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EVALUATING THE GLUCOSE TOLERANCE TEST IN MICE

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ABSTRACT

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 weeks. Variables tested were: fasting duration (0, 3, 6, overnight and 24 hrs fasting), route of administration (intraperitoneal vs oral) load of glucose given (2 g/kg, 1 g/kg or 0.5 g/kg and fixed 50 mg dose) and state of consciousness. Basal glucose concentrations were increased in high-fat compared to chow fed mice following 6 hrs 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 hrs of fasting (1973 ± 96 vs. 1248 ± 83 mmol/l x 120min). 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 to 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 to their chow counterparts whether tested under conscious or anesthetized conditions. We conclude that a 2 g/kg of glucose administered orally following 6 hrs of fasting is best to assess glucose tolerance in mice under these conditions.

Keywords: intraperitoneal glucose tolerance test, oral glucose tolerance test, fasting, high-fat feeding, C57BL/6J

Abbreviations: IPGTT – intraperitoneal glucose tolerance test, OGTT – oral glucose tolerance test, AUC – area under the curve, HOMA-IR – homeostasis model assessment for insulin resistance, NZO – New Zealand Obese, NOD – Non-obese diabetic

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INTRODUCTION

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 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 down-regulated in a tissue-specific manner. As such, the glucose tolerance test has become one of the most widely used physiological tests to characterise 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 approximately 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 to humans and more akin to starvation. Furthermore, the administration of glucose via an intraperitoneal injection means that there is no incretin response, which 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 g/kg (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 on 100 articles in 2007 using the keywords “glucose tolerance and mouse” and found the following information: route of administration - 73 administered the glucose intraperitoneally, while 24 performed oral glucose tolerance tests (3 articles used both); fasting duration – 73 studies fasted animals for 12 hours or longer (overnight), 5 studies used 8-10 hour fast, 18 studies fasted mice for 4-6 hours while the remaining 4 either did not state the time or did not fast the mice; dose – 51 articles reported a 2 g/kg, 26 articles used 1 g/kg, while the remaining 23 articles reported doses ranging from 0.5 g/kg – 3 g/kg; and anesthesia – 5 articles did studies in anesthetized mice, 8 conducted experiments in conscious animals, while 87 articles either did not report the state of consciousness or referred to previous publications.

This has led to some conflicting data. For example, in a recent article the deletion of endoplasmic reticulum chaperone $P58^{IPK}$ 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 re-evaluate 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 pathologic model that we chose to work with was the C57BL/6J high-fat fed mouse, as 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 weeks of age from the Walter and Eliza Hall Institute of Medical Research (Kew, Vic, Australia) and were allowed to acclimatize to their surroundings for 3 weeks. 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 (Nnt) (7). The origin of these mice is the Jackson Laboratories and this is denoted by the J. Artificial lighting was maintained on a 12hr light/dark cycle. Room temperature was kept constant at 21°C. At 11 weeks of age mice were randomly divided into the two diet groups, the control group receiving a diet containing (w/w) 7% fat, 18% protein and 75% carbohydrate. The high-fat group received a diet containing (w/w) 60% fat, 18% protein and 22% carbohydrate. Both diets were otherwise identical and manufactured by Glen Forrest Specialty Feeds, Glen Forrest, WA, Australia. The composition of the diets has been previously reported (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.

Body Weight and Food intake

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

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Intraperitoneal/Oral glucose tolerance test

The intraperitoneal (IPGTT) and oral (OGTT) glucose tolerance tests were performed following 8 weeks of diet feeding as previously described (21). Briefly, after a designated fasting period animals were anesthetized with an intraperitoneal injection (100 mg/kg) of sodium pentobarbitone (Therapon Pty Ltd, Burwood Vic Australia) and a silastic catheter filled with heparinised 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 injected either into the intraperitoneal cavity or delivered into the stomach by a gavage needle (20G – 38 mm, 2 ¼ mm diameter, curved. Able Scientific, Canning Vale 6155 Western Australia) and 200 µl of blood was sampled at 0, 15, 30, 60, and 120 min for plasma glucose and insulin analyses. Blood was immediately centrifuged, the plasma separated and stored at –20 °C until assayed. The red blood cells were resuspended in an equal volume of heparinised saline and reinfused into the animal via the carotid artery prior to the collection of the next blood sample to prevent anemic shock.

Experiment One: Fasting Duration

To determine an appropriate level of fasting, 10 mice per group were deprived of food from 8 am for 0, 3, 6, and 24 hours at which time the mice were anesthetized and an IPGTT was performed as previously described above. In a separate group of animals, the fast was performed overnight (18 hrs) with the food withdrawn at around 4 pm. This was done for comparison, as the overnight fast is most widely used in animal studies assessing glucose tolerance.

Experiment Two: Route of Administration

After a 6 hour fast, 9 mice per group were either subjected to an IPGTT or an OGTT (as previously described above).

Experiment Three: Dosage of Glucose

Dosage of glucose was determined by giving 7 mice per group a bolus of 2 g/kg, 1 g/kg or 0.5 g/kg of glucose orally. In a separate group of mice the dose of glucose delivered was 50 mg regardless of body weight.

Experiment Four: Impact of anesthesia on oral glucose tolerance

To assess the impact of anesthesia on the measured difference in glucose tolerance between our two study groups, 6-hour fasted chow and high-fat fed mice were either anesthetized using an intraperitoneal injection of sodium pentobarbitone (100 mg/kg) or were maintained awake. All mice were gavaged with 5 mg of glucose and blood for measurement of glucose was taken by tail-vein sampling.

Experiment Five: Glucose Tolerance of Six Inbred Mouse Strains

Ten week old male C57BL/6J, FVB/n, Non obese diabetic (NOD/Lt), 129T2/SV, DBA/2 and New Zealand Obese (NZO) mice that were being maintained on a normal chow diet were obtained from the Walter and Eliza Hall Institute of Medical Research. On the day of experimentation mice were fasted for 6 hours (from 8 am) and an oral glucose tolerance test was performed using a 2 g/kg glucose bolus.

Measurement of plasma glucose and insulin concentrations

Blood glucose concentrations were measured on a Medisense Optium glucometer. The glucose oxidase method was used to determine plasma glucose levels using a GM7 Analox glucose analyzer (Helena Laboratories, Mount Waverley Vic, Australia). Plasma insulin concentrations were determined by a commercially available radioimmunoassay specific for rodent insulin (Linco Research Immunoassay, St Charles, MO, USA).

Statistical analysis

Data are expressed as means ± S.E.M. The trapezoidal rule was used to determine the area under the curve. HOMA-IR was calculated as (fasting glucose level x fasting insulin level)/22.4. The level of statistical significance was determined using student's two-tailed t-test when considering difference between the means of two populations. For differences in plasma glucose or insulin levels during the glucose tolerance tests, an ANOVA was performed and significance determined using Tukey's post-hoc test. A P value less than 0.05 was considered statistically significant.

RESULTS

Male C57BL/6J mice only were used throughout this study. Mice on a high-fat diet gained significantly more weight than chow-fed mice (Table 1). While the amount of food consumed was significantly less, high-fat fed mice had an increase in daily caloric intake due to the higher energy content of the diet (Table 1).

To determine the effect of fasting on glucose tolerance, C57BL/6J chow and high-fat fed mice were fasted for 0, 3, 6, 24 and 18 (overnight) hours and an intraperitoneal glucose tolerance test (IPGTT) was performed using 2 g/kg glucose (Figure 1). While there was a trend for increased basal plasma glucose and insulin levels this was only significantly different after 6 hours and 24 hours of fasting indicating impaired fasting glucose (Table 2). It is important to note that fasting reduced basal plasma insulin levels in both chow and high-fat fed mice by 3-fold and 5-fold in the 24 hour fasted compared with their respective controls fasted for 0 hours (Table 2). During the IPGTT, there was a significant increase in plasma glucose levels in high-fat fed versus control fed mice only after 6 hours of fasting (Figure 1E). This was also illustrated with the area under the curve (AUC) measurement (Figure 1K). Interestingly, plasma insulin levels during the IPGTT were also significantly elevated following 6 hours of fasting (Figure 1F) as well as after no fasting (Figure 1B), indicative of insulin resistance, and this was reflected in the AUC data (Figure 1L). There was no effect of fasting on AUCglucose (Figure 1K) or AUCinsulin (Figure 1L) in the chow fed group. In the high-fat diet group the 6 hour AUCglucose was significantly higher than the 3 hour and 24 hour AUCglucose (Figure 1K), while the 6 hour AUCinsulin was significantly higher than the 3 hour, 24 hour and overnight fasted AUCinsulin (Figure 1L).

When HOMA-IR was calculated, there was a significant increase in the high-fat group only after 6 hours of fasting (Figure 2A). There was also a significant positive correlation between AUCinsulin and HOMA-IR ($r=0.77$, $p<0.0001$) when all the data was considered. Furthermore, when only the 6-hour fasting data was regressed, there was still a significant positive correlation between these two measures of insulin sensitivity (Figure 2B, $r=0.73$, $p<0.01$), while this association was lost after overnight (18 hours) fasting ($r=0.10$, $p=0.76$). A significant positive correlation was also found between AUCinsulin/AUCglucose and HOMA-IR following a 6-hour fast ($r=0.69$, $p<0.02$). Fasting overnight and for 24 hours reduced HOMA-IR in both the chow and high-fat fed groups (Figure 2A). Based on this data we decided to conduct the subsequent experiments of this study by fasting the mice for 6 hours.

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-hour fast (Figure 3). It is immediately clear that both the glucose and insulin excursions were significantly different depending on how glucose was delivered. In the IPGTT (Figure 3A) there was a rise in plasma glucose levels above 30 mmol/L with the peak occurring at 15 minutes in chow fed mice, while there was a sustained-peak at 15 and 30 minutes in high-fat fed mice. In contrast, during the OGTT (Figure 3B), peak plasma glucose levels occurred at 15 minutes 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 (Figure 3C) showed a peak at 30 minutes with chow feeding, while there was a sustained peak at 30 and 60 minutes in the high-fat fed group. On the contrary, during the OGTT (Figure 3D) the peak plasma insulin levels occurred at 15 minutes in both groups of mice, with a significant increase at 15 and 30 minutes in the high-fat versus chow group.

When considering the AUC data, during both the IPGTT and OGTT the high-fat fed group displayed glucose intolerance (Figure 3E). Interestingly, while there was a trend for increased AUCinsulin during the IPGTT, this parameter was significantly higher during the OGTT (Figure 3F). The OGTT was associated with lower AUCglucose (Figure 3E) and AUCinsulin (Figure 3F) for both chow and high-fat fed mice. For comparison we performed OGTT following an overnight fast (Figure 4). While there was a significant increase in plasma glucose levels (Figure 4A) and AUCglucose (Figure 4C) in the high-fat fed mice compared with chow fed mice, there was little difference in plasma insulin levels (Figure 4B) and the AUCinsulin (Figure 4D) was similar between the two groups of mice, confirming that a 6-hour fast is more appropriate. This data suggests that delivering the glucose load orally following a 6-hour fast results in significant increases in glucose and insulin profiles in high-fat fed versus chow fed C57BL/6J mice.

To determine the effect of glucose dose on glucose tolerance in chow and high-fat fed mice, we performed OGTTs following 6 hours of fasting and compared 0.5 g/kg to 1 g/kg to 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 Figure 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 g/kg (Figure 5A) or 1 g/kg (Figure 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 (Figure 5E). Similarly, there were no differences in plasma insulin levels in response to either the 0.5 g/kg (Figure 5B) or 1 g/kg (Figure 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 minutes compared with chow fed mice (Figure 5F). There was

a significant increase in AUCglucose at the 2 g/kg glucose dose only (Figure 5G). AUCinsulin was also only increased following the 2 g/kg glucose dose (Figure 5H). AUCinsulin was significantly higher in the high-fat fed 2 g/kg treated mice compared to the other two doses in this diet group (Figure 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, $n=15$ $p<0.05$). We therefore conducted OGTTs in which the chow and high fat diet mice received a fixed dose of 50 mg of glucose (regardless of their body weight) and the data shown in Figure 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 (Figure 6A) and insulin excursion (Figure 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 AUCglucose (Figure 6C) and AUCinsulin (Figure 6D) data.

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

From the above experiments we concluded that 2 g/kg glucose delivered orally following a 6 hour 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, FVB/n, the Type 1 diabetes model NOD and the obese and insulin resistant model NZO. For clarity we present the AUC data in Figure 8. Interestingly, there was no difference in glucose tolerance (AUCglucose) among any of these strains (Figure 8A). However, we did find significant differences in AUCinsulin, with DBA/2 and NZO mice having higher insulin responses than the other strains, while the 129T2 mice had the lowest response (Figure 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 Introduction 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-hour 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 hours) resulted in enhanced insulin sensitivity such that the rate of glucose disappearance was higher and endogenous glucose production was completely suppressed compared to 5 hours of fasting, as assessed using the euglycemic-hyperinsulinemic clamp (8). Liver glycogen levels have been shown to be near depleted while liver triglycerides were 6-fold higher following overnight fasting compared with 4-5 hours 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 hours. 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 hour and the standard overnight (18 hour) fast compared with

shorter fasting times. In fact we found that following 6-hours of fasting there was a clear indication of glucose intolerance in the high-fat fed compared with chow animals. We therefore 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 hours) 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 hours of fasting and this correlated well with AUCinsulin (r=0.73) and AUCinsulin/AUCglucose (r=0.69) during the glucose tolerance test. We therefore suggest that determining HOMA-IR following a 6-hour fast may be a useful non-invasive 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-hour fasted values to calculate HOMA-IR would need to be validated with the clamp performed on 6-hour 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 hours (22). Perhaps a stronger correlation may have been achieved following 6-hours 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 versus 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; (2) the plasma insulin levels increased sharply and peaked at 15 minutes, while during the IPGTT plasma insulin increased at a slower rate reaching peak levels at 30-60 minutes. This results in the significant increase at 15 minutes and AUCinsulin in high-fat compared with chow diet during the OGTT, while there were trends for increased plasma insulin levels and AUCinsulin during the IPGTT. Similar data in sharp increases in insulin levels were recently obtained by Ahren and colleagues when comparing intravenous infusion of glucose to match an oral glucose load in chow and high-fat fed mice and were attributed to the incretin response (3). 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/6 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 g/kg 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, as the high-fat fed mice were heavier and this 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 glucose regardless of body weight and found glucose intolerance and higher plasma insulin levels in the high-fat compared with chow fed animals. This suggests that under these experimental conditions a fixed dose of glucose can discriminate glucose tolerance. In summary we suggest that when administered orally, 2 g/kg glucose is an appropriate amount to show a difference between chow and high-fat fed C57BL/6J mice.

The final variable tested was state of consciousness. We found that glucose intolerance was present whether the high-fat fed mice compared with their chow fed counterparts were anesthetized or conscious. This suggests that as long as the “control” and “test” mice are treated equally, differences in glucose tolerance can be discerned.

In the last set of experiments we applied what we had learnt to mouse strains that have previously been shown to have differences in glucose tolerance. Interestingly, we saw little difference in the area under the glucose curve among the six strains tested. However, we did see the expected changes in plasma insulin levels. In particular, as we and others have previously shown the 129T2 mice had lower insulin levels compared with DBA/2 and C57BL/6J mice (12, 19). Furthermore, DBA/2 mice had higher insulin levels compared with C57BL/6J mice and we have recently shown that this associated with an increase in the mitochondrial proton pump, nicotinamide nucleotide transhydrogenase (Nnt) (7). The highest insulin levels were observed in the NZO mouse and this is the expected outcome since it is a polygenic model of obesity, glucose intolerance and insulin resistance (9, 30). These data highlight the importance of measuring plasma insulin levels during the glucose tolerance test.

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In conclusion, we have evaluated the glucose tolerance test in the mouse and have determined that administering a 2 g/kg body weight of glucose orally following 6 hours 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 a standard technique for assessing glucose tolerance in mice be agreed upon.

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DUALITY OF INTEREST

The authors wish to declare that there are no conflicts of interest associated with this study.

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TABLES

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

	Chow Diet (n=40)	High-fat Diet (n=40)
Body weight gain (g)	1.39 ± 0.01	3.32 ± 0.01#
Food intake (g/d)	3.41 ± 0.01	2.20 ± 0.01#
Energy intake (KJ/d)	54.87 ± 0.12	61.54 ± 0.25#

Data are presented as mean ± SEM. # $P<0.005$ vs chow diet

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 weeks. The start time of the fast is shown in the brackets.

	Chow Diet	**High-fat Diet**
	Plasma glucose levels (mmol/l)	
0 hr (8 am)	6.73 ± 0.45	6.91 ± 0.70
3 hr (8 am)	7.03 ± 0.42	8.56 ± 0.39
6 hr (8 am)	7.88 ± 0.42	9.10 ± 0.30*
18 hr (4 pm)	6.13 ± 0.22	6.28 ± 0.51
24 hr (8 am)	5.03 ± 0.32	6.26 ± 0.35*
	Plasma insulin levels (ng/ml)	
0 hr (8 am)	0.90 ± 0.69	1.03 ± 0.31
3 hr (8 am)	0.89 ± 0.17	1.27 ± 0.45
6 hr (8 am)	0.72 ± 0.13	1.28 ± 0.25*
18 hr (4 pm)	0.22 ± 0.08	0.40 ± 0.08
24 hr (8 am)	0.17 ± 0.06	0.30 ± 0.11*

Data are presented as mean ± SEM. *P<0.05, # P<0.005 vs chow diet.

Figure Legends

Figure 1: Plasma glucose and insulin concentrations during the intraperitoneal glucose tolerance test (2 g/kg) following fasting for 0 hr (A,B), 3 hrs (C,D), 6 hrs (E,F) 24 hrs (G,H) and overnight (18 hrs) (I,J) in C57BL/6J mice fed a standard chow (■) or high-fat (□) diet for 8 weeks. Area under the curve for glucose (K) and insulin (L) was calculated using the trapezoidal rule. Results are the mean ± SEM of (n=8-10). * $p<0.05$, # $p<0.005$ chow vs. high-fat, †$p<0.05$ vs 6 hour fasting high fat diet group.

Figure 2: A. HOMA-IR following fasting for 0 hr, 3 hrs, 6 hrs 24 hrs and overnight (18 hrs) in C57BL/6J mice fed a standard chow (■) or high-fat (□) diet for 8 weeks. B. Correlation between HOMA-IR and AUCinsulin during the intraperitoneal glucose tolerance test (2 g/kg) in mice fasted for 6 hours. Results are the mean ± SEM of (n=8-10). * $p<0.05$ chow vs. high-fat, †$p<0.05$ vs the other time-points in their respective diet groups.

Figure 3: Plasma glucose and insulin concentrations during the intraperitoneal glucose tolerance test (IPGTT, 2 g/kg) (A,C) or oral glucose tolerance test (OGTT, 2 g/kg) (B,D) following 6 hrs of fasting in C57BL/6J mice fed a standard chow (■) or high-fat (□) diet for 8 weeks. Area under the curve for glucose (E) and insulin (F) was calculated using the trapezoidal rule. Results are the mean ± SEM of (n=8-10). * $p<0.05$, # $p<0.005$ chow vs. high-fat, †$p<0.05$ vs IPGTT.

Figure 4: Plasma glucose (A) and insulin (B) concentrations during the oral glucose tolerance test (OGTT, 2 g/kg) following overnight (18 hrs) of fasting in C57BL/6J mice fed a standard chow (■) or high-fat (□) diet for 8 weeks. Area under the curve for glucose (C)

and insulin (D) was calculated using the trapezoidal rule. Results are the mean ± SEM of (n=8-10). * $p<0.05$, # $p<0.005$ chow vs. high-fat.

Figure 5: Plasma glucose and insulin concentrations during the oral glucose administration of 0.5 g/kg (A,B) or 1.0 g/kg (C,D) or 2 g/kg (E,F) glucose, following 6 hrs of fasting in C57BL/6J mice fed a standard chow (■) or high-fat (□) diet for 8 weeks. Area under the curve for glucose (G) and insulin (H) was calculated using the trapezoidal rule. Results are the mean ± SEM of (n=8-10). * $p<0.05$ chow vs. high-fat, †$p<0.05$ vs 2 g/kg high-fat diet group.

Figure 6: Plasma glucose (A) and insulin (B) concentrations during the oral glucose tolerance test (OGTT) using a fixed glucose dose of 50 mg per mouse, following 6 hrs of fasting in C57BL/6J mice fed a standard chow (■) or high-fat (□) diet for 8 weeks. Area under the curve for glucose (C) and insulin (D) was calculated using the trapezoidal rule. Results are the mean ± SEM of (n=7-8). * $p<0.05$ chow vs. high-fat.

Figure 7: Blood glucose concentrations during the oral glucose tolerance test (OGTT) in the anesthetized (A) or conscious (B) state using a fixed glucose dose of 50 mg per mouse, following 6 hrs of fasting in C57BL/6J mice fed a standard chow (■) or high-fat (□) diet for 8 weeks. Area under the curve for glucose (C) was calculated using the trapezoidal rule. Results are the mean ± SEM of (n=4). * $p<0.05$ chow vs. high-fat.

Figure 8: Area under the glucose (AUCglucose) (A) and insulin (AUCinsulin) (B) curves during the oral glucose tolerance test (OGTT, 2 g/kg) following 6 hrs of fasting in 10 week old C57BL/6J, DBA/2, 129T2, NOD, FVB/n and NZO male mice.

Figure 1

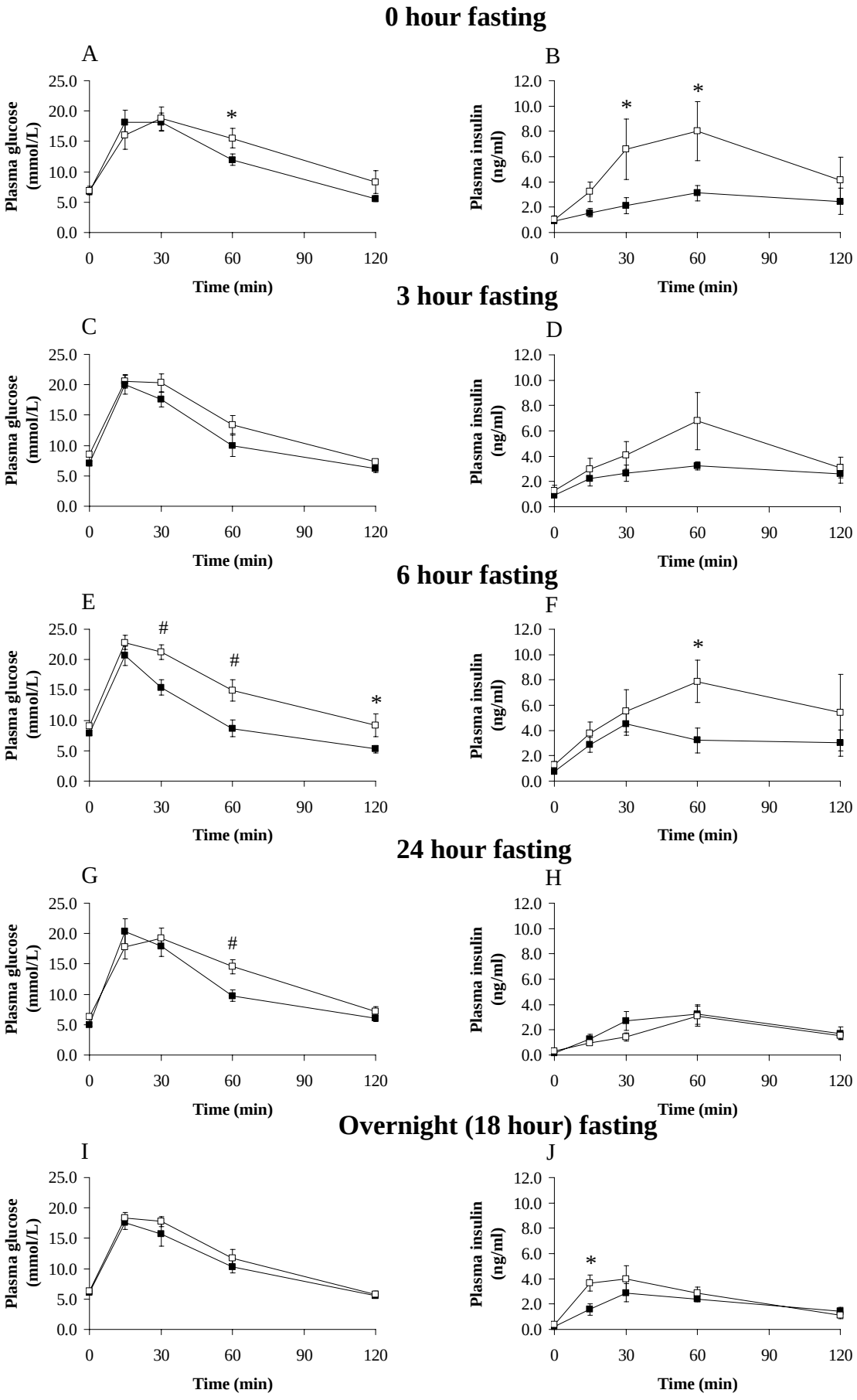

0 hour fasting
A
B
3 hour fasting
C
D
6 hour fasting
E
F
24 hour fasting
G
H
Overnight (18 hour) fasting
I
J
Plasma glucose (mmol/L)
Plasma insulin (ng/ml)
Time (min)
0
30
60
90
120
*
#

Figure 1

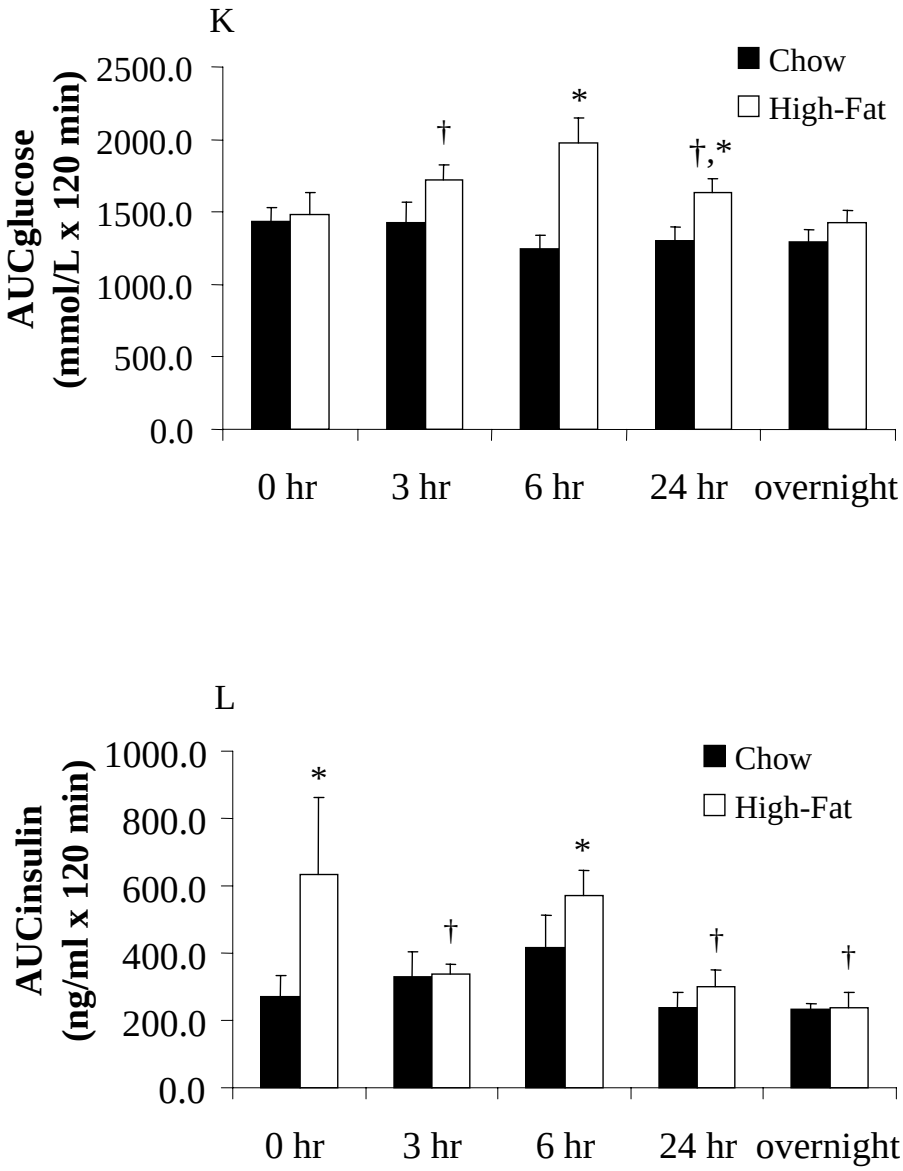

K
AUCglucose (mmol/L x 120 min)
2500.0
2000.0
1500.0
1000.0
500.0
0.0
Chow
High-Fat
†
*
†,*
0 hr
3 hr
6 hr
24 hr
overnight
L
AUCinsulin (ng/ml x 120 min)
1000.0
800.0
600.0
400.0
200.0
0.0
Chow
High-Fat
*
†
*
†
†
0 hr
3 hr
6 hr
24 hr
overnight

Figure 2

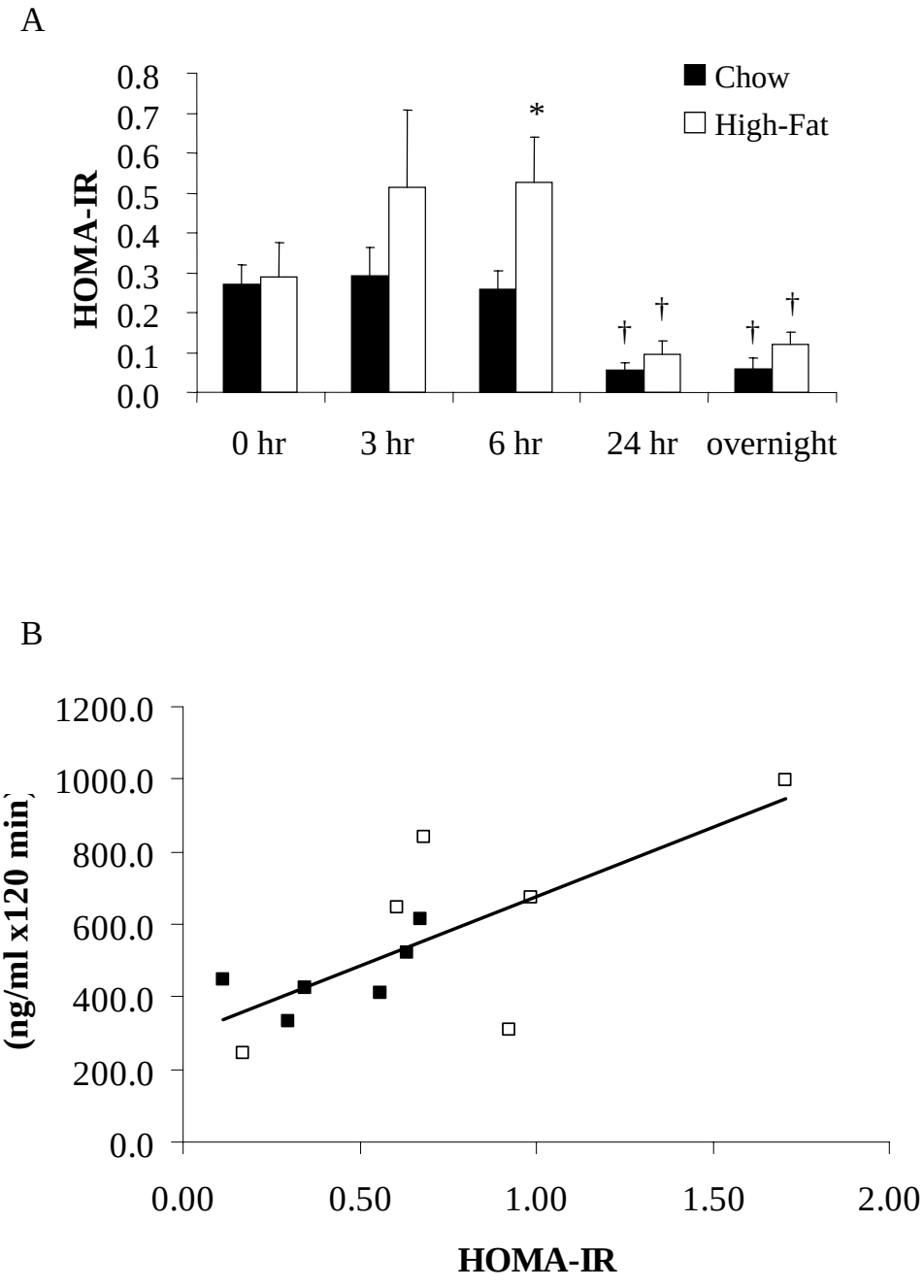

A
HOMA-IR
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Chow
High-Fat
*
†
†
†
†
0 hr
3 hr
6 hr
24 hr
overnight
B
AUCinsulin
(ng/ml x120 min)
1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
0.00
0.50
1.00
1.50
2.00
HOMA-IR

Figure 3 **IPGTT** **OGTT**

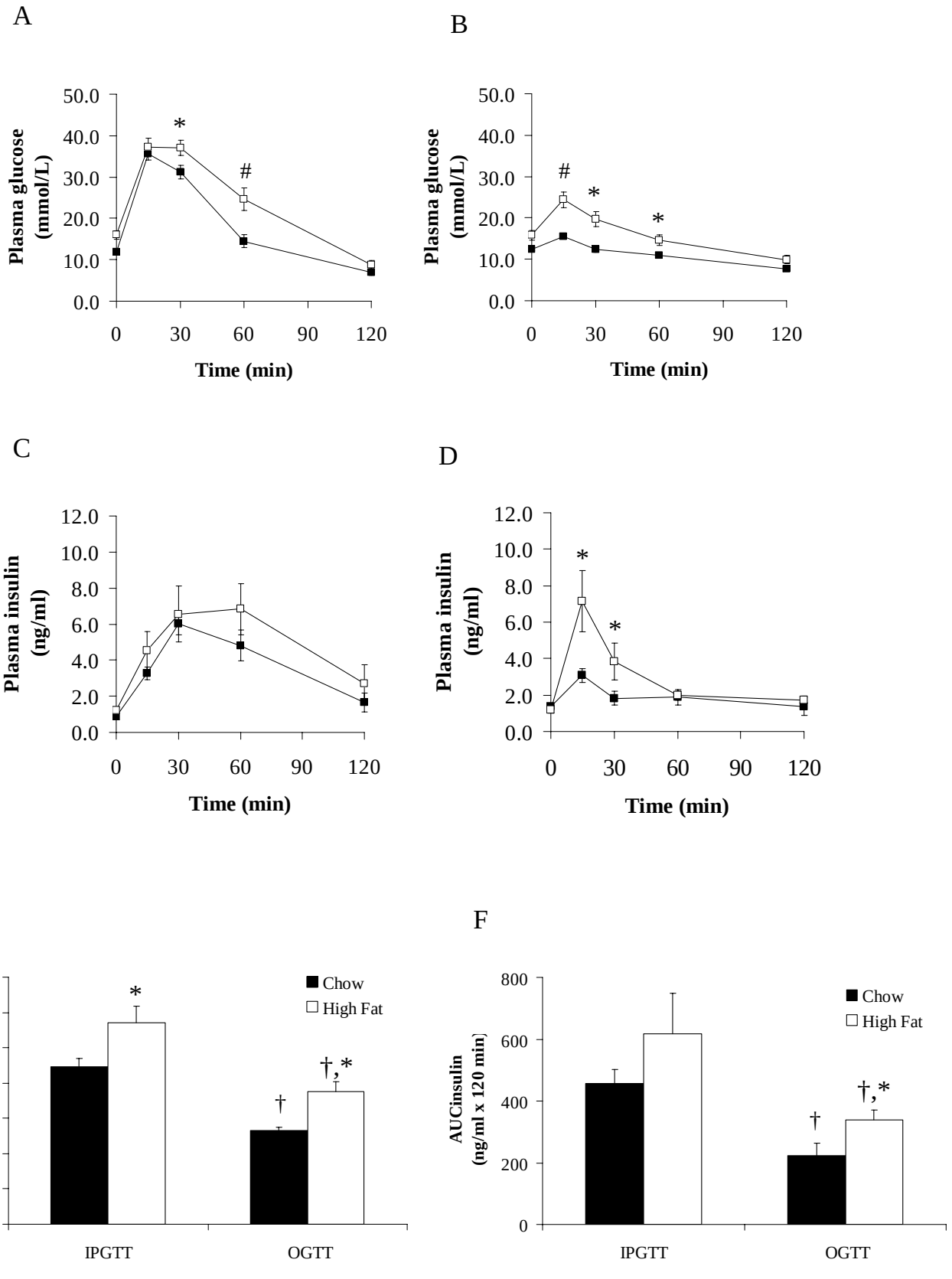


Figure 4

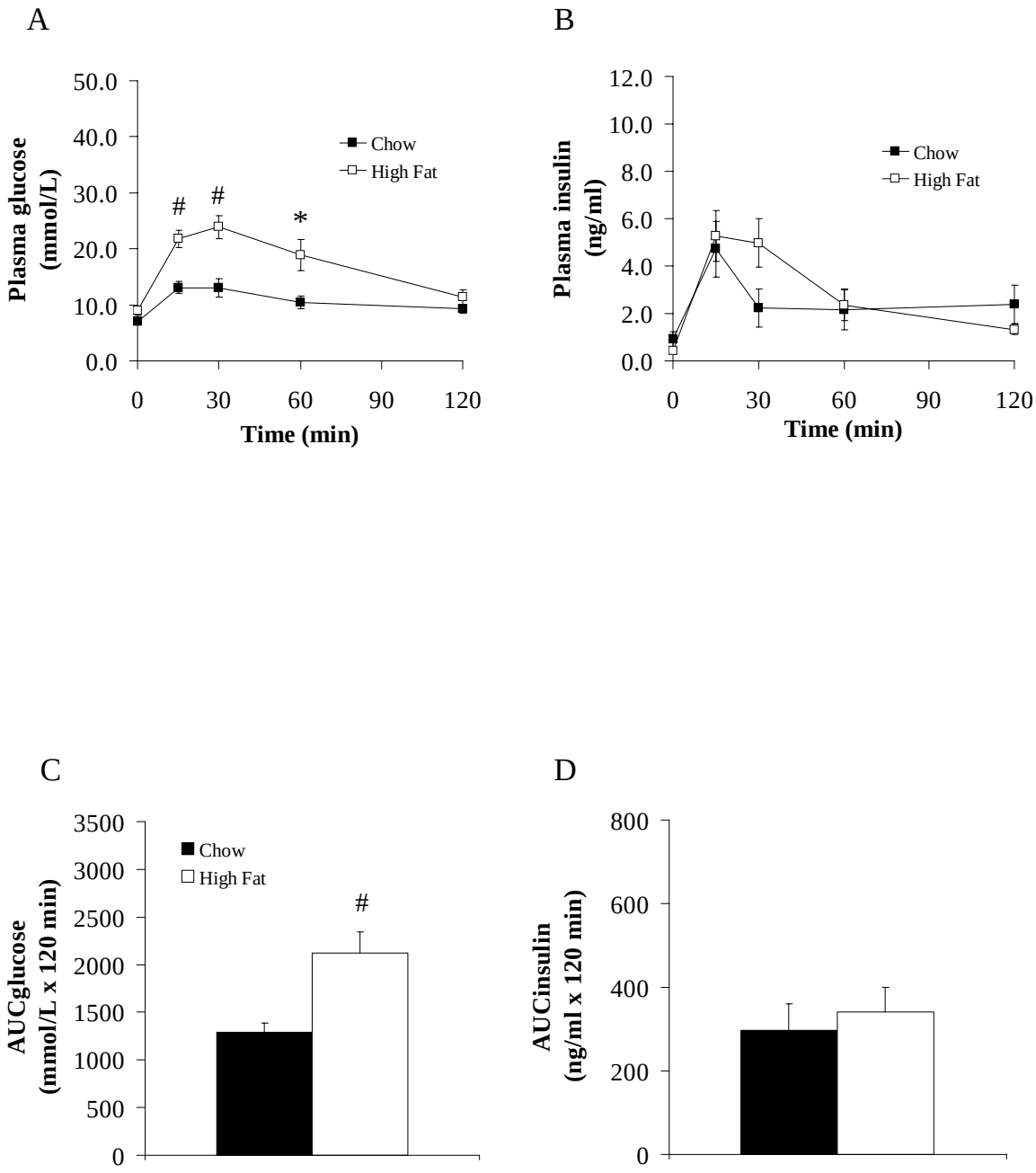

A
Plasma glucose (mmol/L)
50.0
40.0
30.0
20.0
10.0
0.0
Chow
High Fat
#
#
*
0
30
60
90
120
Time (min)
B
Plasma insulin (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Chow
High Fat
0
30
60
90
120
Time (min)
C
AUCglucose (mmol/L x 120 min)
3500
3000
2500
2000
1500
1000
500
0
Chow
High Fat
#
D
AUCinsulin (ng/ml x 120 min)
800
600
400
200
0

Figure 5

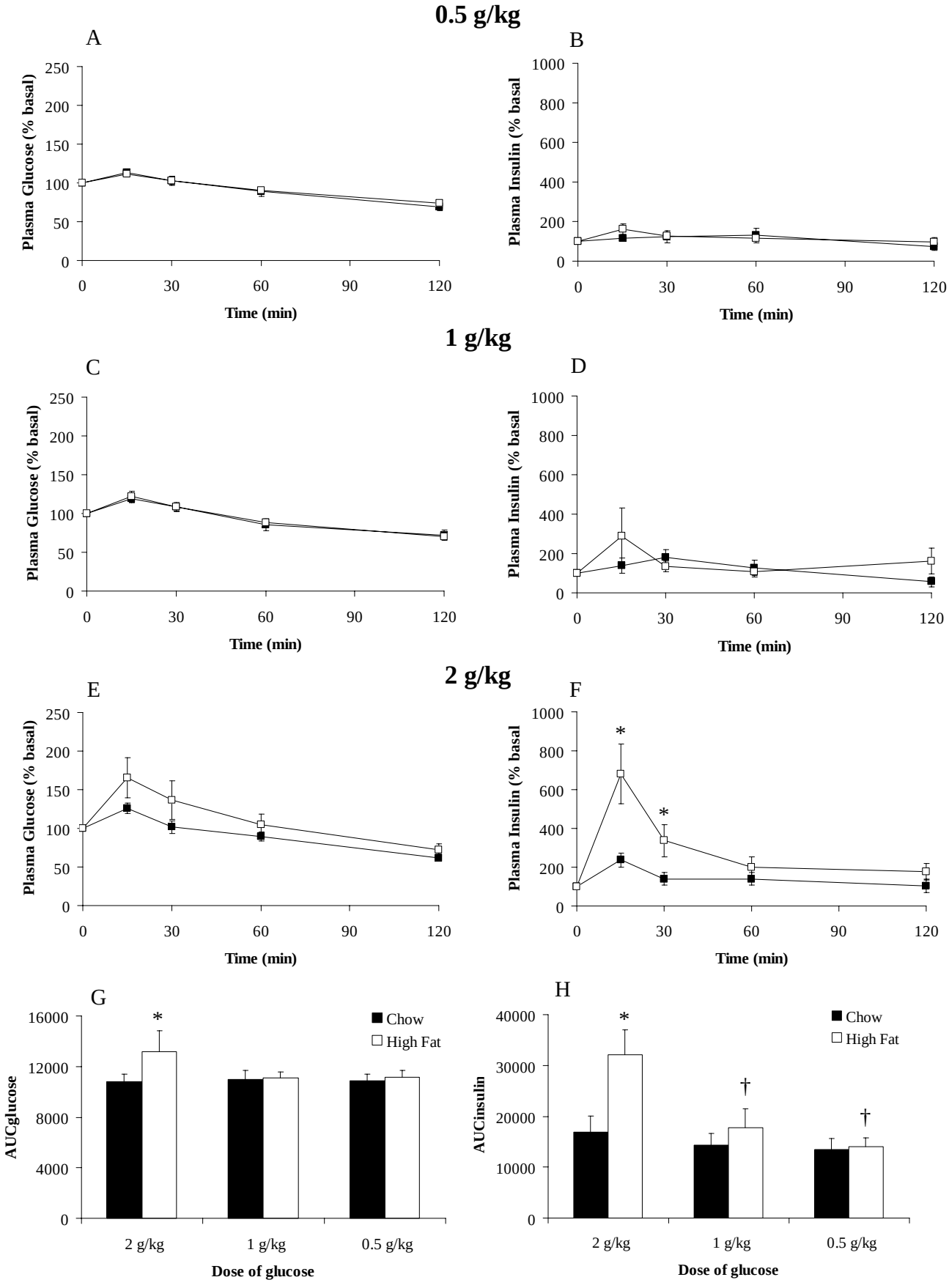

0.5 g/kg
A
B
Plasma Glucose (% basal)
Plasma Insulin (% basal
Time (min)
1 g/kg
C
D
2 g/kg
E
F
*
G
H
Chow
High Fat
AUCglucose
AUCinsulin
†
2 g/kg
1 g/kg
0.5 g/kg
Dose of glucose

Figure 6

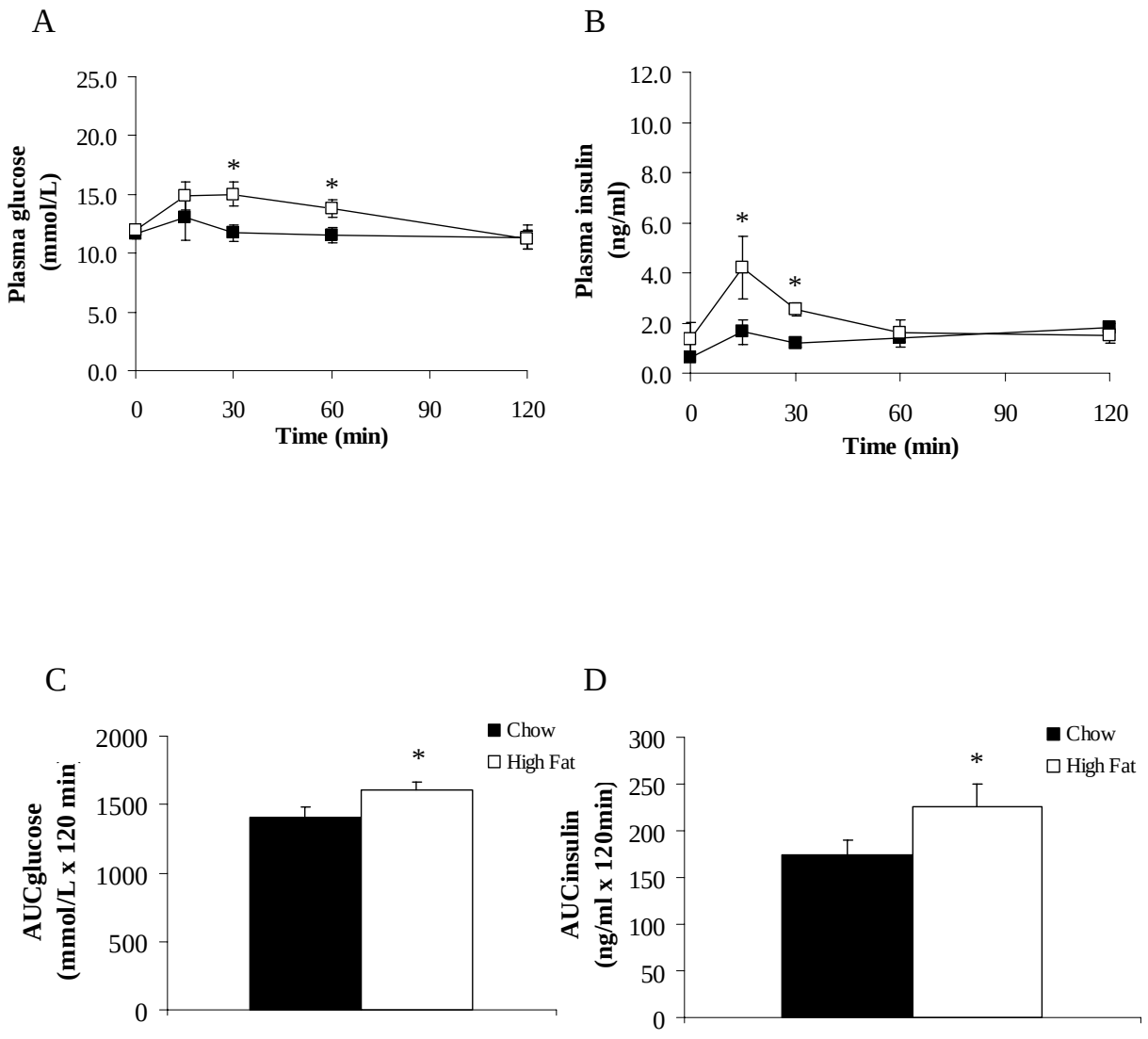

A
Plasma glucose (mmol/L)
25.0
20.0
15.0
10.0
5.0
0.0
0
30
60
90
120
Time (min)
*
*
B
Plasma insulin (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
30
60
90
120
Time (min)
*
*
C
AUCglucose (mmol/L x 120 min)
2000
1500
1000
500
0
Chow
High Fat
*
D
AUCinsulin (ng/ml x 120min)
300
250
200
150
100
50
0
Chow
High Fat
*

Figure 7

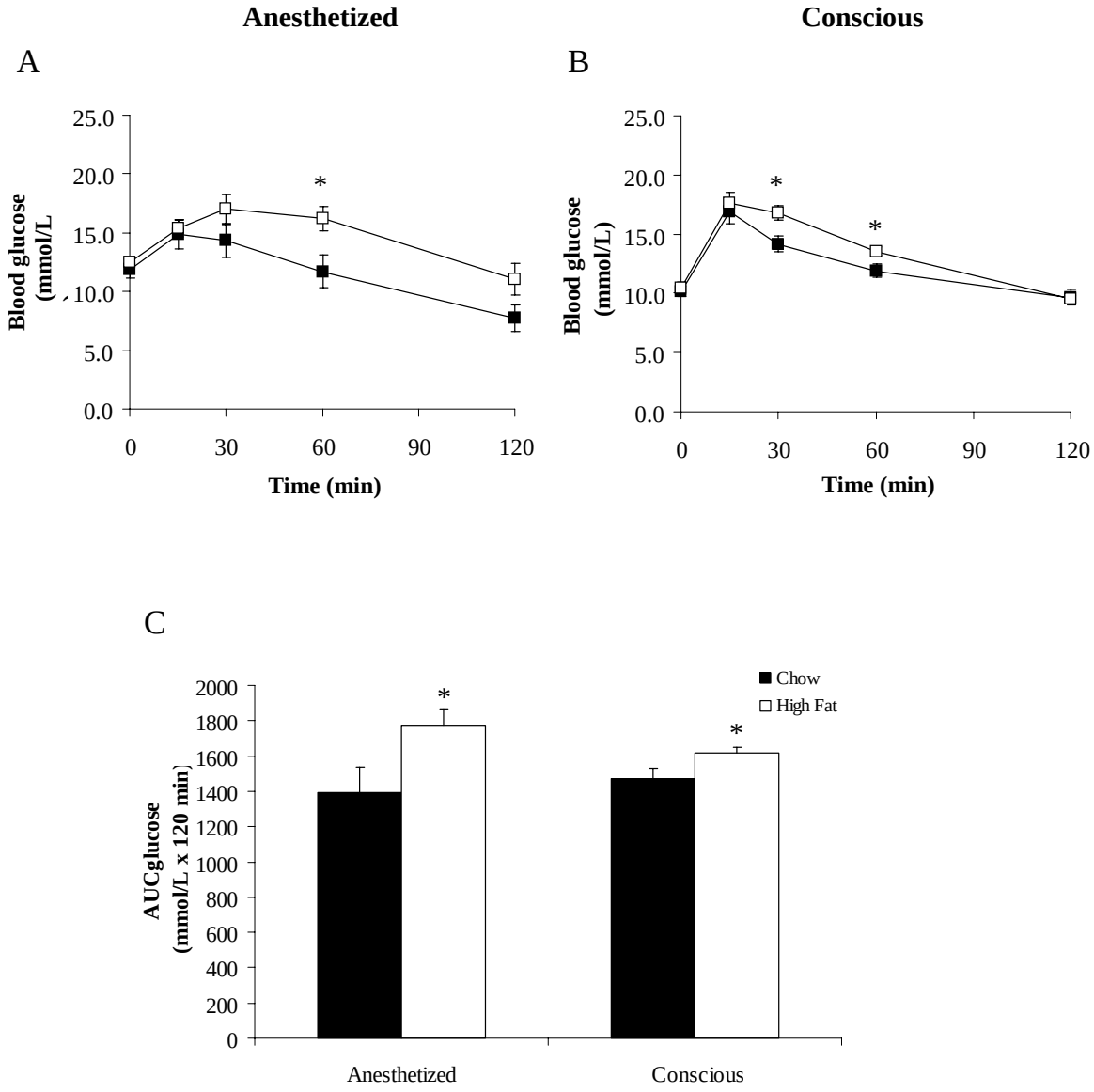
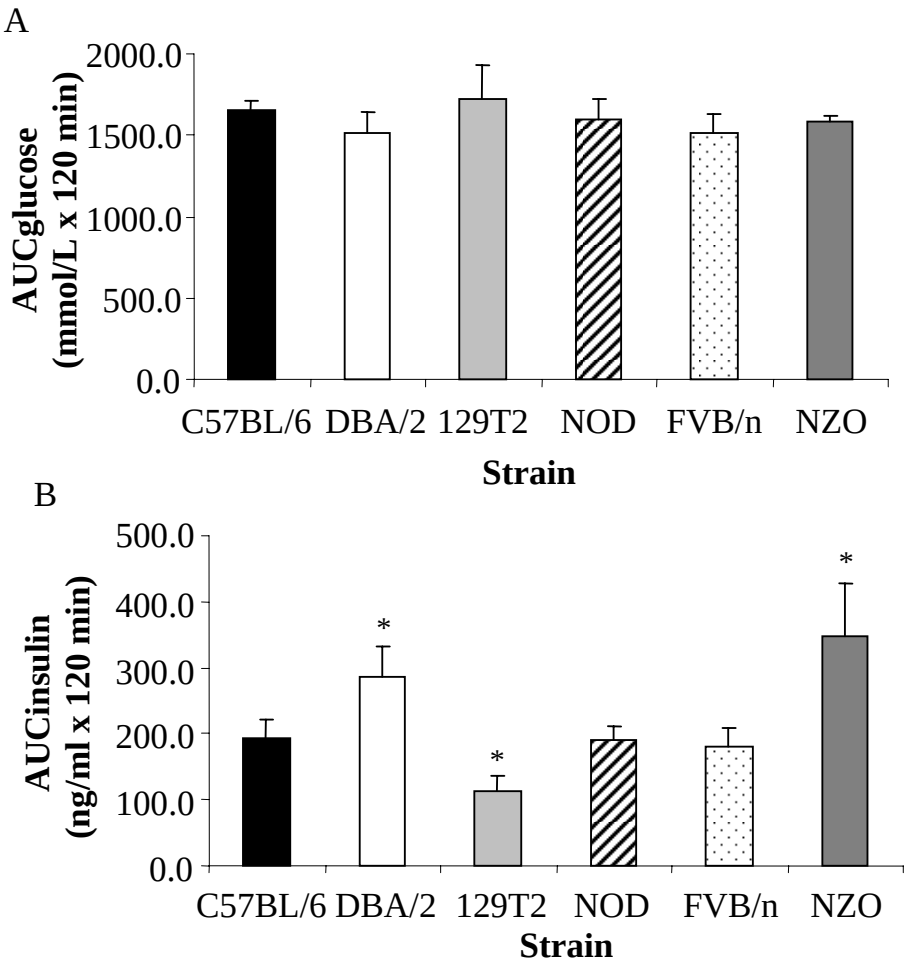

Anesthetized
Conscious
A
B
Blood glucose (mmol/L)
25.0
20.0
15.0
10.0
5.0
0.0
0
30
60
90
120
Time (min)
*
C
AUCglucose (mmol/L x 120 min)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Chow
High Fat
Anesthetized
Conscious

Figure 8


A
AUCglucose (mmol/L x 120 min)
2000.0
1500.0
1000.0
500.0
0.0
C57BL/6
DBA/2
129T2
NOD
FVB/n
NZO
Strain
B
AUCinsulin (ng/ml x 120 min)
500.0
400.0
300.0
200.0
100.0
0.0
*
*
*
C57BL/6
DBA/2
129T2
NOD
FVB/n
NZO
Strain