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The Glucose Tolerance Test as a Laboratory Tool with Clinical Implications

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1. Introduction

Metabolic syndrome is a cluster of abnormalities that are often associated with prediabetes, a condition present prior to the onset of diabetes (1). The hallmark of both metabolic syndrome and prediabetes is insulin resistance, an impairment of insulin action within tissues at the level of the insulin receptor and subsequent cellular events. Insulin resistance resides mainly within muscle, liver and adipose tissues (2). Type 2 diabetes arises from a combination of insulin resistance and a relative impairment of insulin secretion in response to meal ingestion. Type 2 diabetics differ in the relative contributions of insulin resistance and impaired insulin secretion to the development of hyperglycemia (3). Some diabetic patients predominantly have a secretory defect, while others may have normal or even excessive insulin secretion. The latter subgroup of diabetics has insulin resistance as the primary defect in their glucose homeostasis. Hyperglycemia by itself does not indicate whether there is an insufficiency of insulin secretion or a deficit in insulin action. Type 2 diabetics lie along a continuum between these two extremes, with a variable mix of secretory and cell signaling deficits. During the progression from normal glucose tolerance to impaired glucose tolerance to prediabetes to diabetes to severe diabetes over a number of years, individuals show a progressive decline in pancreatic insulin secretion in response to a glucose load(3). Insulin resistance may show progressive deterioration, but generally insulin resistance is the major defect early in the development of diabetes whereas late in the course of the disease pancreatic islet cell failure is more notable.

In Type 2 diabetes, determining the relative contribution of insulin secretion versus action has clinical implications. Knowledge of the predominant abnormalities in glucose homeostasis may well affect the choice of therapeutic agent for the treatment of diabetes. For example, metformin acts to reduce insulin resistance, particularly within the liver (4), and therefore is of considerable utility in early stages of Type 2 diabetes where insulin resistance

is predominant. Sulfonylureas such as glyburide, in contrast, promote the secretion of endogenous insulin in response to a meal (5) and might therefore be more useful when insulin secretion is declining.

However, some have alleged that sulfonylureas may accelerate beta cell exhaustion over time and thereby accelerate the deterioration of diabetes (5). The incretin mimetic agents such as exendin and the DPP-IV inhibitors also promote insulin release from pancreatic islets but may also be cytoprotective within pancreatic islets and thereby delay the progression of diabetes (6). As shown in the Results section below, DPP-IV inhibitors are also effective in reversing metabolic syndrome prior to the onset of diabetes.

Diabetes typically manifests itself in the context of other abnormalities, especially the principal components of metabolic syndrome, namely hypertension and hyperlipidemia (2). The SHROB or Koletsky rat is a leading animal model of metabolic syndrome (7;8), as summarized in Table 1.

Trait	SHR(7)	SHROB(7)	Human Metabolic Syndrome(9)
Hypertension	SBP = 195	SBP = 185	SBP > 130
Insulin resistance	Absent	Profound	Present
Fasting glucose	Normal	Normal to slightly high	Normal to slightly high
Glucose tolerance	Normal	Impaired	Impaired
Triglycerides	Absent	>200 mg/dL	>150 mg/dL
Total Cholesterol	Normal	Slightly elevated	>200 mg/dL
Visceral obesity	Absent	Present	Present
Fasting insulin	Normal	Elevated 20-fold	Elevated
Fasting glucagon	Normal(10)	Elevated 2-fold(10)	Elevated(11;12)

Table 1. Characteristics of human metabolic syndrome compared with the SHROB model

Each of the primary abnormalities expressed in human metabolic syndrome are present in the SHROB rat. Other obese rodents fail to show these characteristics. In particular, the SHROB is only rodent model that incorporates hypertension and related cardiovascular changes together with insulin resistance and abnormal glucose homeostasis. The SHROB has marked hyperinsulinemia without hyperglycemia. Other rodent models show diabetes that ranges from mild (Zucker fatty rat) to fulminant Type 2 diabetes (db/db mouse) (8;13). In contrast, the SHROB is a model for the prediabetic state accompanied by hypertension and hyperlipidemia as well as obesity. Prediabetes can lead to secondary disease complications even in the absence of fasting hyperglycemia (14). The SHROB model can be used to understand the complex physiologic processes that contribute to metabolic syndrome. Dietary and pharmacologic interventions in this model may help identify the best prevention strategies and treatment combinations for metabolic syndrome as well.

Premise: The oral glucose tolerance test (OGTT) is vital for the characterization of metabolic syndrome, the natural progression from prediabetes to Type 2 diabetes, and characterization of the metabolic actions of cardiovascular and metabolic drugs. Although the OGTT is seldom used as a diagnostic test for Type 2 diabetes (15), it is extensively used as a sensitive indicator of gestational diabetes (16). As we demonstrate in the present work, the OGTT is an important laboratory tool in preclinical studies. In both humans and animals, the OGTT provides an indication of the relative roles of insulin secretion and insulin resistance in the progression of glucose intolerance. The impact of diet composition and pharmacologic interventions can be tested and possible target tissues identified. This *in vivo*, real time, whole body test can be used to identify the best treatments and possibly delay or prevent development of Type 2 diabetes.

Insulin release in response to a glucose load occurs in two phases in humans and in rodents. The early phase peaks within the first 15-30 min and is responsible for limiting the initial rise in glucose upon meal ingestion. The late phase of insulin secretion occurs later than 30 min after a meal, and may persist for several hours. This delayed burst of insulin secretion is responsible for returning glucose to baseline fasting levels. In the face of insulin resistance, the late phase of insulin secretion persists for an extended period and contributes to excessive insulin levels even after a return to the fasted state, resulting in fasting hyperinsulinemia (2).

Insulin and glucagon, derived from beta and alpha pancreatic islet cells, respectively, are the two most important hormones involved in glucose homeostasis. Together they determine whether food is utilized immediately for energy or put into storage for later use. Abnormalities in the release or actions of either or both of these hormones can be involved in the development of hyperglycemia and diabetes. Glucagon levels may influence glucose tolerance (11), although this hormone is seldom considered in the context of oral glucose tolerance. Glucagon raises glucose levels, and a higher level of insulin is needed to control glucose in the presence of glucagon (10).

The phases of insulin secretion in response to the oral ingestion of glucose are cephalic, gastric and intestinal. The cephalic phase begins with the sight of food and is strongly triggered by sweet taste receptors on the tongue (17). The cephalic and gastric phases are considered part of the early response, while the intestinal response is later. Interestingly, sweet taste receptors are also present in the small intestine and may shape the physiological response to carbohydrates (18;19). Sweet taste receptors in the small intestine not only regulate the rate of glucose absorption through glucose transporters (19), but also trigger the release of GLP-1 and cholecystokinin (18). GLP-1 and gastric inhibitory peptide (GIP) are incretins, gut hormones secreted from enteroendocrine cells into the blood in response to meal ingestion. Importantly, incretin secretion only occurs with oral ingestion of nutrients. Incretin secretion is not triggered in response to intravenous feeding, as the intestinal receptors are not activated when glucose is delivered through the circulation. Indeed, incretins were discovered as a result of the observation that the insulin stimulation and glucagon suppression by glucose was much greater when glucose was delivered orally or

into the gut rather than by any other route (20). Thus, the normal physiological response to food ingestion can only be elicited through the oral route of administration.

Both incretins are rapidly deactivated by a degradative enzyme called dipeptidyl peptidase 4 (DPP4) (21). Since 2005, two new classes of drugs based on incretin action have been approved for Type 2 diabetes therapy: incretin analogs and incretin enhancers, which inhibit DPP4. DPP4 inhibitors act by slowing the breakdown of endogenous incretin peptides, thus extending their actions.

An OGTT is used clinically to diagnose impaired glucose tolerance and as a standardized test of carbohydrate metabolism and insulin secretion. The test is based on oral administration of glucose and subsequently following plasma glucose and insulin levels over time. A prolonged elevation (>120min) in both plasma glucose and insulin constitutes impaired glucose tolerance and insulin resistance and can be used in conjunction with fasting hyperglycemia in diagnosing Type 2 diabetes. A prolonged elevation in glucose with minimal changes in insulin secretion would be suggestive of impaired glucose stimulated insulin secretion. This procedure is directly applicable to rats and mice.

The OGTT was introduced first as a diagnostic tool for diabetes. Later studies focused on identifying patterns of the glucose curve during the OGTT that predicted the onset of diabetes years later. An OGTT profile during pregnancy can accurately predict which women will later develop Type 2 diabetes when they reach middle age (22). Clinically, the use of OGTT has fallen off because of the need for glucose loading, which many patients perceive as unpleasant. Furthermore, 4 to 6 separate blood samples are necessary. In the modern clinic, the OGTT has generally been supplanted by measuring fasting blood glucose, random post prandial blood glucose and glycosylated hemoglobin values.

The OGTT is influenced by many factors including age, diet, state of health, GI function, medications and emotional state. As age increases, the excursion of glucose during the OGTT progressively increases as a consequence of declining insulin sensitivity of target tissues, such as in muscle, fat and liver. The dose of glucose and standardization of measured responses including glucose levels and insulin have been debated over the years. When used in large population studies the OGTT test overestimates the prevalence of diabetes. In other words, it has a high false positive rate.

The intravenous glucose tolerance test is not sensitive for the diagnosis of diabetes and is used mainly as an assessment purely of glucose disposal i.e. the time required for glucose to clear circulation. It can be used as an index of residual beta cell function as well. The test bypasses the GI tract and eliminates the influence of intestinal hormones, especially incretins, on insulin and glucagon levels and subsequent glucose disposal. The endocrine system of the small intestine is important in the development of diabetes and has spawned new drugs to treat diabetes. The relative advantages of OGTT over other methods of assessing glucose homeostasis in the laboratory are summarized in Table 2.

The hyperinsulemic clamp has been described by some authors as “the gold standard” yet has a number of limitations. Indwelling catheters are required, necessitating some trauma.

Even with time for recovery, glucose homeostasis can be altered by ongoing wound healing and pain. For highly insulin resistant individuals, insulin must be elevated to very high levels. For example, insulin levels in SHROB rats must be raised to levels that would result in convulsions and death from hypoglycemia in a lean animal (results not shown). The OGTT allows all of the normal stages of insulin secretion and glucose processing to take place in sequence without causing stress or trauma to the subject.

	OGTT	IVGTT	Hyperinsulinemic Clamp
Incorporates cephalic phase	Yes	No	No
Incorporates gut-pancreatic axis	Yes	No	No
First pass through liver and portal circulation	Yes	No	No
Raises insulin to nonphysiologic levels	No	No	Yes
Requires surgery	No	Yes	Yes

Table 2. Advantages of OGTT over IVGTT and hyperinsulinemic clamps

2. Methods

Special diets are fed for 30 to 90d prior to the OGTT. Antihypertensive or antidiabetic medications are given in the drinking water or incorporated into the food and given for 42 to 90d. We carry out the OGTT by fasting animals for 18h, taking a baseline 200 μ L blood sample from the tail under local anesthesia, and then gavaging with 12 mL/kg of a 50% glucose solution, which delivers 6g of glucose per kg of body weight. Blood samples are taken 30, 60, 120, 180, 240 and 360 minutes after the glucose meal and analyzed for blood glucose with a clinical meter. Plasma samples are frozen for later analysis of rat insulin by ELISA. The 240 and 360 minute time points are not typically used in OGTT, but the SHROB is so profoundly glucose intolerant that extended sampling is required to reliably establish the area under the curve. Area under the curve is determined for each subject using curve fitting software (Prism 5.0, GraphPad software, San Diego, CA).

Procedures:

1. Weigh the rats to the nearest 0.5g and place them into clean cages with new bedding. Rodents are coprophagic, so new cages will assure true fasting conditions over the next 18h.
2. Dextrose (D-glucose) solution: A 50% dextrose solution is made by weighing out the needed amount of dextrose and heating and stirring with water (be sure not to overheat solution or caramelization will take place). For example, if you need 50mL of workable solution, you would weigh out 25g of dextrose and add that to approximately 28mL of water then stir and heat until dextrose is in solution. Once in solution, pour into a graduated cylinder and dilute up to 50mL and allow cooling at room temperature prior to using.

3. Dose: We administer a 6g/kg body weight of a 50% dextrose solution for our studies to assure a significant challenge to glucose metabolism, but 2-4g/kg body weight is frequently reported by others.
4. Oral gavaging: Discharge rate should be slow so as not to cause a pressure induced "dumping syndrome". This will be apparent about 30-60min after gavaging with the presence of diarrhea.
5. Blood Collection: This is done by tail bleed. We recommend that blood be collected at least five times: 0, 30, 60, 120 and 180 min. However, we collect additional time points of 240 and 360 min in our SHROB rats, as this represents the delayed insulin peak response and allows an accurate estimate of the area under the curve for glucose, as glucose returns to fasting levels only very slowly. We also dip the cut tail tip into the local anesthetic, bupivacaine (Marcaine, 0.5%), after the first tail bleed and after each sample thereafter to minimize pain associated with the procedure.
6. Glucose measurement: A clinical glucose meter (One Touch Ultra, Lifescan) is used with 5 μ L of whole blood.
7. Insulin and glucagon ELISA: Rodent-specific kits are available from several suppliers, including ALPCO (Salem, NH). The coefficient of variation is less than 10% in repeat measures and the sensitivity is 0.1 ng/mL. Plasma samples from our insulin resistant rats must be diluted 1:10 in assay buffer before addition to the assay plate.
8. Statistical analysis: Glucose and insulin data are processed through an analysis of variance with repeated measures to determine treatment effects, time effects, and treatment by time interaction terms by using the Prism 5.0 statistical and graphing program (GraphPad Software, Sand Diego, CA). Newman-Keuls post-hoc tests are used to determine significance of individual comparisons. Area under the curve measurement are also determined for each animal, expressed as mg*min/dL for glucose and ng*min/mL for insulin, and analyzed as a single datum.

3. Results

We review data from several contrasting interventions in SHROB rats, which includes diets and drug treatments with both beneficial and harmful effects on glucose tolerance. We contrast the use of OGTT with other measures such as fasting glucose and glucose to insulin ratios.

A relatively normal OGTT profile is illustrated by the data from lean SHR. Glucose starts at a low fasting level and rises only to a maximum of about 100 mg/dL at 60 min. Insulin levels are low in the fasted state and show a maximum 4-fold rise around 60 min and then return to baseline by 240 min. Not shown is the profile for normotensive Wistar or Sprague-Dawley control rats, which would show a similar profile but with even lower levels of insulin owing to a small amount of insulin resistance associated with hypertension (23).

SHROB on a control diet show a normal fasting level of glucose, but glucose ascends to a much higher peak around 60 min and takes much longer to return to baseline. Fasting insulin levels in the SHROB are 40-fold elevated compared to lean SHR littermates, and the

further rise in insulin in response to a glucose load is delayed and prolonged. Dietary obese genetically lean SHR rats are only about 25% overweight compared to lean SHR on normal chow, in contrast to genetically obese SHROB which are about 100% overweight compared to lean SHR on normal chow (24). Despite the lesser degree of obesity, the dietary obese genetically lean SHR showed about the same degree of glucose intolerance as SHROB. Insulin levels at fasting and after the glucose load were unchanged relative to the SHR fed control diet. SHROB fed a high sucrose diet showed unchanged glucose tolerance. In contrast, the insulin response to a glucose load was greatly increased and prolonged beyond that observed for SHROB on a control diet. To our knowledge, the insulin levels in sucrose-fed SHROB during the OGTT, which exceed 100 ng/mL, are the highest ever recorded in a rodent. The data in Figure 1 illustrate the value of the OGTT. High sucrose input affected glucose levels in genetically lean animals without affecting insulin. In contrast, in genetically obese SHROB rats, high sucrose affected insulin without affecting glucose levels. None of this could be learned by obtaining fasting levels alone.

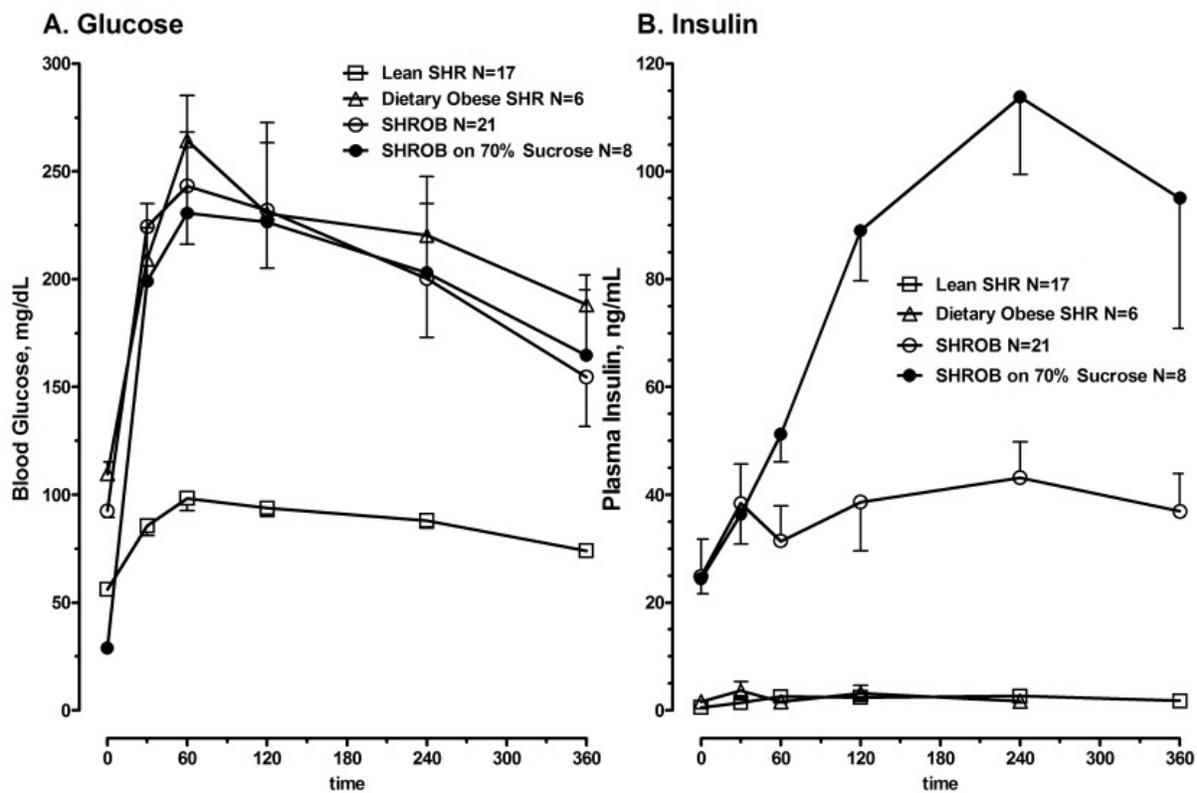


Figure 1. Glucose tolerance test: SHR versus SHROB on normal versus high sucrose diets. A: Glucose. B: Insulin. SHR were fed a supplementary sweet drink to induce dietary obesity in these genetically lean rats. SHROB were fed a high sucrose diet (70% sucrose by weight).

Figure 2 includes only the genetically obese SHROB fed a control diet, which were given vehicle or drug in their drinking water. Two oral antihyperglycemic agents, the insulin secretagogue glyburide (mg/kg) and the DPP4 inhibitor sitagliptin (mg/kg), were contrasted with the widely used antihypertensive hydrochlorothiazide (50 mg/kg). None of the agents had any effect on fasting glucose. The two antihyperglycemic agents, as expected, lowered

the excursion of glucose in response to an oral load. The antihypertensive agent dramatically increased glucose levels following the oral load, with glucose rising to levels associated with frank diabetes (>400 mg/dL).

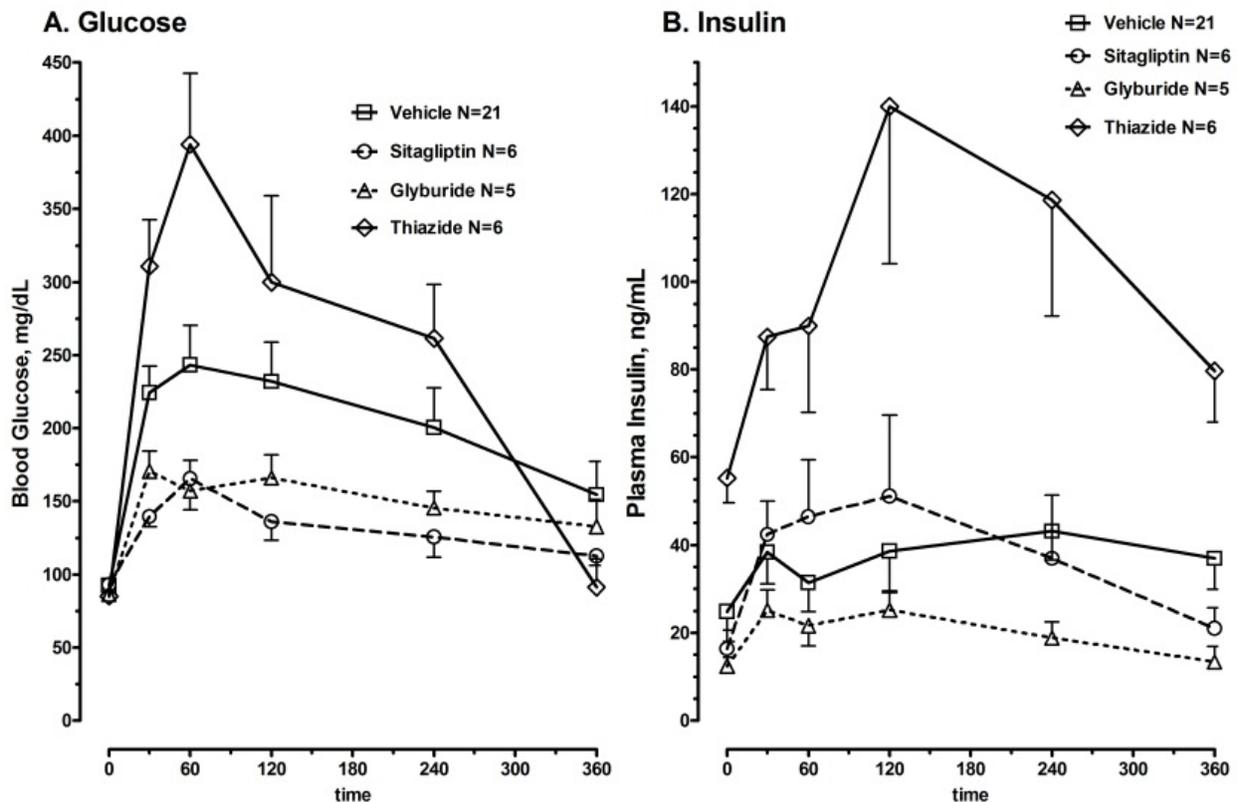


Figure 2. Glucose tolerance test: untreated SHROB versus SHROB treated with antihyperglycemic agents and one antihypertensive agent. A: Glucose. B: Insulin

Fasting insulin levels in vehicle controls (Figure 2) were very high, similar to those observed before in Figure 1. The oral antihyperglycemic drugs both reduced fasting insulin levels, although not to levels seen in lean SHR. Remarkably, hydrochlorothiazide further elevated fasting insulin. The post-load insulin curve in glyburide treated SHROB closely paralleled vehicle treated controls, but at a lower level. The insulin curve for the sitagliptin treated group showed a higher net response to the glucose load, especially at 30 min. This is consistent with the hypothesized role of incretin hormones in facilitating the insulin response to glucose. Hydrochlorothiazide greatly increased insulin levels, reminiscent of the effect of dietary sucrose in Figure 1.

Other hormones and metabolites can be measured during OGTT, not just glucose and insulin. Figure 3 illustrates changes in plasma glucagon during the first hour after an oral glucose load. Homeostasis predicts that glucagon would be high in the fasting state and decline when the fed state is restored. Glucagon levels are unchanged in SHR animals, possibly because the fall in glucagon is mainly mediated by gut hormones such as GLP-1 and GIP which are secreted later after the load has reached the distal small intestine where the L-cells that secrete these hormones reside. Remarkably, SHROB rats show a dramatic

increase in glucagon from already high fasting levels. This paradoxical increase in glucagon in response to a glucose meal has been reported in humans with Type 2 diabetes (12).

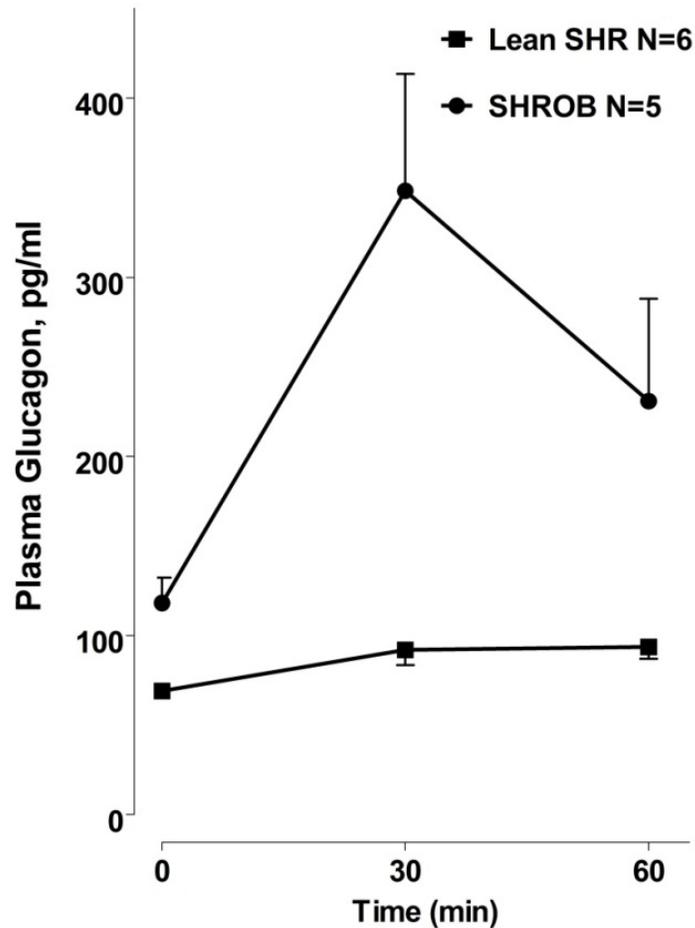


Figure 3. Changes in plasma glucagon level during an OGTT in SHROB and lean SHR littermates.

Besides glucose, another plasma substrate that can be assayed is the level of free fatty acids. We found that free fatty acids fall after glucose load in lean SHR, indicating reduced lipolysis when an abundant supply of glucose is present (10). Remarkably, the level of free fatty acids in SHROB rats stayed at the elevated level found in fasting (data not shown). This finding illustrates the multiple abnormalities in the whole body handling of metabolic fuels that can be uncovered in an OGTT.

4. Discussion

These data show that OGTT is much more sensitive than are other measures in identifying the loci of insulin resistance and its modulation by different interventions. Moreover, the results of OGTT testing in the SHROB model tracks closely with efficacy against metabolic syndrome and Type 2 diabetes in clinical trials. Thus, the OGTT yields laboratory data with greater relevance to the prevention and treatment of human disease.

The glucose tolerance test determines the rate at which glucose is cleared from blood. Glucose is administered either orally or intravenously and serial samples of glucose, insulin and glucagon are measured over time. Hypoglycemic medications used to treat diabetes have been developed that modify the actions of glucagon and insulin directly. Medications that are used to treat high blood pressure and lipid disturbances can also modify the responses to these hormones, sometimes improving the clearance of glucose and sometimes making it worse. Comparisons of the responses of glucose, insulin and glucagon levels during glucose tolerance testing with and without medications used to treat metabolic syndrome will lead to a better understanding of what combinations of these medications are the most beneficial and least toxic.

The current literature has many examples of inadequate assessments of glucose metabolism. Many authors report only on “random glucose” values, taken without regard to whether the animal had just eaten or had voluntarily fasted for several hours. If food is available ad libitum, then some animals may be postprandial and others will be fasted as a result of their sleep cycle. The glucose and insulin values obtained under these circumstances are highly variable and often irreproducible. A somewhat better approach is to determine fasting glucose and insulin levels. Various ratios between glucose and insulin can be calculated, with the most popular being the Homeostasis Model Assessment (HOMA). The mathematical derivation of the HOMA equation is sophisticated, but it is still based on only two numbers: fasting glucose and fasting insulin. The OGTT, by contrast, reflects the in vivo physiological response to a glucose meal and integrates the homeostatic response to a meal over time. Thus, the OGTT more accurately mirrors daily life.

The OGTT is useful as a research tool. It does allow for a more physiologic assessment of metabolism as it assesses a global response to the disposition of a meal. Neurologic and hormonal responses that influence the function of the gastric tract, absorption, transportation and processing of food in the liver and transport to and from storage tissue can be assessed. These include newly recognized hormones secreted from the GI tract in the presence of food known as incretins and previously recognized hormones such as insulin and glucagon. The OGTT is particularly useful in characterizing the actions of drugs which can be either beneficial or detrimental to glucose homeostasis.

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