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# Quantifying rates of glucose production in vivo following an intraperitoneal tracer bolus.

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*Each made equally critical contributions to the development of the work discussed here.

**Running title:** Coupling stable isotope tracers and mass spectrometry-based analyses.

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**Abstract**

Aberrant regulation of glucose production makes a critical contribution to the impaired glycemic control that is observed in Type 2 diabetes. Although isotopic tracer methods have proven to be informative in quantifying the magnitude of such alterations, it is presumed that one must rely on venous access to administer glucose tracers which therein presents obstacles for the routine application of tracer methods in rodent models. Since intraperitoneal injections are readily used to deliver glucose challenges and/or dose potential therapeutics, we hypothesized that this route could also be used to administer a glucose tracer. The ability to then reliably estimate glucose flux would require attention towards setting a schedule for collecting samples and choosing a distribution volume. For example, glucose production can be calculated by multiplying the fractional turnover rate by the pool size. We have taken a step-wise approach to examine the potential of using an intraperitoneal tracer administration in rat and mouse models. First, we compared the kinetics of [U-$^{13}$C]glucose following either an intravenous or an intraperitoneal injection. Second, we tested whether the intraperitoneal method could detect a pharmacological manipulation of glucose production. Finally, we contrasted a potential application of the intraperitoneal method against the glucose-insulin clamp. We conclude that it is possible to (i) quantify glucose production using an intraperitoneal injection of tracer and (ii) derive a "glucose production index" by coupling estimates of basal glucose production with measurements of fasting insulin concentration; this yields a proxy for clamp-derived assessments of insulin sensitivity of endogenous production.

## Introduction

Type 2 diabetes is typically characterized by the presence of fasting hyperglycemia, which largely results from a disruption in the control of basal glucose production (9; 10). Numerous investigators have aimed to quantify glucose production using isotopic tracers in order to better understand the pathophysiology surrounding Type 2 diabetes and states of insulin resistance (27; 28; 38). In that regard, emphasis has been placed on coupling tracer kinetics with the use of a "glucose-insulin clamp" in order to address questions regarding the insulin sensitivity of endogenous glucose production (10; 16; 38). Given the increase in obesity and the prevalence of insulin resistance it comes as no surprise that many methods have been adapted for use in rodent models where one can readily manipulate gene expression and/or examine the efficacy and mechanism of action of novel therapeutics (1-3; 21). Unfortunately, studies are typically low throughput since animals require catheterization. Herein we have considered an alternative modality for quantifying glucose production in vivo. Namely, we suspected that one could quantify glucose flux using an intraperitoneal bolus of a tracer which would then allow investigators to envision higher throughput studies in rodent models while helping to conserve resources. The rationale for considering this approach is outlined below.

Rates of glucose production can be quantified by administering an isotopically labeled tracer and then measuring its dilution (28; 41). If one restricts questions surrounding glucose production to conditions of a metabolic steady-state, e.g. determine rates of glucose production in a post-absorptive period, than investigators must contend with two central issues when planning a study. For example, one must consider (i) what type of tracer should be given and (ii) how should it be administered?

Classic studies relied on the use of radiolabeled tracers and generated interesting data regarding how the type of tracer (i.e. $^{3}H$ vs $^{14}C$) and the positional labeling could affect one's interpretation of the metabolic rate(s) (18-20). For example, it was recognized that specific glucose tracers would experience different dilutions and that this could be explained via glucose cycling (18-20; 41). The adaptation of stable isotope tracers confirmed the early observations and allowed investigators to simultaneously mix multiple isotopes in order to quantify the magnitude of the different flux rates (31). Since mass spectrometry and NMR can separate the different species, it is possible to envision the routine implementation of such methods (13).

As our understanding of physiology has advanced, in part due to the application of mathematical models, it became clear that one could use different approaches for administering a tracer. For example, one could dose a tracer via a single intravenous injection, a continuous infusion or a primed-infusion (27; 28; 41). Glucose flux rates are then determined by measuring the dilution of the isotope and estimating the pool size (e.g. when using the intravenous bolus approach) or by simply measuring the rate of tracer dilution (e.g. when using either a constant infusion and/or a primed-infusion) (28; 38). Note that this logic assumes a single (well-mixed) compartment model, which appears to be reasonably valid in rodent models and in basal conditions in humans (4; 5; 14; 30).

Given the extensive history in this field, investigators have several choices regarding the design of preclinical studies. However, it is unfortunate that none of the described protocols are well suited for rapid (user friendly) phenotyping of rodent models. Namely, the presumption is that investigators must rely on a surgical manipulation to implant catheters and/or utilize protocols which require a certain degree of care in order to make delicate tail vein injections. We hypothesized that one could quantify rates of glucose flux by administering a glucose tracer via an intraperitoneal bolus. This approach would be analogous to the use of an intravenous (e.g. tail vein) injection of a tracer but could, in principle, be easier to implement by less specialized associates and/or facilitate larger scale studies. Indeed, the use of intraperitoneal administration is not necessarily new (42), one can expect a reasonably rapid distribution of substances into the circulation (40). Herein we discuss the development and use of an intraperitoneal bolus method for quantifying glucose flux in rodents.

## Materials and Methods

*Biological.* A pilot study was conducted in 5 week old female ZDF (fZDF) rats that had been maintained on a high fat diet (D10111701, Research Diets, New Brunswick, NJ; 48% kcal from fat, 18% kcal from protein and 34% kcal from carbohydrates, 4.8 kcal / g food); tracer studies were performed following a 3 week feeding period. Food was removed at 8AM on the day of the study, 5 hours later rats were dosed with [U-$^{13}$C]glucose (0.04 g per kg) using either a tail vein injection or intraperitoneal bolus (the dosing volume was ~ 5 ml per kg). The concentration of circulating glucose was immediately determined using a glucometer, a small sample of whole blood (~ 5 ul) was collected into 5 ul 0.1N citrate buffer at various time points and used to determine the isotopic labeling. The isotopic enrichment of circulating glucose was determined as follows: during the intravenous bolus protocol samples were collected at 0, 5, 15, 30, 45, 60, 75, 90, 120 and 150 minutes and during the intraperitoneal bolus protocol samples were collected at 0, 15, 30, 45, 60, 75, 90, 120 and 150 minutes post tracer administration.

A second study was conducted in fZDF rats following treatment with Rosiglitazone. This study relied on the same diet and conditions as noted above, Rosiglitazone or 0.5% methylcellulose (vehicle) was administered using an oral gavage (10 mg drug per kg body weight per day, the total dosing ran for 5 days over the last week of the high fat feeding period). The glucose tracer protocol and sampling scheme followed the same outline as above for the intraperitoneal method.

Studies were run to quantify glucose production in mouse models using the following protocols. Male C57Bl/6J mice started on a high fat diet at 6 weeks of age (D12492, Research Diets, New Brunswick, NJ; 60% kcal from fat, 20% kcal from protein and 20% kcal from carbohydrates, 5.2 kcal / g food), they were maintained on this diet for 16 weeks. To estimate glucose production, food was removed at 8AM and mice were dosed at 1PM with [U-$^{13}$C]glucose (0.05 g per kg) using the tail vein injection or intraperitoneal bolus (the dosing volume was ~ 5 ml per kg), i.e. a similar protocol to that used in rats. Whole blood (~ 5 ul) was collected into 5 ul 0.1N citrate buffer at 5, 15, 30, 45, 60, 75, 90 and 120 minutes post-injection, the 5 minutes sample was not collected for the intraperitoneal protocol.

A second study was conducted in ob/ob mice following treatment with Pioglitazone. In all cases we studied 10 week old male animals. The ob/ob mice were randomized and either dosed with 0.5% methylcellulose (vehicle) or 10 mg per kg of Pioglitazone (orally, QD at ~ 8-9AM) for 5 days, control mice (ob/+) were dosed with 0.5% methylcellulose (vehicle) for the same period. To estimate glucose production, food was removed at 8AM and mice were dosed at 1PM with [U-$^{13}$C]glucose (0.05 g per kg) using an intraperitoneal bolus (the dosing volume was ~ 5 ml per kg), i.e. a similar protocol to that used in studies noted earlier. Whole blood (~ 5 ul) was collected into 5 ul 0.1N citrate buffer at 0, 30, 60, 90 and 120 minutes post-tracer.

Glucose-insulin clamps were performed using the following protocol. Rodents (5 week old fZDF rats) were purchased from Charles River and fed a high-fat diet (Research Diets, D10111701) for 3 weeks to induce hyperinsulinemia and hyperglycemia. Seven days prior to the clamps, rats were anesthetized and dual indwelling catheters (carotid artery and jugular vein) were surgically implanted. Starting 2 days after the surgery, rats were dosed with either 0.5% methycellulose (vehicle) or Rosiglitazone (5 mg per kg, orally, QD at ~ 8-9 AM) for 5 days. After 5 days of treatment, rats were fasted for ~ 6 hours and hyperinuslinemic-euglycemic clamps were performed as described previously (39). Briefly ~ 4 hours into the fast, a baseline blood sample was drawn (t = -90 min) and a 100 µg per minute bolus infusion of [U-$^{13}C_6$]glucose was given for 10 minutes, this was followed by a continuous infusion for 80 minutes (at 25 µg per minute) to achieve a steady-state. Two blood draws (t = -10 and 0 minutes) were made to measure basal glucose turnover. After the blood collection at t = 0 minutes, insulin (Humulin, 25 mU per min per kg) was infused and blood glucose was maintained at ~ 120 to 140 mg per dl by variable infusion of 25% dextrose solution (this solution was enriched to ~ 2.5% with [U-$^{13}C_6$]glucose). Blood samples were collected at various time points (t = 90, 100, 110 and 120 minutes) during the steady-state phase of the clamp and stored until further analysis.

Mice (5 week old male ob/ob mice) were purchased from Jackson Laboratories and allowed to acclimate for one week, they were then fed regular chow or chow mixed with Pioglitazone (300 mg per kg diet). After 3 weeks on the respective diets, mice were surgically implanted with dual indwelling catheters (carotid artery and jugular vein) and allowed to recover. On day 7 following the surgery, mice were fasted for ~ 6 hours and hyperinuslinemic-euglycemic clamps were performed as described above.

A final group of rats was studied to determine if we could quantify differences in short- vs long-term fasting. In this case, Sprague-Dawley rats were maintained on normal carbohydrate-based chow. Food was removed for 4 hours before an intraperitoneal bolus of [U-$^{13}C_6$]glucose was given (0.015 g per kg), blood was sampled at various times over the next 150 minutes. Rats were allowed to recover and then maintained again for 1 week in their standard cages with free access to their carbohydrate-based chow, the tracer study was then repeated however following an 18 hour fast. [U-$^{13}C_6$]Glucose was again given via an intraperitoneal bolus and samples were collected over the same period as in the study with short-term fasting.

*Analytical.* The enrichment of glucose was determined as follows. Samples were vigorously mixed after the addition of 100 ul methanol, following a brief centrifugation the supernatant was removed and evaporated to dryness under a stream of nitrogen. The residue was reacted with 50 ul of hydroxylamine hydrochloride (25 mg per ml of pyridine) at 65C for 30 min, 50 ul acetic anhydride was then added and the heating was continued for another 30 min. Samples were then evaporated to dryness sunder a stream of nitrogen and the residue was dissolved in 70 ul ethyl acetate prior to GCMS analyses. Samples were analyzed in an Agilent 5973 mass spectrometer coupled with a 6890N GC (HP 5 column, 175°C initial temperature, hold for 0 min and ramp to 300°C at 35°C per min, hold for 1 min). Data were acquired using selected ion monitoring under electron impact ionization (m/z 314 to 319, 10 ms dwell per ion).

Note that although M+6 glucose is administered this analytical method actually measures the enrichment using the M+5 isotopomer. We have determined that there is negligible recycling of $^{13}C$ during this period which therein avoids any potential interference. For example, the enrichment in M+1, 2 or 3 isotopomers is less than 2% at its maximum (this was determined using the same derivative but running the analyses using chemical ionization in order to quantify the full isotope spectrum, unpublished observations). We specifically chose this derivatization and analytical scheme since the chemical workup is straightforward and robust and the analytics can be performed with a somewhat basic mass spectrometer operated in a simple mode.

Insulin concentrations were determined using a standard kit (AlphaLISA Insulin, PerkinElmer).

*Calculations.* The fractional turnover rate of glucose was estimated by measuring the decrease in glucose enrichment, data were fit using a linear regression analyses (i.e. the y-axis labeling was converted to a natural log scale and the slope was determined using GraphPad Prism). The glucose pool size was estimated by measuring the concentration of glucose and then assuming a volume of distribution that was equivalent to 20% of body weight. In cases when [U-$^{13}C$]glucose was administered using an intravenous bolus the glucose pool size was determined from the initial enrichment, typically using samples that were collected 5 minutes after the tracer was administered, i.e. pool size (mg) = dose of tracer (mg) / initial labeling (30; 41). Absolute rates of glucose production were determined by multiplying the fractional turnover by the pool size (30; 41).

In cases where glucose-insulin clamps were used to study glycemic control, glucose flux was determined using steady-state analyses. Namely, basal rates of appearance (Ra) of glucose are equal to rates of endogenous glucose production (EGP) and determined using the standard equation:

$$Ra\ (mg \times kg^{-1} \times min^{-1}) = tracer\ infusion\ (mg \times kg^{-1} \times min^{-1}) \times [(IE_{infusate} / IE_{plasma}) - 1]$$

where IE represents isotopic enrichment. During the hyperinsulinemic clamp period the tracer infusion again yields the Ra however EGP is determined after subtracting the glucose infusion rate using the equation:

$$EGP = Ra - glucose\ infusion\ rate$$

where all flux rates are expressed as mg x $kg^{-1}$ x $min^{-1}$.

## Results

Figure 1 demonstrates the temporal profile of glucose concentration and isotopic enrichment in high-fat fed fZDF rats and high-fat fed C57Bl/6J mice following either an intravenous or intraperitoneal bolus of [U-$^{13}$C]glucose. As shown, the glucose concentration remained constant regardless of the modality that was used to administer the tracer dose of [U-$^{13}$C]glucose in rats (Panel A) but there was transient increase in glucose concentration following the intravenous tracer administration in mice (Panel C). Regardless of the model, and as expected, there is a more rapid rise in plasma enrichment following the intravenous tracer bolus; the slower rise following the intraperitoneal tracer injection is consistent with the hypothesis of delayed tracer entry into the central compartment (Panel B and D).

Table 1 contains the critical parameters that are derived from the primary concentration and enrichment data (contained in Figure 1) and demonstrates the apparent rates of endogenous glucose production. In the rat model, glucose turnover was determined using data that were collected from 45 to 120 minutes post-tracer administration. These time points were chosen based on the data obtained in animals that had received an intraperitoneal bolus. Clearly, the delayed entry of tracer following the intraperitoneal bolus requires that the sampling be shifted to a region where one can observe a steadily decreasing enrichment, i.e. time points that are sampled after the tracer has moved from the intraperitoneal space and into the central compartment. The data in the rat model are relatively consistent regardless of the modality that is used for administering the tracer. Note that we observed comparable fractional rates of turnover and equivalent estimates of pool size, therefore we calculated nearly identical rates of endogenous glucose production in fZDF rats (Table 1).

Regardless of the modality that was used to administer the tracer the mouse model also showed comparable fractional rates of glucose turnover, however, there are clear differences between the apparent pool size. For example, if we used the initial dilution method, i.e. pool size (mg) = tracer dose (mg) / initial labeling, we would conclude that the glucose pool is 43 ± 4 mg (mean ± sem). This value overestimates the pool size that is inferred by measuring the baseline concentration and assuming a distribution volume that is ~ 20% of body weight (i.e. ~ 24 mg as shown in Table 1). The difference is ~ 1.8-fold (i.e. ~ 43 vs ~ 24 mg) and is consistent with the acute change in glucose concentration that is seen 5 minutes after the intravenous bolus is administered, i.e. the glucose concentration increased ~ 1.6-fold (from ~ 195 to ~ 315 mg per dl). The stress that is associated with the intravenous tracer injection and the effect this had on estimating the initial tracer dilution suggested to us that it is best to estimate the pool size by assuming a distribution volume that is ~ 20% of body weight. As shown in Table 1, we observed reasonably good agreement between the apparent rates of endogenous glucose production regardless of how the tracer was administered. Obviously, we obtained a slightly higher value in mice receiving an intravenous bolus, which is not surprising given the transient perturbation in glucose homeostasis. Note that the glucose turnover (i.e. % of the pool per min) was nearly identical demonstrating that quantitation of tracer dilution was not affected by the intraperitoneal injection.

Figure 2 demonstrates the time-dependent measurements of glucose concentration and isotopic enrichment in rodents following treatment with a PPAR-γ activator. Regardless of the model or the specific compound, we observed a dramatic decrease in blood glucose (Panel A and C). Based on the previous data that established a reasonable equivalence between the modalities for administering a tracer (Figure 1 and Table 1), [U-$^{13}$C]glucose was dosed here via an intraperitoneal injection. In the case where we studied fZDF rats, we again observed a transient increase in plasma labeling as the tracer slowly enters the systemic circulation (Panel B), this is not seen in ob/ob mice (Panel D) and reflects a faster movement of tracer from the intraperitoneal space to the systemic circulating.

The effect of PPAR-γ treatment on the rate of endogenous glucose production was calculated from the parameter estimates (i.e. pool size and fractional turnover rates) shown in Table 2. Treatment of fZDF rats with Rosiglitazone substantially reduced endogenous glucose production (i.e. ~ 18%, $p < 0.05$), consistent with reports in the literature (22). As expected, Rosiglitazone led to a marked reduction in fasting insulin concentration, which dramatically influenced the calculated "glucose production index" (37). Similar observations were made in ob/ob mice following treatment with Pioglitazone. As expected, Pioglitazone led to a dramatic decrease in blood glucose and endogenous glucose production (i.e. ~ 28%, $p < 0.01$) (22), note that in this case glucose production approached values that were observed in control (ob+) mice ($p = 0.12$). Consistent with data that were obtained in the rat model, treatment of ob/ob mice with Pioglitazone resulted in a marked reduction in the fasting insulin concentration, which led a sizeable decrease in the "glucose production index".

Another set of experiments was considered in both rodent models to cross-validate the data observed to this point and to determine whether the calculated "glucose production index" would reflect clamp-derived estimates of insulin sensitivity of endogenous glucose production, as suggested in humans (37). Figure 3 demonstrates the primary data that were obtained when euglycemic-hyperinsulinemic clamps were used to characterize the rats (Panel A - C) and mice (Panel D - F). An infusion of tracer amounts of [U-$^{13}$C]glucose was initiated during the basal period, to minimize a large shift in enrichment during the clamp period a small amount of [U-$^{13}$C]glucose was spiked into the exogenous glucose infusate. In all cases we could clearly quantify the [$^{13}$C]labeling of glucose, Table 3 contains the glucose flux rate data that are derived from the primary data contained in Figure 3. As expected, there was a marked impairment in the ability of insulin to suppress endogenous glucose production in the vehicle-treated disease models however both "glitazones" improved the insulin-mediated control of glucose production in the respective models.

A final set of experiments was conducted to determine the potential of this approach in normal (control) rats. For example, to this point most experiments had considered studies following the modulation of glucose flux in a disease model, herein we asked whether the method could detect "normal" glucose flux responses. Since it is known the glucose production will decrease with extended fasting (8), we measured glucose production in short- vs long-term fasted states. Figure 5 demonstrates that extended fasting leads to a somewhat lower basal glucose concentration, although the tracer administration tended to increase the concentration of circulating glucose this was consistent across the groups. Based

on the enrichment that was achieved (~ 6%), and as expected based on the load of tracer that was given (0.015 mg per kg), one expects that the transient rise is glucose (Figure 5, Panel A) is due to some degree of stress that likely results from handling of the animals and not from a substrate-mediated perturbation. As summarized in Table 4, it was possible to obtain estimates of glucose flux that compare with values that one might expect, as well, we were able to observe a decrease in glucose production in the overnight vs the 4 hour fasted states.

## Discussion

Despite the wide-spread number of studies that are aimed at quantifying rates of endogenous glucose production, there is a gap in the field when it comes to accessing a simple tracer method(s) for use in rodent models in vivo. Unfortunately, the path to successful drug discovery and development is often impeded by several challenges when implementing tracer methods. For example, while it is possible to implant catheters in rodents (3; 32), it is often not practical to consider this in broader experimental plans. This is especially true in cases where one aims to screen potential therapeutic compounds and/or contrast various dosing schemes. Academic laboratories are faced with similar hurdles. For example, although a given laboratory may aim to characterize a limited number of animal models and/or conditions, the requisite expertise to implant catheters (or perform tail vein injections) may not exist. Many academic centers, and indeed even the NIH, have sponsored core facilities to help support such studies with better efficiency (2; 3; 21). Nevertheless, extra resources are required to transfer animals to specialized laboratories.

Some investigators have appreciated the issues noted above and therein explored different modalities for quantifying metabolic flux and phenotyping rodents. For example, Kurland and colleagues have shown that one can deliver tracers using osmotic pumps (43). While we have found this logic to be valuable in some instances (6), the use of mini-pumps imposes an extra cost and requires that animals undergo a mild sedation (to place the device) which may affect acute readouts of glucose flux. Jones and colleagues have been able to derive information regarding metabolic phenotypes using a variety of novel approaches that also circumvent the use of catheters (11; 12; 23). In some cases certain novel approaches can unmask phenotypes that are missed by “gold-standard” methods (36). If one considers the seemingly unlimited number of models and/or therapeutic probes that can be generated, it is expected that the development of simplified (but reliable) methods may yield new insight into factors which regulate glucose homeostasis and affect the loss of glycemic control that is seen in Type 2 diabetes and insulin resistant states.

We hypothesized that one could quantify glucose flux by using an intraperitoneal injection to deliver a glucose tracer. This is conceptually similar to the use of an intravenous bolus for administering a tracer with two obvious caveats. First, one would need to obtain some knowledge regarding when to collect samples. Ideally, one should wait until the tracer has completely entered (and mixed with) the plasma compartment. Second, one would have to estimate a glucose pool size by assuming a volume of distribution for glucose. For example, although it is possible to determine the pool size following an intravenous bolus (i.e. pool size = mass injected tracer / initial labeling) (30; 41), this logic would not

hold in cases where the tracer slowly enters the circulation. We previously demonstrated that one could obtain comparable rates of endogenous glucose production when using an infusion method vs a bolus method in rats (25). Those studies assumed a volume of distribution that was equivalent to ~ 20% of body mass. Provided that pool sizes do not dramatically change over the course of an experiment, it is not surprising that one would obtain some agreement between the different approaches for administering the glucose tracers (27; 28).

A particularly important caveat to consider is whether the amount of [U-$^{13}C_6$]glucose would be comparable to a true tracer load or whether it would perturb the metabolism under investigation. Based on the rationale noted above one could derive an algorithm to examine the tracer load. For example, the pool size can be estimated from the body weight and the basal glucose concentration, i.e. total mg of glucose ~ body weight (g) x 0.2 x glucose concentration (mg per ml). For a 25 g mouse with "normal" fasting glucose (i.e. ~ 1.25 mg per ml) one would expect a pool size of ~ 6.25 mg glucose. If we wanted to reach a reasonable enrichment, e.g. ~ 5%, we would multiply 6.25 x 0.05 and determine that the tracer dose should be ~ 0.3 mg, or ~ 0.0125 mg per kg body weight. In cases where the basal glucose concentration is higher than normal the tracer dose would simply be scaled accordingly (in proportion to the change in glucose concentration) to ensure that one could reliably quantify the isotopic enrichment and yet minimize the chance of altering the endogenous glucose concentration.

The studies outlined herein determined if we could reliably quantify rates of glucose production when a tracer (e.g. [U-$^{13}C$]glucose) is administered using an intraperitoneal bolus injection. Our initial set of experiments aimed to directly contrast the use of an intravenous vs an intraperitoneal bolus of tracer. Recognizing that investigators may use rats and mice interchangeably we thought to consider both models. As expected, there is an exponential dilution of [U-$^{13}C$]glucose following intravenous tracer administration, note that the maximum enrichments were typically below 10% (when administering 0.05 g [U-$^{13}C$]glucose per kg body weight in obese animals). Although those relatively low levels of enrichment imply a minimum tracer-induced perturbation of metabolism it is obvious that the glucose concentration was not always stable. For example, in the mouse model (Figure 1C) we detected an increase in the concentration of circulating glucose immediately following the intravenous bolus. We primarily attribute this to stress which likely results from the brief restraint that is required to perform the tail vein injection. In our experience, some rat models are more stable to handling and manipulation therefore these transient excursions in circulating glucose concentrations are typically not seen (Figure 1A).

As noted above, in cases where we administered the glucose tracer via an intraperitoneal injection we assumed a volume of distribution that was equal to ~ 20% of the respective body weight. This yielded a glucose pool size which was virtually identical to the pool size that was determined from the initial dilution of [U-$^{13}C$]glucose following the intravenous bolus in fZDF rats (Table 1). Glucose production rates were then calculated by multiplying the respective pool sizes by the corresponding fractional turnover rates (Table 1); those data suggested comparable estimates of flux regardless of the route by which the tracer was administered. The absolute values that we obtained (i.e. ~ 6.75 mg x $kg^{-1}$ x $min^{-1}$)

are in reasonable agreement with the literature which suggested that the intravenous approach would have merit in our rat model (7; 15; 29; 33-35).

Studies in a mouse model of insulin resistance and obesity led to similar overall conclusions regarding the potential merit of using an intravenous bolus injection (Table 1) with one caveat. For example, we observed comparable rates of tracer dilution (i.e. fractional turnover of the glucose pool) regardless of the modality that was used to administer the tracer. The obvious caveat in the mouse model centers on the sudden increase in glucose concentration following the intravenous injection (Figure 1C). As noted earlier, if we had used the initial dilution method (i.e. pool size (mg) = tracer dose (mg) / initial labeling) we would overestimate the glucose pool as compared to what we would infer by measuring the baseline concentration and assuming a distribution volume that is ~ 20% of body weight (i.e. ~ 43 vs ~ 24 mg shown Table 1). The ~ 1.8-fold difference in the apparent pool size is very consistent with the acute ~ 1.6-fold change in glucose concentration that is seen within 5 minutes of giving an intravenous tracer bolus, this most likely reflects some degree of stress that is associated with that modality.

When we estimated the volume of distribution, by assuming that it represents ~ 20% of body weight, we calculated rates of glucose production that were similar (albeit statistically different) between mice which were given an intravenous vs an intraperitoneal bolus of tracer (Table 1). Although the current data set could not definitively explain the difference between the rates of glucose production between the studies, we expect that the initial perturbation of glucose concentration following the intravenous injection directly altered the metabolism under investigation. In our experience, the use of an intraperitoneal injection typically leads to smoother profiles of circulating glucose concentration therein providing the initial motivation for us to explore this route of tracer administration as a means of facilitating studies. The absolute values of glucose production that we observed in the mouse study (i.e. ~ 11 mg x $kg^{-1}$ x $min^{-1}$, Table 1) are comparable to reports in the literature (1; 2). Obviously there is a caveat that the use of [U-$^{13}$C]glucose would typically yield flux rates that are, if anything, lower than reports that rely on [3-$^{3}$H]glucose (3; 21) given the different metabolic fates of the respective labeled glucose molecules (18-20).

Based on the agreement between the intravenous and intraperitoneal modes of tracer administration in both rat and mouse studies, we were encouraged to examine the possibility of detecting changes in glucose flux. It is well known that “glitazones” can modulate endogenous glucose production, although the magnitude of those changes is not necessarily large the data are generally consistent across studies (22). Therefore, rodents were either treated with Rosiglitazone or Pioglitazone for a given period and glucose flux was examined following an intraperitoneal tracer administration (Figure 2). In cases where we examined the fZDF rat model, we observed reasonable reductions in glucose production following treatment with Rosiglitazone (Table 2). The magnitude of this effect was slightly greater in the Pioglitazone-treated ob/ob mouse model, which approached values of glucose production that were close to control animals (Table 2). From these studies we concluded that the use of an intraperitoneal route of tracer administration should have some merit in helping investigators quantify rates of glucose production in vivo. Specifically, we could observe different flux rates between lean and obese mice and

we could detect an improvement in the control of glucose homeostasis following drug treatment in rat and mouse models (Table 2). Collectively, these data strongly suggest that the approach has merit.

Another set of experiments was initiated to contrast our intraperitoneal method for estimating glucose production with data that would be obtained from comparable rodent models under euglycemic-hyperinsulinemic clamp conditions. Specifically, attention was directed towards a comparison of the logic that has been described by Vangipurapu et al (37). Briefly, although glucose-insulin clamps represent a "gold-standard" for studying glucose homeostasis, it is obviously not practical to routinely perform clamps especially as resources become limited. Since Vangipurapu et al have shown that it is possible to derive an insulin sensitivity index of glucose production by simultaneously measuring glucose production and prevailing insulin concentrations (37) we aimed to apply their logic in our preclinical setting.

Our rodent models were subjected to glucose-insulin clamps in order to confirm the existence of dysregulated glucose production in fZDF rats and ob/ob mice (Figure 3); those studies demonstrate a substantially blunted suppression of glucose production in each model (Table 3). Since the data compare very well with the calculated glucose production index for the respective models, our observations imply that the "glucose production index" provides a useful surrogate of clamp-derived insulin sensitivity of endogenous glucose production in rodent models (Figure 4). Clearly, the ability to derive this production index (Table 2) is facilitated by the ability to easily determine rates of glucose production, which we believe can be achieved via an intravenous route of tracer administration.

A final set of experiments aimed to quantify glucose flux under more normal conditions. For example, the studies that have been considered to this point were primarily aimed at studying the fundamental underlying concepts surrounding the tracer kinetics and to demonstrate the application in disease models following drug treatment. As noted in Figure 2 and Table 2 this approach could quantify differences in glucose flux between obese mice and lean controls, as well as determine the pharmacological efficacy. Consistent with those observations, we were able to detect differences in glucose production in short- vs long-term fasted rats (Figure 5 and Table 4). For example, it is generally well known that extended fasting will result in lower glucose flux, i.e. hepatic glycogen stores become depleted and glucose flux is dominated by the gluconeogenic contribution (8). Beylot and colleagues clearly demonstrated the fasting will change total glucose flux (and the contribution of the respective intra-hepatic flux rates) in rodents as seen in humans (24), our data (Figure 5 and Table 4) are in strong agreement with the expected changes in glucose production. One point that is worth mentioning centers on the absolute values of EGP reported here, which are somewhat lower than those reported in other studies (17). A likely explanation has to do with the body mass, our rats were approximately twice the size of those used in other studies. Since data are normalized to body weight this variable could contribute to the lower rate of EGP.

To summarize our findings, we have considered the use of a novel modality for administrating a glucose tracer(s) which therein allows one to estimate rates of endogenous glucose production. We have

compared this approach, using direct experimentation, with previously accepted methods for quantifying glucose kinetics in vivo. Our data demonstrate reasonable agreement between this new approach and (i) the use of an intravenous route of tracer administration and (ii) the ability to detect alterations in glucose production via glucose-insulin clamps. For example, we could couple these simple estimates of basal glucose production and measurements of insulin levels and therein calculate a glucose production index (37). Those values compare favorably with direct determinations of insulin-mediated control of glucose production suggesting that one may be able to circumvent the use of glucose-insulin clamps in some circumstances.

In conclusion, although intraperitoneal injections are typically used in studies of glucose tolerance and as a route of drug delivery, it is also possible to administer glucose tracers using this route and therein estimate endogenous flux rates. Presumably the approach that is outlined here will facilitate studies of glucose production however one should still consider the fact that metabolic perturbations may occur in some cases when a glucose tracer is administered using an intraperitoneal injection. The two other important factors to consider are centered on assumptions regarding (i) when to collect samples and (ii) how to estimate the volume of distribution. As noted, this can have an obvious impact on the absolute rates of glucose production. In our experience it seems that rats and mice can be sampled ~ 45 and ~ 30 min after the tracer is injected, respectively. This delay between the tracer injection and initial sampling should ensure that enrichment is being measured after the tracer has entered the systemic circulation. Obviously, investigators can modify the sampling schedule as needed. Likewise, one would need to consider whether any factor(s) might influence the entry of label into the plasma. For example, the presence of ascites might slow the movement of tracer. As shown herein, even in cases where we have studied obese (e.g. NAFLD/NASH-like) animals we were able to apply the logic and quantify glucose flux.

It is important to note that although the discussion to this point reflects in vivo data which support our logic, we have also considered questions surrounding the timing of sample collection using a more general modeling and simulation strategy (see Appendix). The exercise considered a case in which in the analytical error is ~ 1% of the measured enrichment (this is in the range of the general assay conditions that we have used). Namely, if we expect the initial enrichment to reach 5%, an analytical error of ±1% could lead to estimates of enrichment ranging from 4.95 to 5.05% (26). Since we have used [U-$^{13}C_6$]glucose and there is no detectable natural background labeling, the overall method can tolerate considerable analytical error (simulations demonstrated that reasonably good fits of the tracer dilution could be obtained even if the analytical error approached 10%, not shown). We remind the reader that mass spectrometry error in measuring enrichments is not the only factor to consider, for example, noise in the glucose pool size (i.e. measurements of glucose concentration) would also contribute to variability in the apparent glucose flux; therefore, attention to animal handling is also important. Presumably one can most easily probe for differences between groups if body size and/or compositional changes are minimal. Nevertheless, we were able to observe differences between ob/ob and ob/+ control mice. We hope that the approach developed here can be of use to investigators who are focused on understanding and manipulating glucose production.

493

494

495 **Figure legends**

496 **Figure 1. Characteristics of glucose concentration and isotopic enrichment in rodent models.** Post-
497 absorptive blood samples were collected to determine the fasting glucose concentration. A tracer dose
498 of [U-$^{13}$C]glucose was administered using an intravenous (IV) or intraperitoneal (IP) bolus injection (t =
499 0), samples were collected thereafter to measure the temporal profiles of glucose concentration and
500 enrichment. Panel A and B contain profiles that were obtained in fZDF rats whereas Panel C and D
501 contain data that were obtained in high-fat fed C57Bl/6J mice. Data are shown as mean ± sem.

502

503 **Figure 2. Glitazone-mediated modulation of glucose homeostasis in rodents.** Rosiglitazone or vehicle
504 (methylcellulose) was mixed with the diet and fed for 5 days to the respective groups of fZDF rats, ob/ob
505 mice were treated with Pioglitazone under a comparable paradigm. Post-absorptive blood samples were
506 then collected to determine the fasting glucose concentration. A tracer dose of [U-$^{13}$C]glucose was
507 administered using an intraperitoneal (IP) bolus injection (t = 0), samples were collected thereafter to
508 measure the temporal profiles of glucose concentration and enrichment. Panel A and B contain data
509 from fZDf rats whereas Panel C and D contain data from ob/ob mice. Data are shown as mean ± sem.

510

511 **Figure 3. Glucose-insulin clamps in rodents.** Rosiglitazone (5 mg per kg) or vehicle (methyl-cellulose)
512 was orally dosed in fZDF rats for 5 days and ob/ob mice were treated with Pioglitazone (30 mg per kg)
513 mixed in the diet for 4 weeks. Post-absorptive blood samples were then collected to determine the
514 fasting glucose concentration. An infusion of [U-$^{13}C_6$]glucose was used to estimate glucose flux during a
515 baseline period (sampled at t = -10 and 0 minutes) and during a hyperinsulinemic-euglycemic clamp
516 period (starting at t = 0 minutes and sampled for enrichment measurements over the last 40 min of the
517 study). Data are shown as the mean ± sem for rats (Panels A-C) and mice (Panels D-F). Data are shown as
518 mean ± sem.

519

520 **Figure 4. Measuring insulin sensitivity of endogenous glucose production: Comparison of a glucose-**
521 **insulin clamp vs a glucose production index.** Data from the respective studies discussed herein were
522 compared against each other. As explained in the Materials and Methods, the glucose production index
523 reflects the product of endogenous glucose production (EGP) and circulating insulin concentration. The
524 % suppression of EGP reflects the change in EGP from baseline vs the steady-state period of the clamp.
525 Comparisons are shown using mean ± sem, square and circles represent data in ob/ob mice and fZDF
526 rats, respectively, solid and open symbols represent baseline untreated disease models and "glitazone"
527 treated, respectively.

528

529 **Figure 5. Effect of nutritional status on glucose flux in vivo.** Sprague-Dawley rats were given on
530 intraperitoneal tracer dose of [U-$^{13}$C]glucose, samples were collected thereafter to measure the
531 temporal profiles of glucose concentration (Panel A) and enrichment (Panel B). The overall glucose
532 concentration profile was lower with longer fasting, enrichment profiles matched the general trend that
533 is seen in other studies, i.e. there is a delay phase of entry over the initial ~ 30 min followed by a dilution
534 of the labeling to the end of the study. Data are shown as mean ± sem (n = 8 per group).

535

**List of Tables.**

Table 1. Glucose production in rodent models. Kinetic parameters including glucose turnover and pool size were estimated from the primary data (Figure 1). Rates of endogenous glucose production (EGP) were calculated as described in Materials and Methods. Statistical analyses were performed using 2-tailed t-tests and assuming equal variance, no differences were observed in any measurements for fZDR rats, * represents p = 0.02 for EGP in high-fat (HF) fed mice.

| | fZDF rats (n = 6-7 per group) | | HF-fed C57BL/6J mice (n = 7-8 per group) | |
|---|---|---|---|---|
| | IV bolus | IP bolus | IV bolus | IP bolus |
| Body weight (g) | 289 ± 5 | 286 ± 4 | 54.2 ± 1.1 | 53.7 ± 0.6 |
| Turnover (% pool per min,45→120 min) | 1.83 ± 0.29 | 1.86 ± 0.25 | 2.70 ± 0.16 | 2.36 ± 0.10 |
| Pool size (mg) | 112 ± 11 | 111 ± 15 | 24.5 ± 1.2 | 24.1 ± 1.0 |
| EGP (mg/kg/min) | 6.91 ± 0.81 | 6.55 ± 0.58 | 11.9 ± 0.5 | 10.5 ± 0.3* |

Table 2. Glucose production in rodents following treatment with either Rosiglitazone or Pioglitazone. Kinetic parameters including glucose turnover and pool size were estimated from the primary data (Figure 2), rates of endogenous glucose production (EGP) were calculated as described in Materials and Methods. The glucose production index was calculated as described (37). For studies in fZDF rats, statistical analyses were performed using 2-tailed t-tests and assuming equal variance where * represents $p < 0.05$. For studies done in obese mice, statistical analyses were done using ANOVA with Tukey's post-hoc testing across all groups where * represents $p < 0.05$ between Vehicle and Pioglitazone of ob/+ vehicle and # $p < 0.05$ between Pioglitazone and ob/+ vehicle.

| | fZDF rats (n = 6-9 per group) | | ob/ob mice (n = 7-8 per group) | | |
|---|---|---|---|---|---|
| | Vehicle | Rosiglitazone | Vehicle | Pioglitazone | ob/+, vehicle |
| Body weight (g) | 302 ± 3 | 342 ± 6* | 45.9 ± 0.9 | 46.2 ± 0.7 | 26.3 ± 0.4* |
| Turnover (% pool per min,45→120 min) | 1.67 ± 0.19 | 3.43 ± 0.25* | 1.93 ± 0.09 | 2.39 ± 0.19* | 2.17 ± 0.05 |
| Pool size (mg) | 165 ± 24 | 77 ± 7* | 27.6 ± 1.4 | 16.3 ± 0.7* | 9.2 ± 0.3*# |
| EGP (mg/kg/min) | 9.33 ± 0.56 | 7.58 ± 0.54* | 11.8 ± 0.9 | 8.4 ± 0.5* | 7.5 ± 0.2* |
| Insulin (ng/ml) | 5.81 ± 0.45 | 1.04 ± 0.12* | 27.9 ± 2.3 | 5.4 ± 1.4* | 0.1 ± 0.02*# |
| Glucose production index (EGP x [insulin]) | 54.6 ± 9.5 | 7.0 ± 0.6* | 316 ± 27 | 46 ± 12* | 0.92 ± 0.02*# |

Table 3. Glucose kinetics as determined using a tracer infusion protocol coupled with a euglycemic-hyperinsulinemic clamp. Glucose flux rates, including the rate of appearance (Ra) and endogenous glucose production (EGP) were determined in different rodent models following treatment with the respective PPAR-γ activators or methyl-cellulose vehicle. Statistical analyses were performed using 2-tailed t-tests and assuming equal variance, * represents $p = 0.05$.

| | fZDF rats (n = 6-8 per group) | | ob/ob mice (n = 6-8 per group) | |
|---|---|---|---|---|
| | Vehicle | Rosiglitazone | Vehicle | Pioglitazone |
| Body weight (g) | 322 ± 6 | 349 ± 8* | 47.5 ± 0.4 | 50.1 ± 1.3 |
| Basal Ra (mg/kg/min) | 8.3 ± 0.5 | 7.8 ± 0.4 | 43.5 ± 9.3 | 32.8 ± 5.1 |
| Clamp Ra (mg/kg/min) | 10.9 ± 3.2 | 29.8 ± 2.5* | 29.3 ± 4.1 | 43.4 ± 3.1* |
| Clamp EGP (mg/kg/min) | 5.1 ± 1.2 | 2.8 ± 0.6* | 24.3 ± 5.1 | 2.4 ± 1.2* |
| % suppression of EGP | 38 ± 13 | 69 ± 7# | 37 ± 7 | 95 ± 4* |

559 Table 4. Effect of nutritional status on glucose production. Kinetic parameters including glucose turnover and pool size were estimated from the
560 primary data (Figure 5). Rates of endogenous glucose production (EGP) were calculated as described in Materials and Methods. Statistical
561 analyses were performed using a paired t-test, * represents $p < 0.01$ and ** $p = 0.05$.
562

| | Sprague-Dawley rats (n = 8 per study) | |
|---|---|---|
| | 4 hour fast | overnight fast |
| Body weight (g) | 535 ± 3 | 519 ± 3* |
| Turnover (% pool per min,45→150 min) | 1.74 ± 0.21 | 1.39 ± 0.11** |
| Pool size (mg) | 117 ± 5 | 95 ± 3* |
| EGP (mg/kg/min) | 3.8 ± 0.5 | 2.6 ± 0.2* |

563

564 Reference List

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**Appendix. Application of modeling and simulation to validate the logic and optimize protocol designs.**
We used a modeling and simulation approach to assess the propagation of error in this overall method, and to understand how the sampling parameters and data analysis methods could impact studies. Figure 1 demonstrates that the absorption and distribution of the intraperitoneal [U-$^{13}$C]glucose can be modeled using a two-compartment model.

The analytical solutions to the differential equations describing this system are given by:

$$[G_c^{lbl}(t)] = \frac{a}{a-k}\left(e^{-k\cdot t} - e^{-a\cdot t}\right)\frac{F\cdot G_d^{lbl}(t=0)}{V_c}$$

$$EGP(t) = V_c\left\{\frac{d}{dt}[G_c(t)] + k\cdot[G_c(t)]\right\}$$

where "a" and "k" are rate constants, "F" is the bioavailability, and "$G^{lbl}$" and "G" represent the concentrations of $^{13}$C-labeled and endogenous (naturally labeled) glucose, respectively. Here we see that the key parameter for the calculation of EGP is the decay constant "k", as the concentrations of the labeled and unlabeled glucose pools are, in principle, measurable through traditional analytical techniques. This decay constant can be determined by fitting the data with a double or single exponential function, depending on whether the plasma glucose data is taken early (where the absorption coefficient "a" dominates) or late (where the decay constant "k" dominates).

Using this model, we performed simulations to examine how error propagation through this system would impact the outcomes. We considered the use of two fitting methods to determine the optimal sampling protocol, i.e. that which yields a value of "k" closest to the theoretical value and with the least variance. The following parameters were used in the model: "a" = 0.1 min$^{-1}$, "F" = 95%, "$V_d$" = 200 ml per kg, tracer dose = 50 mg per kg. The errors in the total and $^{13}$C-glucose measurements were modeled using values for precision that are found in typical studies and based on our previous experience. Furthermore we assumed animal handling considerations would allow a maximum of ~ 7 plasma samples at a minimum interval of 15 minutes. Using these model assumptions, values for the theoretical time course of plasma $^{13}$C-glucose were calculated for three values of "k" (i.e. 0.015, 0.030 and 0.045 per min) which span a reasonably expected physiological range. A Monte Carlo approach was then used to add error to each $^{13}$C-glucose value and "k" was estimated using both a single and double exponential fit. This process was repeated 10,000 times and the resulting distribution of "k" values was used to calculate the mean value along with 95% confidence intervals for "k" (Figure 2).

The overall feasibility of using the intraperitoneal route to deliver a glucose tracer is reasonable. For example, Figure 2 demonstrates that the single and double exponential fits each yield reliable estimates of glucose turnover. As expected, the results suggest that for early sampling times, the single exponential fit will underestimate the correct value for "k". The double exponential fit, while producing more accurate estimates for "k", results in larger variance. From these simulations we concluded that a reasonable protocol (i.e. that which will most likely yield an accurate value for "k" with least variance) is

to sample ~ 45 to 150 minute after administering the glucose tracer and to use a single decaying exponential fit to estimate “k”. Obviously, a strict adherence to these conditions is not essential, it is expected that investigators will likely need to adjust the protocol as needed since there may be other constraints that affect the design. The model and simulations outlined here give some general guidance regarding the applicability of this method.

**Appendix Figure Legends.**

**Figure 1. Mathematical model of glucose metabolism following an intraperitoneal bolus administration.** Different compartments are used to describe the absorption and metabolism.

**Figure 2. Effect of error on estimates of glucose turnover.** The fractional turnover of glucose was assumed to range from 0.015 to 0.045 pools per min. It was expected that a tracer would be injected using an intraperitoneal bolus at t = 0 minutes and that enrichment could be measured using samples that were collected for up to 180 minutes. Panels A vs B reflect the apparent fractional turnover (mean ± standard deviation) if we used a single vs double exponential fit, respectively.

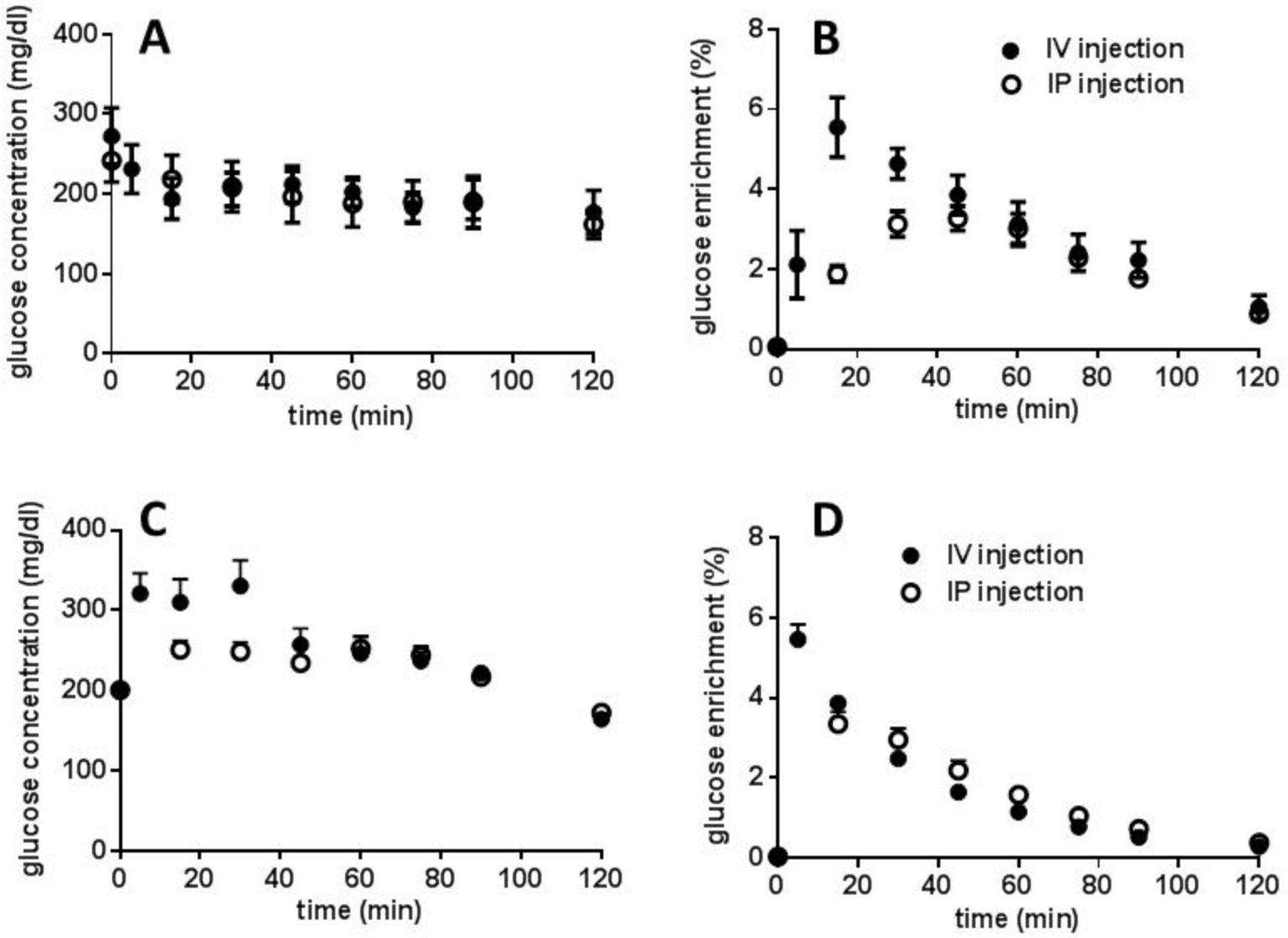

A
glucose concentration (mg/dl)
400
300
200
100
0
0
20
40
60
80
100
120
time (min)
B
glucose enrichment (%)
8
6
4
2
0
IV injection
IP injection
time (min)
C
glucose concentration (mg/dl)
time (min)
D
glucose enrichment (%)
IV injection
IP injection
time (min)

Figure 1

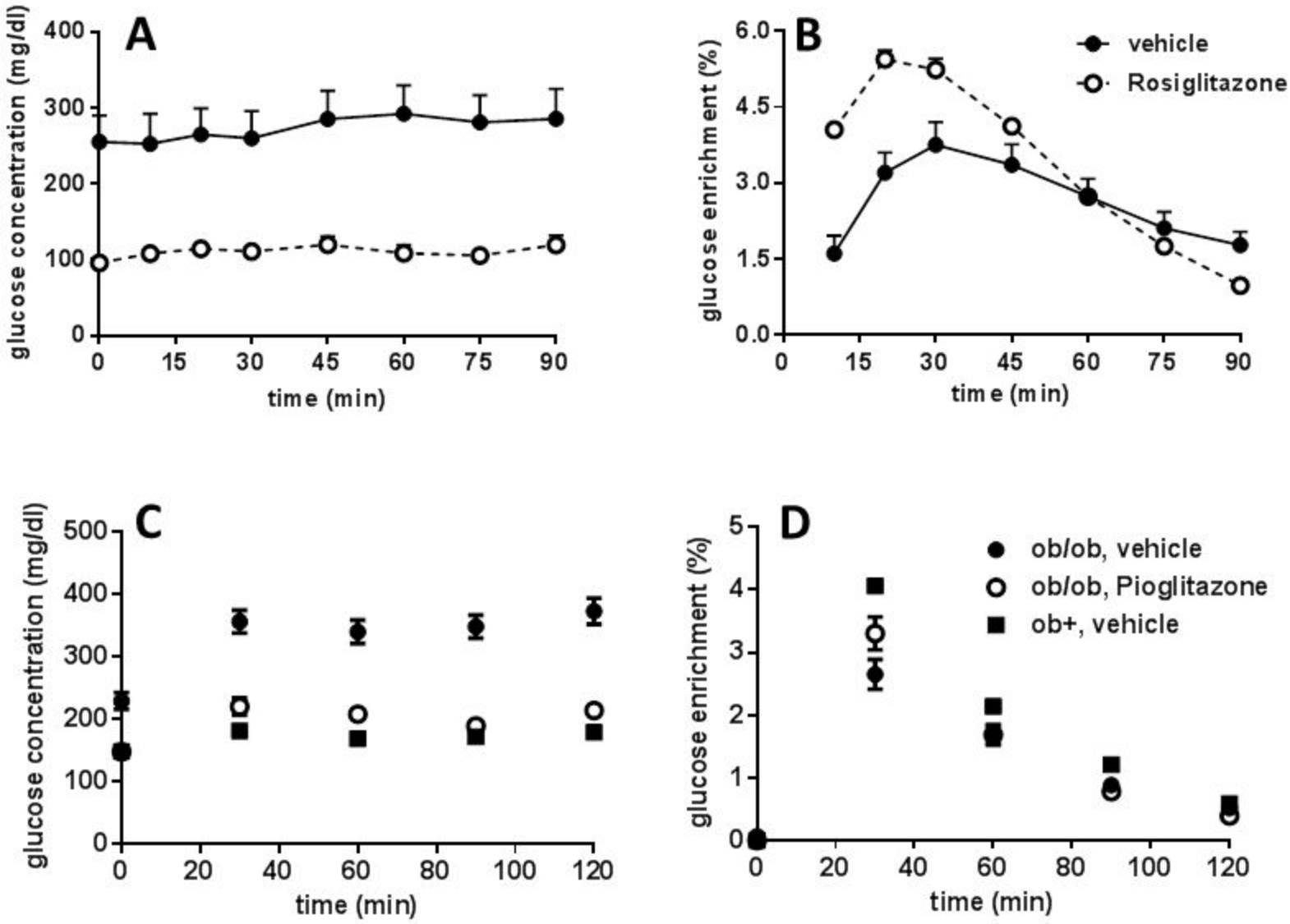

A
glucose concentration (mg/dl)
400
300
200
100
0
0
15
30
45
60
75
90
time (min)
B
glucose enrichment (%)
6.0
4.5
3.0
1.5
0.0
vehicle
Rosiglitazone
time (min)
C
glucose concentration (mg/dl)
500
400
300
200
100
0
0
20
40
60
80
100
120
time (min)
D
glucose enrichment (%)
5
4
3
2
1
0
ob/ob, vehicle
ob/ob, Pioglitazone
ob+, vehicle
time (min)

Figure 2

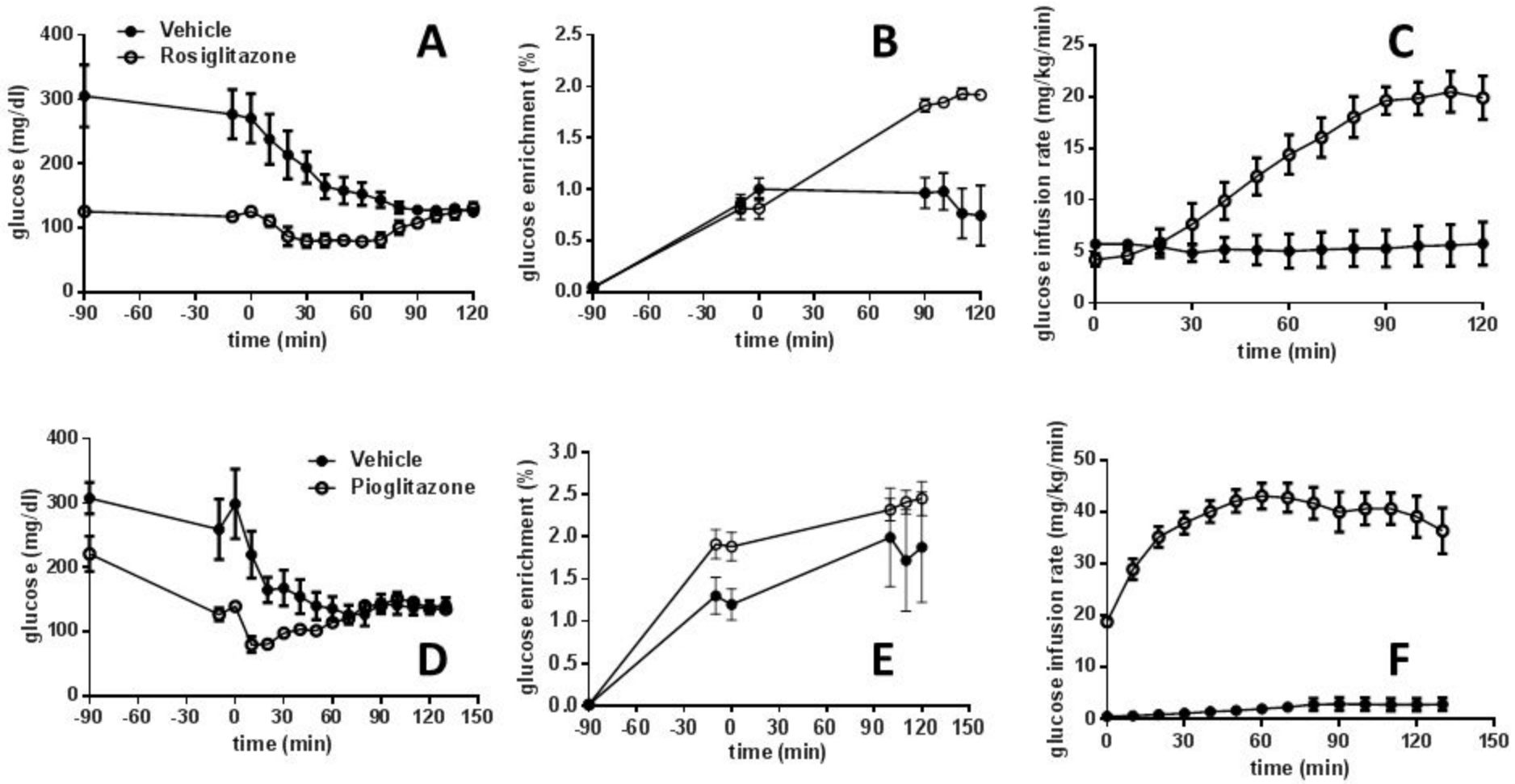

A
Vehicle
Rosiglitazone
glucose (mg/dl)
time (min)
B
glucose enrichment (%)
time (min)
C
glucose infusion rate (mg/kg/min)
time (min)
D
Vehicle
Pioglitazone
glucose (mg/dl)
time (min)
E
glucose enrichment (%)
time (min)
F
glucose infusion rate (mg/kg/min)
time (min)

Figure 3

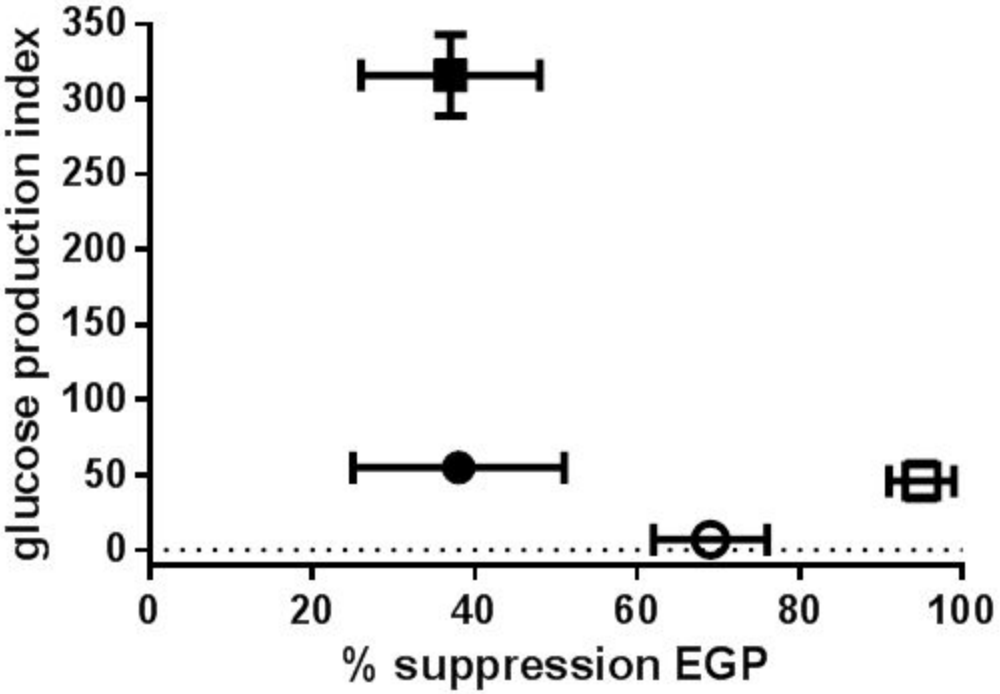

glucose production index
350
300
250
200
150
100
50
0
0
20
40
60
80
100
% suppression EGP

Figure 4

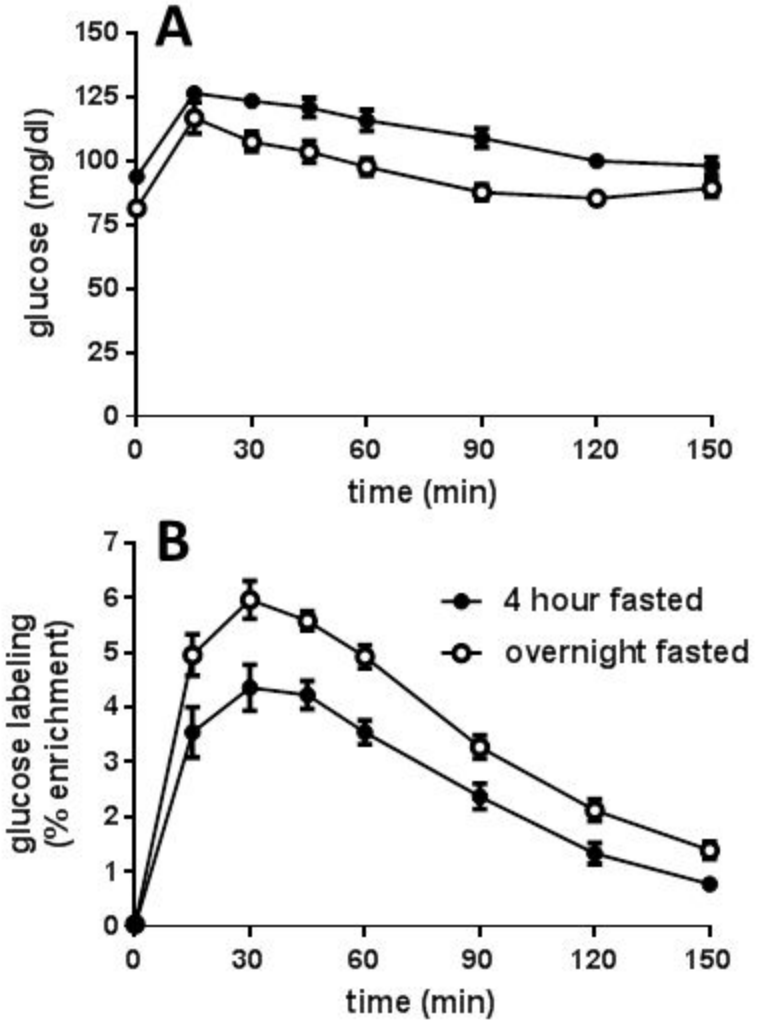
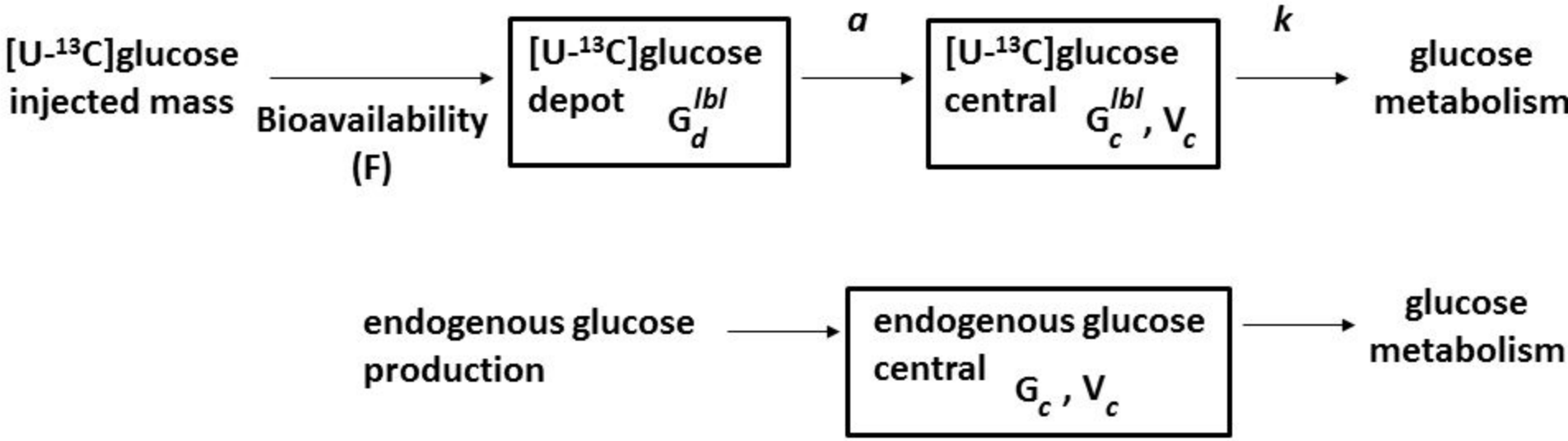
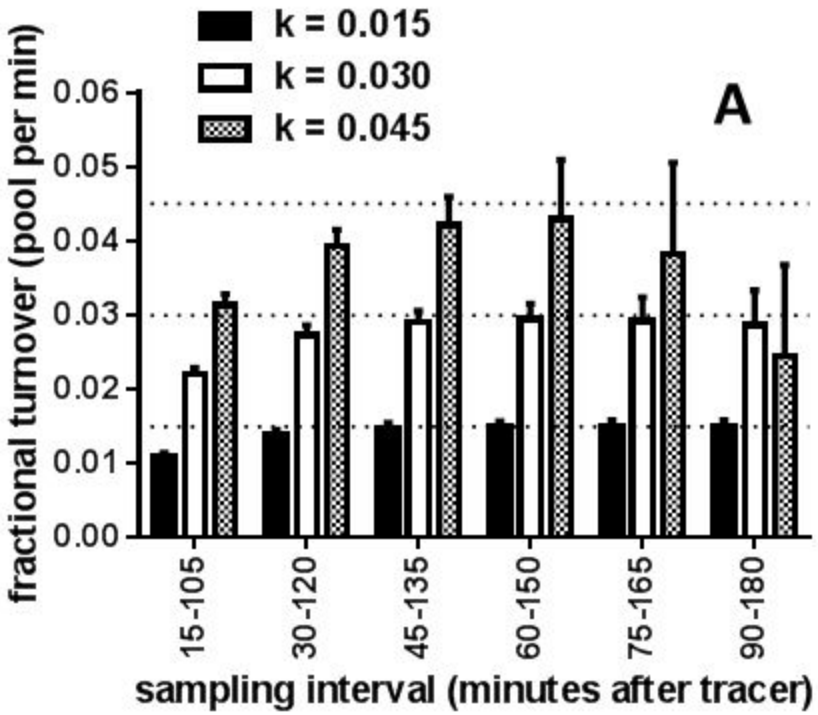
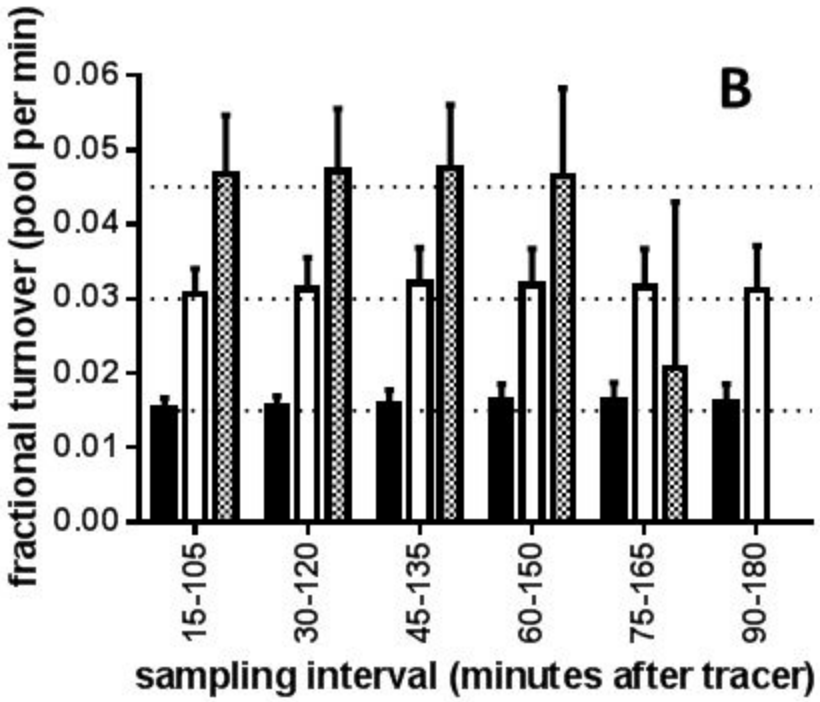

A
150
125
100
75
50
25
0
glucose (mg/dl)
0
30
60
90
120
150
time (min)
B
7
6
5
4
3
2
1
0
glucose labeling
(% enrichment)
4 hour fasted
overnight fasted
0
30
60
90
120
150
time (min)

Figure 5


[U-13C]glucose injected mass
Bioavailability (F)
[U-13C]glucose depot $G_d^{lbl}$
a
[U-13C]glucose central $G_c^{lbl}$, $V_c$
k
glucose metabolism
endogenous glucose production
endogenous glucose central $G_c$, $V_c$
glucose metabolism

Appendix 1


k = 0.015
k = 0.030
k = 0.045
A
fractional turnover (pool per min)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
15-105
30-120
45-135
60-150
75-165
90-180
sampling interval (minutes after tracer)


B
fractional turnover (pool per min)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
15-105
30-120
45-135
60-150
75-165
90-180
sampling interval (minutes after tracer)

Appendix 2