Specifically *ob/ob* mice had significantly higher baseline blood glucose levels after an overnight fast, as well as significantly higher levels at all time points following glucose administration (A) and higher area under curve values (B). Mean \pm s.e.m., $n=6$, * $P<0.05$ vs lean control.

regular intervals thereafter. The GTT is the simplest, and usually the first, test applied to an animal model, and it provides a physiological overview of any changes in glucose tolerance without determining the causative mechanisms. GTT results can be expressed as both a time course of absolute blood glucose measurements and as the area under the curve (AUC). Generally, a two-way repeated measures ANOVA would be appropriate for the statistical comparison of the time course of glucose levels, whilst AUC data may be compared using the *t*-test. In cases where there is a difference in baseline fasting glucose levels, the absolute blood glucose levels should still be presented, but glucose tolerance can be assessed through comparison of baseline corrected AUC values. A representative example of GTT data from lean control mice and from obese leptin-deficient *ob/ob* mice (Fig. 2A and B) highlights some of the advantages and disadvantages of this type of test. Thus, a relatively simple procedure demonstrates the significantly impaired glucose tolerance in *ob/ob* mice compared with lean controls, either through the time course of elevated plasma glucose (Fig. 2A) or the cumulative AUC plasma glucose data (Fig. 2B). Alone these data clearly identify a pathological phenotype for further study, but provide no further information on the mechanisms underlying the phenotype, and further, more detailed studies are required for mechanistic insights. For example, additional blood samples could be collected during the GTT for the measurement of plasma insulin levels.

GTTs are the most widely used test in the literature for assessing glucose homeostasis in rodents, but there is remarkably little consensus on a specific protocol, although several recent studies have attempted to evaluate the GTT and to encourage standardisation of optimal protocols

between studies (Andrikopoulos *et al.* 2008, Ayala *et al.* 2010). Even a relatively simple method like the GTT can be influenced by several variables that must be considered when designing the study, including the length of the fasting period, the glucose dose and its route of administration. In addition, when comparing between studies, factors such as age, strain and sex of the animals used should be considered. Different commonly used mouse strains are known to have variations in glucose metabolism, which further complicates direct comparisons between studies (Andrikopoulos *et al.* 2008, Berglund *et al.* 2008), and there is also an increase in insulin resistance with ageing in both mice and rats (Bailey & Flatt 1982, Carvalho *et al.* 1996), resulting in impaired glucose tolerance. Furthermore, differences exist in glucose metabolism between male and female animals (Shi *et al.* 2008, Macotela *et al.* 2009), and this should be considered, both in normal laboratory animals and particularly when studying novel genetically modified mice in which the phenotype may differ between the sexes. Finally, even when using the same animal strain, it is worth noting that changing animal source may introduce variability as different levels of insulin secretion have been reported in C57BL/6 mice from different suppliers (Freeman *et al.* 2006, Mekada *et al.* 2009). Therefore it is essential that all studies of glucose homeostasis should be carried out in age-matched animals of the same sex and strain, and sourced from the same supplier where possible, and these details should be reported.

Fasting

Some period of fasting is required before glucose administration to provide stable baseline measurements and to obtain consistent excursions in plasma glucose after glucose loading. Typically fasting periods are either an overnight fast (ca. 16 h) or a fast of ~6 h, starting in the morning. Overnight fasting is most commonly used in published studies for GTTs in both mice and rats, and has the advantage of producing low, stable baseline blood glucose and insulin levels (Heikkinen *et al.* 2007, Muniyappa *et al.* 2008). However, several recent studies have expressed concern that overnight fasting in mice is not ideal because, as nocturnal animals, they consume most of their daily calories overnight. This, combined with their relatively high metabolic rate, means that an overnight fast is a relatively long time for mice to be deprived of food, and it may induce a state more similar to starvation than to an overnight fast in humans (Andrikopoulos *et al.* 2008). It is also worth noting that prolonged fasting inhibits insulin-stimulated glucose uptake in humans, but

the reverse is true in mice, where overnight fasting increases insulin sensitivity (Ayala *et al.* 2006).

Route and dose of glucose administration

The most common methods for the administration of glucose as part of GTT in rodents are by oral gavage and single i.p. injection. Both routes of administration are widely accepted as appropriate methods for administering glucose, and are similar in terms of both technical difficulty and severity for the animal. I.p. injection is subject to error as a result of injection into the intestines or stomach rather than the intraperitoneal space (Arioli & Rossi 1970), which affects the rate at which glucose appears in the circulation, but it is a simple and reliable technique which is available to most laboratories. However, gavage should not be ignored as a route of glucose administration for GTT because it can generate additional information to intraperitoneal glucose delivery. Figure 3 highlights the effect that the route of glucose administration can have on GTT results, by showing blood glucose (Fig. 3A) and AUC (Fig. 3B) data from mice administered the same dose of glucose (2 g/kg) by either oral gavage or i.p. injection. Peak plasma glucose levels in response to an oral GTT are significantly lower when compared with the same glucose dose administered as an i.p. injection, and this is reflected in statistically significant differences at 15, 30 and 60 min time post-glucose loading, as well as in the glucose AUC values. In accordance with the different plasma glucose profiles, there are also differences in the dynamics of the plasma insulin response to oral and intraperitoneal delivery, with oral glucose resulting in a more rapid plasma insulin response peaking at 15 min,

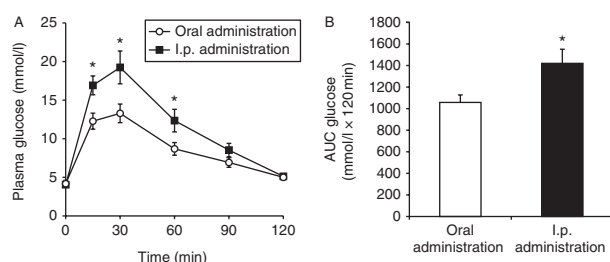


Figure 3 Comparing i.p. and oral glucose administration. In male ICR mice, i.p. glucose administration (2 g/kg) caused a significantly greater increase in blood glucose levels at 15, 30 and 60 min post-injection when compared with the same dose of glucose administered by oral gavage (A). Furthermore, the glucose AUC values over 2 h were significantly higher in animals administered glucose via i.p. injection rather than by oral gavage (B). Mean \pm s.e.m., $n=8$, $*P<0.05$ vs oral administration.

whilst intraperitoneal glucose results in a slower response and a delayed peak at 30 min (Andrikopoulos *et al.* 2008). It is well established that absorption of glucose from the gut leads to the incretin effect – the release of gastrointestinal hormones, primarily GLP1 from the intestinal L-cells – which in turn potentiates glucose-induced insulin release (Drucker 2013). Thus, the incretin effect following oral glucose delivery results in an elevated insulin secretory response with consequently lower blood glucose levels relative to i.p. glucose injection, where the incretin effect is absent. It is important to be aware of these differences when designing GTTs, in which the main objective is to study hormonal changes in response to nutrient intake. It is also worth briefly noting the difference in the extent of glucose excursion following i.p. administration of an equivalent glucose dose in Fig. 2 compared with Fig. 3. C57BL/6 mice, the background strain for obese *ob/ob* mice, were used to collect the lean data in Fig. 2, whilst the data in Fig. 3 was collected from ICR mice. ICR mice are ~5 g heavier than equivalent age-matched C57BL/6 mice, and there are established differences in glucose metabolism between the two strains, particularly in models of diabetes (Luo *et al.* 1998, Burgess *et al.* 2005, Shimizu *et al.* 2012). Thus this is most likely due to variations in glucose metabolism between different mouse strains as mentioned previously (Andrikopoulos *et al.* 2008, Berglund *et al.* 2008), and reinforces the point that rodent strain is an important factor to be taken into consideration when comparing between studies.

I.v. administration of glucose is used much less commonly than the oral or intraperitoneal routes because unless the researcher is skilled in the technique it can be both more difficult and more stressful for the animal. Whilst i.v. administration into the tail vein is occasionally used for GTT tests (Akerblom *et al.* 2007), it is more frequently used when experimental animals are implanted with intravenous catheters, usually for sampling purposes. In animals in which intravenous catheters are implanted, i.v. administration of glucose can be a useful route of administration, circumventing the gut-derived incretin effect and thus generating a reduced insulin response and a higher peak in blood glucose, similar to intraperitoneal glucose loading (Ahren *et al.* 2008). However, the dynamics of the responses to i.p. glucose administration are different to those when glucose is given by i.v. administration: after i.p. administration blood glucose levels peak within 15–30 min, while i.v. administration of glucose causes an immediate peak in blood glucose concentrations which return to baseline levels over approximately 30 min. Thus blood sampling patterns

need to be tailored to the route of glucose administration in GTTs, with more samples taken at frequent early time points after i.v. glucose administration.

The dose of glucose administered for GTT experiments is an additional source of variability, with either 1 or 2 g/kg glucose being typically used in the literature (Muniyappa *et al.* 2008), irrespective of the route of administration. This may be important in studies of insulin resistance as impaired glucose tolerance may only be revealed in response to the higher glucose loading (2 g/kg; Andrikopoulos *et al.* 2008). This approach of dosing animals based on body weight is adequate assuming weight and body composition is approximately equivalent between groups. However, in comparisons involving groups of different weights, such as comparing lean mice with obese mice maintained on a high-fat diet, body composition should be taken into account. In many high-fat models, the animals increased weight is predominantly additional fat mass without a similar increase in muscle or liver, the primary tissues involved in lowering blood glucose. Thus animals may be administered a higher glucose based on weight, despite lacking a proportionate increase in lean mass to process this additional glucose, biasing results towards showing impaired glucose tolerance in the high-fat group. Thus, if possible when comparing animals with different body compositions, it is desirable to calculate the dose based on lean body mass as opposed to total body weight (McGuinness *et al.* 2009).

Stress and anaesthesia

It is important to minimise stress to the experimental animal in all *in vivo* studies of glucose homeostasis because it is well established that activating the stress response, with the subsequent elevations in adrenaline and nor-adrenaline, has major physiological effects on glucose handling (Nonogaki 2000, Ziegler *et al.* 2012), which will complicate the interpretation of the experimental data. This is particularly relevant in protocols that involve restraint or repeated handling of the animal, which are acknowledged stressful stimuli (Balcombe *et al.* 2004, Buynitsky & Mostofsky 2009). The potential influence of stress during experimental protocols can be assessed by measuring plasma catecholamines or corticosterone (Balcombe *et al.* 2004), but this may be impractical because of sample volume, as discussed earlier.

One mechanism to minimise stress in experimental studies *in vivo* is to perform the studies on anaesthetised animals, but this may not offer an ideal solution depending on the anaesthetic used. Some anaesthetics lower heart rate

and blood flow, which may influence nutrient homeostasis. Whilst, several anaesthetic regimens such as pentobarbital and fentanyl–ketamine–midazolam have been shown to have little effect on either blood glucose or insulin release (Guarino *et al.* 2013, Zuurbier *et al.* 2014), other anaesthetics can cause insulin resistance and hyperglycaemia in both mice and rats (Pomplun *et al.* 2004, Brown *et al.* 2005, Tanaka *et al.* 2009, Guarino *et al.* 2013, Sato *et al.* 2013), which could potentially confound interpretation of experimental data generated using anaesthetised animals. For example isoflurane anaesthesia results in significantly suppressed plasma insulin levels, whilst ketamine–medetomidine–atropine anaesthesia results in almost complete suppression of insulin release and hyperglycaemia (Zuurbier *et al.* 2014). Thus, the precise effects on glucose metabolism will obviously depend on the anaesthetic regimen (Guarino *et al.* 2013, Sato *et al.* 2013). Investigating whole-body glucose homeostasis under anaesthesia may be a viable technical approach, but requires careful consideration of the anaesthetic regimen to be used.

Time of day

Circadian rhythms have been shown to affect glucose metabolism in both mice and rats with variations in glucose plasma hormone levels across a 24 h period (Kohsaka & Bass 2007). Thus the time of day that experiments are conducted can be an important factor for consideration and all experiments in a given study should be carried out at the same time of day and this should be reported. Similarly the light–darkness cycle of the room in which the animals were kept should be reported.

Islet function

Measurement of blood glucose requires small blood volumes (typically 5 µl) that can be obtained through a needle prick to the tip of the tail, but assessment of islet function requires larger blood volumes for hormone assay. Analysis of islet secretory function *in vivo* primarily involves the measurement of basal plasma insulin levels and the increase in insulin release from the islets in response to a defined glucose challenge. However, depending on the aims of the study, it may also be desirable to measure other circulating hormones such as glucagon, somatostatin and C-peptide. The development of commercially available sensitive assays now allows the detection of hormones in relatively small plasma volumes, but a blood sample of ~50 µl is still required for the accurate measurement of plasma insulin (with replicates),

and this volume cannot be easily collected from a needle prick to the tail. These larger blood volumes can be sampled from the mouse tail by incision over the tail vein or by clipping the distal 1–2 mm of the tail tip, with blood samples being collected into capillary tubes after massaging the tail. However, obtaining frequent and multiple blood samples through this route is difficult and stressful for the animal, so alternative experimental strategies are required to monitor minute-to-minute changes in circulating hormone levels in rodent models.

Surgical catheterisation

Chronic implantation of indwelling catheters is a time-consuming and technically demanding surgical procedure, but it has the major advantages of (i) allowing studies be carried out with minimal handling of the animals, thus reducing their levels of stress (see above); (ii) allowing access to the vascular space for administration and sampling. Thus, catheters allow for both i.v. infusion and the collection of blood samples in conscious unrestrained animals in, for example, hyperglycaemic or hyperinsulinaemic–euglycaemic clamp studies which require frequent blood sampling and for the monitoring of plasma hormone levels to assess islet function. Whilst the implantation of catheters is too complex for initial studies such as GTTs, it offers a follow-on procedure for mechanistic *in vivo* studies.

There are species differences in catheterisation approaches using rodents. In the mouse, intravenous catheters are usually implanted into the femoral or jugular vein for infusion of experimental substances, whilst arterial catheters are implanted into the carotid or femoral arteries for the collection of blood samples for analysis. In contrast, in the rat venous catheters are usually implanted into the jugular vein, where they are routinely used for both infusion and collection of blood samples through the same single catheter. The ability to both infuse and collect samples from the same catheter, the capacity to take considerably larger blood samples and the relative ease of catheter implantation in rats makes them a more attractive model for studies requiring surgical catheterisation. In both mice and rats, the catheters are generally externalised at the back of the head and are either fixed in place or attached to a tether to ensure that they cannot be interfered with by the experimental animals.

Hyperglycaemic clamp

The hyperglycaemic clamp is a powerful tool for analysing how the islets cope with hyperglycaemia, but its

complexity means that it is a less frequently used technique for assessing islet function *in vivo*. In brief, an initial dose of glucose is delivered via an intravenous catheter to induce hyperglycaemia and a variable rate of glucose is then infused to maintain a pre-determined level of hyperglycaemia for the duration of the experiment. Frequent measurements of blood glucose inform the maintenance of the pre-determined hyperglycaemia by adjusting the rate of glucose infusion, with blood samples being taken for the measurements of insulin content when appropriate. Frequent sampling during the early stages of the hyperglycaemic clamp also permits the analysis of both first-phase and second-phase insulin secretion under optimal conditions due to the steady level of elevated blood glucose. Whilst frequent early samples can be taken following a single bolus administration of glucose, the changing blood glucose levels across the sampling period makes both data interpretation and comparison between first phase and second phase insulin release more difficult. The larger sampling volumes available from rats permit the measurement of other hormones such as glucagon, somatostatin or incretins to further dissect the hormonal control of glucose homeostasis.

Insulin resistance

As discussed earlier, any detailed analysis of glucose homeostasis *in vivo* should include some measurement of insulin resistance to complement measurements of glucose tolerance and hormone secretion. There are a number of different methods for the evaluation of insulin resistance in animal models *in vivo*, ranging in complexity from surrogate measures of insulin resistance, such as the homeostasis model assessment of insulin resistance (HOMA-IR) and the quantitative insulin check index of insulin sensitivity (QUICKI), to the insulin tolerance test (ITT) and to the hyperinsulinaemic–euglycaemic clamp, which is a technically complex technique (Radziuk 2000). As might be expected, the more complex methods provide more detailed information, and the choice of methodology should be informed by the predicted outcomes of the study.

HOMA-IR and QUICKI

HOMA-IR and QUICKI are surrogate measures of insulin resistance that are routinely used in clinical studies of human glucose homeostasis, and they are calculated from fasting blood glucose and fasting plasma insulin levels, which are routinely measured in GTT studies (see above).

Though both indices were derived independently in humans, and validated against hyperinsulinaemic–euglycaemic clamp measurements, they are related mathematically. The principle difference between the two indices is that the equation for calculating HOMA-IR (insulin (mU/l) × glucose (mmol/l)/22.5) includes a normalising factor specific to its use in humans, whilst the equation for calculating QUICKI (1/(log(insulin) + log(glucose))) does not (Mather 2009).

Whilst not a replacement for direct measurement of insulin resistance, HOMA-IR and QUICKI provide an acceptable substitute whereas other techniques are not possible. Formal assessments of the relationship between surrogate measures using indices and clamp measures of insulin resistance confirm that both HOMA-IR and QUICKI provide a reasonably reliable approximation of direct measures of insulin resistance in both rats and mice as in humans (Cacho *et al.* 2008, Lee *et al.* 2008). Surrogate indices adequately reflect the differences in hyperinsulinaemic–euglycaemic clamp data for insulin resistance seen during pregnancy in rat (Cacho *et al.* 2008) and transgenic mice (Lee *et al.* 2008), validating the continued use of these measures in animal studies (Fig. 4D).

Insulin tolerance test

The ITT is technically very similar to the GTT as it involves monitoring blood glucose levels over time, but in response to insulin administration rather than glucose loading. ITTs are usually carried out in 6 h fasted animals and blood glucose levels are monitored every 15–30 min for 60–90 min following insulin administration. The degree to which blood glucose levels fall in response to insulin administration is indicative of insulin sensitivity, primarily in liver and skeletal muscle.

Many of the technical considerations that apply to GTTs also apply to ITTs, though with some additional factors. Insulin administration in fasted animals carries the risk of inducing hypoglycaemia, so we recommend a fasting time of 6 h to reduce the possibility of hypoglycaemia associated with overnight (16 h) fasted animals. Insulin can be administered as either a bolus i.v. or i.p. injection, while oral gavage is not an option for the administration of insulin. The half life of insulin in the circulation is <10 min, therefore blood glucose measurements in ITTs are usually limited to within 90 min of insulin administration as any later effects are unlikely to be due to the administered insulin. As with GTTs, results can be expressed as both a time course of blood glucose measurements and the AUC (see Fig. 4E and F).

Hyperinsulinaemic–euglycaemic clamp

The hyperinsulinaemic–euglycaemic clamp was first described in 1979 (DeFronzo *et al.* 1979) and is generally considered to be the ‘gold standard’ for the specific evaluation of whole-body insulin sensitivity *in vivo*. The primary advantage of the hyperinsulinaemic–euglycaemic clamp over other techniques is that, through infusion of insulin and glucose, it eliminates the effects of endogenous glucose and insulin, allowing accurate quantification of insulin action with minimal interference from endogenous control

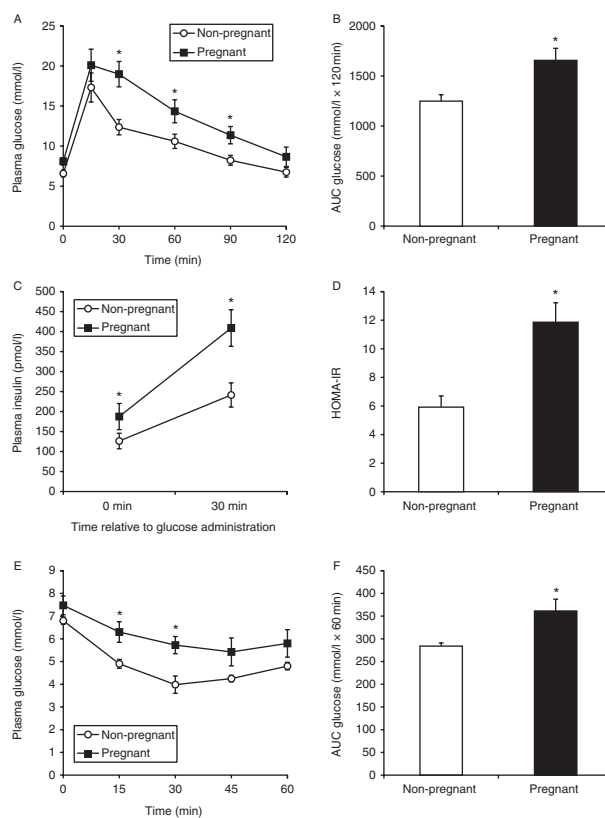


Figure 4

Scenario 1 – assessing glucose homeostasis in pregnant mice. At day 16 of pregnancy mice fasted for 6 h had significantly impaired glucose tolerance following i.p. glucose administration (2 g/kg) when compared with non-pregnant controls, as determined by both comparison of individual time points (A) and glucose AUC (B) over the course of the test. Pregnant mice also had significantly increased fasting plasma insulin levels and significantly greater insulin release in response to i.p. glucose administration (2 g/kg) after 30 min when compared with non-pregnant controls (C). Fasting blood glucose and plasma insulin levels were used for HOMA-IR calculations, indicating that the pregnant mice were significantly more insulin resistant than non-pregnant mice (D). At day 18, pregnant mice fasted for 6 h had significantly reduced plasma glucose response to i.p. insulin administration (0.75 IU/kg) when compared with non-pregnant controls, again through both comparison of individual time points (E) and glucose AUC (F) over the course of the test. Mean ± s.e.m., $n=6$, * $P<0.05$ vs non-pregnant control.

mechanisms. In brief, exogenous insulin is infused into fasted animals through an intravenous catheter to maintain steady hyperinsulinaemia, which suppresses endogenous glucose production and stimulates glucose uptake by insulin target tissues. Glucose is then infused at a variable rate in order to maintain euglycaemia. Regular blood glucose measurements are made in order to ensure euglycaemia and the rate of glucose infusion adjusted as necessary. Animals with enhanced insulin sensitivity will require a greater rate of glucose infusion to maintain euglycaemia, whereas insulin resistant animals will require lower rates of glucose infusion.

Despite the high quality of the data that may be obtained using this technique, the hyperinsulinaemic–euglycaemic clamp has several disadvantages which prevent its widespread adoption for experimental studies of glucose homeostasis. First, because of the invasive surgical approach many hyperinsulinaemic–euglycaemic clamp experiments have been conducted under anaesthesia (Auvinen *et al.* 2013, Tanaka *et al.* 2013), with the potential caveats outlined above. Second, the hyperinsulinaemic–euglycaemic clamp is a technically demanding approach, which represents a relatively severe procedure for the experimental animal, and the fact remains that the small animal surgery involved in this model is technically challenging, and may not be an option for all groups interested in studying glucose homeostasis. However, whilst not appropriate for a preliminary assessment, the hyperinsulinaemic–euglycaemic clamp remains the ‘gold standard’ for detailed, controlled assessment of insulin resistance. Recently, reports of the application of this technique in conscious, unrestrained mice have increased and have the potential to widen its use to many transgenic mouse models of metabolic disorders, as covered in further detail by several recent studies (Ye *et al.* 2008, Ayala *et al.* 2011).

Experimental scenarios

One aim of this review is to give practical guidance about how best to assess glucose homeostasis *in vivo* in rodent models. To illustrate how we currently assess glucose homeostasis in mice and rats, we here present representative *in vivo* data from two studies, in which we are currently measuring a range of experimental parameters. We include these experimental scenarios to highlight both the practical considerations when designing experiments to measure glucose homeostasis in rodents and the differences between the information obtained from a relatively non-invasive mouse model (scenario 1) and from a surgically-invasive rat model (scenario 2).

Scenario 1 – assessing glucose homeostasis in pregnant mice

Despite their inherent limitations, mice are by far the most commonly used animal model for the study of glucose homeostasis, so this scenario will represent an initial characterisation of glucose homeostasis in a mouse model. In this case, the pregnant mouse is used as an example of a model in which glucose homeostasis is altered, but similar techniques are applicable to investigate glucose homeostasis in other mouse models. It is well established that during pregnancy, mice become progressively more insulin resistant, inducing a compensatory increase in β -cell mass that leads to an increased capacity for insulin release and maintenance of relatively normal glucose homeostasis (Sorenson & Brelje 1997, Rieck & Kaestner 2010). The mechanisms underlying this adaptation of islets to pregnancy are currently poorly understood, and the study of glucose homeostasis in pregnant mice *in vivo* is likely to provide a critical tool moving forward in this area of research. However, during pregnancy there are also additional considerations beyond those for non-pregnant animals that must be taken into account in the experimental design. As such this scenario offers illustrative practical guidelines for one approach to the assessment of glucose homeostasis in pregnant mice, along with the justification for this design.

Assessment of glucose homeostasis in pregnant mice used a GTT and an ITT on 6 h fasted mice, which were carried out at day 16 and 18 of pregnancy, separated by a day to allow recovery of the animals. Both the GTT and ITT were carried out following a 6 h fast. As discussed previously, there is no universally agreed optimum length of fast before measuring glucose homeostasis. Overnight fasting is most commonly used, and is a reasonable choice for most scenarios. However, in pregnancy where the developing foetuses require a constant supply of nutrients and are placing a heavy metabolic demand on the mother, an overnight fast was considered to be too severe; therefore a 6 h fast was chosen instead. Furthermore, the route of glucose administration was an additional consideration. The presence of the foetuses must be considered when injecting intraperitoneally in pregnant mice to minimise the risk of damaging a foetus. Where the intention is to use GTTs alone in pregnant mice, administration by oral gavage may be the safer choice to reduce any risk of foetal injury. However, oral administration of insulin is not an option for an ITT, so for consistency both tests were

carried out using the i.p. administration route. During GTTs, in addition to blood glucose measurements, further 50 μ l blood samples were taken by capillary from a superficial incision on the tail vein at both baseline and 30 min after glucose administration for the separation of plasma and measurement of insulin levels. Other studies have previously taken additional plasma samples during mouse GTTs for the measurement of plasma insulin (Ahren *et al.* 2008, Andrikopoulos *et al.* 2008, Barbosa-Sampaio *et al.* 2013), and modern insulin assays allow the measurement of insulin levels in plasma samples as small as 5 μ l. However, for the purposes of this study, the critical information required was the baseline fasting plasma insulin level and the peak insulin secretion in response to the glucose challenge (30 min). Although it was technically possible to collect additional samples for monitoring the return of plasma insulin to baseline levels, in this particular case it was considered that this extra information did not justify the additional stress to the mice.

Intraperitoneal GTTs (IPGTTs) in pregnant mice revealed impaired glucose tolerance, although the fasted baseline blood glucose levels were similar in pregnant and non-pregnant mice, as shown in Fig. 4. There was no significant difference in plasma glucose levels at 15 min after glucose administration, but pregnant mice were significantly less able to clear the glucose and took longer to bring their blood glucose back down to normal levels (Fig. 4A). This impaired glucose tolerance was also observed in the significantly higher glucose AUC over the course of the GTT (Fig. 4B). Despite similar fasting blood glucose levels, pregnant mice had significantly higher basal plasma insulin levels compared with non-pregnant controls, suggesting insulin resistance (Fig. 4C). Indeed, HOMA-IR calculations based on fasting blood glucose and plasma insulin levels suggest significant insulin resistance in pregnant mice (Fig. 4D). Furthermore, despite their impaired glucose tolerance, pregnant mice had a significantly greater increase in plasma insulin levels in response to glucose administration at 30 min, again consistent with insulin resistance. This was confirmed by the results of the ITT, as shown in Fig. 4E and F. Again, both pregnant and non-pregnant mice had similar fasting blood glucose levels, but insulin administration was significantly less effective in lowering blood glucose levels in pregnant mice when comparing both individual time points and glucose AUC with non-pregnant mice (Fig. 4E and F).

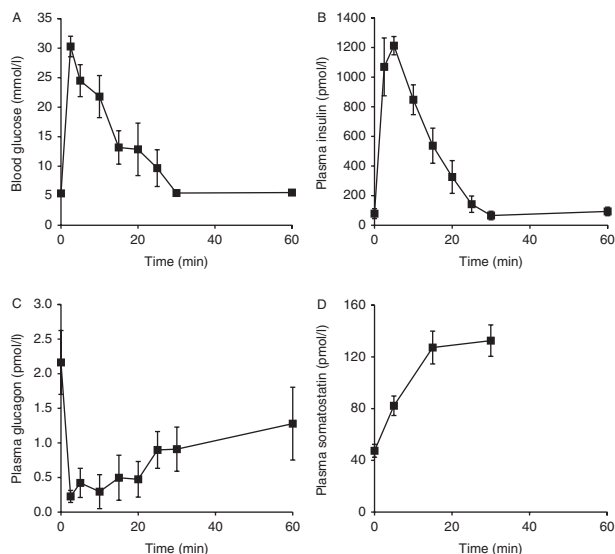
In summary, these relatively simple tests demonstrated that the glucose tolerance of pregnant mice was

impaired in comparison with non-pregnant mice as a result of increased insulin resistance, as assessed by both the direct ITT and HOMA-IR. The insulin resistance was not severe enough to cause elevations in fasting blood glucose, so the pregnant mice are not diabetic, but the impaired glucose tolerance becomes apparent following glucose challenge. To compensate for the insulin resistance in target tissues, the islet β -cells release more insulin both at baseline and in response to glucose, resulting in elevated plasma insulin under both circumstances.

Scenario 2 – assessing islet hormone release *in vivo* in rats

Rat models offer several technical advantages over mice due to their larger size, as discussed earlier. Of particular interest in studies of glucose homeostasis is the capacity to take multiple relatively large blood samples over the course of a single experiment. The contribution of islet function to maintain glucose homeostasis is often simplistically reduced to measure insulin release as an experimental end-point, but the other islet hormones – glucagon and somatostatin – are also involved in glucose homeostasis, and other non-islet hormones, such as GLP1, can also influence glucose handling. The availability of larger blood samples from rats allows measurement of multiple hormone levels over the time course of a GTT or ITT to investigate the complex interplay between endocrine regulators of glucose homeostasis.

In this representative scenario, the aim was to collect detailed measurements of islet hormone levels in the blood in response to a GTT. While studies in humans have investigated plasma levels of insulin, glucagon and somatostatin simultaneously in response to GTTs, along with many other circulating hormones (Jacobsen *et al.* 2012), there is little equivalent data in animal models. Male Wistar rats were chronically implanted with intravenous catheters into the jugular vein, allowing for both infusion and regular blood sample collection in conscious un restrained rats without handling. Following recovery, rats were fasted overnight before GTT. Whilst there is some concern in the literature regarding the severity of overnight fasting in mice, rats are better able to cope with this metabolic stress, and overnight fasting is routinely used in rats for GTTs. Baseline blood samples were taken before i.v. infusion of 1 g/kg glucose and further samples taken at 2.5, 5, 10, 15, 20, 25, 30 and 60 min after glucose for the measurement of blood glucose, plasma insulin and plasma glucagon. Additional blood was taken at baseline, 5, 15 and 30 min for the measurement of plasma somatostatin.

**Figure 5**

Scenario 2 – assessing islet hormone release *in vivo* in rats. I.v. glucose administration (1 g/kg) in overnight fasted male Wistar rats caused an immediate increase in plasma glucose levels, before returning to baseline after 30 min. This rapid rise in plasma glucose levels was matched by similarly increased plasma insulin levels. In contrast, plasma glucagon levels, which were originally high in fasted rats, immediately dropped following glucose administration, whilst plasma somatostatin levels rose more slowly in response to glucose administration. Mean \pm S.E.M., $n=5-6$.

As expected, i.v. glucose administration resulted in an immediate increase in blood glucose, which peaked at 2 min and then gradually returned to baseline over the following 30 min (Fig. 5A). Plasma insulin levels approximately mirrored blood glucose levels, peaking at 5 min in response to elevated blood glucose and gradually returning to baseline levels over 30 min as glucose homeostasis was restored (Fig. 5B). Baseline plasma glucagon levels were high to mobilise glycogen stores in the liver and maintain normal blood glucose levels during fasting. In response to glucose administration, plasma glucagon levels dropped immediately as the islet α -cells responded to elevated glucose levels by reducing glucagon secretion. Glucagon levels then slowly started to rise over the course of 60 min as blood glucose levels returned to normal (Fig. 5C). Release of somatostatin *in vivo* in response to a GTT is less studied. Somatostatin release from the islet δ -cells is stimulated by glucose and acts to inhibit insulin secretion from the β -cells, consistent with plasma somatostatin increasing as plasma insulin levels start to drop (Fig. 5D). However, it is also worth noting that plasma somatostatin may be derived from either the islet δ -cells or from the gut. The assay used in this study is unable to differentiate between the two sources, thus these changes in plasma

somatostatin in response to a GTT may partly reflect effects on the gut.

In summary, the larger blood volume of the rat, combined with the relative ease of implanting catheters, allows for dynamic measurements of multiple hormones in the same animal. This more complex experimental protocol allows investigations into the interactions between different hormones, and highlights the fact that the endocrine control of glucose homeostasis is not restricted to the release and actions of insulin but is a more subtle, multifactorial control system.

Conclusion

Rodent models are increasingly being used in *in vivo* studies of glucose homeostasis. The choice of species – rat or mouse – and the choice of experimental protocol should be influenced by the system being studied, the expected outcomes, the technical and financial resources available and the degree of severity of the procedure to the experimental animal. As might be expected, data obtained from the simpler, non-invasive models are less informative than those obtained using more complex, surgically-invasive models, but this needs to be balanced against the difficulty and effort required to generate the data, and against the severity of the procedure for the experimental animals. It is therefore important before choosing the experimental model to have a clear assessment of the minimum level of detail required in the end-point measurements, because this will enable informed decision making about how best to design and execute the experiments and to interpret the experimental data.

Methods

Animals

For the mouse data presented here female ICR mice (25 g), male *ob/ob* mice (35 g) or lean C57BL/6 mice (20 g) (Harlan, Bicester, UK) at 8 weeks of age were used. For experiments involving pregnant mice, females were housed with a male and checked daily for the presence of a vaginal plug. The day a vaginal plug was observed and designated as day 0 of pregnancy. For rat experiments, male Wistar rats (Harlan) at 10 weeks of age were used. Animals were housed under controlled conditions (1400 h light:1000 h darkness; lights on at 0700 h; temperature at 22 ± 2 °C) and provided with food and water *ad libitum*. All animal procedures were undertaken in accordance with the United Kingdom Home Office Regulations.

Glucose tolerance tests

Following an overnight 16 h fast, mice were administered with glucose (2 g/kg) via either an i.p. injection or by oral gavage. Small blood samples were taken by tail prick at 0, +15, +30, +60, +90 and +120 min relative to glucose administration for the measurement of blood glucose levels using a StatStrip glucose meter (Nova Biomedical, Waltham, MA, USA).

Pregnant mice

On day 16 of pregnancy, an IPGTT was carried out. Following a 6 h fast, mice were administered with glucose (2 g/kg) via i.p. injection. Small blood samples (5 µl) were taken by tail prick at 0, +15, +30, +60, +90 and +120 min relative to glucose administration for the measurement of blood glucose levels using a StatStrip glucose meter (Nova Biomedical). In addition, two larger plasma samples (50 µl) were taken from the tail vein before glucose administration and 30 min post-glucose administration for the measurement of plasma insulin levels. Plasma was separated by centrifugation (1500 g, 5 min at 4 °C) and frozen for later assay of islet hormone content. HOMA-IR was calculated using the following formula: $\text{HOMA-IR} = (\text{fasting serum insulin (mU/l)}) \times (\text{fasting serum glucose (mmol/l)}) / 22.5$.

At day 18 of pregnancy, an intraperitoneal ITT (IPITT) was carried out on all mice. Again, following a 6 h fast, mice were administered with insulin (0.75 IU/kg, Sigma) via i.p. injection. Small blood samples were taken by tail prick at 0, +15, +30, +45 and +60 min relative to insulin administration for the measurement of blood glucose levels.

Intravenous cannulation in rats

Surgical procedures were performed under ketamine (100 mg/kg i.p.; Pharmacia and Upjohn Ltd) and Rompun (10 mg/kg i.p.; Bayer) anaesthesia. The rats were fitted with two indwelling cardiac catheters via the jugular veins as described previously (Bowe *et al.* 2009). The catheters were exteriorised at the back of the head and secured to a cranial attachment: the rats were fitted with a 30 cm long metal spring tether (Instec Laboratories, Inc., Boulder, CO, USA). The distal end of the tether was attached to a fluid swivel (Instec Laboratories), which allowed the rat freedom to move around the enclosure. Experimentation commenced 3 days later.

Before experimental tests, the rats were fasted for 16 h overnight. Blood samples (100 µl) were withdrawn with

heparinised syringes via cardiac catheters at baseline, 2.5, 5, 10, 15, 20, 25, 30, and 60 min following glucose administration (1 g/kg) for the measurement of blood glucose, insulin and glucagon. At baseline, 5, 15 and 30 min, additional blood was collected for the measurement of somatostatin. Blood glucose concentration was measured using a StatStrip glucose meter (Nova Biomedical) after which the plasma was separated by centrifugation (1500 g, 5 min at 4 °C) and frozen for later assay of islet hormone content.

The hormone content of plasma samples was assessed by ELISA, using commercially available kits (insulin: Millipore, Watford, UK; glucagon: Mercodia, Uppsala, Sweden; somatostatin: USCN Life Science, Inc., Wuhan, China).

Statistical analyses

All data were expressed as mean and standard error of the mean (S.E.M.). For comparison between two groups an un-paired Student's *t*-test was used. For analysis of IPGTT and IPITT data, a two-way repeated measures ANOVA was used. Differences with $P < 0.05$ were considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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