Evaluating the glucose tolerance test in mice

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Submitted 21 July 2008; accepted in final form 18 September 2008

Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J. Evaluating the glucose tolerance test in mice. Am J Physiol Endocrinol Metab 295: E1323–E1332, 2008. First published September 23, 2008; doi:10.1152/ajpendo.90617.2008.—The objective of this study was to determine the optimal conditions under which to assess glucose tolerance in chow- and high-fat-fed C57BL/6J mice. Mice were fed either chow or high-fat diet for 8 wk. Variables tested were fasting duration (0-, 3-, 6- and 24-h and overnight fasting), route of administration (intraperitoneal vs. oral) load of glucose given (2, 1, or 0.5 g/kg and fixed 50-mg dose), and state of consciousness. Basal glucose concentrations were increased in high-fat- compared with chow-fed mice following 6 h of fasting (9.1 ± 0.3 vs. 7.9 ± 0.4 mmol/l P = 0.01). Glucose tolerance was most different and therefore significant (P = 0.001) in high-fat-fed mice after 6 h of fasting (1,973 ± 96 vs. 1,248 ± 83 mmol-l⁻¹·120 min⁻¹). The difference in glucose tolerance was greater following an OGTT (142%), in contrast to an IPGTT, with a 127% difference between high fat and chow. We also found that administering 2 g/kg of glucose resulted in a greater level of significance (P = 0.0008) in glucose intolerance in high-fat-compared with chow-fed mice. A fixed dose of 50 mg glucose regardless of body weight was enough to show glucose intolerance in high-fat- vs. chow-fed mice. Finally, high-fat-fed mice showed glucose intolerance compared with their chow-fed counterparts whether they were tested under conscious or anesthetized conditions. We conclude that 2 g/kg glucose administered orally following 6 h of fasting is best to assess glucose tolerance in mice under these conditions.

intraperitoneal glucose tolerance test; oral glucose tolerance test; fasting; high-fat feeding; C57BL/6J

Type 2 diabetes is characterized by glucose intolerance, which is contributed to by peripheral (muscle, fat, and liver) insulin resistance as well as islet β-cell dysfunction (17). The glucose tolerance test is used in clinical practice and research to identify individuals with normal or impaired glucose tolerance and patients with type 2 diabetes. It is important to acknowledge that the glucose tolerance test is the only means of identifying impaired glucose tolerance. The glucose tolerance test is performed with the individual drinking a 75-g flavored glucose drink following an overnight fast (an evening meal is allowed but nothing else thereafter).

The molecular and biochemical mechanisms contributing to type 2 diabetes are not fully known. In an effort to understand these mechanisms, researchers have increasingly used genetically modified mouse models in which a gene is up- or downregulated in a tissue-specific manner. As such, the glucose tolerance test has become one of the most widely used physiological tests to characterize transgenic and knockout mouse models of diabetes. In the mouse, the glucose tolerance test is routinely performed following an overnight fast, with the food being removed around 4 PM and the glucose load administered via an intraperitoneal injection. However, mice are nocturnal feeders, with ~70% of their daily caloric intake occurring during the dark cycle (8), and their metabolic rate is much higher than humans. Therefore, an overnight fast is a comparatively long time compared with humans and more akin to starvation. Furthermore, the administration of glucose via an intraperitoneal injection means that there is no incretin response that is known to significantly potentiate the glucose-mediated insulin response. Finally, there is no consistency on the amount of glucose administered, with studies using 1 (5, 25, 27) or 2 g/kg (7, 15, 18, 23, 32) or both doses to determine glucose tolerance in their genetically modified mice (13, 29).

In fact, these inconsistencies are evident from a survey we conducted in 2007 on 100 articles, using the keywords “glucose tolerance and mouse,” where we found the following information: route of administration: in 73 articles the glucose was administered intraperitoneally, whereas in 24 articles oral glucose tolerance tests (OGTT) were performed [in 3 articles both OGTT and intraperitoneal glucose tolerance tests (IPGTT) were used]; fasting duration: in 73 studies animals were fasted for 12 h or longer (overnight), in five studies an 8–to 10-h fast was used, in 18 studies mice were fasted for 4–6 h, and in the remaining four studies either the time was not stated or the mice were not fasted; dose: in 51 articles a dose of 2 g/kg was reported, in 26 articles a dose of 1 g/kg was used, and in the remaining 23 articles doses ranging from 0.5 to 3 g/kg were reported; anesthesia: in five articles studies were done on anesthetized mice, in eight articles experiments were conducted on conscious animals, and in 87 articles either the state of consciousness was not reported or the articles referred to previous publications.

This has led to some conflicting data. For example, in a recent article the deletion of endoplasmic reticulum chaperone P58IPK resulted in β-cell failure and diabetes, yet glucose tolerance (following an overnight fast with the bolus administered ip) was unchanged (20). Similarly, mice with a complete deletion of muscle glycogen synthase were reported to have improved glucose tolerance despite reduced muscle and fat glucose uptake during a euglycemic hyperinsulinemic clamp (26). These inconsistencies have prompted us to reevaluate the glucose tolerance test to determine the most appropriate fasting time, route of administration, and dose of glucose to administer. In addition, the impact of anesthesia on oral glucose tolerance was also assessed. The pathological model that we chose to work with was the C57BL/6J high-fat-fed mouse, as

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this is a widely used model to precipitate glucose intolerance (1, 2, 4, 10, 12, 31).

MATERIALS AND METHODS

Animals. Male C57BL/6J mice were obtained at 8 wk of age from the Walter and Eliza Hall Institute of Medical Research (Kew, Victoria, Australia) and were allowed to acclimate to their surroundings for 3 wk. It is important to note that the substrain of C57BL/6J used throughout this study has the recently described 5-exon deletion in nicotinamide nucleotide transhydrogenase (7). The origin of these mice is the Jackson Laboratories, and this is denoted by the letter J in C57BL/6J. Artificial lighting was maintained on a 12:12-h light-dark cycle. Room temperature was kept constant at 21°C. At 11 wk of age, mice were randomly divided into the two diet groups, the control group receiving a diet containing (wt/wt) 7% fat, 18% protein, and 75% carbohydrate. The high-fat group received a diet containing (wt/wt) 60% fat, 18% protein, and 22% carbohydrate. Both diets were otherwise identical and manufactured by Glen Forrest Specialty Feeds (Glen Forrest, Western Australia, Australia). The composition of the diets has been reported previously (4, 11). Ethics approval was obtained from the Austin Health Animal Ethics Committee, and all experiments were in accordance with the guidelines for the care and use of laboratory animals specified by this committee.

Body weight and food intake. Mice were housed in pairs, and body weight and food intake were determined weekly for 8 wk during the midportion of the light cycle. Preweighed food was placed in the food hoppers and measured on a per-cage basis. Food intake was determined as grams consumed per day.

IPGTT/OGTT. The IPGTT and OGTT were performed following 8 wk of diet feeding, as described previously (21). Briefly, after a designated fasting period, animals were anesthetized with an intraperitoneal injection (100 mg/kg) of pentobarbitone sodium (Therapond, Burwood, Victoria, Australia), and a silastic catheter filled with heparinized saline (20 U/ml) was inserted into the left carotid artery. Mice also underwent a tracheotomy to aid with breathing. A bolus of glucose was either injected into the intraperitoneal cavity or delivered into the stomach by a gavage needle (20-gauge, 38 mm long curved, 1/16-
inch outside diameter) prior to the collection of the next blood sample to prevent anemic shock. To determine an appropriate level of fasting, 10 mice/group were deprived of food beginning at 8 AM for 0, 3, 6, 10, 12, 24, and 18 (overnight) h, and an IPGTT was performed using 2 g/kg glucose (Fig. 1). Although there was a trend for increased basal plasma glucose and insulin levels, this was not significantly different after 6 and 24 h of fasting, indicating impaired fasting glucose tolerance (Table 2). It is important to note that fasting reduced basal plasma insulin levels in both chow- and high-fat-fed mice by three- and fivefold, respectively, in the 24-h-fasted mice compared with their respective controls, which were fasted for 0 h (Table 2). During the IPGTT, there was a significant increase in plasma glucose levels in high-fat-fed vs. control mice only after 6 h of fasting (Fig. 1E). This was also illustrated with the AUC measurement (Fig. 1K). Interestingly, plasma insulin levels during the IPGTT were also significantly elevated following 6 h of fasting (Fig. 1F) as well as after no fasting (Fig. 1B), indicative of insulin resistance, and this was reflected in the AUC data (Fig. 1L). There was no significant difference in plasma insulin levels in high-fat-fed vs. control mice only after 6 h of fasting (Fig. 1E).

Table 1. Body weight gain and daily food and energy intake (following a 6-h fast) of C57BL/6J mice fed a chow or high-fat diet for 8 wk

<table>
<thead>
<tr>
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<th>Chow Diet</th>
<th>High-Fat Diet</th>
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<tbody>
<tr>
<td>Body weight gain, g</td>
<td>1.39 ± 0.01</td>
<td>3.32 ± 0.01*</td>
</tr>
<tr>
<td>Food intake, g/day</td>
<td>3.41 ± 0.01</td>
<td>2.20 ± 0.01*</td>
</tr>
<tr>
<td>Energy intake, KJ/day</td>
<td>54.87 ± 0.12</td>
<td>61.54 ± 0.25*</td>
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Data are presented as means ± SE; n = 40. *P < 0.005 vs. chow diet.
Fig. 1. Plasma glucose and insulin concentrations during the intraperitoneal glucose tolerance test (2 g/kg) following fasting for 0 (A and B), 3 (C and D), 6 (E and F), 24 (G and H), and 18 h (overnight; I and J) in C57BL/6J mice fed a standard chow (■) or high-fat (□) diet for 8 wk. Area under the curve for glucose (AUC_{glucose}; K) and insulin (AUC_{insulin}; L) was calculated using the trapezoidal rule. Results are means ± SE (n = 8–10). *P < 0.05; #P < 0.005, chow vs. high fat; †P < 0.05 vs. 6-h fasting high-fat diet group.
The next step was to determine the effect of route of administration on glucose tolerance by delivering a 2 g/kg glucose bolus either by intraperitoneal injection or orally via gavage after a 6-h fast (Fig. 2). It is immediately clear that both the glucose and insulin excursions were significantly different depending on how glucose was delivered. In the IPGTT (Fig. 3A), there was a rise in plasma glucose levels >30 mmol/l, with the peak occurring at 15 min in chow-fed mice, whereas there was a sustained peak at 15 and 30 min in high-fat-fed mice. In contrast, during the OGTT (Fig. 3B), peak plasma glucose levels occurred at 15 min in both groups of animals, with a mean of 15.5 ± 0.6 mmol/l in the chow- and 24.4 ± 1.9 mmol/l in the high-fat-fed mice. Likewise, the plasma insulin levels during the IPGTT (Fig. 3C) showed a peak at 30 min with chow feeding, whereas there was a sustained peak at 30 and 60 min in the high-fat-fed group. On the contrary, during the OGTT (Fig. 3D) the peak plasma insulin levels occurred at 15 min in both groups of mice, with a significant increase at 15 and 30 min in the high-fat vs. chow group. When considering the AUC data, during both the IPGTT and OGTT the high-fat-fed group displayed glucose intolerance (Fig. 3E). Interestingly, although there was a trend for increased AUCinsulin during the IPGTT, this parameter was significantly higher during the OGTT (Fig. 3F). The OGTT was associated with lower AUCglucose (Fig. 3E) and AUCinsulin (Fig. 3F) for both chow- and high-fat-fed mice. For comparison, we performed OGTT following an overnight fast (Fig. 4). Although there was a significant increase in plasma glucose levels (Fig. 4A) and AUCglucose (Fig. 4C) in the high-fat-fed mice compared with chow-fed mice, there was little difference in plasma insulin levels (Fig. 4B), and the AUCinsulin (Fig. 4D) was similar between the two groups of mice, confirming that a 6-h fast is more appropriate. These data suggest that delivering the glucose load orally following a 6-h fast results in significant increases in glucose and insulin profiles in high-fat-fed vs. chow-fed C57BL/6J mice.

To determine the effect of glucose dose on glucose tolerance in chow- and high-fat-fed mice, we performed OGTT following 6 h of fasting and compared 0.5 g/kg with 1–2 g/kg. Because the basal plasma glucose level was significantly higher in the high-fat-fed compared with chow-fed mice, all data in Fig. 5 are shown as percent change from basal. Plasma glucose levels were not different between chow- and high-fat-fed mice when challenged with either 0.5 (Fig. 5A) or 1 g/kg (Fig. 5C) glucose. It was only following the 2 g/kg dose that there was a separation in plasma glucose levels between the chow and high-fat diet group (Fig. 5E). Similarly, there were no differences in plasma insulin levels in response to either the 0.5 (Fig. 5B) or 1 g/kg (Fig. 5D) glucose dose between chow- and high-fat-fed mice. However, in response to 2 g/kg glucose there was a significant increase in insulin release in both groups of mice, with the high-fat animals showing a significantly higher response at 15 and 30 min compared with chow-fed mice (Fig. 5F). There was a significant increase in AUCglucose following the various fasting periods in C57BL/6J mice fed a chow or high-fat diet for 8 wk.

Table 2. Basal plasma glucose and insulin concentrations following the various fasting periods in C57BL/6J mice fed a chow or high-fat diet for 8 wk

<table>
<thead>
<tr>
<th>Plasma levels, mmol/l</th>
<th>Chow Diet</th>
<th>High-Fat Diet</th>
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<tbody>
<tr>
<td>0 h (8 AM)</td>
<td>6.73 ± 0.45</td>
<td>6.91 ± 0.70</td>
</tr>
<tr>
<td>3 h (8 AM)</td>
<td>7.03 ± 0.42</td>
<td>8.56 ± 0.39</td>
</tr>
<tr>
<td>6 h (8 AM)</td>
<td>7.88 ± 0.42</td>
<td>9.10 ± 0.30*</td>
</tr>
<tr>
<td>18 h (4 PM)</td>
<td>6.13 ± 0.22</td>
<td>6.28 ± 0.51</td>
</tr>
<tr>
<td>24 h (8 AM)</td>
<td>5.03 ± 0.32</td>
<td>6.26 ± 0.35*</td>
</tr>
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<table>
<thead>
<tr>
<th>Plasma insulin levels, ng/ml</th>
<th>Chow Diet</th>
<th>High-Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h (8 AM)</td>
<td>0.90 ± 0.69</td>
<td>1.03 ± 0.31</td>
</tr>
<tr>
<td>3 h (8 AM)</td>
<td>0.89 ± 0.17</td>
<td>1.27 ± 0.45</td>
</tr>
<tr>
<td>6 h (8 AM)</td>
<td>0.72 ± 0.13</td>
<td>1.28 ± 0.25*</td>
</tr>
<tr>
<td>18 h (4 PM)</td>
<td>0.22 ± 0.08</td>
<td>0.40 ± 0.08</td>
</tr>
<tr>
<td>24 h (8 AM)</td>
<td>0.17 ± 0.06</td>
<td>0.30 ± 0.11*</td>
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</table>

Data are presented as means ± SE. *P < 0.05. The start time of the fast is shown in parentheses.

**Fig. 2.** A: homeostasis model assessment of insulin (HOMA-IR) following fasting for 0, 3, 6, 24, and 18 h (overnight) in C57BL/6J mice fed a standard chow (●) or high-fat (□) diet for 8 wk. B: correlation between HOMA-IR and AUCinsulin during the intraperitoneal glucose tolerance test (2 g/kg) in mice fasted for 6 h. Results are means ± SE (n = 8–10). *P < 0.05, chow vs. high fat; †P < 0.05 vs. the other time points in their respective diet groups.
at the 2 g/kg glucose dose only (Fig. 5G). AUC\textsubscript{insulin} was also increased only following the 2 g/kg glucose dose (Fig. 5H). AUC\textsubscript{insulin} was significantly higher in the high-fat-fed 2 g/kg-treated mice compared with the other two doses in this diet group (Fig. 5H).

One can argue that it is not surprising that high-fat-fed mice have greater glucose excursions given that the glucose dose is given per kilogram body weight and they receive more glucose in absolute terms. Indeed, in our study, high-fat-fed mice were significantly heavier than chow-fed animals on the day of study (30.0 ± 0.8 vs. 27.9 ± 0.8 g, n = 15, P < 0.05). Therefore, we conducted OGTT in which the chow- and high-fat-fed mice received a fixed dose of 50 mg of glucose (regardless of their body weight; data shown in Fig. 6). This dose was based on a 25-g mouse receiving 2 g/kg of glucose. There was a small but significant increase in both the glucose (Fig. 6A) and insulin excursion (Fig. 6B) during the OGTT in the high-fat- compared with chow-fed mice. This increase in the high-fat-fed mice was also seen in the AUC\textsubscript{glucose} (Fig. 6C) and AUC\textsubscript{insulin} (Fig. 6D) data.

In all of the above experiments, the mice were anesthetized before the glucose tolerance tests were conducted. To deter-
mine whether consciousness would affect glucose tolerance, we conducted OGTT in mice that were either conscious or anesthetized, using a fixed dose of 50 mg glucose/mouse. For these experiments, we did not cannulate the mice and thus only measured blood glucose levels using a hand-held glucometer from blood sampled from the tail vein. It is clear from Fig. 7 that regardless of whether the mice were anesthetized (Fig. 7, A and C) or conscious (Fig. 7, B and C), high-fat feeding caused glucose intolerance compared with chow feeding.

From the above experiments, we concluded that 2 g/kg glucose delivered orally following a 6-h fast was the most appropriate set of conditions to determine glucose tolerance. To test this, we determined glucose tolerance in C57BL/6J, DBA/2, 129T2, FVBN, the type 1 diabetes model NOD, and the obese and insulin-resistant model NZO. For clarity, we present the AUC data in Fig. 8. Interestingly, there was no difference in glucose tolerance (AUCglucose) among any of these strains (Fig. 8A). However, we did find significant differences in AUCinsulin, with DBA/2 and NZO mice having higher insulin responses than the other strains, whereas the 129T2 mice had the lowest response (Fig. 8B).

**DISCUSSION**

In rodent research, the glucose tolerance test is the most widely used test to determine whether a genetically engineered (e.g., transgenic or knockout) or dietary-induced mouse is glucose intolerant and diabetic. As stated in the introductory section, it is evident that variables such as fasting duration, route, and amount of glucose administration, as well as state of consciousness, may have a large impact on the measured glucose tolerance in mice. Given the almost mandatory use of the glucose tolerance test, we are surprised that an attempt has not previously been made to validate the various variables of this test in mice.

As expected, our data showed that fasting duration had a major impact on both the plasma glucose and insulin levels. The overnight- and 24-h-fasted mice had significantly suppressed basal plasma glucose and insulin levels such that there were no differences between high-fat-fed and chow animals. Furthermore, there was a major suppressive effect on plasma insulin levels during the glucose tolerance test in both groups of animals, suggesting that overnight fasting in the mouse is a considerably long period of time. It has previously been shown that fasting C57BL/6J mice overnight (18 h) resulted in enhanced insulin sensitivity such that the rate of glucose disappearance was higher and endogenous glucose production was completely suppressed compared with 5 h of fasting, as assessed using the euglycemic hyperinsulinemic clamp (8). Liver glycogen levels have been shown to be nearly depleted, whereas liver triglycerides were sixfold higher following overnight fasting compared with 4–5 h fasting (8, 16). Furthermore, transgenic mice overexpressing GLUT4 (28) or hexokinase II (14) in muscle showed increased insulin sensitivity following an overnight fast but not after shorter fasting periods of 3–5 h. It is therefore not surprising to find in our study that the differences between chow- and high-fat-fed mice were less evident following a 24-h and the standard overnight (18 h) fast compared with shorter fasting times. In fact, we found that following 6 h of fasting there was a clear indication of glucose intolerance in the high-fat-fed compared with chow animals. Therefore, we chose this time point to conduct the subsequent tests of this study.

The fasting duration study allowed us to calculate HOMA-IR, a surrogate marker of insulin resistance. It is clear that prolonged fasting (overnight and 24 h) caused a significant reduction in HOMA-IR in both chow- and high-fat-fed mice such that there was no difference between the two groups. A significant difference was seen following 6 h of fasting, and
this correlated well with AUC\textsubscript{insulin} ($r = 0.73$) and AUC\textsubscript{insulin}/AUC\textsubscript{glucose} ($r = 0.69$) during the glucose tolerance test. Therefore, we suggest that determining HOMA-IR following a 6-h fast may be a useful noninvasive way of determining insulin sensitivity in mice. It is important, however, to note that the euglycemic hyperinsulinemic clamp is considered the “gold standard” method of measuring insulin sensitivity, and our data using 6-h-fasted values to calculate HOMA-IR would need to be validated with the clamp performed on 6-h-fasted mice. Interestingly, a recent study found that there was only a weak correlation between a number of surrogate markers of insulin resistance (including HOMA-IR; $r = -0.44$) and glucose infusion rate during the clamp, but the mice were fasted for 15 h (22). Perhaps a stronger correlation might have been achieved following 6 h of fasting.

The next variable we tested was the route of administration and to determine whether delivering the glucose orally in mice provides better glucose tolerance data than the intraperitoneal injection. It has previously been shown that there is a 10–20% rate of error with intraperitoneal injections as a result of the needle piercing the lumen of the intestines or the stomach (6, 24), which would obviously impact the rate and amount of glucose appearing in the circulation. Thus one may expect that the intraperitoneal vs. oral glucose delivery would result in different dynamics in the plasma glucose and insulin excursions, which is exactly what we found. There were two striking features of the OGTT: 1) the plasma glucose levels were significantly lower compared with the IPGTT, and 2) the plasma insulin levels increased sharply and peaked at 15 min, whereas during the IPGTT plasma insulin increased at a slower rate, reaching peak levels at 30–60 min. This results in the significant increase at 15 min and AUC\textsubscript{insulin} in high-fat compared with chow diet during the OGTT, whereas there were trends for increased plasma insulin levels and AUC\textsubscript{insulin} dur-
The IPGTT. Similar data in sharp increases in insulin levels were recently obtained by Ahren et al. (3) when intravenous infusion of glucose to match an oral glucose load in chow- and high-fat-fed mice was compared and were attributed to the incretin response. Therefore, we suggest that the OGTT is a more sensitive technique to discern differences in plasma glucose and insulin levels in chow compared with high-fat-fed C57BL/6J mice.

The third variable that we tested was the amount of glucose to administer to the mice. It is evident from a survey of the literature that, in rodent research, there is no consistent amount of glucose given to determine glucose tolerance, with both 1 and 2 g/kg used. In our study, we compared these two doses as well as a third dose of 0.5 g/kg to determine which of these doses would result in the greatest difference in glucose and insulin levels between chow- and high-fat-fed C57BL/6J mice. It is clear that the two lower doses did not result in any difference between chow- and high-fat-fed mice in either glucose or insulin levels. It is only when 2 g/kg glucose was administered that a difference between chow and high fat was robustly evident. These results may not be surprising because the high-fat-fed mice were heavier and received more glucose in absolute terms compared with chow-fed mice. Consequently we conducted a series of experiments in which we delivered a fixed dose of 50 mg of glucose regardless of body weight and found glucose intolerance and higher plasma insulin levels in high-fat- compared with chow-fed mice. This suggests that under these experimental conditions a fixed dose of glucose can discriminate glucose tolerance. In conclusion, we have evaluated the glucose tolerance test in the mouse and have determined that administering a 2 g/kg body wt of glucose orally following 6 h of fasting will result in robust differences in plasma glucose and insulin levels in chow compared with high-fat-fed C57BL/6J mice. For the sake of comparison of the severity of glucose intolerance in different metabolic states and gene manipulations, we suggest that a standard technique for assessing glucose tolerance in mice be agreed upon.

ACKNOWLEDGMENTS

We acknowledge Specialty Feeds for donating the feed for this project. We thank Christian Rantzau for excellent technical assistance.

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