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Leucine acutely potentiates glucose-stimulated insulin secretion in fetal sheep

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Abstract

A 9-day infusion of leucine into fetal sheep potentiates fetal glucose-stimulated insulin secretion (GSIS). However, there were accompanying pancreatic structural changes that included a larger proportion of β -cells and increased vascularity. Whether leucine can acutely potentiate fetal GSIS *in vivo* before these structural changes develop is unknown. The mechanisms by which leucine acutely potentiates GSIS in adult islets and insulin-secreting cell lines are well known. These mechanisms involve leucine metabolism, including leucine oxidation. However, it is not clear if leucine-stimulated metabolic pathways are active in fetal islets. We hypothesized that leucine would acutely potentiate GSIS in fetal sheep and that isolated fetal islets are capable of oxidizing leucine. We also hypothesized that leucine would stimulate other metabolic pathways associated with insulin secretion. In pregnant sheep we tested *in vivo* GSIS with and without an acute leucine infusion. In isolated fetal sheep islets, we measured leucine oxidation with a [$1\text{-}^{14}\text{C}$] L-leucine tracer. We also measured concentrations of other amino acids, glucose, and analytes associated with cellular metabolism following incubation of fetal islets with leucine. *In vivo*, a leucine infusion resulted in glucose-stimulated insulin concentrations that were over 50% higher than controls ($P < 0.05$). Isolated fetal islets oxidized leucine. Leucine supplementation of isolated fetal islets also resulted in significant activation of metabolic pathways involving leucine and other amino acids. In summary, acute leucine supplementation potentiates fetal GSIS *in vivo*, likely through pathways related to the oxidation of leucine and catabolism of other amino acids.

Key Words

- ▶ leucine
- ▶ insulin
- ▶ pancreas
- ▶ islet
- ▶ β -cell
- ▶ fetus
- ▶ sheep

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Introduction

Metabolism of nutrients by the β -cell in the pancreatic islet is key to nutrient-stimulated insulin secretion. This is described by the fuel-mediated hypothesis of insulin release (Malaisse *et al.* 1979). The fuel-mediated hypothesis posits that nutrient-stimulated insulin release depends on the capacity of nutrients to be metabolized by the β -cell as an energy source. In the adult islet, leucine increases its own oxidative metabolism. Leucine also stimulates glucose metabolism by allosterically activating glutamate dehydrogenase (GDH). GDH converts glutamate to

α -ketoglutarate, thereby increasing the activity of the tricarboxylic acid pathway (Fajans *et al.* 1967, Malaisse *et al.* 1980, Sener & Malaisse 1980, McClenaghan *et al.* 1996, Gao *et al.* 2003, Li *et al.* 2003). Therefore, consistent with the fuel-mediated hypothesis of insulin secretion leucine stimulates insulin release from adult islets by increasing cellular energy production (Malaisse *et al.* 1979).

Among the various amino acids that can impact β -cell metabolism and function, leucine is particularly relevant to study in the fetal period as it has the potential for

stimulating pancreatic islet growth, development, and insulin secretion (Rozance & Hay Jr 2016, Boehmer *et al.* 2017). We have demonstrated that leucine potentiates GSIS in vitro in isolated fetal sheep islets (Rozance *et al.* 2006, Benjamin *et al.* 2017). We also have demonstrated that a 9-day infusion of leucine into fetal sheep at a rate adjusted to double fetal arterial plasma leucine concentrations potentiated fetal GSIS without increasing non-glucose-stimulated fetal insulin concentrations (Boehmer *et al.* 2020). After the 9-day infusion of leucine, insulin⁺ β -cells comprised a larger proportion of the pancreas and pancreatic islet. There also was higher pancreatic and pancreatic islet vascularity (Boehmer *et al.* 2020). Therefore, it was not clear whether potentiation of GSIS by leucine was due to these structural changes or if leucine can acutely potentiate fetal GSIS in vivo. Furthermore, whether the same leucine stimulated metabolic signaling pathways observed in adult islets are active in fetal islets is unknown. Therefore, we used fetal sheep to demonstrate that leucine acutely potentiates fetal GSIS in vivo. We also used isolated fetal sheep islets to test the hypothesis that these islets have the capacity to oxidize leucine and that leucine increases the products of cellular metabolism in vitro.

Materials and methods

In vivo leucine potentiated GSIS

All animal experiments were conducted at the Perinatal Research Center, University of Colorado School of Medicine, Aurora, Colorado, in compliance with ARRIVE guidelines. The Institutional Animal Care and Use Committee approved this study. The Perinatal Research Center Center is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. *In vivo* leucine potentiated GSIS studies were conducted in five Columbia–Rambouillet sheep with singleton pregnancies. Animals were anesthetized at 121 ± 1 days of gestational age (dGA) and surgeries performed to place indwelling catheters into the fetal abdominal aorta, fetal femoral vein, and maternal femoral vein and artery as previously described (Rozance *et al.* 2006). Before insulin secretion was measured, animals were allowed to recover for at least 5 days.

GSIS was measured with a 90-min variable rate, square-wave, fetal hyperglycemic clamp initiated at time 0 min (Gadhia *et al.* 2013). At -105 and -100 min, fetal blood was sampled for baseline hormone, nutrient and blood gas concentrations. At -90 min an experimental

infusion into a fetal vein was started. The infusion was either leucine ($752 \mu\text{mol/h}$; Sigma-Aldrich) or saline (0.3 mL/h to match the sodium delivery in the leucine infusion). Fetal blood was then sampled at -15 and -10 min to measure the response to the experimental leucine infusion and to determine insulin and glucose concentrations prior to the hyperglycemic clamp. The hyperglycemic clamp was then started and fetal blood samples were taken at minutes 5, 10, 15, 20, 30, 45, 60, 75, and 90 to measure insulin and glucose concentrations. The total blood volume removed for each study was between 13 and 15 mL. Experimental infusions were continued for the duration of the hyperglycemic clamp. Animals had GSIS measured multiple times, always with at least 48 h between measurements. The experimental infusion (leucine or saline) was alternated and fetuses had leucine-potentiated GSIS measured twice and GSIS following the saline infusion measured one to three times. The rationale for measuring GSIS multiple times during the leucine and saline infusions is that we did not have preliminary data indicating the effect size of leucine on the potentiation of GSIS. We also did not have an estimate of the within animal variability for leucine potentiated GSIS. Therefore, we prospectively measured leucine potentiated GSIS and GSIS without a leucine infusion multiple times in each animal. Results from this study were analyzed in a mixed models ANOVA in SAS v. 9.2 with terms to account for repeated measures within the same fetus and within the same study. Time, experimental infusion (leucine or saline), and their interaction were included as fixed terms. Individual means were compared with Fisher's least squares difference test. P -values ≤ 0.05 were accepted as significant. For Fig. 1 and Table 1 the concentrations for an animal during either the leucine or saline infusion studies were averaged, and these means were used to calculate group means and variances for leucine and saline potentiated GSIS. Blood was sampled from arterial catheters except for one animal's studies. For this fetus blood could only be sampled from a venous catheter and so these biochemical values are only included in the glucose and insulin concentration analyses during the GSIS studies (Fig. 1). No data points were excluded as outliers.

Biochemical analyses

Biochemical analysis was performed as previously described (Culpepper *et al.* 2016). Blood was analyzed immediately after collection for pH, O_2 , CO_2 , and hemoglobin (ABL825; Radiometer America Inc.,

Figure 1

In vivo fetal GSIS is potentiated by leucine. Mean $\pm$ S.E.M. fetal arterial plasma glucose (A) and insulin (B) concentrations are shown relative to the start of a primed continuous variable rate fetal hyperglycemic clamp (time = 0 min). At time -90 an experimental infusion of leucine (752 μ mol/h, closed circles) or saline (0.3 mL/h, open squares) was started. Means are calculated from experiments conducted in five animals in which the experimental infusion was alternated. Animals had GSIS measured multiple times, always with at least 48 h between measurements. All fetuses had leucine potentiated GSIS measured twice and GSIS following the saline infusion measured one to three times. The concentrations for a single animal during either the leucine or saline infusion studies were averaged, and these means were used to calculate group means and variances in the figure. Results were analyzed in a mixed models ANOVA and individual means were compared with Fisher's least squares difference test. *Significantly higher glucose-stimulated insulin concentrations during the leucine infusion compared to the saline infusion ($P \leq 0.05$; with the exception of minute 15, $P = 0.056$).

Brea, CA, USA), as well as plasma concentrations of glucose and lactate (YSI 2700 biochemistry analyzer; Yellow Springs Instruments, Yellow Springs, OH, USA). Additional plasma was stored at -80°C for measurement of insulin (intra- and inter-assay CVs 5.6 and 4.7%, respectively), insulin-like growth factor-1 (IGF-I; intra- and inter-assay CVs 3.1 and 5.6%, respectively), and cortisol (intra- and inter-assay CVs 5.6 and 4.7%, respectively) by ELISA (ALPCO Immunoassays, Salem, NH, USA); glucagon by RIA (intra- and inter-assay CVs 4.8 and 11.7%, respectively; Millipore) and norepinephrine by HPLC (intra- and inter-assay CVs 9.2 and 9.0%, respectively).

Pancreatic islet isolation

Pancreatic islets were isolated from four of the five animals subjected to measurement of *in vivo* leucine-potentiated GSIS at least 1 day after the last *in vivo* study. These islets were used to determine the impact of supplemental leucine on a wide range of pancreatic analytes.

We also isolated pancreatic islets from four additional late gestation fetal sheep. These islets were used to measure leucine oxidation. All four of these fetuses underwent a surgery to place maternal and fetal catheters. Two of these fetuses had an acute hyperinsulinemic-euglycemic clamp performed and two had baseline physiologic studies performed without acute clamp studies prior to collection of pancreatic islets. Their gestational age at the time of pancreatic islet collection was 131 ± 2 . All islets were isolated by collagenase digestion, as previously described (Rozance *et al.* 2006). The animals were anesthetized with ketamine (10–25 mg/kg) and diazepam (0.1–0.3 mg/kg) administered intravenously to the ewe. After the umbilical cord was clamped and divided, the ewe was euthanized with an overdose of Fatal Plus pentobarbital solution. The fetus also was euthanized with an overdose of Fatal Plus given intravenous or intracardiac.

Pancreatic islet leucine oxidation

Fetal sheep pancreatic islet leucine oxidation rates were determined as previously described for glucose oxidation with the exception that we used [$1\text{-}^{14}\text{C}$] L-leucine instead of [$U\text{-}^{14}\text{C}$] D-glucose (Rozance *et al.* 2007). Briefly, fetal sheep islet leucine oxidation rates were determined for four different fetuses. Islets were placed in a 1 mL Nunc CryoTube (1 mL; Nalge Nunc International, Naperville, IL, USA) affixed with epoxy inside a 20 mL glass scintillation vial ($n = 3\text{--}5$ per incubation condition, 25 islets each) and sealed with a Suba-Seal Rubber Septa (Sigma-Aldrich). Islets were incubated in Krebs–Ringer bicarbonate buffer with 0.5% BSA (w/v) containing 1.1 mmol/L D-glucose and 0.4, 4, or 10 mmol/L L-leucine with [$1\text{-}^{14}\text{C}$] L-leucine (8, 12 or 16 $\mu\text{Ci/mL}$, respectively (New England Nucleotides, Boston, MA, USA) for 2 h. Prior to the addition of isolated islets, the buffer was pre-equilibrated in 95% O_2 :5% CO_2 . Blank reactions without islets were included. At the end of the 2 h incubation reaction vials were quickly cooled and the reaction stopped with 100 μL of 1 mol/L HCl. Solvable (0.5 mL, Perkin Elmer) was added to the base of the scintillation vial to trap the CO_2 while the vials were incubated at 37°C with gentle agitation for 90 min. The rubber stopper and reaction tube were removed, Ultima Gold scintillation fluid (Perkin Elmer) was added, and the captured $^{14}\text{CO}_2$ disintegrations per minute (DPM) were determined with a Packard Tri-Carb 2300TR Liquid Scintillation Analyzer. The rate of leucine oxidation (pmol/islet/h) was calculated by dividing the difference between the DPMs from the islet incubations and the blank incubations by the specific activity of leucine in

Table 1 Fetal nutrient, hormone and blood gas measurements prior to in vivo hyperglycemic clamp study.

	Saline		Leucine	
	Pre	Post-75 min	Pre	Post-75 mins
Plasma substrates and hormones				
Leucine ($\mu\text{mol/L}$)	153 $\pm$ 25	146 $\pm$ 21	133 $\pm$ 3	287 $\pm$ 16 ^b
Glucose (mmol/L)	0.9 $\pm$ 0.1	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1	1.1 $\pm$ 0.1
Insulin (ng/mL)	0.35 $\pm$ 0.08	0.36 $\pm$ 0.09	0.36 $\pm$ 0.07	0.46 $\pm$ 0.10 ^b
Glucagon (pg/mL)	80.4 $\pm$ 18.7	87.3 $\pm$ 18.9	59.8 $\pm$ 11.4	81.4 $\pm$ 17.6
IGF-1 (ng/mL)	98.4 $\pm$ 22.6	94.4 $\pm$ 20.3	97.4 $\pm$ 19.9	101.1 $\pm$ 19.9
Norepinephrine (pg/mL)	343.4 $\pm$ 44.3	338.2 $\pm$ 40.4	433.9 $\pm$ 57.7	380.3 $\pm$ 59.8
Cortisol (ng/mL)	22.4 $\pm$ 6.1	22.0 $\pm$ 6.5	18.8 $\pm$ 5.6	16.9 $\pm$ 5.6
Lactate (mmol/L)	1.77 $\pm$ 0.08	1.80 $\pm$ 0.04	1.91 $\pm$ 0.24	2.15 $\pm$ 0.28 ^a
Alanine ($\mu\text{mol/L}$)	238.2 $\pm$ 14.5	230.4 $\pm$ 14.7	248.7 $\pm$ 10.8	238.0 $\pm$ 12.9
Arginine ($\mu\text{mol/L}$)	93.3 $\pm$ 11.2	95.5 $\pm$ 9.7	84.2 $\pm$ 11.5	88.8 $\pm$ 9.0
Asparagine ($\mu\text{mol/L}$)	38.9 $\pm$ 3.7	38.8 $\pm$ 2.6	37.2 $\pm$ 3.9	34.1 $\pm$ 4.0
Aspartate ($\mu\text{mol/L}$)	17.3 $\pm$ 2.2	17.5 $\pm$ 2.8	19.9 $\pm$ 2.5	19.8 $\pm$ 3.5
Citruline ($\mu\text{mol/L}$)	187.0 $\pm$ 52.2	184.9 $\pm$ 48.7	198.4 $\pm$ 32.4	186.7 $\pm$ 43.4
Cysteine ($\mu\text{mol/L}$)	11.3 $\pm$ 1.8	11.1 $\pm$ 1.7	13.3 $\pm$ 3.1	10.1 $\pm$ 1.1
Glutamate ($\mu\text{mol/L}$)	27.9 $\pm$ 7.3	26.6 $\pm$ 7.1	33.1 $\pm$ 7.3	34.4 $\pm$ 6.0
Glutamine ($\mu\text{mol/L}$)	324.2 $\pm$ 10.9	311.8 $\pm$ 8.4	331.8 $\pm$ 15.9	339.2 $\pm$ 15.8
Glycine ($\mu\text{mol/L}$)	347.0 $\pm$ 58.1	331.8 $\pm$ 51.0	334.0 $\pm$ 41.1	302.9 $\pm$ 36.2
Histidine ($\mu\text{mol/L}$)	52.7 $\pm$ 1.6	50.4 $\pm$ 4.5	50.8 $\pm$ 5.0	46.7 $\pm$ 1.9
Isoleucine ($\mu\text{mol/L}$)	128.4 $\pm$ 10.0	127.0 $\pm$ 10.4	110.7 $\pm$ 5.1	106.2 $\pm$ 4.5
Lysine ($\mu\text{mol/L}$)	82.8 $\pm$ 7.0	78.6 $\pm$ 4.3	79.4 $\pm$ 10.7	77.5 $\pm$ 11.0
Methionine ($\mu\text{mol/L}$)	96.2 $\pm$ 10.3	90.3 $\pm$ 11.9	89.9 $\pm$ 3.7	84.4 $\pm$ 4.6
Ornithine ($\mu\text{mol/L}$)	74.3 $\pm$ 11.4	73.6 $\pm$ 10.7	71.2 $\pm$ 8.4	76.3 $\pm$ 10.5
Phenylalanine ($\mu\text{mol/L}$)	95.0 $\pm$ 9.1	92.7 $\pm$ 9.0	89.7 $\pm$ 8.3	88.5 $\pm$ 10.1
Proline ($\mu\text{mol/L}$)	135.4 $\pm$ 6.6	127.4 $\pm$ 4.8	125.3 $\pm$ 9.8	116.3 $\pm$ 7.9
Serine ($\mu\text{mol/L}$)	382.6 $\pm$ 27.2	365.2 $\pm$ 26.2	402.8 $\pm$ 35.2	385.5 $\pm$ 36.5
Taurine ($\mu\text{mol/L}$)	41.6 $\pm$ 6.1	40.8 $\pm$ 9.3	42.8 $\pm$ 7.6	38.9 $\pm$ 7.4
Threonine ($\mu\text{mol/L}$)	217.3 $\pm$ 54.7	204.3 $\pm$ 53.6	221.4 $\pm$ 52.0	202.7 $\pm$ 47.7
Tryptophan ($\mu\text{mol/L}$)	40.0 $\pm$ 4.2	36.6 $\pm$ 2.8	43.4 $\pm$ 3.6	35.8 $\pm$ 3.0
Tyrosine ($\mu\text{mol/L}$)	131.2 $\pm$ 10.2	126.8 $\pm$ 14.0	121.6 $\pm$ 13.4	112.7 $\pm$ 12.0
Valine ($\mu\text{mol/L}$)	428.8 $\pm$ 30.2	411.5 $\pm$ 27.3	388.8 $\pm$ 13.4	361.8 $\pm$ 12.1
Blood gas measurements				
pH	7.35 $\pm$ 0.00	7.35 $\pm$ 0.00	7.35 $\pm$ 0.00	7.34 $\pm$ 0.00
PaCO ₂ (mmHg)	49.6 $\pm$ 1.2	49.1 $\pm$ 1.4	50.5 $\pm$ 0.5	50.6 $\pm$ 0.8
PaO ₂ (mmHg)	19.6 $\pm$ 0.7	19.1 $\pm$ 1.2	20.1 $\pm$ 0.8	19.6 $\pm$ 1.2
Hemoglobin (g/dL)	6.48 $\pm$ 0.32	6.47 $\pm$ 0.32	6.55 $\pm$ 0.31	6.49 $\pm$ 0.29
SaO ₂ (%)	47.8 $\pm$ 2.5	46.1 $\pm$ 4.2	48.7 $\pm$ 3.2	46.6 $\pm$ 4.7
O ₂ content (mmol/L)	3.01 $\pm$ 0.07	2.91 $\pm$ 0.15	3.12 $\pm$ 0.19	2.98 $\pm$ 0.32

Values are the mean $\pm$ S.E.M. *P* values are from a mixed models ANOVA with individual means comparisons leucine infusion with both the pre-leucine infusion and post-75 min saline infusion concentrations. ^b*P* < 0.0001 for the comparison between post-75 min leucine infusion and pre-leucine infusion concentrations, and *P* < 0.05 for the comparison between post-75 min leucine infusion with the post-75 min saline infusion concentrations. ^a*P* < 0.0001 for the comparison between 75 min post-leucine infusion with pre-leucine infusion concentrations. *n* = 4 animals that were studied for each infusion type.

the buffer. Oxidation rates were log transformed before being analyzed with repeated measures ANOVA in SAS v. 9.2. Individual means were compared with Fisher's least squares difference test. *P*-values ≤ 0.05 were accepted as significant. Incubation in 4 mmol/L L-leucine was not performed for one of the sets of islets. No data points were excluded as outliers.

Pancreatic islet analyte measurement

Pancreatic islets were isolated from four fetal sheep and separated into equal batches of at least 250 islets,

as previously described (Rozance *et al.* 2006). Islets were incubated for 24 h in RPMI 1640 (Sigma-Aldrich) supplemented with glucose (2.8 mmol/L), BSA (0.5%, w/v), and $\pm$ leucine (10 mmol/L) at 37°C in 95% O₂–5% CO₂. Following this, islets and conditioned media were separated by centrifugation. The islets were resuspended in PBS and separated into individual cells with a 3-min trypsin digest. PBS was added to the cell suspension such that a final concentration of 2×10^6 cells/mL was achieved. RPMI 1640 with supplements noted above was added to the conditioned media samples to normalize for numbers of cells that were present in the

overnight incubations. Samples were submitted to the Biological Mass Spectrometry Facility (University of Colorado School of Medicine) for measurement of 140 hydrophilic analytes, as previously described (Nemkov *et al.* 2015, 2017). Analytes were extracted from cell pellets in ice-cold methanol:acetonitrile:water (5:3:2, v/v/v) at a ratio of 2×10^6 cells/mL. Islet lysates and conditioned media were agitated for 30 min and vortexed for 10 min at 4°C. Samples were then centrifuged for 10 min at 10,000 *g* and 4°C, supernatants were removed and stored at –80°C. Cell extracts and conditioned media (20 and 5 μ L, respectively) were injected into Vanquish Ultra High Performance Liquid Chromatography (UHPLC) system coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific) and separated through a 3-min isocratic elution on a Kinetex C18 column (150 $\times$ 2.1 mm i.d., 1.7 μ m particle size; Phenomenex, Torrance, CA, USA) at 250 μ L/min (mobile phase: 5% acetonitrile, 95% water and 0.1% formic acid; column temperature: 25°C). The Q Exactive mass spectrometer was operated in both positive and negative ion mode. Scanning was performed in Full MS mode (2 μ scans) from 60 to 900 *m/z* at 70,000 resolution with 4 kV spray voltage, 15 sheath gas, and 5 auxiliary gas. Calibration was performed prior to analysis using Pierce™ Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Data for leucine and isoleucine obtained via UHPLC were combined due to co-elution of peak areas. Additionally, because UHPLC measurements of amino acids are not as accurate as amino acid concentrations measured by high performance liquid chromatography (HPLC), we also used HPLC to measure concentrations in separate aliquots of conditioned media as previously described (Brown *et al.* 2012). Leucine and isoleucine were quantified separately via HPLC. Glucose concentrations in the media were measured with the YSI 2700 biochemistry analyzer. For reference, glucose and amino acid concentrations were also measured in media that was not used to incubate islets. No data points were excluded as outliers.

Comparison of analytes in islets and media was performed by Paired *T*-test. This was done in GraphPad Prism V8.2 for HPLC and YSI measurements and in MetaboAnalyst for UHPLC measurements. For the UHPLC measurements, false discovery rate (FDR) was determined using Benjamini–Hochberg procedure (Hochberg & Benjamini 1990). Analyte annotation was performed using the KEGG database. Pathway analyses was performed using Metaboanalyst using the KEGG database for annotation with enrichment of

relevant pathways determined by the global test method and topological analysis determined using relative betweenness centrality. Analytes with a nominal *P* value ≤ 0.05 and an FDR < 0.25 were used for pathway analysis. Statistical significance for glucose concentrations and amino acid concentrations measured by HPLC and for pathway analysis was set at $P \leq 0.05$. Media glycine, leucine, and serine concentrations determined by HPLC were log transformed for statistical analysis. No data points were excluded as outliers.

Results

Fetal leucine potentiated GSIS

Fetal sheep were subjected to a square-wave hyperglycemic clamp with or without a direct fetal leucine infusion. Averaged fetal arterial blood gases and plasma nutrient and hormone concentrations from the –105 and –100 min blood draws and the –15 and –10 min blood draws are shown in Table 1. As each fetus was subjected to multiple tests of GSIS, we analyzed minute –105 and –100 fetal hemoglobin concentrations as a function of the order of the GSIS test and found that between the first and second time GSIS was measured there was a consistent 7% reduction in fetal hemoglobin ($P < 0.05$). There was no further reduction in fetal hemoglobin after the second GSIS test. This small reduction in fetal hemoglobin did not impact GSIS. The leucine infusion resulted in plasma leucine concentrations that were almost twice as high as in the saline infused fetuses ($P < 0.0001$). No other amino acid concentrations were impacted by the leucine infusion. Glucose concentrations also were not impacted by the leucine infusion, though lactate concentrations increased 12.5% with the leucine infusion ($P < 0.0001$) but not with the saline infusion. Plasma insulin concentrations increased with the leucine infusion ($P < 0.0001$) and were about 25% higher compared to insulin concentrations with the saline infusion ($P < 0.05$).

During the hyperglycemic clamp, insulin concentrations were greater with the concurrent leucine infusion starting at minute 10 of the hyperglycemic clamp ($P < 0.05$; Fig. 1) compared to insulin concentrations with the saline infusion. Glucose concentrations were similar for the duration of the hyperglycemic clamp with the exception of minute 5, when they were 5% greater during the leucine infusion compared to the saline infusion ($P < 0.05$).

Figure 2

Isolated fetal pancreatic islets oxidize leucine. Pancreatic islets were isolated from four late gestation fetal sheep and incubated with 1.1 mmol/L glucose and increasing concentrations of leucine. Data points with the same symbol were derived from a single animal's islets. Median and 95% confidence intervals are shown. Leucine oxidation was measured by including [1-¹⁴C] L-leucine in the incubation buffer and measuring [¹⁴C] CO₂ production. Incubations in 0.4 and 10 mmol/L leucine include islets from all four fetuses and incubations in 4 mmol/L include islets from three of the four fetuses. For statistical analysis, results were log transformed and then analyzed with a repeated measures ANOVA. Individual means of the log transformed data were compared with Fisher's least squares difference test. *Significantly greater leucine oxidation rate with incubations in 4 and 10 mmol/L leucine compared to 0.4 mmol/L ($P < 0.05$).

Pancreatic islet leucine oxidation

In order to demonstrate that isolated fetal pancreatic islets have the capacity to oxidize leucine, islets were incubated with various concentrations of [1-¹⁴C] L-leucine and the production of [¹⁴C] CO₂ was measured (Fig. 2). Islet leucine oxidation rates were greater with incubations in 4 and 10 mmol/L leucine compared to 0.4 mmol/L leucine ($P < 0.05$). There was no statistical difference between the oxidation rates with incubations in 4 and 10 mmol/L leucine, perhaps indicating a maximal oxidative capacity for leucine.

Analytes following islet incubation with supplemental leucine

HPLC analysis demonstrated the targeted 25-fold higher leucine concentrations in media from islets treated with supplemental leucine (9498 ± 92 leucine supplemented vs 367 ± 2 non-supplemented nmol/mL, $P < 0.0001$). The media concentrations of glucose and almost all other amino acids were between 5–30% lower in media from leucine supplemented islets compared to non-leucine supplemented islets ($P < 0.05$; Fig. 3). Proline was not different between experimental conditions. Isoleucine was 15% higher in leucine supplemented media compared to non-supplemented media ($P < 0.0001$, Fig. 3). Insulin concentrations in the media did not consistently differ between treatments (158 ± 90 leucine supplemented vs 118 ± 50 non-supplemented ng/mL).

Figure 3

Supplemental leucine results in lower concentrations of other amino acids after incubation with isolated fetal islets. Fetal islets from four sheep were divided into equal aliquots and incubated in control media (open squares) or control media supplemented with leucine (10 mmol/L, closed circles). Media was then subjected to measurement of amino acids or glucose and results were normalized to the concentrations of these nutrients in reference media that was not exposed to isolated islets. Data are mean $\pm$ S.E.M. *Significant difference between media from islets that were supplemented with leucine vs media from islets that were not supplemented with leucine ($P < 0.05$) by paired *t*-test. ARG, arginine; ASP, aspartate; ASPG, asparagine; CYS, cysteine; GLN, glutamine; GLU, glutamate; GLY, glycine; HIS, histidine; ILE, isoleucine; LYS, lysine; MET, methionine; PHE, phenylalanine; PRO, proline; SER, serine; THR, threonine; TYR, tyrosine; VAL, valine.

UHPLC/mass spectrometry analysis measured 140 analytes in the islets and 120 analytes in the media (Table 2 and Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). Principal component analyses (PCA) confirmed a segregation of analytes from leucine supplemented and non-supplemented islets (Fig. 4A). This segregation was more distinct in the conditioned media (Fig. 4B). Of the analytes measured in islets and media, KEGG database annotations were available for 130 and 107, respectively, and thus these analytes were used for pathway analyses if they were significantly different between groups (nominal P Value ≤ 0.05). For analytes obtained from the islets, nine achieved statistical significance ($P \leq 0.05$; Table 2). Notable islet analytes with significant differences included L-leucine (seven-fold higher, $P < 0.05$) and its related catabolite propionyl-carnitine being 80% lower ($P < 0.05$) in leucine supplemented islets (Table 2), thus confirming that leucine entered the islet cells and underwent cellular catabolism. However, no pathways demonstrated significant enhancement using analytes from the islets with a nominal P value of ≤ 0.05 and an FDR < 0.25 .

For analytes obtained from the media, 39 achieved statistical significance ($P \leq 0.05$; Table 2). Seven pathways demonstrated significant enrichment using analytes from the conditioned media with a nominal P Value ≤ 0.05 and an FDR < 0.25 (Table 3). Consistent with the lower media concentrations of almost all amino acids as measured by HPLC, the pathway analysis indicated significant involvement of metabolic processes involving all

Table 2 Analyses.

	Control		Leucine		Control		Leucine		Fold change	T-value	P-value	FDR	VIP score
	Median	Mean $\pm$ S.D.	Median	Mean $\pm$ S.D.	Median	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.					
Islets													
Threo-3-Hydroxy-L-aspartate	6.57×10^5	$6.19 \times 10^5 \pm 8.20 \times 10^4$	2.95×10^5	$2.67 \times 10^5 \pm 7.15 \times 10^4$					0.45	7.16	0.006	0.56	0.0022
sn-Glycero-3-phosphoethanolamine	1.85×10^5	$1.62 \times 10^5 \pm 4.31 \times 10^4$	1.21×10^6	$1.03 \times 10^6 \pm 2.27 \times 10^5$					6.54	4.71	0.02	0.56	0.0055
L-Leucine	2.07×10^8	$1.89 \times 10^8 \pm 3.36 \times 10^7$	1.57×10^9	$1.53 \times 10^9 \pm 3.77 \times 10^8$					7.57	3.72	0.03	0.56	8.3656
6-Aminohexanoate	2.07×10^8	$1.89 \times 10^8 \pm 3.36 \times 10^7$	1.57×10^9	$1.53 \times 10^9 \pm 3.77 \times 10^8$					7.57	3.72	0.03	0.56	8.3656
Propionyl-L-carnitine	1.90×10^5	$1.87 \times 10^5 \pm 2.96 \times 10^4$	2.37×10^4	$3.42 \times 10^4 \pm 1.46 \times 10^4$					0.12	3.71	0.03	0.56	0.0001
4-Pyridoxate	4.65×10^5	$4.04 \times 10^5 \pm 8.21 \times 10^4$	3.11×10^5	$2.66 \times 10^5 \pm 4.85 \times 10^4$					0.67	3.63	0.04	0.56	0.0009
Oxalosuccinate	4.09×10^5	$3.58 \times 10^5 \pm 5.65 \times 10^4$	2.92×10^5	$2.72 \times 10^5 \pm 5.05 \times 10^4$					0.71	3.17	0.05	0.56	0.0005
Ornithine	6.93×10^6	$7.26 \times 10^6 \pm 1.28 \times 10^6$	5.60×10^6	$4.99 \times 10^6 \pm 1.14 \times 10^6$					0.81	3.14	0.05	0.56	0.0142
L-Aspartate	2.68×10^6	$2.48 \times 10^6 \pm 5.01 \times 10^5$	2.81×10^6	$2.60 \times 10^6 \pm 5.36 \times 10^5$					1.05	3.12	0.05	0.56	0.0008
Conditioned media													
sn-Glycero-3-phosphoethanolamine	1.83×10^5	$1.73 \times 10^5 \pm 1.77 \times 10^4$	1.81×10^6	$1.81 \times 10^6 \pm 6.44 \times 10^4$					9.86	29.78	<0.001	0.01	0.0069
L-Phenylalanine	2.74×10^7	$2.73 \times 10^7 \pm 1.03 \times 10^6$	1.87×10^7	$1.90 \times 10^7 \pm 9.80 \times 10^5$					0.68	24.47	<0.001	0.01	0.0347
L-Carnitine	8.80×10^5	$8.78 \times 10^5 \pm 1.61 \times 10^4$	4.27×10^5	$4.22 \times 10^5 \pm 1.03 \times 10^4$					0.49	20.09	<0.001	0.01	0.0019
L-Leucine	3.07×10^8	$3.16 \times 10^8 \pm 2.14 \times 10^7$	2.84×10^9	$2.91 \times 10^9 \pm 1.62 \times 10^8$					9.24	17.73	<0.001	0.01	10.952
N-carbamyl-L-glutamate	2.66×10^5	$2.93 \times 10^5 \pm 3.72 \times 10^4$	2.92×10^6	$2.87 \times 10^6 \pm 1.94 \times 10^5$					10.97	14.72	<0.001	0.02	0.0109
Butanoyl-L-carnitine	1.34×10^5	$1.40 \times 10^5 \pm 9.23 \times 10^3$	3.43×10^4	$3.26 \times 10^4 \pm 3.69 \times 10^3$					0.26	13.29	<0.001	0.02	0.0005
N-Amidino-L-aspartate	9.22×10^6	$9.12 \times 10^6 \pm 3.07 \times 10^5$	5.46×10^7	$5.50 \times 10^7 \pm 4.05 \times 10^6$					5.93	12.19	0.001	0.02	0.1937
Protein 5-hydroxylysine	4.89×10^5	$4.94 \times 10^5 \pm 1.12 \times 10^4$	1.34×10^5	$1.39 \times 10^5 \pm 2.60 \times 10^4$					0.27	11.83	0.001	0.02	0.0015
Dopamine	1.78×10^6	$1.76 \times 10^6 \pm 2.20 \times 10^5$	1.63×10^7	$1.61 \times 10^7 \pm 1.46 \times 10^6$					9.12	10.45	0.002	0.02	0.0607
Ornithine	2.06×10^6	$2.92 \times 10^6 \pm 9.89 \times 10^5$	9.34×10^6	$9.47 \times 10^6 \pm 4.10 \times 10^5$					4.54	10.45	0.002	0.02	0.0277
dCMP	7.66×10^3	$1.01 \times 10^4 \pm 2.95 \times 10^3$	1.75×10^5	$1.81 \times 10^5 \pm 1.52 \times 10^4$					22.92	10.21	0.002	0.02	0.0007
L-Valine	4.59×10^7	$4.58 \times 10^7 \pm 1.57 \times 10^6$	3.21×10^7	$3.19 \times 10^7 \pm 2.57 \times 10^6$					0.7	8.53	0.003	0.03	0.059
Acetyl-L-carnitine	2.25×10^5	$2.29 \times 10^5 \pm 1.36 \times 10^4$	6.39×10^4	$6.91 \times 10^4 \pm 9.19 \times 10^3$					0.28	8.43	0.004	0.03	0.0007
Creatinine	2.05×10^6	$2.05 \times 10^6 \pm 1.27 \times 10^4$	1.56×10^6	$1.56 \times 10^6 \pm 5.42 \times 10^4$					0.76	7.3	0.005	0.04	0.0021
4-Acetamidobutanoate	5.89×10^4	$5.95 \times 10^4 \pm 6.26 \times 10^3$	3.03×10^5	$3.22 \times 10^5 \pm 3.87 \times 10^4$					5.15	7.28	0.005	0.04	0.0011
L-Aspartate	1.45×10^6	$1.44 \times 10^6 \pm 1.33 \times 10^5$	1.07×10^6	$1.10 \times 10^6 \pm 1.08 \times 10^5$					0.74	7.13	0.006	0.04	0.0014
UMP	9.10×10^5	$9.21 \times 10^5 \pm 7.75 \times 10^4$	5.61×10^5	$5.88 \times 10^5 \pm 5.28 \times 10^4$					0.62	6.78	0.007	0.05	0.0014
L-1-Pyrroline-3-hydroxy-5-carboxylate	2.12×10^7	$2.15 \times 10^7 \pm 1.44 \times 10^6$	1.36×10^7	$1.38 \times 10^7 \pm 6.98 \times 10^5$					0.64	6.26	0.008	0.05	0.0325
Pyridoxal	1.03×10^5	$1.02 \times 10^5 \pm 9.26 \times 10^3$	2.70×10^4	$2.99 \times 10^4 \pm 7.01 \times 10^3$					0.26	6.23	0.008	0.05	0.0003
L-Methionine	1.22×10^7	$1.20 \times 10^7 \pm 9.47 \times 10^5$	9.01×10^6	$9.17 \times 10^6 \pm 6.17 \times 10^5$					0.74	6.19	0.009	0.05	0.0121
L-Selenomethionine	3.10×10^5	$3.07 \times 10^5 \pm 2.65 \times 10^4$	2.23×10^5	$2.18 \times 10^5 \pm 1.14 \times 10^4$					0.72	5.72	0.01	0.06	0.0004
4-Pyridoxate	3.93×10^5	$3.99 \times 10^5 \pm 1.71 \times 10^4$	1.94×10^5	$1.80 \times 10^5 \pm 2.44 \times 10^4$					0.49	5.66	0.01	0.06	0.0009
L-Proline	5.56×10^7	$5.58 \times 10^7 \pm 2.61 \times 10^6$	4.63×10^7	$4.75 \times 10^7 \pm 2.51 \times 10^6$					0.83	5.44	0.01	0.06	0.0349
Creatine	3.06×10^6	$3.08 \times 10^6 \pm 1.80 \times 10^5$	2.08×10^6	$2.06 \times 10^6 \pm 1.61 \times 10^5$					0.68	5.41	0.01	0.06	0.0043
Glucosinolates	3.07×10^5	$3.09 \times 10^5 \pm 3.46 \times 10^4$	2.25×10^5	$2.25 \times 10^5 \pm 2.36 \times 10^4$					0.73	5.4	0.01	0.06	0.0004
N-methylethanolamine phosphate	3.09×10^5	$3.15 \times 10^5 \pm 2.51 \times 10^4$	1.97×10^5	$1.95 \times 10^5 \pm 1.76 \times 10^4$					0.64	4.77	0.02	0.08	0.0005
cis-p-Coumarate	1.55×10^6	$1.54 \times 10^6 \pm 1.13 \times 10^5$	1.11×10^6	$1.11 \times 10^6 \pm 4.61 \times 10^4$					0.71	4.68	0.02	0.08	0.0018
(5-L-Glutamyl)-L-glutamine	3.90×10^4	$3.89 \times 10^4 \pm 4.69 \times 10^2$	2.67×10^4	$2.66 \times 10^4 \pm 2.44 \times 10^3$					0.69	4.62	0.02	0.08	<0.0001
D-Glucosamine	7.52×10^4	$7.62 \times 10^4 \pm 5.74 \times 10^3$	5.55×10^4	$5.66 \times 10^4 \pm 5.94 \times 10^3$					0.74	4.15	0.03	0.11	<0.0001
L-Glutamine	2.07×10^7	$2.07 \times 10^7 \pm 6.77 \times 10^5$	1.71×10^7	$1.69 \times 10^7 \pm 4.09 \times 10^5$					0.82	3.91	0.03	0.12	0.016
Propionyl-L-carnitine	1.23×10^5	$1.19 \times 10^5 \pm 2.02 \times 10^4$	4.42×10^4	$4.21 \times 10^4 \pm 5.28 \times 10^3$					0.36	3.58	0.04	0.14	0.0003

(Continued)

Table 2 Continued.

	Control		Leucine		Fold change	T-value	P-value	FDR	VIP score
	Median	Mean ± s.d.	Median	Mean ± s.d.					
Islets									
L-Serine	1.54 × 10 ⁶	1.56 × 10 ⁶ ± 9.82 × 10 ⁴	1.11 × 10 ⁶	1.14 × 10 ⁶ ± 4.58 × 10 ⁴	0.72	3.54	0.04	0.14	0.0018
L-Threonine	2.88 × 10 ⁶	2.89 × 10 ⁶ ± 1.62 × 10 ⁵	2.29 × 10 ⁶	2.27 × 10 ⁶ ± 1.08 × 10 ⁵	0.79	3.37	0.04	0.15	0.0026
5-Aminopentanoate	3.74 × 10 ⁶	3.73 × 10 ⁶ ± 9.47 × 10 ⁴	3.08 × 10 ⁶	3.11 × 10 ⁶ ± 1.83 × 10 ⁵	0.82	3.36	0.04	0.15	0.0026
L-Glutamate	2.87 × 10 ⁶	2.85 × 10 ⁶ ± 2.25 × 10 ⁵	2.20 × 10 ⁶	2.34 × 10 ⁶ ± 2.07 × 10 ⁵	0.76	3.36	0.04	0.15	0.0021
Nicotinamide	5.32 × 10 ⁶	5.06 × 10 ⁶ ± 4.03 × 10 ⁵	3.52 × 10 ⁶	3.66 × 10 ⁶ ± 2.15 × 10 ⁵	0.66	3.32	0.04	0.15	0.0059
g-Oxalo-crotonate	1.96 × 10 ⁵	1.88 × 10 ⁵ ± 1.61 × 10 ⁴	8.98 × 10 ⁴	9.91 × 10 ⁴ ± 2.49 × 10 ⁴	0.46	3.24	0.05	0.15	0.0004
(S)(+)-Allantoin	4.88 × 10 ⁴	4.76 × 10 ⁴ ± 7.81 × 10 ³	2.30 × 10 ⁴	2.56 × 10 ⁴ ± 5.99 × 10 ³	0.47	3.19	0.05	0.15	< 0.0001
5-Hydroxyisourate	6.62 × 10 ⁵	6.31 × 10 ⁵ ± 6.39 × 10 ⁴	9.14 × 10 ⁵	9.80 × 10 ⁵ ± 7.50 × 10 ⁴	1.38	3.18	0.05	0.15	0.0015

Analytes measured by UHPLC/mass spectrometry from islets and conditioned media after incubation in control or leucine supplemented media (*n* = 4 sets of islets split and incubated in each condition). Analytes are included in this table if their nominal *P* value is ≤ 0.05. Analytes not meeting this criteria are included in the Supplementary Table 1.

Figure 4

Fetal islet and islet media principal component analysis. Fetal sheep islets from four animals were divided into two equal aliquots and incubated in control media (C1–4) or control media supplemented with leucine (10 mmol/L; L1–4). UHPLC was used to determine the relative abundance of 140 analytes in the islets and 120 analytes in the media. Principal component (PC) analysis of the islet analytes (A) and media analytes (B) are shown.

amino acids, not just leucine. Of the seven significantly enriched pathways ($P \leq 0.05$ and an FDR < 0.25), six are directly involved in amino acid metabolism such as nitrogen

Table 3 Metaboanalyst pathway analysis of analytes from the conditioned media.

Pathway	Total compounds	Hits	P-value (raw)	Log(p) × -1	Holm P-value	FDR	Impact
Aminoacyl-tRNA biosynthesis	48	10	4.29E-08	16.965	3.60E-06	3.60E-06	0.1667
Arginine and proline metabolism	38	6	0.00016443	8.713	0.013648	0.005833	0.3393
Arginine biosynthesis	14	4	0.0002083	8.4765	0.017081	0.005833	0.1777
Valine, leucine and isoleucine biosynthesis	8	3	0.00059553	7.4261	0.048238	0.012506	0
Nitrogen metabolism	6	2	0.0074218	4.9033	0.59374	0.1039	0
D-Glutamine and D-glutamate metabolism	6	2	0.0074218	4.9033	0.59374	0.1039	0.5
Vitamin B6 metabolism	9	2	0.017046	4.0718	1	0.20456	0.4902
Alanine, aspartate and glutamate metabolism	28	3	0.025307	3.6767	1	0.26572	0.5345
Glyoxylate and dicarboxylate metabolism	32	3	0.035962	3.3253	1	0.32708	0.0423
Glycine, serine and threonine metabolism	33	3	0.038938	3.2458	1	0.32708	0.2171
Nicotinate and nicotinamide metabolism	15	2	0.045562	3.0887	1	0.34793	0.1943
Histidine metabolism	16	2	0.051323	2.9696	1	0.35926	0
Pyrimidine metabolism	39	3	0.059332	2.8246	1	0.38338	0.1204
Pantothenate and CoA biosynthesis	19	2	0.070037	2.6587	1	0.42022	0
Phenylalanine, tyrosine and tryptophan biosynthesis	4	1	0.0898	2.4102	1	0.50288	0.5
Glutathione metabolism	28	2	0.13613	1.9941	1	0.71469	0.0197
Cysteine and methionine metabolism	33	2	0.17718	1.7306	1	0.8755	0.1263
Phenylalanine metabolism	10	1	0.20998	1.5608	1	0.97988	0.3571
Valine, leucine and isoleucine degradation	40	2	0.23735	1.4382	1	1	0
Butanoate metabolism	15	1	0.29821	1.21	1	1	0
Selenocompound metabolism	20	1	0.37683	0.97596	1	1	0.1591
Beta-alanine metabolism	21	1	0.39149	0.93779	1	1	0
Sphingolipid metabolism	21	1	0.39149	0.93779	1	1	0
Lysine degradation	25	1	0.44686	0.80551	1	1	0
Purine metabolism	65	2	0.45092	0.79646	1	1	0
Porphyrin and chlorophyll metabolism	30	1	0.50922	0.67488	1	1	0
Glycerophospholipid metabolism	36	1	0.57506	0.55328	1	1	0.015
Amino sugar and nucleotide sugar metabolism	37	1	0.58516	0.53586	1	1	0
Tyrosine metabolism	42	1	0.63228	0.45842	1	1	0.1297

Pathways identified by metaboanalyst in the conditioned media after incubation in control or leucine supplemented media ($n = 4$ sets of islets split and incubated in each condition).

metabolism ($P < 0.01$) and the most significantly enriched pathway, the aminoacyl-tRNA biosynthesis pathway ($P < 5 \times 10^{-8}$; Table 3). Key analytes which met the criteria of a nominal P value ≤ 0.05 (Table 2) included leucine (nine-fold higher in leucine supplemented conditioned media, $P < 0.001$) consistent with the experimental design and the HPLC results. Also consistent with the HPLC results were lower phenylalanine, valine, aspartate, methionine, proline, glutamine, serine, threonine, and glutamate. Additionally, analytes such as butanoyl-L-carnitine, acetyl-carnitine, and propionyl-carnitine were all significantly lower in the leucine supplemented group ($P < 0.05$, Table 2). Dopamine was nine-fold higher in the media obtained from leucine supplemented islets compared to non-supplemented islets ($P < 0.005$; Fig. 5). There was a tendency for higher dopamine (five-fold) and lower acetylcholine in leucine supplemented islets as well ($P \leq 0.07$; Fig. 5).

Discussion

The primary goal of this study was to test whether leucine acutely potentiates in vivo fetal GSIS. This goal complements our previous work demonstrating potentiated fetal GSIS with a chronic 9 days leucine infusion (Boehmer *et al.* 2020). That study showed important structural changes in the fetal pancreas. These may have been responsible for the increased GSIS. That study did not determine the impact of an acute fetal leucine infusion on GSIS, nor did it test the impact of supplemental leucine on in vitro fetal islet metabolism (Boehmer *et al.* 2020). Therefore, we also sought to determine if isolated fetal sheep islets have the capacity to oxidize leucine and if leucine can increase cellular metabolism in isolated fetal islets. Our results indicate that isolated fetal sheep islets oxidize leucine as evidenced

Figure 5

Leucine increases islet production of dopamine and decreases acetylcholine. (A) Schematic representation of pathways related to dopamine and acetylcholine regulation of insulin secretion. Underlined text represents higher or lower abundance in leucine supplemented conditions relative to non-supplemented conditions as indicated by the preceding arrow. Abundance of dopamine was higher following leucine supplementation relative to non-supplemented conditions in islets (B) and media (C), relative to non-supplemented conditions. Islet abundance of acetylcholine was lower in leucine supplemented conditions relative to non-supplemented conditions (D). Data were analyzed in Metaboanalyst and presented as means $\pm$ s.e.m. *P* values for paired t-test on four sets of islet incubations are presented as insets in the graphs. #*P* < 0.1, ****P* < 0.005.

by production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ L-leucine in a 2 h incubation. Incubating isolated fetal sheep islets overnight in supplemental leucine demonstrated metabolic effects beyond leucine oxidation. Concentrations of almost all amino acids and glucose in the media obtained following islet incubations without supplemental leucine were lower than the control media that was not exposed to islets. Importantly, supplemental leucine resulted in amino acid and glucose concentrations that were even lower. It is reasonable to assume that the supplemental leucine stimulated cellular amino acid and glucose uptake from the media. The notable exception to this was isoleucine. Isoleucine concentrations were higher in media following overnight incubation with supplemental leucine relative to non-supplemented media. This likely reflects the competitive inhibition of isoleucine transport into the cells by leucine as these two branched chain amino acids (BCAAs) enter cells through similar sets of amino acid transporters (Javed & Fairweather 2019). Our analysis with UHPLC/mass spectrometry is in agreement with the results and demonstrates a pattern consistent with an overall increase in cellular amino acid metabolism.

To demonstrate the capacity of leucine to acutely potentiate GSIS *in vivo*, we performed fetal insulin secretion studies in pregnant sheep. GSIS was measured in five sheep fetuses with a 90-min variable rate, square-wave, fetal hyperglycemic clamp with or without a concurrent acute leucine infusion that began 90 min prior to the hyperglycemic clamp. Prior to the hyperglycemic clamp, the leucine infusion minimally increased insulin

concentrations compared to the control saline infusion. However, the difference in insulin concentrations between the leucine infusion and control saline infusion was pronounced during the hyperglycemic clamp. These data are consistent with our previous reports demonstrating that supplemental leucine on its own is a weak fetal insulin secretagogue (Rozance *et al.* 2006, Boehmer *et al.* 2020), and are also consistent with the minimal changes in media insulin concentrations following islet incubation with supplemental leucine observed in the current study. However, when combined with supplemental glucose, leucine robustly potentiates fetal GSIS.

The relative expression of analytes demonstrated that pathways most impacted by supplemental leucine involved amino acid metabolism, and not just leucine or BCAA metabolism. Over half of the significantly impacted pathways identified with Metaboanalyst using the KEGG database for annotation are directly involved in amino acid metabolism. Most of these pathways are associated with amino acids other than leucine. Amino acid, tricarboxylic acid cycle, and pyrimidine metabolic pathways have all been implicated in the glucose-stimulated insulin secretory response in insulin-secreting cell lines (Huang & Joseph 2012, Lorenz *et al.* 2013). Other interesting findings from the UHPLC/mass spectrometry analysis were decreased acetylcholine and increased dopamine concentrations following incubation with supplemental leucine. Acetylcholine modifies β -cell calcium signaling to enhance insulin secretion, and low intracellular acetylcholine concentrations inhibit insulin secretion

(Gilon & Henquin 2001). Enriched dopamine signaling implicates a dopamine feedback mechanism that also is inhibitory for insulin secretion. Dopamine is co-secreted with insulin from β -cells, and serves as an autocrine signal to inhibit insulin secretion (Shankar *et al.* 2006, Garcia-Tornadu *et al.* 2010, Ustione *et al.* 2013). While these inhibitory signals may seem counter to the potentiation of in vivo and in vitro GSIS, it may be that without excess glucose these pathways are partly responsible for the lack of a consistent or large effect of supplemental leucine only on media insulin concentrations following the overnight islet incubations and on fetal insulin concentrations prior to the commencement of the hyperglycemic clamp despite active metabolism of leucine and other substrates. Once supplemental glucose is provided, these inhibitory pathways are overcome by pathways which stimulate GSIS.

The implications of our findings for the regulation of fetal growth and metabolism by the fetal nutrient supply are several fold. Insulin is a dominant fetal growth hormone, especially during the latter part of gestation (Fowden 1992). Similar to adults, human fetuses also are characterized by the capacity to respond to a hyperglycemic challenge with increased insulin concentrations (Nicolini *et al.* 1990). Numerous studies in fetal sheep have also demonstrated not only GSIS, but also amino acid stimulated insulin secretion (Fowden 1980, Aldoretta *et al.* 1998, Rozance *et al.* 2006, Brown *et al.* 2016) and amino acid potentiated GSIS (Gadhia *et al.* 2013, Brown *et al.* 2016). Therefore, the fetal β -cell can be seen as matching secretion of an anabolic growth hormone (insulin) with the fetal nutrient supply and nutrient availability (Boehmer *et al.* 2017). Disorders of fetal growth, such as intrauterine growth restriction from placental insufficiency, are characterized by lower rates of fetal nutrient supply and lower concentrations of insulin (Nicolini *et al.* 1990, Marconi *et al.* 1999, Paolini *et al.* 2001, Limesand *et al.* 2006, Wai *et al.* 2018). Strategies to improve fetal growth which directly target the pancreatic β -cell to produce more insulin have been considered (Brown *et al.* 2011). We have previously demonstrated that infusion of a complete amino acid mixture, both acutely and chronically, potentiates fetal GSIS in normal and IUGR fetuses (Gadhia *et al.* 2013, Brown *et al.* 2016). In the current study we chose to focus on leucine because of its potential for stimulating pancreatic islet growth, development, and insulin secretion (Rozance & Hay Jr 2016, Boehmer *et al.* 2017); and because of its ability to potentiate fetal GSIS following a chronic 9-day infusion (Boehmer *et al.* 2020). Our results show that supplemental leucine without supplementation of other nutrients has a

minimal impact on fetal insulin concentrations. Strategies which chronically increase the fetal leucine supply with other nutrients like glucose hold more promise for increasing fetal insulin secretion.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0243>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All authors approved the submission of the manuscript and contributed as follows: B H B and P J R conceived of the experimental design; B H B, P R B, and P J R conducted experiments and statistical analyses; B H B and P J R wrote the first draft of the manuscript; B H B, P R B, L D B, S R W, and P J R participated in data analysis, proofread the manuscript, and approved the final manuscript.

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