Glycerol induces G6pc in primary mouse hepatocytes and is the preferred substrate for gluconeogenesis both in vitro and in vivo

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Running Title: Glycerol induces G6PC and is the preferred substrate

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Abstract

Gluconeogenesis (GNG) is de novo production of glucose from endogenous carbon sources. Although it is a commonly studied pathway, particularly in disease, there is a lack of consensus about substrate preference. Moreover, primary hepatocytes are the current gold standard for in vitro liver studies but no direct comparison of substrate preference at physiologic fasting concentrations has been performed. We show that mouse primary hepatocytes prefer glycerol to pyruvate/lactate in glucose production assays and ¹³C-isotope tracing studies at the high concentrations commonly used in the literature as well as at more relevant fasting, physiologic concentrations. In addition, when glycerol, pyruvate/lactate, and glutamine are all present, glycerol is responsible for over 75% of all glucose carbons labeled. We also found that glycerol can induce a rate-limiting enzyme of GNG, glucose-6-phosphatase. Lastly, we suggest that glycerol is a better substrate than pyruvate to test in vivo production of glucose in fasting mice. In conclusion glycerol is the major carbon source for GNG in vitro and in vivo, and should be compared to other substrates when studying GNG in the context of metabolic disease states.

Introduction

The liver maintains euglycemia during fasting by first releasing glycogen stores and subsequently by inducing gluconeogenesis (GNG). More importantly, in diseases such as diabetes mellitus (DM), dysregulated GNG is believed to be the major cause of fasting hyperglycemia (1,2). GNG is regulated by several hormones including glucagon, insulin, thyroid hormone, and glucocorticoids through changes in gluconeogenic enzyme expression, activity as well as substrate availability. During fasting, glucagon is secreted by pancreatic α-cells, binds to the glucagon receptor, and activates protein kinase A (PKA) which phosphorylates CREB and stimulates gluconeogenic enzyme expression. The most significant enzymes are PCK1, which converts oxaloacetate to phosphoenolpyruvate, and G6PC, which converts glucose-6-phosphate to glucose (3,4). G6PC is the terminal enzyme in GNG, and glucose-6-phosphate, regardless of how it was synthesized, must be acted upon by G6PC to become glucose. G6PC expression is high in the liver and renal cortex, where glucose is produced and absent in other tissues, such as muscle and fat, where glucose is utilized (5). What remains unclear is if expression levels of these enzymes or others can explain the observed increases in hepatic glucose production in certain diseases such as
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DM (6,7). Another important factor controlling gluconeogenesis is substrate availability. Biochemistry textbooks suggest that the Cori Cycle generates pyruvate and lactate from glucose metabolism in the periphery, which are then used by the liver for GNG. Lactate is rapidly oxidized to pyruvate in the liver by reducing NAD⁺ to NADH (lactate dehydrogenase), which then enters the mitochondrion and is carboxylated to oxaloacetate by pyruvate carboxylase. After reduction to malate, the four-carbon unit is transported to the cytoplasm and eventually becomes glucose. Although the malate-aspartate shuttle generates NADH from NAD⁺ in the mitochondrion, it regenerates NAD⁺ in the cytoplasm as malate is oxidized to oxaloacetate. Rapid transport of malate to the cytoplasm at the beginning of GNG is thought to limit its entry into the TCA cycle (8).

On the other hand, glycerol has a much shorter pathway to generate glucose via GNG. In fasting, glycerol derived from lipolysis of triglycerides in adipose tissue is released into the circulation and then taken up by the liver to enter the GNG (9). Hepatic glycerol kinase encoded by the X-chromosome converts glycerol to glycerol 3-phosphate (G3P) which requires ATP for its phosphorylation. G3P is then oxidized to dihydroxyacetone phosphate, which enters the middle of GNG (10).

Although pyruvate and lactate have been suggested as the most important sources of endogenous glucose production, the importance of glycerol as a significant source of glucose is less clear. For example, glycerol is elevated in T2DM and predicts the worsening of hyperglycemia and insulin resistance (11-13). Livers of diet-induced obese rats also show higher rate of GNG from glycerol than from pyruvate and lactate (14), suggesting that glycerol may be a preferred substrate to pyruvate and lactate under some conditions.

Another factor that could potentially alter glucose production in primary hepatocytes is the presence of free fatty acids (FFAs). FFA metabolism results in formation of acetyl-CoA which is a major regulator of pyruvate carboxylase (15). Whether FFA alter GNG in vitro remains a question as studies have reported contradicting data (16,17).

The majority of studies on substrate contribution to GNG were done in the 1960s (18-21). Although these were extremely thorough for the tools available at the time, new technologies have emerged that allow for a more sophisticated analysis. Thus, we used mouse primary hepatocytes to systematically determine the substrate contribution of pyruvate/lactate, glycerol and glutamine in GNG using LC-MS measurements of 13C isotope labeled metabolites. Through extensive study of primary hepatocytes, we show that glycerol is the preferred substrate for glucose production in all cases and is able to induce expression of G6pc, the key terminal enzyme in GNG. Our studies suggest that when investigating GNG in primary hepatocytes, substrates delivered in the most appropriate concentrations are critical to obtain the most physiologically relevant data. In addition, our in vivo mixed substrate tolerance test in wild type (WT) mice also showed that majority of glucose carbon labeling comes from glycerol. We propose that the traditionally used pyruvate tolerance test is not the most appropriate method for studying GNG either in vitro or in vivo.

Results

Primary hepatocytes produce more glucose from glycerol than from pyruvate and lactate

In order to compare glucose production from different gluconeogenic substrates, we isolated primary hepatocytes from age-matched C57BL/6J-albino female mice fed a regular chow diet. After a 24-hour recovery in complete media, the cells were serum starved for 3 hours followed by a substrate challenge (Fig. 1A). Hepatocytes were treated with either pyruvate/lactate (1:10 molar ratio) over a range of high concentrations (1mM pyruvate/10mM lactate to 5mM pyruvate/50mM lactate) or glycerol (1mM to 5mM). Basal glucose production from amino acids from the media as well as glutamine were subtracted from each group based on substrate free control group. Treatment with pyruvate and lactate in a ratio of 1:10 was employed based on physiological ratio
of these substrates and their known, rapid interconversion (22). Glycerol treatment at any concentration resulted in significantly more glucose production compared to various pyruvate/lactate concentrations (Fig 1B). To confirm that the increased glucose production came primarily from glycerol and not from the release of other sources such as glycogen or amino acids, we treated cells with $^{13}$C$_3$ glycerol or $^{13}$C$_3$ pyruvate/$^{13}$C$_3$ lactate. Based on our glucose production assays we first chose to use maximum concentrations for labeling studies of glucose production in vitro. We observed that glycerol treatment produced significantly higher labeled carbon enrichment fraction compared to the pyruvate/lactate treatment in both glucose (Fig. 1C) and glucose-6-phosphate (Fig. 1F), even though approximately ten times more $^{13}$C$_3$ was present in the pyruvate/lactate mixture. These results demonstrated that about 60% of labeled glucose carbon was derived from glycerol compared to 30% from pyruvate/lactate labeling, suggesting that glycerol is the preferred carbon source for the generation of glucose in hepatocytes at high, non-physiologic concentrations typically used in in vitro glucose production assays.

$^{13}$C$_3$ labeling shows glycerol to be a direct source of glucose

We next examined the labeling pattern of glucose using $^{13}$C$_3$ labeled glycerol or pyruvate/lactate. $^{13}$C$_3$ labeled pyruvate/lactate showed a variety of labeling patterns in glucose, suggesting carbon loss or exchange in other pathways such as the TCA cycle (Fig. 1D, blue bars). In contrast, $^{13}$C$_3$ glycerol produced approximately 80% labeling in glucose at m+3 and m+6 positions (Fig. 1E). This suggests that the three-carbon glycerol is a direct carbon source for glucose production without cycling within other metabolic pathways such as TCA cycle. Labeling of glucose-6-phosphate showed a similar pattern with glycerol having over an 80% m+3 and m+6 contribution and pyruvate/lactate a mixed distribution of labeled carbons (Fig. 1G and 1H). These results suggested that glycerol is both a preferred as well as direct substrate for GNG in vitro compared to pyruvate and lactate.

**Glycerol is the primary substrate for glucose production in the presence of pyruvate, lactate and glutamine**

To determine whether glycerol is the preferred substrate in the context of a more physiologically relevant experiment, primary hepatocytes were treated with overnight fasting serum concentration of gluconeogenic substrates: glutamine (0.5mM), pyruvate (0.05mM), lactate (2.5mM) and glycerol (0.33mM) (23). Since glutamine is an essential component of cell culture media it was also investigated due to its potential to be a gluconeogenic substrate entering through the TCA cycle. We first characterized individually labeled substrates (glutamine, pyruvate/lactate or glycerol) at physiological fasting concentrations (Fig. 2A-C). Glutamine (Gln) and pyruvate/lactate (Pyr/Lac) showed a variety of glucose carbon labeling (Fig 2A, 2B) while glycerol (Gro) produced a clear m+3 and m+6 carbon pattern in glucose labeling (Fig. 2C) as previously observed in the higher concentration treatments (Fig. 1E).

We next treated primary hepatocytes with fasting serum physiological concentrations of all substrates, mimicking the presence of all substrates in vivo (Fig. 2D-F). In the first group, we combined $^{13}$C$_5$ labeled glutamine and non-labeled pyruvate/lactate and glycerol (Fig. 2D). The other two groups had $^{13}$C$_3$ labeled pyruvate/lactate (Fig. 2E) or $^{13}$C$_3$ labeled glycerol along with other non-labeled substrates (Fig. 2F). We observed that only about 10% of glucose was labeled by $^{13}$C$_5$ glutamine (Fig. 2D) or $^{13}$C$_3$ labeled pyruvate/lactate (Fig. 2E) when all substrates are combined at physiological concentrations. Most interestingly, however, when hepatocytes were treated with $^{13}$C$_3$ glycerol along with other non-labeled substrates, about 80% of glucose was labeled, suggesting that glycerol is the dominant substrate in glucose production in hepatocytes, even in the presence of all known substrates (Fig. 2F). The carbon-labeling pattern also showed the same m+3 and m+6 pattern reflecting incorporation of either one or two intact, labeled $^{13}$C$_3$ glycerol molecules.
Glycerol is directly incorporated into glucose while pyruvate and lactate first circulate in the TCA cycle

To investigate further the metabolic fates of each substrate, we used single labeled substrates (Fig. 3B and 3D) as well as combination groups with individually labeled substrates (Fig. 3C and 3E). We tracked the metabolic fate of the labeled substrates in GNG and the TCA cycle by looking at various intermediates as shown (Fig. 3A). When examining the GNG intermediates phosphoenolpyruvate (PEP), 3-phosphoglycerate (3-PG), and glucose-6-phosphate (G6P) and glucose, we found that $^{13}$C$_3$ glycerol alone contributed on average approximately 80% of labeled carbon to GNG intermediates and glucose (Fig. 3B). In the presence of three substrates, glycerol was also predominately used in GNG contributing over 70% of labeled carbon to each of these intermediates and glucose (Fig. 3C). $^{13}$C$_3$ glutamine and $^{13}$C$_3$ pyruvate/lactate, in contrast, labeled intermediates and glucose at a much lower level, especially in the context of all three substrates (Fig. 3B, C). Our second question focused on the amount of labeled carbon entering the TCA cycle as reflected in labeling of malate, citrate, fumarate, α-ketoglutarate and aconitate. We observed that only $^{13}$C$_5$ glutamine and $^{13}$C$_3$ pyruvate/lactate carbons contributed significantly to labeling TCA cycle intermediates (Fig. 3D and 3E). As expected, the high labeling was derived from $^{13}$C$_5$ glutamine due to its entry into the TCA cycle directly through α-ketoglutarate (Fig. 3D and E). Somewhat unexpectedly, $^{13}$C$_3$ pyruvate/lactate also significantly labeled TCA intermediates. These data suggest that glycerol is a preferred substrate for GNG and directly contributes to glucose production without generating TCA cycle intermediates.

Presence of FFAs does not alter glycerol’s contribution to glucose carbon

Based on glucose production assays seen in Figure 1A, we treated primary hepatocytes with high concentrations of pyruvate/lactate (5/50mM) and glycerol (5mM) with or without palmitate and oleate. No significant differences in glucose production were observed after the addition of FFA. Next, using the same experimental conditions as in Figure 3, we treated mouse primary hepatocytes with labeled substrate mixtures in presence or absence of a mixture of 200 µM palmitate and 200 µM oleate conjugated to BSA (Fig 4B-D). We chose these concentrations because FFA fasting serum concentrations range between 300 to 600 µM (24-28). We also observed no significant differences in labeling fractions between these two groups. Since FFAs were dissolved in ethanol prior to application to primary cultures, we also determined the NADH/NAD$^+$ and DHAP/G3P ratios, which could be artificially altered by the presence of ethanol. No significant changes were observed in either ratios, indicating that the residual ethanol in the tissue culture medium did not affect basic energy pathways in the primary hepatocyte cultures (Fig. S1).

Next, we developed and validated a mathematical model for primary mouse hepatocyte glucose production in the presence of fasting concentrations of glycerol, pyruvate/lactate, and glutamine (Fig S3). Based on this model, we calculated the contribution of each substrate to final glucose carbons. In presence of all GNG substrates, 76% of glucose label was derived from glycerol, 18% was from other substrates such as glycogen and amino acids present in the medium and only 6% was derived from pyruvate/lactate (Fig. 4E). The presence of FFAs did not significantly alter these percentages (Fig. 4F).

To validate this method, we first compared the simulated and observed labeling patterns of glucose, G6P, PEP and pyruvate when $^{13}$C$_3$ lactate or glycerol was used as tracer (Fig S4A, S4B). The simulated and observed patterns are consistent, suggesting a good estimation of the fluxes based on this model. Since all the fluxes are calculated using $^{13}$C$_3$ glycerol and $^{13}$C$_3$ lactate data, it is important to know whether these fluxes also predict the labeling patterns from other tracers. Therefore, we simulated the labeling patterns under $^{13}$C$_5$ glutamine tracer and found that simulated and observed patterns are consistent (Fig S4C). Overall, these data suggest our method can be used to accurately estimate GNG fluxes in primary hepatocytes cultured with physiological concentration of substrates.
**Glycerol induces G6pc and reduces Pck1 expression in primary hepatocytes**

In order to investigate why glycerol is a preferred substrate in GNG, we measured gene expression of key gluconeogenic enzymes (**G6pc** and **Pck1**) after glycerol and pyruvate/lactate treatment using RT-qPCR. A significant induction of **G6pc** expression, the terminal enzyme in GNG, was observed after an 8-hour glycerol treatment in mouse primary hepatocytes over a range of concentrations (Fig. 5A), plateauing around 2mM. There was no significant change in gene expression after pyruvate/lactate treatment compared to control base media (Fig. 5B). Next, we examined expression of **Pck1**, an enzyme in the early part of GNG that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, and is required for glucose production from pyruvate/lactate. Interestingly, glycerol suppressed the expression of **Pck1** (Fig. 5C), while pyruvate/lactate treatment did not alter **Pck1** expression compared to the control hepatocytes (Fig. 5D). We also tested whether hormones such as insulin and glucagon would affect gene expression. Insulin significantly reduced **G6pc** and **Pck1** expression in pyruvate/lactate treated hepatocytes (Fig. S2A,B). In the glycerol group, insulin only significantly inhibited **G6pc** expression (Fig. S2A,B). Glucagon on the other hand, stimulated **G6pc** and **Pck1** similarly in the base media and pyruvate/lactate groups. Glycerol had a much larger increase in **G6pc** expression and much smaller reponse to **Pck1** compared to the other groups (Fig. S2C,D).

**Glycerol becomes the dominant substrate for glucose production in wild type mice**

Next, we wanted to study how glycerol would label glucose after a bolus injection of a substrate mixture. Similar to our experiment in Figure 3, we set up the following combination groups: 1) 1mM $^{13}$C$_3$ pyruvate - 1mM glycerol; 2) 0.1mM/0.9mM $^{13}$C$_3$ pyruvate/$^{13}$C$_3$ lactate- 1mM glycerol and 3) 1mM pyruvate – 1mM $^{13}$C$_3$ glycerol. Traditionally, a pyruvate tolerance test is done at a 1mM concentration, therefore we wanted to keep the substrates in that range and observe which would dominate in a glucose production assay. Fifteen minutes post injection (Fig. 6A), we observed a similar pattern of label fractions as seen in Figure 3. Pyruvate and pyruvate/lactate mixture groups showed a range of the labeled carbon while glycerol primarily showed a m+3 and m+6 label. At 60 minutes (Fig. 6B), pyruvate and pyruvate/lactate mixture groups stayed consistent while glycerol’s carbon label increased and other labeling patterns were observed. Next, we looked at the enrichment fraction of glucose from these groups. We found that pyruvate and pyruvate/lactate groups labeled only about 10% of glucose at both time points (Fig 6C, D). Glycerol however, labeling almost 30% of glucose at 15 min and slightly more at 60 min (Fig 6E).

**Discussion**

It is conventionally accepted that pyruvate and lactate are the main substrates for GNG and these substrates are extensively used to test hepatic glucose production both *in vitro* and *in vivo* and are employed generally at supraphysiologic concentrations (29-35). There are no studies, however, showing glucose production using physiologic fasting concentrations of substrates or studies using a combination of substrates. In this study we show: 1) glycerol is the main source of glucose carbon by itself as well as in the presence of other GNG substrates; 2) pyruvate and lactate contribute to TCA cycle intermediates; 3) the presence of FFAs in media does not alter glycerol’s glucose contribution; and 4) glycerol but not pyruvate/lactate induces expression of G6PC and inhibits expression of PCK1.

Early studies suggested that pyruvate/lactate was the main substrate for glucose production in the liver (36-38). Based on these and other observations, *in vitro* and *in vivo* pyruvate tests have been used to measure hepatic glucose production (3,39,40). However, our analysis shows that glycerol alone, or in combination with other major substrates, accounts for the majority of the labeled glucose carbon in mouse primary hepatocytes. We suggest that glycerol is a more powerful substrate than previously estimated. Previous work also supports a critical role for glycerol. For example, elevated serum glycerol is found in obese Type 2 diabetes
mellitus (T2DM) patients and predicts the development of hyperglycemia (11,25). Furthermore, glucose production from glycerol in Type II DM subjects displays a 1.7-fold increase compared to normal subjects (41); and glycerol incorporation into glucose was reported to have a 3-fold increase in Type II DM compared to controls (27).

Pyruvate enters GNG after conversion to oxaloacetate in the mitochondrion, which is then transported to the cytosol for glucose production via the malate shuttle (4,42,43). However, our studies show similar labeling of TCA intermediates to that observed from glutamine - a substrate that must label TCA intermediates. Our findings suggest that a significant amount of pyruvate in primary hepatocytes enters the TCA cycle prior to GNG and this event could limit glucose production.

A factor normally absent in cell culture media that could potentially alter substrate preference for glucose is free fatty acids (FFAs). Acetyl CoA is produced from β-oxidation of FAs and allosterically activates pyruvate carboxylase, which then promotes the entry of pyruvate into the GNG pathway (15). For this reason, we added oleic and palmitic acid conjugated to BSA to primary hepatocyte media; and similar to Figure 3, we measured the contribution of each GNG substrate to glucose carbons. The addition of FFA did not increase glucose production or shift substrate preference in primary hepatocytes, confirming that hepatocytes prefer glycerol even in the presence of FFA. A major concern with using FFAs in culture media is the presence of ethanol solvent, which when metabolized could alter NADH/NAD⁺ ratios. We determined the NADH/NAD⁺ and DHAP/G3P ratio but did not observe any differences after FFA treatment (Fig. S1).

Fatty acids are said to play a role in the development of insulin resistance in muscle and liver (44) but the role of FFAs on hepatic glucose production remains unclear. Collins et al. (16) reported that oleate in concentrations ranging from 100µM to 1.5mM induces glucose production in primary hepatocytes. On the other hand, a different study reported that oleate and palmitate attenuated GNG in primary hepatocytes at 1mM (45). One of the hypotheses for glucose production involves generation of succinate from free acetate produced by FFAs (17). Although we observed elevated succinate levels in FFA treated hepatocytes (data not shown), this elevation did not result in any alteration in glucose carbon preference.

Another finding in this study is that glycerol metabolism increases G6pc expression. Previously, glycerol was shown to slightly increase levels of G6pc in a rat hepatoma cell line (46); however, an effect in primary hepatocytes has not been reported. The importance of elevated G6pc mRNA levels, however, in mediating hyperglycemia is still unclear. Clore et al. (25) reported that in morbidly obese patients with T2DM, G6PC activity was significantly increased compared to controls. The same phenomenon was observed in diabetic rats (47). In another study, increased G6pc activity was correlated with hyperglycemia (48). On the other hand, other work showed that G6PC expression is not elevated in patients with T2DM (6). A minimal glycerol concentration required for G6PC induction or acute versus chronic exposure to glycerol could be other reasons why data are conflicting in patients with T2DM. In this study, we observed an approximately 8-fold, concentration-dependent increase in G6pc expression (Fig. 5) in primary mouse hepatocytes, which correlated with the marked increase in glucose production we observed (Fig. 1).

In contrast to induction of G6pc expression, Pck1 expression was inhibited by glycerol treatment. In fact, primary hepatocytes treated with glycerol showed a strong, concentration-dependent inhibition in Pck1 expression. In obese humans, serum and liver glycerol are significantly increased (11) and PCK1 expression is lower compared to controls (6). Intriguingly, it has been shown that mice with a liver-specific knockout of Pck1 develop hepatic steatosis (49), while Pck1 overexpression in skeletal muscle reduced weight gain and had a
positive effect on metabolism and energy in mice (50). Hepatic steatosis is found in as many as 75% of individuals with T2DM (51), and our studies suggest that glycerol inhibition of Pck1 might contribute to the development of fatty liver.

The activation of gluconeogenesis often results from glucagon signaling. We observed that in hepatocytes treated with both glucagon and glycerol, G6pc expression increased even further compared to basal and pyruvate/lactate media (Fig S2C). Consistent with these results, T2DM patients have fasting hyperglycemia due to inappropriate glucagon secretion and increased GNG (2,52). Interestingly, Pck1 expression was lower in the glycerol group compared to basal and pyruvate/lactate media. While the mechanism of glycerol’s activation of G6pc and suppression of Pck1 remains unanswered, these data provide a direction for future mechanistic investigations.

While our model primarily investigates the role of glycerol in primary hepatocytes, we also observed that it applies to in vivo physiology. In Figure 6, we investigated substrates utilization in GNG in overnight WT fasted mice. We tried to mimic a traditional pyruvate tolerance test (PTT) but also used a combination of substrates to observe which one would dominate (similar to our in vitro experiment from Figure 3). It is clear from these studies that glycerol is the preferred in vivo substrate. A PTT is commonly used to assess gluconeogenic capacity in a number of mouse models (39). Our critical finding suggests that compared to a traditionally PTT, glycerol might be a better substrate for evaluating GNG in mice.

The primary hepatocyte system allows an investigation of GNG without the contribution of the Cori Cycle or the influence of serum hormones. While there is an advantage to studying pathways with this approach, it is of course limited. Our findings are meant to better approximate but not recapitulate in vivo metabolism. However, due to the feasibility and popularity of using primary hepatocyte cultures as a model system, we suggest that it is critical to the field to provide a more physiologically valid in vitro model. We believe our findings provide such a model.

We report a previously underestimated importance of glycerol in glucose production in primary mouse hepatocytes as well as in vivo glucose production assays. As a substrate for GNG, glycerol is capable of producing more glucose than other known substrates. It also induces the terminal enzymatic step in the GNG pathway (G6pc) and inhibits an early GNG enzyme (Pck1) that is critical for entry of pyruvate and substrates metabolized to pyruvate into GNG. We believe that glycerol’s importance in GNG may be underappreciated especially in the context of current in vitro and in vivo assays used to measure hepatic glucose production.

Experimental Procedures

Animal Experiments

All animal protocols were approved by the Institutional Animal Care and Use Committee of Rutgers University. B6(Cg)-Tyr-2j/J or B6 albino mice were obtained from Jackson Laboratory, Maine USA. Mice were fed ad libitum standard chow and kept at 12 hr light, 12 hr/dark cycles.

Primary Hepatocyte Isolation

Age-matched C57/B6J albino females between 3-4 months were used for primary culture isolation. Mice were first anesthetized using a ketamine/xylazine mixture (Henry Schein). To obtain primary hepatocytes, the hepatic portal vein was cannulated and perfused with Kreb’s Ringer containing EGTA for 10 min at 37 C. After the first wash, a second Kreb’s Ringer wash containing CaCl and Liberase™ (Roche) was used for 10 minutes at 37 C. Hepatocytes were filtered through a gauze mesh and resuspended in plating media: William’s Media E (Sigma): 10 % FBS (Sigma), 200 nM dexamethasone (Sigma), 5mL penicillin/streptomycin (Fisher) and 2 mM L-glutamine (Fisher). Cells were plated at a density of 0.3x10⁶ on six well collagen (Sigma) coated plate. Hepatocytes were allowed to recover overnight and experiments were started 24 h post isolation.
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Glucose Production Assays
Prior to each glucose production assay, cells were serum starved for 3 hours. The assay was done in basal media DMEM (Gibco) containing no glucose or pyruvate but supplemented with L-glutamine (2mM) unless otherwise stated, HEPES (1.76 g) and penicillin/streptomycin (5mL). For substrate glucose production assays, basal media was supplemented with L-glutamine, glycerol or a sodium pyruvate/lactate mixture. For labeled experiments, the same conditions were used but uniformly labeled stable isotope substrates were obtained from Cambridge Isotopes: $^{13}$C-sodium pyruvate (CLM-2440), $^{13}$C-sodium lactate (CLM-10768), $^{13}$C-L-glutamine (CLM-1822) and $^{13}$C-glycerol (CLM-1510). Free fatty acids were dissolved in ethanol and then conjugated with 2% BSA (0.3 mM). Glucose measurements were done enzymatically with Glucose Assay Kit (Abcam).

Real time qRT-PCR analysis
Total RNA was isolated from primary hepatocytes and mouse livers using TriZol method. cDNA was obtained using iScript (Biorad) and then subjected to qRT-PCR analysis using SYBR Green (BioRad) according to manufacturer’s protocol. The primers used for the analysis were the following: G6pc Forward 5’-CAGCAAGGTAGATCCGGGA-3’ Reverse 5’-AAAAAGCCAACGTATGGATTCCG-3’; Pck1 Forward 5’-AGCATTCAACGCCAGGTTC-3’ Reverse 5’-CGAGTCTGTCAGTTCAATACCAA-3’; Actb Forward 5’-CCAGTTGGTAAACATGCCATG-3’ Reverse 5’-GGCTGTATTCCCCTCCATCG-3’.

Western Blot
Hepatocytes were lysed in Laemmli Buffer with B-mercaptoethanol. 8uL of cell lysate was run by a BioRad Western Blot protocol. G6pc antibody was generously provided by Dr. Fabienne Rajas. Cyclophilin B was used as a loading control. Protein expression was normalized to its loading control and subsequently to fold change of Base Media control. Densitometric analysis was performed on Image Lab software (BioRad).

Metabolite extraction for LC-MS
Fresh primary hepatocytes were treated with 40:40:20 methanol:acetonitrile:water solution with 0.1% formic acid, followed by incubation on ice for 5 min, and neutralized by NH$_4$HCO$_3$ addition. Then they were centrifuged at 4°C and 16,000g for 10 min. The supernatant was transferred to a clean tube and stored at -80°C until analysis.

Metabolite Isotope Tracing
Conditions were optimized on an HPLC-ESI-MS system fitted with a Dionex UltiMate 3000 HPLC and a Thermo Q Exactive Plus MS. The HPLC was fitted with a Waters XBridge BEH Amide column (2.1mm × 150mm, 2.5µm particle size, 130Å pore size) coupled with a Waters XBridge BEH XP VanGuard cartridge (2.1mm x 5mm, 2.5µm particle size, 130Å pore size)guard column. The column over temperature was set to 25°C. The solvent A consisted of water/acetonitrile (95:5, V/V) with 20mM NH$_3$AC and 20mM NH$_3$OH at ph=9. The solvent B consisted of acetonitrile/water (80:20, V/V) with 20mM NH$_3$AC and 20mM NH$_3$OH at ph=9 in the following solvent B percentages over time: 0 min, 100%; 3 min, 100%; 3.2 min, 90%; 6.2 min, 90%; 6.5 min, 80%; 10.5 min, 80%; 10.7 min, 70%; 13.5 min, 70%; 13.7 min, 45%; 16 min, 45%; 16.5 min, 100%. The flow rate was set to 300 µL/min with an injection volume 5 µL. The column temperature was set at 25 °C. MS scans were obtained in negative ion mode with a resolution of 70,000 at m/z 200, in addition to an automatic gain control target of 3 x 10$^6$ and m/z scan range of 72 to 1000. Metabolite data was obtained using the MAVEN software package (cite: PMID 21049934) with each labeled isotope fraction (mass accuracy window: 5 ppm).The isotope natural abundance and tracer isotopic impurity were corrected using AccuCor (cite: PMID 28471646).

Quantification of gluconeogenic fluxes in primary hepatocytes
The gluconeogenic fluxes are calculated by an elementary metabolite units (EMU) based methods (53-55). In brief, a simplified flux network was constructed and is described in supplementary data.
Bolus injection study
Age matched male mice were fasted overnight and IP injected with the mixture of substrates in the following groups: 1 mM $^{13}$C$_3$ Pyruvate – 1 mM Glycerol; 0.1mM/0.9mM $^{13}$C$_3$ Pyruvate/$^{13}$C$_3$ Lactate- 1mM Glycerol; and 1mM Pyruvate – 1mM $^{13}$C$_3$ Glycerol. Blood was sampled at 15 and 60 mins and results were analyzed on the LC-MS.

Statistics and Analysis
All analysis and graphs were done on GraphPad Prism software. Statistics involved a student’s t-test or ANOVA analysis as appropriate.
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Figure 1: Primary hepatocytes produce more glucose from glycerol than from pyruvate/lactate
(a) Primary hepatocytes were isolated from 3-4 month old C57B6J-albino female mice. Hepatocytes were recovered for 24 hours in William’s Media E supplemented with 10% FBS for 24 hours. The following day, the cells were serum starved in William’s Media E for 3 hours. Glucose production assays were conducted over an 8 hour period in glucose-free media supplemented with the indicated substrate. (b) cells were treated with pyruvate/lactate 1:10 mixture and glycerol over a range of concentration and adjusted for basal glucose production
(c,f) enrichment fraction of glucose and glucose-6-phosphate in cell extract was calculated from 5/50mM $^{13}$C$_3$ pyruvate/$^{13}$C$_3$ lactate and 5mM $^{13}$C$_3$ glycerol treated cells (d,g) carbon label fraction pattern of glucose and glucose-6-phosphate after 5/50mM $^{13}$C$_3$ pyruvate/$^{13}$C$_3$ lactate treatment and (e,h) 5mM $^{13}$C$_3$ glycerol treatment from their cell extracts.
Figure 2: In presence of substrates at physiologic fasting concentration, glycerol is the main source of $^{13}$C labeled glucose
(a) Glucose label fraction from cellular extract after treatment with $^{13}$C$_3$ glutamine alone and (d) $^{13}$C$_3$ glutamine in presence of nonlabelled pyruvate/lactate and glycerol (b) glucose label fraction from cellular extract after treatment with $^{13}$C$_3$ pyruvate/lactate alone and (e) $^{13}$C$_3$ pyruvate/lactate in presence of nonlabelled glutamine and glycerol (c) glucose label fraction from cellular extract after treatment with $^{13}$C$_3$ glycerol alone and (f) $^{13}$C$_3$ glycerol in presence of nonlabelled glutamine and pyruvate/lactate
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Figure 3: Glycerol carbon mainly produces gluconeogenic intermediates and enters the TCA cycle to a minimal extent

(a) Simplified overview of substrate entry into gluconeogenesis and TCA cycle (b) gluconeogenic intermediates (phosphoenolpyruvate, 3-phosphoglycerate, glucose-6-phosphate and glucose) and (d) TCA intermediates (malate, isocitrate, fumarate, α-ketoglutarate, aconitate) cellular extract enrichment fraction from single $^{13}$C labeled substrates at their physiologic concentration (c) gluconeogenic and (e) TCA intermediates cellular extract enrichment fraction of all substrate groups with first $^{13}$C$_5$ glutamine in presence of unlabelled pyruvate/lactate and glycerol, then $^{13}$C$_3$ pyruvate/lactate in presence of unlabelled glutamine and glycerol and finally $^{13}$C$_3$ glycerol in presence of unlabelled glutamine and pyruvate/lactate
Glycerol induces G6PC and is the preferred substrate for glucose production. The presence of free fatty acids does not change the substrate preference for glycerol. (a) Glucose production with high concentrations of glycerol (5mM) and pyruvate/lactate (5/50mM) in the absence of palmitate and oleate conjugated to BSA. (b-d) Glucose enrichment fraction from physiologic fasting concentrations of substrates with or without palmitate (200 µM) and oleate (200 µM) conjugated to BSA: (b) $^{13}$C$_5$ glutamine in presence of unlabelled pyruvate/lactate and glycerol; (c) $^{13}$C$_3$ pyruvate/lactate in presence of unlabelled glutamine and glycerol; and (d) $^{13}$C$_3$ glycerol in presence of unlabelled glutamine and pyruvate/lactate. (e, f) Final glucose carbon contributions determined using the mathematical model without (e) and with (f) FFA supplementation.
Glycerol induces G6PC and is the preferred substrate

Figure 5: Glycerol induces G6pc and suppresses Pck1 expression in primary mouse hepatocytes. (a) G6pc mRNA expression after glycerol fold substrate free base media (BM) and (b) pyruvate/lactate treatment over increasing concentrations. (c) Pck1 mRNA expression after glycerol and (d) pyruvate/lactate treatment over increasing concentrations. (e) Western blot of G6pc protein in glycerol (5mM) and pyruvate/lactate (5/50mM) treated samples. Values (arbitrary units) indicate expression of G6pc normalized to Cyclophilin B.
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Figure 6: Glycerol becomes the dominant substrate for glucose production in WT mice. Label fraction of glucose (a) 15 min and (b) 60 min after injection of a mixture of substrates in the following groups: 1 mM $^{13}$C$_3$ Pyruvate – 1 mM Glycerol; 0.1 mM/0.9 mM $^{13}$C$_3$ Pyruvate/$^{13}$C$_3$ Lactate – 1 mM Glycerol; and 1 mM Pyruvate – 1 mM $^{13}$C$_3$ Glycerol (c-e) Enrichment fraction of glucose in the same groups n=3 per group of overnight fasted WT males.
Glycerol induces G6pc in primary mouse hepatocytes and is the preferred substrate for gluconeogenesis both in vitro and in vivo

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