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Effects of Visceral Adiposity on Glycerol Pathways in Gluconeogenesis

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Abstract

Objective—To determine the feasibility of using oral ¹³C labeled glycerol at assess effects of visceral adiposity on gluconeogenic pathways in obese humans.

Research Design and Methods—Obese (BMI ≥ 30 kg/m²) participants without type 2 diabetes underwent visceral adipose tissue (VAT) assessment and stratification by median VAT into high VAT-fasting (n=3), low VAT-fasting (n=4), and high VAT-refed (n=2) groups. Participants ingested [U-¹³C₃] glycerol and blood samples were subsequently analyzed at multiple time points over 3 hours by NMR spectroscopy. The fractions of plasma glucose (enrichment) derived from [U-¹³C₃] glycerol via hepatic gluconeogenesis, pentose phosphate pathway (PPP), and tricarboxylic acid (TCA) cycle were assessed using ¹³C NMR analysis of glucose. Mixed linear models were used to compare ¹³C enrichment in glucose between groups.

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Relationship with Industry

Nothing to disclose.

Disclosures

The authors have no conflicts of interest to disclose.

Author Contributions

All authors contributed to the design and conduct of the study, data collection and analysis, data interpretation, and manuscript writing. Dr. Neeland takes full responsibility for the contents of this article.

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Results—Mean age, BMI, and baseline glucose were 49 years, 40.1 kg/m², and 98 mg/dl, respectively. Up to 20% of glycerol was metabolized in the TCA cycle prior to gluconeogenesis and PPP activity was minor (<1% of total glucose) in all participants. There was a 21% decrease in ¹³C enrichment in plasma glucose in the high VAT-fasting compared with low VAT-fasting group ($p=0.03$), suggesting dilution by endogenous glycerol. High VAT-refed participants had 37% less ¹³C enrichment in glucose compared with high VAT-fasting ($p=0.02$). There was a trend toward lower [1,2-¹³C₂] (via PPP) and [5,6-¹³C₂]/[4,5,6-¹³C₃] (via TCA cycle) glucose in high VAT versus low VAT groups.

Conclusions—We applied a simple method to detect gluconeogenesis from glycerol in obese humans. Our findings provide preliminary evidence that excess visceral fat disrupts multiple pathways in hepatic gluconeogenesis from glycerol.

Keywords

visceral fat; glycerol; gluconeogenesis; NMR; pentose phosphate pathway; tricarboxylic acid cycle

1.0 Introduction

Abdominal obesity and excess visceral fat, termed “visceral adiposity”, have strong associations with insulin resistance, hyperglycemia and type 2 diabetes [1–5]. However the amount of visceral adipose tissue (VAT) in most individuals represents only a relatively small fraction of body fat burden, generally less than 15% [6]. The disproportionate influence of visceral fat on systemic metabolism has been attributed to resistance of mesenteric fat cells to the anti-lipolytic effects of insulin [3]. Consequently, persistent turnover of mesenteric triglycerides in spite of hyperinsulinemia delivers glycerol and fatty acids directly into the portal circulation, providing both a gluconeogenic substrate and energy for gluconeogenesis in the liver [7, 8]. Glycerol contributes about 10% of total glucose production after an overnight fast in healthy non-obese participants [9–11], but little is known about the contribution of glycerol to glucose production in participants with visceral adiposity.

This knowledge gap is due to the complexity of glycerol metabolism and the limited applicability of arterial and hepatic vein cannulation for clinical research [8, 12, 13]. Glycerol enters gluconeogenesis/glycolysis after phosphorylation via glycerol kinase to generate glycerol 3-phosphate which rapidly exchanges with the trioses, dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GA3P). Subsequent metabolism yields pyruvate, if flux through glycolysis is active, or glucose, if gluconeogenesis is dominant. Studies of ¹³C-labeled glycerol in humans have assumed that glycerol is converted directly to glucose and that the products of glycerol metabolism do not pass through the pentose phosphate pathway (PPP) or tricarboxylic acid (TCA) cycle prior to glucose export [14]. However, other studies demonstrated appearance of ¹⁴CO₂ from ¹⁴C-enriched glycerol which would not be expected from the direct conversion of glycerol to glucose [15]. Previs and colleagues found that a significant fraction of glucose derived from [U-¹³C] glycerol in rodents and non-human primates passed through the oxaloacetate pool in the TCA cycle prior to gluconeogenesis [16]. More recent studies in rodents [17] and healthy humans [18] demonstrated that most glycerol is metabolized directly to glucose but that a modest fraction

enters the TCA cycle prior to re-synthesis to glucose and a small fraction of glycerol carbons are redistributed in the oxidative arm of the PPP. These observations are consistent with reports of bidirectional metabolism in the gluconeogenic and glycolytic pathways [19, 20]. Since glycerol biochemistry *in vivo* is complex, analytical methods must be sensitive to these pathways. Compared to either mass spectrometry or radiotracer methods, the use of ^{13}C , a nonradioactive tracer detected by NMR, offers more detail about the pathways involved in glucose production. This advantage arises from information about site-specific enrichment and ^{13}C - ^{13}C spin-spin coupling. $[\text{U-}^{13}\text{C}_3]$ glycerol can be applied to quantify the fraction of glucose derived from glycerol in these pathways from a single ^{13}C NMR spectrum [10, 21].

The purpose of this pilot study was to evaluate the feasibility of using orally-administered $[\text{U-}^{13}\text{C}_3]$ glycerol in obese participants to probe the effects of visceral adiposity on various glucose production pathways *in vivo*. We hypothesized that participants with high VAT would have lower ^{13}C enrichment in glucose, signifying greater endogenous adipose contribution of glycerol to hepatic gluconeogenesis, compared with participants with low VAT, independent of fasting blood glucose level or body mass index (BMI). We also examined the effects of refeeding on these gluconeogenic pathways in participants with high VAT.

2.0 Materials and Methods

2.1 Study population and variable ascertainment

Participants were recruited through the Dallas Heart Study (DHS), a multiethnic, probability-based, population cohort study of Dallas County adults, as well as through patient referrals from community physicians. Detailed methods of the DHS have been described previously [22]. For inclusion in the study, participants had to be age ≥ 18 years, obese (defined as a BMI $\geq 30 \text{ kg/m}^2$ at both the time of visceral fat imaging and at enrollment), without a diagnosis of type 2 diabetes mellitus (both by self-reported medical history and fasting plasma glucose $<126 \text{ mg/dl}$ measured at enrollment), and have an assessment of VAT by either magnetic resonance imaging (MRI) or dual x-ray absorptiometry (DXA). Participants were excluded if they were pregnant (by urine pregnancy test at the time of enrollment) or breastfeeding, incarcerated, non-obese at the time of visceral fat imaging or enrollment, had a $>10\%$ change in body weight between the time of visceral fat imaging and enrollment, or had donated blood within 6 weeks of enrollment.

Participants were stratified by median VAT level into high VAT and low VAT groups. Among those with high VAT, participants were randomly assigned to fasting (no food or drink for 8 hours prior to $[\text{U-}^{13}\text{C}_3]$ glycerol ingestion) or re-fed: given a standardized mixed meal containing 880 kcal with 80g carbohydrates (45%), 37g fat (21%), and 59g protein (34%) within 60 minutes of $[\text{U-}^{13}\text{C}_3]$ glycerol ingestion. All low VAT participants were fasting. Age, sex, and race/ethnicity were self-reported. Weight and height were measured using standard scales and BMI was calculated as weight in kilograms divided by height in meters squared. Waist circumference was measured 1 cm above the iliac crest and hip circumference at the widest circumference of the buttocks at the area of the greater

trochanters. A total of 10 participants were enrolled but one participant withdrew consent from the study before any study procedures were performed so the present analysis includes a final sample size of 9 participants. All participants provided written informed consent, and the protocol was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center.

2.2 Visceral fat measurements

Seven participants underwent whole body composition analysis with a GE Lunar Prodigy Advance iDXA scanner (GE Healthcare, Madison, WI) and images were analyzed with CoreScan enCORE software version 14.10.022. This software enables estimation of VAT mass in the android region of a total body DXA scan. The methodology for DXA VAT measurement has been described previously [23]. Briefly, the CoreScan computer algorithm uses the lateral abdominal subcutaneous adipose tissue (SAT) seen in the DXA image to determine the anterior and posterior abdominal SAT, allowing VAT to be estimated from the total abdominal fat measured (Total abdominal fat - total abdominal SAT = VAT, expressed in kg). Two participants were imaged with a 1.5 Tesla MRI scanner (Intera, Philips Medical Systems, Best, The Netherlands) using a prospectively designed and validated method of fat mass prediction from a single MRI slice at the L2-L3 inter-vertebral level that has been shown to be highly concordant with total abdominal fat mass measured at all intervertebral levels ($R^2=0.85-0.96$) [24]. The DXA based method of VAT mass quantification has been shown to be highly accurate and comparable to the MRI based method [25].

2.3 Study Procedures

Participants arrived in the morning after an overnight fast. Vital signs and anthropometric data were acquired. A peripheral intravenous catheter was inserted and 0.5 ml of blood was drawn for glucose analysis using an YSI 2300 Biochemistry Analyzer (Xylem Inc., Yellow Springs, OH). Participants in the high VAT-refed arm were then provided a standardized mixed meal as detailed above. $[U-^{13}C_3]$ glycerol (Cambridge Isotope Laboratories, Tewksbury, MA) was then prepared in $\frac{1}{2}$ cup of filtered water using the following formula: $\text{Weight (kg)} \times 0.05 \text{ g/kg} \times 1 \text{ ml/1.302 g} = [U-^{13}C_3] \text{ glycerol (ml)}$. Participants drank the glycerol followed by 1 cup of filtered water to rinse the bottle. Blood samples were subsequently drawn at +15, +30, +60, +90, +120, +150, and +180 minutes for analysis. For each sample 0.5 ml of blood was used for blood glucose analysis and 40 ml of each sample was immediately centrifuged and stored on ice to be used for nuclear magnetic resonance (NMR) analysis as described below. After completion of the study procedures, the participants were discharged.

Plasma glucose was converted to monoacetone glucose (MAG) as previously described [26], because MAG has a wide chemical shift dispersion and each ^{13}C signal is easily resolved. Unlike glucose, the spectrum is not complicated by anomers, and MAG provides an internal standard, the natural-abundance ^{13}C signal in the methyl carbons of the acetone group added through glucose derivatization. All NMR spectra were acquired using a Varian INOVA 14.1 T spectrometer (Agilent, Santa Clara, CA) equipped with a 3-mm broadband probe with the observe coil tuned to ^{13}C (150 MHz). ^{13}C NMR spectra were collected using a 60° pulse, a 36,765-Hz sweep width, 110,294 data points, and a 1.5-s acquisition time with 1.5-s

interpulse delay at 25 °C. Proton decoupling was performed using a standard WALTZ-16 pulse sequence. Spectra were averaged over ~7000–23,000 scans requiring ~6–20 hours. All NMR spectra were analyzed using the ACD/Labs PC-based NMR spectral analysis program (Advanced Chemistry Development, Inc., Toronto, Canada). The fraction of plasma glucose derived from [U-¹³C₃] glycerol was determined by measuring the total area of each distinct multiplet referenced to the natural abundance ¹³C signals from methyl groups of MAG [10] (Figure 1). Correction for T1 differences and nuclear Overhauser effects were negligible.

3.0 Theory/Calculation

3.1 Glycerol pathways in gluconeogenesis

Glycerol-gluconeogenesis is directly interrogated by determining the fraction of ¹³C enrichment in blood glucose using NMR spectroscopic quantification of ¹³C-labeled glucose isotopomers. Total ¹³C enrichment in plasma glucose is measured by the sum of all glucose isotopomers with excess ¹³C. Additional information about specific pathways is derived from specific glucose isotopomers [17, 18]. Initially, glycerol is phosphorylated in the liver by glycerol kinase and is converted to DHAP and GA3P. Direct production of glucose via gluconeogenesis occurs by condensation of DHAP and GA3P to form carbons 1–3 and 4–6 of glucose, respectively (Figure 2). Therefore, quantification of [1,2,3-¹³C₃] glucose and of [4,5,6-¹³C₃] glucose reflects primarily direct hepatic gluconeogenesis from ingested [U-¹³C₃] glycerol. Passage of [1,2,3-¹³C₃] glucose 6-phosphate (G6P, ¹³C from DHAP) through the PPP produces [1,2-¹³C₂] fructose 6-phosphate and subsequently [1,2-¹³C₂] glucose so that the quantification of [1,2-¹³C₂] glucose reflects PPP activity (Figure 2). Metabolism of [U-¹³C₃] glycerol to [U-¹³C₃] pyruvate followed by either decarboxylation to [U-¹³C₂] acetyl-CoA or carboxylation to [1,2,3-¹³C₃] oxaloacetate allows rearrangement of ¹³C in the TCA cycle. Two possible phosphoenolpyruvate (PEP) isotopomers through glycerol metabolism in the TCA cycle are [1,2-¹³C₂]- and [2,3-¹³C₂]PEP [18]. The PEP metabolism to glucose will produce [4,5-¹³C₂]- or [5,6-¹³C₂] glucose (Figure 2). Since the PPP does not disrupt carbon-carbon bonds in carbons 4–6 of glucose, the presence of [4,5-¹³C₂]- or [5,6-¹³C₂] glucose indicates metabolism of [U-¹³C₃] glycerol through the TCA cycle prior to gluconeogenesis.

3.2 Statistical analysis

Participants were stratified into 3 groups based on their VAT measurement and feeding state: low VAT-fasting, high VAT-fasting, and high VAT-refed. Pairwise characteristics were compared using Wilcoxon rank sum or signed rank tests as appropriate. The mean ± standard error percent ¹³C enrichment in blood glucose versus time for each group was plotted and mixed linear models were used to compare excess ¹³C enrichment (%) in blood glucose between low VAT-fasting and high VAT-fasting groups and between high VAT-fasting and high VAT-refed groups over all time points (primary outcomes). The trapezoidal rule was used to calculate the area under each enrichment curve in all groups. Secondary outcomes included plasma glucose over time in all groups, enrichment of [1,2-¹³C₂] glucose (μmol/L, PPP), and the ratio (%) of [5,6-¹³C₂]/[4,5,6-¹³C₃] in glucose as a measure of the portion of [U-¹³C₃] glycerol metabolism through the TCA cycle compared with direct gluconeogenesis from glycerol (TCA cycle/mitochondria) in blood glucose over time

between groups. For all statistical testing, a 2-sided p -value <0.05 was considered statistically significant. The study was designed to achieve 80% power at a two-sided alpha level of 0.05 to detect at least a 5% difference (2% standard deviation) in enrichment between low VAT-fasting and high VAT-fasting groups (requiring at least 3 participants in each arm) and to achieve 90% power at a two sided alpha of 0.05 to detect at least a 9% difference in enrichment between high VAT-fasting and high VAT-refed groups (requiring at least 2 participants in each arm). All statistical analyses were performed using SAS version 9.4 software (SAS Corporation, Cary, NC).

4.0 Results

There were 4 participants in the low VAT group, 3 participants in the high VAT-fasting group, and 2 participants in the high VAT-refed group. Characteristics of the study population are presented in Table 1. Mean (standard deviation, SD) age, BMI, and baseline plasma glucose of the overall study cohort were 49 (7) years, 40.1 (5.1) kg/m², and 98 (12) mg/dl, respectively. Mean (SD) visceral fat mass in the low VAT group was 1.62 (0.40) kg, in the high VAT-fasting group 2.99 (0.42) kg, and in the high VAT-refed group 3.0 (0.57) kg ($p=0.03$ for low VAT, high VAT-fasting comparison, and $p=0.56$ for high VAT-fasting, high VAT-refed comparison). There were no statistically significant differences in age, BMI, or baseline plasma glucose between groups. Mean plasma glucose for all groups decreased over the study period from 98 (12) mg/dl at baseline to 89 (4) mg/dl at time +180 minutes ($p=0.02$) (Figure 3).

4.1 ¹³C NMR Analysis of Plasma Glucose

High quality ¹³C NMR spectra were obtained in all participants (Figure 1, for example). The ¹³C signal from carbon 5 shows a quartet from [4,5,6-¹³C₃] glucose, indicating primarily the direct conversion of [U-¹³C₃] glycerol to glucose. The presence of [4,5-¹³C₂] glucose and [5,6-¹³C₂] glucose demonstrates, in all participants, metabolism of [U-¹³C₃] glycerol in the TCA cycle prior to re-synthesis to triose and then glucose. The dominant signal from [5,6-¹³C₂] glucose arises because, after metabolism to [U-¹³C₃] pyruvate, carboxylation and backwards scrambling in the succinate and fumarate pool yield an equal mixture of [1,2,3-¹³C₃] oxaloacetate and [2,3,4-¹³C₃] oxaloacetate. Gluconeogenesis from the former is indistinguishable from direct gluconeogenesis from [U-¹³C₃] glycerol while gluconeogenesis from the latter yields [5,6-¹³C₃] glucose. These signals were easily detected in all participants, indicating metabolism of glycerol in multiple pathways to gluconeogenesis.

4.2 Low Visceral Fat versus High Visceral Fat

There was a statistically significant 21% decrease in ¹³C enrichment in glucose in the high VAT-fasting group compared with the low VAT-fasting group ($p=0.03$, Figure 4, **Panel A**). The high VAT-fasting group had a lower and longer duration to enrichment compared with the low VAT-fasting group, although enrichment became equilibrated at time +180 minutes. Results were similar after additional adjustment for baseline plasma glucose and BMI. In all participants, there was a small amount of plasma glucose ($<1\%$ of total glucose) that underwent rearrangement due to the PPP and we observed a trend toward less amount of

[1,2- $^{13}\text{C}_2$] glucose representing PPP activity in the high VAT-fasting group compared with the low VAT-fasting group (Figure 4, **Panel B**). Furthermore, a significant amount of glycerol (up to 20%) passed through the TCA cycle prior to gluconeogenesis in all participants and we observed a trend toward less [5,6- $^{13}\text{C}_2$]/[4,5,6- $^{13}\text{C}_3$] in glucose (%) representing [U- $^{13}\text{C}_3$] glycerol metabolism through the TCA cycle in mitochondria in the high VAT-fasting group compared with the low VAT-fasting group (Figure 4, **Panel C**). The proportion of gluconeogenesis related to ingestion of $^{13}\text{C}_3$ -glycerol did not differ between the high VAT-fasting group and the low VAT-fasting group (e.g. mean amount of plasma glucose produced from $^{13}\text{C}_3$ -glycerol at the 15 minute interval= 6.3 vs. 6.7 mg/dl, respectively, $p=0.99$; with similar results across other time points).

4.3 Fasting versus Refeeding

There was a statistically significant 37% decrease in ^{13}C enrichment in glucose in the high VAT-refed group compared with the high VAT-fasting group ($p=0.02$, Figure 5, **Panel A**). The high VAT-refed group also had a lower and longer duration to enrichment compared with the high VAT-fasting group and enrichment remained lower in the high VAT-refed group throughout the study period. Results were also similar after additional adjustment for baseline plasma glucose and BMI. We also observed a trend toward less amount of [1,2- $^{13}\text{C}_2$] glucose representing PPP activity (Figure 5, **Panel B**) and a trend toward less [5,6- $^{13}\text{C}_2$]/[4,5,6- $^{13}\text{C}_3$] in glucose (%) representing [U- $^{13}\text{C}_3$] glycerol metabolism through the TCA cycle in mitochondria in the high VAT-refed group compared with the high VAT-fasting group.

5.0 Discussion

These studies demonstrate that a recently described method to detect gluconeogenesis from glycerol was easily extended to obese humans. Since the study involves only oral administration of a stable isotope tracer and venous blood sampling, the method is highly acceptable to patients. Although relatively large amounts (40 mL) of blood were drawn at each time point, both the volume and frequency of blood samples can be reduced in future studies with more detailed knowledge of the time-course of plasma enrichment. Using this novel method, we quantified the effects of excess visceral adiposity on the fraction of glucose derived from glycerol (“glycerol-gluconeogenesis”) in multiple pathways in hepatic gluconeogenesis. Up to 20% of glucose from glycerol passed through the TCA cycle prior to gluconeogenesis and bidirectional metabolism of glycerol was detected in all participants. Interestingly, the fraction of blood glucose derived from labeled glycerol was lower in participants with high visceral fat, consistent with dilution due to unlabeled endogenous substrates contributing to gluconeogenesis. Glucose produced during gluconeogenesis underwent carbon rearrangements due to flux through the oxidative branch of the PPP, but the fraction of plasma glucose involved in this pathway was small in all participants. Our study demonstrates that orally-administered [U- ^{13}C] glycerol coupled with venous blood sampling and NMR analysis is a simple tool to detect biomarkers of gluconeogenesis, the PPP, and mitochondrial metabolism in obese persons.

Our findings show preliminary evidence that participants with high VAT had lower ^{13}C enrichment in glucose compared with participants with low VAT, likely reflecting greater VAT contribution of endogenous glycerol to hepatic gluconeogenesis, independent of fasting glucose level and BMI. We also showed that viscerally obese individuals had a trend toward lower ^{13}C -labeled glucose isotopomers produced through the PPP or TCA cycle suggesting a possible link between excess VAT, inflammation, PPP dysregulation, and mitochondrial failure. Furthermore, we observed that refeeding results in lower ^{13}C enrichment in glucose through direct gluconeogenesis from $[\text{U-}^{13}\text{C}_3]$ glycerol, through PPP activity or through the TCA cycle prior to gluconeogenesis, compared with the fasting state, consistent with prior observations in non-obese refed rodents [17]. Overall, our findings provide intriguing evidence that excess VAT may act as a “constitutively fed state” and result in increased risk for hyperglycemia and type 2 diabetes through overstimulation of hepatic gluconeogenesis by chronic delivery of glycerol arising from mesenteric triglyceride turnover directly into the portal circulation and to the liver. These findings should stimulate further study with larger numbers of participants to more comprehensively elucidate the effects of visceral adiposity, independent of obesity, on gluconeogenic pathways.

Stable isotopes have previously been used to estimate global gluconeogenesis by NMR isotopomer distribution analysis of various tracers (e.g. ^{13}C bicarbonate and ^{13}C lactate) [27, 28] and to quantify hepatic gluconeogenic fluxes in humans [14, 29]. However, no prior studies have utilized this method to interrogate the effects of visceral adiposity on multiple pathways in glycerol-gluconeogenesis. We found that the majority of gluconeogenesis occurred directly from $[\text{U-}^{13}\text{C}_3]$ glycerol being incorporated into either DHAP or G3AP to produce $[1,2,3\text{-}^{13}\text{C}_3]$ or $[4,5,6\text{-}^{13}\text{C}_3]$ glucose. Excess VAT most likely suppressed the incorporation of $[\text{U-}^{13}\text{C}_3]$ glycerol into this gluconeogenic pathway by contributing excessive amounts of endogenous adipose-derived glycerol to the liver, although it is possible that other unlabeled gluconeogenic substrates may contribute. It is unlikely that our results were due to less intestinal or hepatic uptake of the exogenous glycerol since 1) intestinal uptake depends on local inhibitory compounds that are not known to differ in obesogenic states [30], and 2) hepatic uptake of glycerol via aquaporin-9 is paradoxically increased in obese animals despite hyperinsulinemia [31]. It is also unlikely that the decreased enrichment in high VAT participants was due to less overall gluconeogenesis with high VAT since blood glucose levels were similar between groups throughout the experiment. These findings provide evidence for a key mechanistic link between the presence of excessive visceral fat and abnormal gluconeogenesis in abdominally obese individuals. Whether interventions targeting reduction or redistribution of visceral fat may alter these pathways and ameliorate the excessive flux of glycerol from stored triglycerides into hepatic gluconeogenesis remains to be determined. This represents an important opportunity for further research using these stable isotope techniques.

A small proportion of $[1,2,3\text{-}^{13}\text{C}_3]$ G6P was directed to the PPP becoming $[1,2\text{-}^{13}\text{C}_2]$ fructose 6-phosphate and ultimately $[1,2\text{-}^{13}\text{C}_2]$ glucose. In contrast, a more significant fraction, up to 20%, of glucose was produced through glycerol metabolism via the TCA cycle prior to gluconeogenesis. Although we observed trends toward less enrichment of $[1,2\text{-}^{13}\text{C}_2]$ glucose (PPP activity) and less enrichment of $[5,6\text{-}^{13}\text{C}_2]$ glucose (TCA cycle) in the high VAT-fasting compared with the low VAT-fasting group, our study was not

sufficiently powered for these exploratory endpoints and these observations should be considered hypothesis-generating. It is possible that differential enrichment of [5,6- $^{13}\text{C}_2$] glucose may reflect abnormal mitochondrial function [32, 33]. For example, downregulation of several genes in the electron transport chain (an integral component of cellular respiration in mitochondria linked to the TCA cycle) were found in visceraally obese women with diabetes, and was in part mediated by expression of TNF- α , an important inflammatory cytokine implicated in the pathogenesis of type 2 diabetes [34]. A separate study also found that both mitochondrial biogenesis and multiple markers essential to the TCA cycle pathway were downregulated in acquired obesity in monozygotic twins [35]. However, these studies examined mitochondrial function in VAT, not the liver where gluconeogenesis occurs. The effects on TCA cycle activity in the liver among obese individuals with insulin resistance remains an active area of investigation [36, 37]. Further studies utilizing assays specific for mitochondrial function will inform the mechanistic underpinnings of glycerol flux through the TCA cycle related to excess VAT. Unlike direct gluconeogenesis and the PPP in which a relative plateau of ^{13}C enrichment was observed toward the end of the study, the proportion of ^{13}C enrichment signifying TCA cycle activity appeared to rise continually throughout the study. This finding may be due to preferential shifting of glycerol metabolism to oxidation in the TCA cycle in response to declining energy stores over a prolonged fast.

Strengths of this study include the application of a novel, *in vivo* metabolic tracer technique to directly interrogate multiple hepatic gluconeogenic pathways in obese humans stratified by visceral fat and inclusion of a refeeding arm to compare fasting and fed states among those with high VAT. Limitations also merit comment. The sample size (9 participants) in this pilot study was relatively small and a complete pathophysiological model will require larger studies to confirm and extend these novel findings; however, our study was powered appropriately for both primary outcomes and thus our sample size was valid for statistical comparisons. We did not measure hepatic fat in this study so we are unable to comment on the influence of hepatic fat on these metabolic pathways. However, VAT and hepatic fat are highly correlated [5] so similar findings are likely.

6.0 Conclusions

In conclusion, for the first time in obese humans, we used a simple stable isotope technique to investigate *in vivo* the mechanisms underlying the effects of excess visceral adiposity on gluconeogenesis from glycerol. Future models of glycerol-gluconeogenesis should account for multiple pathways including the PPP and TCA cycle. Our findings implicate visceral fat in the pathogenesis of hyperglycemia and type 2 diabetes via its disruptive effects on these multiple pathways of gluconeogenesis from glycerol. Further studies are needed using these readily applicable and reproducible techniques to further elucidate the interactions between abdominal obesity and metabolic risk vis-à-vis glycerol-glucose metabolism.

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Abbreviations

DHS	Dallas Heart Study
DXA	dual x-ray absorptiometry
MAG	monoacetone glucose
MRI	magnetic resonance imaging
NMR	nuclear magnetic resonance
PPP	pentose phosphate pathway
TCA	tricarboxylic acid
TPI	triose phosphate isomerase
VAT	visceral adipose tissue

8.0 References

1. Bray GA, Jablonski KA, Fujimoto WY, Barrett-Connor E, Haffner S, Hanson RL, et al. Relation of central adiposity and body mass index to the development of diabetes in the Diabetes Prevention Program. *Am J Clin Nutr*. 2008; 87:1212–8. [PubMed: 18469241]
2. Gastaldelli A, Cusi K, Pettiti M, Hardies J, Miyazaki Y, Berria R, et al. Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology*. 2007; 133:496–506. [PubMed: 17681171]
3. Despres JP. Is visceral obesity the cause of the metabolic syndrome? *Ann Med*. 2006; 38:52–63. [PubMed: 16448989]
4. Neeland IJ, Turer AT, Ayers CR, Powell-Wiley TM, Vega GL, Farzaneh-Far R, et al. Dysfunctional adiposity and the risk of prediabetes and type 2 diabetes in obese adults. *JAMA*. 2012; 308:1150–9. [PubMed: 22990274]
5. Neeland IJ, Ayers CR, Rohatgi AK, Turer AT, Berry JD, Das SR, et al. Associations of visceral and abdominal subcutaneous adipose tissue with markers of cardiac and metabolic risk in obese adults. *Obesity (Silver Spring)*. 2013; 21:E439–47. [PubMed: 23687099]
6. Abate N, Garg A, Peshock RM, Stray-Gundersen J, Grundy SM. Relationships of generalized and regional adiposity to insulin sensitivity in men. *J Clin Invest*. 1995; 96:88–98. [PubMed: 7615840]
7. Kuriyama H, Shimomura I, Kishida K, Kondo H, Furuyama N, Nishizawa H, et al. Coordinated regulation of fat-specific and liver-specific glycerol channels, aquaporin adipose and aquaporin 9. *Diabetes*. 2002; 51:2915–21. [PubMed: 12351427]
8. Nielsen S, Guo Z, Johnson CM, Hensrud DD, Jensen MD. Splanchnic lipolysis in human obesity. *J Clin Invest*. 2004; 113:1582–8. [PubMed: 15173884]
9. Baba H, Zhang XJ, Wolfe RR. Glycerol gluconeogenesis in fasting humans. *Nutrition*. 1995; 11:149–53. [PubMed: 7647479]
10. Jin ES, Jones JG, Merritt M, Burgess SC, Malloy CR, Sherry AD. Glucose production, gluconeogenesis, and hepatic tricarboxylic acid cycle fluxes measured by nuclear magnetic resonance analysis of a single glucose derivative. *Anal Biochem*. 2004; 327:149–55. [PubMed: 15051530]

11. Jones JG, Solomon MA, Cole SM, Sherry AD, Malloy CR. An integrated (2)H and (13)C NMR study of gluconeogenesis and TCA cycle flux in humans. *Am J Physiol Endocrinol Metab.* 2001; 281:E848–56. [PubMed: 11551863]
12. Bjorkman O, Felig P, Wahren J. The contrasting responses of splanchnic and renal glucose output to gluconeogenic substrates and to hypoglucagonemia in 60-h-fasted humans. *Diabetes.* 1980; 29:610–6. [PubMed: 6108272]
13. Wahren J, Efendic S, Luft R, Hagenfeldt L, Bjorkman O, Felig P. Influence of somatostatin on splanchnic glucose metabolism in postabsorptive and 60-hour fasted humans. *J Clin Invest.* 1977; 59:299–307. [PubMed: 833277]
14. Hellerstein MK, Neese RA, Linfoot P, Christiansen M, Turner S, Letscher A. Hepatic gluconeogenic fluxes and glycogen turnover during fasting in humans. A stable isotope study. *J Clin Invest.* 1997; 100:1305–19. [PubMed: 9276749]
15. Teng CT, Karnovsky ML, Landau BR, Hastings AB, Nesbett FB. Metabolism of C14-labeled glycerol and pyruvate by liver in vitro. *J Biol Chem.* 1953; 202:705–16. [PubMed: 13061494]
16. Previs SF, Fernandez CA, Yang D, Soloviev MV, David F, Brunengraber H. Limitations of the mass isotopomer distribution analysis of glucose to study gluconeogenesis. Substrate cycling between glycerol and triose phosphates in liver. *J Biol Chem.* 1995; 270:19806–15. [PubMed: 7649990]
17. Jin ES, Sherry AD, Malloy CR. Interaction between the pentose phosphate pathway and gluconeogenesis from glycerol in the liver. *J Biol Chem.* 2014; 289:32593–603. [PubMed: 25288790]
18. Jin ES, Sherry AD, Malloy CR. An oral load of [13C3]glycerol and blood NMR analysis detect fatty acid esterification, pentose phosphate pathway and glycerol metabolism through the tricarboxylic acid cycle in human liver. *J Biol Chem.* 2016; 291:19031–41. [PubMed: 27432878]
19. Phillips JW, Jones ME, Berry MN. Implications of the simultaneous occurrence of hepatic glycolysis from glucose and gluconeogenesis from glycerol. *Eur J Biochem.* 2002; 269:792–7. [PubMed: 11846780]
20. Moreno KX, Satapati S, DeBerardinis RJ, Burgess SC, Malloy CR, Merritt ME. Real-time detection of hepatic gluconeogenic and glycogenolytic states using hyperpolarized [2-13C]dihydroxyacetone. *J Biol Chem.* 2014; 289:35859–67. [PubMed: 25352600]
21. Coggan AR. Use of stable isotopes to study carbohydrate and fat metabolism at the whole-body level. *Proc Nutr Soc.* 1999; 58:953–61. [PubMed: 10817163]
22. Victor RG, Haley RW, Willett DL, Peshock RM, Vaeth PC, Leonard D, et al. The Dallas Heart Study: a population-based probability sample for the multidisciplinary study of ethnic differences in cardiovascular health. *Am J Cardiol.* 2004; 93:1473–80. [PubMed: 15194016]
23. Kaul S, Rothney MP, Peters DM, Wacker WK, Davis CE, Shapiro MD, et al. Dual-energy X-ray absorptiometry for quantification of visceral fat. *Obesity (Silver Spring).* 2012; 20:1313–8. [PubMed: 22282048]
24. Abate N, Garg A, Coleman R, Grundy SM, Peshock RM. Prediction of total subcutaneous abdominal, intraperitoneal, and retroperitoneal adipose tissue masses in men by a single axial magnetic resonance imaging slice. *Am J Clin Nutr.* 1997; 65:403–8. [PubMed: 9022523]
25. Neeland IJ, Grundy SM, Li X, Adams-Huet B, Vega GL. Comparison of visceral fat mass measurement by dual-X-ray absorptiometry and magnetic resonance imaging in a multiethnic cohort: the Dallas Heart Study. *Nutr Diabetes.* 2016; 6:e221. [PubMed: 27428873]
26. Jin ES, Szuszkiewicz-Garcia M, Browning JD, Baxter JD, Abate N, Malloy CR. Influence of liver triglycerides on suppression of glucose production by insulin in men. *J Clin Endocrinol Metab.* 2015; 100:235–43. [PubMed: 25250633]
27. Landau BR, Wahren J, Chandramouli V, Schumann WC, Ekberg K, Kalhan SC. Contributions of gluconeogenesis to glucose production in the fasted state. *J Clin Invest.* 1996; 98:378–85. [PubMed: 8755648]
28. Alves TC, Nunes PM, Palmeira CM, Jones JG, Carvalho RA. Estimating gluconeogenesis by NMR isotopomer distribution analysis of [13C]bicarbonate and [1-13C]lactate. *NMR Biomed.* 2008; 21:337–44. [PubMed: 17683055]

29. Jones JG, Solomon MA, Sherry AD, Jeffrey FM, Malloy CR. ^{13}C NMR measurements of human gluconeogenic fluxes after ingestion of $[\text{U-}^{13}\text{C}]$ propionate, phenylacetate, and acetaminophen. *Am J Physiol*. 1998; 275:E843–52. [PubMed: 9815005]
30. Kato T, Hayashi Y, Inoue K, Yuasa H. Glycerol absorption by Na^{+} -dependent carrier-mediated transport in the closed loop of the rat small intestine. *Biol Pharm Bull*. 2005; 28:553–5. [PubMed: 15744090]
31. Maeda N, Funahashi T, Shimomura I. Metabolic impact of adipose and hepatic glycerol channels aquaporin 7 and aquaporin 9. *Nat Clin Pract Endocrinol Metab*. 2008; 4:627–34. [PubMed: 18852723]
32. Crescenzo R, Bianco F, Mazzoli A, Giacco A, Liverini G, Iossa S. A possible link between hepatic mitochondrial dysfunction and diet-induced insulin resistance. *Eur J Nutr*. 2016; 55:1–6. [PubMed: 26476631]
33. Kim JA, Wei Y, Sowers JR. Role of mitochondrial dysfunction in insulin resistance. *Circ Res*. 2008; 102:401–14. [PubMed: 18309108]
34. Dahlman I, Forsgren M, Sjogren A, Nordstrom EA, Kaaman M, Naslund E, et al. Downregulation of electron transport chain genes in visceral adipose tissue in type 2 diabetes independent of obesity and possibly involving tumor necrosis factor- α . *Diabetes*. 2006; 55:1792–9. [PubMed: 16731844]
35. Heinonen S, Buzkova J, Muniandy M, Kaksonen R, Ollikainen M, Ismail K, et al. Impaired Mitochondrial Biogenesis in Adipose Tissue in Acquired Obesity. *Diabetes*. 2015; 64:3135–45. [PubMed: 25972572]
36. Satapati S, Kucejova B, Duarte JA, Fletcher JA, Reynolds L, Sunny NE, et al. Mitochondrial metabolism mediates oxidative stress and inflammation in fatty liver. *J Clin Invest*. 2015; 125:4447–62. [PubMed: 26571396]
37. Petersen KF, Befroy DE, Dufour S, Rothman DL, Shulman GI. Assessment of Hepatic Mitochondrial Oxidation and Pyruvate Cycling in NAFLD by (^{13}C) Magnetic Resonance Spectroscopy. *Cell Metab*. 2016; 24:167–71. [PubMed: 27411016]

Figure 1. ^{13}C NMR spectrum of Monoacetone Glucose Derived from Plasma Glucose

An overnight fasted participant with high VAT ingested $[\text{U-}^{13}\text{C}_3]$ glycerol and blood was drawn at multiple time points. Glucose was converted to MAG for ^{13}C NMR analysis and the spectrum is from blood drawn at 180 min after the oral load of $[\text{U-}^{13}\text{C}_3]$ glycerol. Signals from $[1,2,3\text{-}^{13}\text{C}_3]$ - and $[4,5,6\text{-}^{13}\text{C}_3]$ glucose were dominant and were produced through gluconeogenesis directly from $[\text{U-}^{13}\text{C}_3]$ glycerol. Gluconeogenesis through $[\text{U-}^{13}\text{C}_3]$ glycerol metabolism via the TCA cycle produced double-labeled ($[1,2\text{-}^{13}\text{C}_2]$, $[2,3\text{-}^{13}\text{C}_2]$, $[4,5\text{-}^{13}\text{C}_2]$ and $[5,6\text{-}^{13}\text{C}_2]$) glucose. Hepatic PPP activity produced additional $[1,2\text{-}^{12}\text{C}_2]$ glucose from $[1,2,3\text{-}^{13}\text{C}_3]$ hexose. The ^{13}C enrichment in each glucose isotopomer was calculated using two methyl resonances from MAG with natural abundance ^{13}C (1.1%). *D12*, doublet from coupling of C1 with C2; *D23*, doublet from coupling of C2 with C3; *Q*, doublet of doublets, or quartet, arising from coupling of C2 with both C1 and C3 or from coupling of C5 with both C4 and C6; *D45*, doublet from coupling of C4 with C5; *D56*, doublet from coupling of C5 with C6; *S*, singlet. MAG=monoacetone glucose; VAT=visceral adipose tissue

Figure 2. Glycerol pathways in gluconeogenesis

Glycerol-gluconeogenesis is directly interrogated by determining the fraction of $^{13}\text{C}_3$ enrichment in blood glucose using NMR spectroscopic quantification of ^{13}C -labeled glucose isotopomers. Total ^{13}C enrichment in plasma glucose is measured by the sum of all glucose isotopomers with excess ^{13}C . Additional information about specific pathways is derived from specific glucose isotopomers. Initially, glycerol is phosphorylated in the liver by glycerol kinase and is converted to DHAP and GA3P. Direct production of glucose via gluconeogenesis occurs by condensation of DHAP and GA3P to form carbons 1–3 and 4–6 of glucose, respectively. Therefore, quantification of $[1,2,3\text{-}^{13}\text{C}_3]$ glucose and of $[4,5,6\text{-}^{13}\text{C}_3]$ glucose reflects primarily direct hepatic gluconeogenesis from ingested $[\text{U-}^{13}\text{C}_3]$ glycerol (*black filled circles*). Passage of $[1,2,3\text{-}^{13}\text{C}_3]$ G6P through the PPP produces $[1,2\text{-}^{13}\text{C}_2]$ F6P and subsequently $[1,2\text{-}^{13}\text{C}_2]$ glucose so that the quantification of $[1,2\text{-}^{13}\text{C}_2]$ glucose reflects PPP activity (*red filled circles*). Metabolism of $[\text{U-}^{13}\text{C}_3]$ glycerol to $[\text{U-}^{13}\text{C}_3]$ pyruvate followed by either decarboxylation to $[\text{U-}^{13}\text{C}_2]$ acetyl-CoA or carboxylation to $[1,2,3\text{-}^{13}\text{C}_3]$ oxaloacetate allows rearrangement of ^{13}C in the TCA cycle to form PEP. Two possible PEP isotopomers through glycerol metabolism in the TCA cycle are $[1,2\text{-}^{13}\text{C}_2]$ - and $[2,3\text{-}^{13}\text{C}_2]$ PEP. Conversion to PEP and metabolism to glucose will produce $[4,5\text{-}^{13}\text{C}_2]$ - or $[5,6\text{-}^{13}\text{C}_2]$ glucose (*green filled circles*). Since the PPP does not disrupt carbon-carbon bonds in carbons 4–6 of glucose, the presence of $[4,5\text{-}^{13}\text{C}_2]$ - or $[5,6\text{-}^{13}\text{C}_2]$ glucose indicates metabolism of $[\text{U-}^{13}\text{C}_3]$ glycerol through the TCA cycle prior to gluconeogenesis. DHAP= dihydroxyacetone phosphate; GA3P= glyceraldehyde 3-phosphate; G6P=glucose 6-phosphate; F6P= fructose 6-phosphate; NMR=nuclear magnetic resonance; PEP= phosphoenolpyruvate; PPP=pentose phosphate pathway, TCA=tricarboxylic acid; TPI= triose phosphate isomerase

Figure 3. Plasma Glucose versus Time for All Study Groups

Mean $\pm$ standard error plasma glucose (mg/dl) versus time for all study groups. Mean plasma glucose for all groups decreased over the study period from baseline to time +180 minutes. VAT=visceral adipose tissue.

Figure 4. Contribution of [U- $^{13}\text{C}_3$] Glycerol to Glucose Production via Multiple Pathways in Low Visceral Fat vs. High Visceral Fat Participants after a Fast

There was a statistically significant 21% decrease in ^{13}C enrichment in glucose in the high VAT-fasting group compared with the low VAT-fasting group (**Panel A**). ^{13}C enrichment in blood glucose demonstrating PPP and TCA cycle activity was observed in all participants. We observed a trend toward less amount of [1,2- $^{13}\text{C}_2$] glucose representing PPP activity in the high VAT-fasting group compared with the low VAT-fasting group (**Panel B**). We also observed a trend toward less [5,6- $^{13}\text{C}_2$]/[4,5,6- $^{13}\text{C}_3$] in glucose (%) representing [U- $^{13}\text{C}_3$] glycerol metabolism through the TCA cycle in mitochondria in the high VAT-fasting group compared with the low VAT-fasting group (**Panel C**). VAT=visceral adipose tissue

Figure 5. Contribution of $[\text{U}-^{13}\text{C}_3]$ Glycerol to Glucose Production via Multiple Pathways in Fasting vs. Refed High Visceral Fat Participants

There was a statistically significant 37% decrease in ^{13}C enrichment in glucose in the high VAT-refed group compared with the high VAT-fasting group (**Panel A**). ^{13}C enrichment in blood glucose demonstrating PPP and TCA cycle activity was observed in all participants. We observed a trend toward less amount of $[1,2-^{13}\text{C}_2]$ glucose representing PPP activity in the high VAT-refed group compared with the high VAT-fasting group (**Panel B**). We also observed a trend toward less $[5,6-^{13}\text{C}_2]/[4,5,6-^{13}\text{C}_3]$ in glucose (%) representing $[\text{U}-^{13}\text{C}_3]$ glycerol metabolism through the TCA cycle in mitochondria in the high VAT-refed group compared with the high VAT-fasting group (**Panel C**). VAT=visceral adipose tissue

Characteristics of the Study Population

Table 1

Group	Age (yrs)	Sex	Race	Weight (kg)	Body mass index (kg/m ²)	Waist Circumference (cm)	Visceral Fat (kg)	Blood Pressure (mmHg)	Plasma Glucose (mg/dl)
Low Visceral Fat									
Subject #1	56	F	White	101.6	42.3	124	1.8	117/77	110
Subject #2	44	F	White	105.2	35.3	101	1.4	130/89	73
Subject #3	47	F	White	87.6	31.2	108	1.2	107/68	86
Subject #4	43	F	White	96.1	40.1	103	2.1	135/85	103
High Visceral Fat									
<i>Fasting</i>									
Subject #1	45	F	White	115.7	45.2	121	3.3	135/90	100
Subject #2	41	M	Black	144.24	37.7	130	2.5	131/88	96
Subject #3	58	M	Black	141.7	38.5	128	3.2	131/63	101
<i>Refed</i>									
Subject #1	58	F	White	138.5	47.8	135	2.6	117/72	113
Subject #2	48	F	White	130.2	42.4	118	3.4	117/77	103