Lipids and ketones dominate metabolism at the expense of glucose control in pulmonary arterial hypertension: a hyperglycaemic clamp and metabolomics study

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Highly technical metabolic approaches show that fasting nutrient metabolism in pulmonary arterial hypertension favours lipid and ketone metabolism and that, in response to hyperglycaemia, pancreatic β -cell function is similar to control participants <http://bit.ly/2uihG2Q>

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ABSTRACT Individuals with idiopathic pulmonary arterial hypertension (PAH) display reduced oral glucose tolerance. This may involve defects in pancreatic function or insulin sensitivity but this hypothesis has not been tested; moreover, fasting nutrient metabolism remains poorly described in PAH. Thus, we aimed to characterise fasting nutrient metabolism and investigated the metabolic response to hyperglycaemia in PAH.

12 participants (six PAH, six controls) were administered a hyperglycaemic clamp, while 52 (21 PAH, 31 controls) underwent plasma metabolomic analysis. Glucose, insulin, C-peptide, free fatty acids and acylcarnitines were assessed from the clamp. Plasma metabolomics was conducted on fasting plasma samples.

The clamp verified a reduced insulin response to hyperglycaemia in PAH (-53% *versus* control), but with similar pancreatic insulin secretion. Skeletal muscle insulin sensitivity was unexpectedly greater in PAH. Hepatic insulin extraction was elevated in PAH ($+11\%$ *versus* control). Plasma metabolomics identified 862 metabolites: 213 elevated, 145 reduced in PAH ($p < 0.05$). In both clamp and metabolomic cohorts, lipid oxidation and ketones were elevated in PAH. Insulin sensitivity, fatty acids, acylcarnitines and ketones correlated with PAH severity, while hepatic extraction and fatty acid:ketone ratio correlated with longer six-min walk distance.

Poor glucose control in PAH could not be explained by pancreatic β -cell function or skeletal muscle insulin sensitivity. Instead, elevated hepatic insulin extraction emerged as an underlying factor. In agreement, nutrient metabolism in PAH favours lipid and ketone metabolism at the expense of glucose control. Future research should investigate the therapeutic potential of reinforcing lipid and ketone metabolism on clinical outcomes in PAH.

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Introduction

Pulmonary arterial hypertension (PAH) is a debilitating disease characterised by remodelling of the pulmonary vascular bed leading to elevated pulmonary arterial pressure requiring progressive compensation by the right ventricle, and culminates in right heart failure. Despite recent clinical advances, prognosis remains poor with estimated 3-year survival rates of 55–69% in newly diagnosed patients [1, 2]. The pathobiology of PAH is incompletely understood and none of the currently available treatment modalities directly targets the underlying pulmonary vascular remodelling [3]. As such, there remains a dire need to identify the underlying pathophysiology of PAH to improve disease management.

Recent epidemiological observations have identified a disproportionate prevalence of obesity and diabetes comorbidities in individuals with PAH; diseases hallmarked by insulin resistance and poor glycaemic control [4, 5]. Corroborating data from *in vitro*, pre-clinical and biological experiments with humans have implicated metabolic disease in PAH pathogenesis [6]. We previously reported metabolic abnormalities in lipid [7] and carbohydrate metabolism [8, 9] which were associated with poorer clinical outcomes. Importantly, patients with PAH exhibited overt glucose intolerance concomitant with reduced circulating insulin levels in response to an oral glucose tolerance test, suggesting impaired pancreatic β -cell function (*i.e.* reduced insulin secretion) in response to hyperglycaemia [9].

Additionally, ketone metabolism has been associated with the metabolic abnormalities in preclinical models of PAH [10] and humans [11]. Ketogenesis is primarily controlled by the liver in response to hormonal, sympathetic and nutritional input, which regulates circulating ketone concentrations, predominantly β -hydroxybutyrate (β OHB). Another upstream regulator of β OHB is the delivery of lipids to the liver to be oxidised and subsequently converted into β OHB. This regulation of β OHB is particularly relevant in PAH pathophysiology due to the characteristic progression of right heart failure, as the failing heart increasingly relies on β OHB for fuel [12]. Yet, plasma β OHB during fasting and in response to hyperglycaemia has not been characterised in PAH.

Further, methodologically rigorous approaches investigating metabolism are lacking in the PAH field. Poor oral glucose control has been reported in PAH [9, 13]; the underlying mechanisms remain to be defined, but may involve pancreatic β -cell function, skeletal muscle insulin sensitivity, ketogenesis or lipid metabolism. Thus, we utilised a combination of the state-of-the-art hyperglycaemic clamp procedure together with unbiased plasma metabolomics to assess β -cell function, insulin sensitivity and nutrient metabolism in two independent cohorts of PAH and Controls. We hypothesised that individuals with PAH would present with impaired pancreatic function and elevated ketone and lipid metabolism.

Methods and materials

Subjects

64 subjects were recruited as patients of the Cleveland Clinic's Respiratory Institute or from the greater Cleveland area (OH, USA); 12 received a hyperglycaemic clamp to assess pancreatic β -cell function, while 52 underwent untargeted metabolomics. PAH designation was based on pre-existing diagnosis [9]. This research was reviewed and approved by the Cleveland Clinic's Institutional Review Board and informed consent was obtained prior to initiating study procedures.

Pancreatic β -cell insulin secretion

Participants reported to the Cleveland Clinic's Clinical Research Unit following stringent control procedures to minimise the influence of diet and physical activity on metabolic testing [14]. A hyperglycaemic clamp was performed in six PAH and six controls [15] to assess the pancreatic response to hyperglycaemia. Briefly, hyperglycaemia ($180 \text{ mg}\cdot\text{dL}^{-1}$) was rapidly achieved with a primed infusion of intravenous glucose (dextrose, 20%) and maintained by variable rate infusion for 3 h. Blood glucose was measured every 5 min (YSI 2300; STAT Plus, Yellow Springs, OH, USA) and the glucose infusion rate was adjusted according to the algorithm by DeFRONZO *et al.* [16]. With hyperglycaemia, the plasma insulin response is well documented to be biphasic with an initial spike and nadir during the first 10 min (0–10 min, first phase) followed by a linear increase over time (10–180 min, second phase). During the first phase, blood was collected every 2 min; during the second phase, blood was collected every 15 min. Samples were processed and stored at -80°C until analysis.

Plasma analytes

Insulin and C-peptide were assessed *via* radio-immunoassay (#HI-14K and #HCP-20K; Millipore Corporation, Billerica, MA, USA). The insulin:C-peptide molar ratio was calculated to assess hepatic insulin extraction. β OHB was quantified *via* colorimetric assay (#700190; Cayman Chemical, Ann Arbor, MI, USA). Free fatty acids (FFA) and acylcarnitines were quantified by liquid chromatography tandem mass spectrometry (LC/MS/MS). Untargeted metabolomic analysis (Metabolon Inc., Durham, NC, USA)

was performed on fasting plasma samples. Additional details on the LC/MS/MS and metabolic approaches are available in the supplementary material.

Glucose disposal and insulin sensitivity

Glucose infusion rates (GIR) were calculated from the glucose required to maintain hyperglycaemia during steady-state (150–180 min) of the clamp. Insulin sensitivity was estimated as glucose metabolism (M ; $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) normalised to the prevailing insulin concentration (M/I ; $\text{mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}\cdot\mu\text{U}^{-1}\cdot\text{mL}^{-1}$) during steady-state [15]. Indirect calorimetry was performed with an automated system (V_{max} Encore, Viasys HealthCare, Yorba Linda, CA, USA) in a semi-darkened, thermoneutral ($22\pm 1^\circ\text{C}$) environment.

Statistical analysis

Statistical analyses were performed using PRISM7 (GraphPad, La Jolla, CA, USA). Group differences were assessed by two-way repeated measures ANOVA (Group $\times$ Time). *Post hoc* multiple comparison tests were optimised for each analysis (detailed in figure legends). Area under the curve (AUC) was calculated using the trapezoidal method. AUC and baseline comparisons were evaluated by unpaired Student's *t*-test. Pearson's correlation assessed relationships among outcome measures of interest. Normality was determined by the Shapiro–Wilk test ($\alpha=0.05$). Non-normal data were log-transformed. Data are expressed as mean $\pm$ SD. Significance accepted at $p<0.05$.

Metabolomic data are presented as fold-change and box-and-whiskers plots of scaled intensity values. Missing values were imputed with the minimum observed value for each compound. Following log-transformation, one-way ANOVA identified metabolites that differed between groups. To account for multiple comparisons, an estimate of the false discovery rate (*q*-value) was calculated. Significance accepted at $q<0.1$.

Results

Hyperglycaemic clamp

Glucose, insulin and C-peptide

Participants (matched for age, BMI and sex) displayed similar fasting glucose, insulin and C-peptide concentrations (table 1). Basal metabolic rate was elevated in PAH, when normalised for height, weight, age and sex, consistent with previous findings [9]. The insulin response to hyperglycaemia was reduced by 53% in PAH (figure 1b). C-peptide, a marker of pancreatic β cell function and insulin secretion, was similar between groups (figure 1c). The glucose infusion rate required to maintain hyperglycaemia was similar for both groups (figure 1d). Insulin sensitivity (M/I) was elevated by 92% in PAH compared with controls (figure 1e).

Ketogenesis (βOHB) and hepatic insulin extraction

Baseline βOHB was elevated in PAH (+40%; $p=0.018$) while both groups reached similar levels during hyperglycaemia (figure 2a). Reductions in βOHB only reached significance in PAH (60 min, 120 min, 180 min; all $p<0.02$), and βOHB remained greater in PAH throughout the clamp (βOHB AUC, PAH: 13.0 ± 0.7 AU, control: 10.4 ± 0.5 AU; $p=0.016$). Hepatic insulin extraction was greater in PAH during the first and second phase insulin response (figure 2b and c).

FFAs and acylcarnitines

Total FFAs were similar between groups at baseline and decreased equivalently throughout the clamp (figure 3a). Palmitate (C16:0) was the most prevalent lipid species, while palmitoleate (C16:1) was elevated in PAH at baseline (figure 3b). Acetylcarnitine was greater at baseline in PAH while both groups decreased with hyperglycaemia. The reduction was greater in PAH (delta, PAH: -6.3 ± 0.4 μM , control: -4.7 ± 0.5 μM ; $p=0.039$) (figure 3c).

Correlational analysis with metabolic measures

Given these novel and unintuitive nutrient metabolism findings, we investigated relationships between the major metabolic pathways: insulin sensitivity, ketogenesis and hepatic extraction. Insulin sensitivity (M/I) correlated with hepatic extraction (figure 4a) and βOHB throughout the clamp (figure 4b). These relationships remained significant when using first phase hepatic extraction or fasting βOHB as predictive factors for clamp-derived M/I (figure S1a and b). Furthermore, the ratio of fasting free fatty acids to ketones (FFA: βOHB) predicted clamp-derived M/I when controlling for BMI and PAH status ($r^2=0.614$, $p=0.045$; multiple linear regression); the presence of PAH contributed significantly to the model ($p=0.044$) while BMI did not. βOHB correlated with second phase hepatic extraction at baseline (figure 4c) and throughout the clamp (figure 4d). In agreement with known physiology of lipid oxidation and ketogenesis, acetylcarnitine correlated with βOHB (figure 4e).

TABLE 1 Subject characteristics

	Control	PAH	p-value
Subjects n	6 (5 female)	6 (5 female)	
Age years	40±15	49±12	0.32
Height cm	171±7	167±8	0.36
Weight kg	90±18	87±12	0.75
BMI kg·m⁻²	31±4	31±5	0.73
Baseline glucose mg·dL⁻¹	90±11.6	86±3.4	0.40
Baseline insulin µU·mL⁻¹	16±7.1	11±2.9	0.18
Baseline C-peptide ng·mL⁻¹	2.2±0.5	2.0±0.6	0.44
Baseline βOHB mM	0.16±0.03	0.22±0.03	0.02
Baseline FFA µM	857±245	961±408	0.61
HOMA-IR	3.7±2.0	2.4±0.5	0.17
BMR kcals·day⁻¹	1484±317	1519±88	0.82
Baseline RER	0.80±0.03	0.79±0.03	0.81
SBP mmHg	130±4	120±8	0.28
DBP mmHg	76±8	69±10	0.25
Heart rate beats·min⁻¹	69±10	81±13	0.11
O₂ saturation %	99.2±1.3	94.5±2.1	<0.01
Liver panel			
Total protein g·dL ⁻¹	6.8±0.5	6.6±0.5	0.65
Albumin g·dL ⁻¹	3.9±0.3	4.0±0.3	0.84
Total bilirubin mg·dL ⁻¹	0.45±0.14	0.52±0.21	0.54
ALP U·L ⁻¹	55±14	72±20	0.12
AST U·L ⁻¹	19±8	14±4	0.20
ALT U·L ⁻¹	24±13	14±7	0.15

Data are presented as mean±SD, unless otherwise stated. PAH: pulmonary arterial hypertension; BMI: body mass index; βOHB: β-hydroxybutyrate; FFA: free fatty acids; HOMA-IR: homeostatic model assessment of insulin resistance; BMR: basal metabolic rate; RER: respiratory exchange ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; O₂ saturation: oxygen saturated haemoglobin relative to total haemoglobin; ALP: alkaline phosphatase; AST: aspartate transaminase; ALT: alanine transaminase.

Palmitate (C16:0) correlated strongly with total FFAs ($r^2=0.943$, $p<0.001$; figure S2a), suggesting palmitate increases in proportion with total FFA concentrations. In contrast, palmitoleate (C16:1) did not correlate with total FFAs ($r^2=0.051$, $p=0.481$; figure S2b), suggesting alternate regulation of palmitoleate outside of global FFA concentrations. Further, palmitoleate was strikingly well-correlated with βOHB (figure 4f) and M/I (figure S1c and d), implicating a potential relationship with nutrient metabolism in agreement with emerging literature on palmitoleate as a bio-active lipid [17].

Correlational analysis with clinical measures

Table 2 details clinical characteristics of PAH participants. Given the unexpected metabolic findings and the prevailing hypothesis that aberrant metabolism is inherent to PAH pathophysiology, we assessed whether the metabolic abnormalities were associated with functional capacity (6-min walk distance) or measures of right heart dysfunction. 6-min walk distance correlated with both the first (figure S3a) and second phase (figure S3b) hepatic extraction, and with FFA: βOHB (figure S3c). Presence of pericardial effusion correlated with greater βOHB during the clamp (figure S3d). Presence of right atrial dilation correlated with lower hepatic extraction (figure S3e). FFAs during the clamp were associated with higher resting heart rate (figure S3f), while baseline FFAs were related to lower O₂ saturation (figure S3g). M/I correlated with both right ventricular systolic pressure (figure S3h) and N-terminal B-type natriuretic peptide (NT-proBNP) (figure S3i).

Plasma metabolomics

To validate the clamp metabolic findings and clinical associations in a larger cohort, untargeted metabolomic analysis was conducted on 21 idiopathic PAH patients and 31 age-, BMI- and sex-matched controls. Subject characteristics of the metabolomics cohort and scaled intensity values of all 862 targets are available elsewhere [11]. We identified 862 metabolites, of which 358 were different in PAH (213 elevated, 145 reduced). A volcano plot illustrating the metabolite responses are displayed in figure 5a. A heat map displaying the top 30 differentially expressed metabolites is illustrated in figure 5b. Notably, βOHB was five-fold higher in PAH ($q=0.001$, figure 5c). 30 metabolites represent fatty acid oxidation

FIGURE 1 Hyperglycaemic clamp. Hyperglycaemic clamp results for glucose [a], insulin [b] and C-peptide [c]. Research participants were effectively clamped at 180 mg·dL⁻¹ glucose. a) 150–180 min, pulmonary arterial hypertension (PAH): 177±4 mg·dL⁻¹, control: 179±1 mg·dL⁻¹; ns. Insulin concentrations were reduced in PAH compared with control. b) 150–180 min, PAH: 55.2±10.3 μU·mL⁻¹, control: 102.8±12.2 μU·mL⁻¹; two-way repeated measures ANOVA (group×time), significant effect of time and interaction. C-peptide concentrations were similar between groups. c) 150–180 min, PAH: 7.4±0.9 ng·mL⁻¹, control: 8.5±0.8 ng·mL⁻¹; two-way repeated measures ANOVA (group×time), significant effect of time. *: Tukey's *post hoc* test *p*<0.05. d) Glucose infusion rate was similar between groups [PAH: 2.7±0.2 mg·kg⁻¹·min⁻¹, control: 2.9±0.5 mg·kg⁻¹·min⁻¹]. e) Insulin sensitivity (M/I) was elevated in PAH compared with controls (M/I, 150–180 min; PAH: 0.025±0.007, control: 0.013±0.008, *p*=0.024. βOHB: β-hydroxybutyrate; M/I, mg glucose/kg body weight·min⁻¹·μU insulin·mL⁻¹.

(acylcarnitines); 21 were elevated in PAH, including acetylcarnitine (1.7-fold, *q*<0.001; figure 5d). Mirroring clamp data, βOHB and acetylcarnitine strongly correlated (figure S4a). Of 38 fatty acid metabolites, 19 were elevated in PAH, including palmitate (figure S4b), while palmitoleate showed the greatest increase (figure S4c). Seven amino acids have ketogenic capacity of which six were reduced in PAH (trp, tyr, ile, thr, lys, leu; all *q*<0.10; figure S4d). Correlation analysis was conducted between metabolites of interest identified from the clamp study and clinical measures. Right ventricular systolic pressure was associated with βOHB (figure S5a), palmitate (figure S5b) and acetylcarnitine (figure S5c). Grade of tricuspid regurgitation correlated with βOHB (figure S5d) and palmitate (figure S5e). Palmitate was negatively associated with myocardial oxygen consumption (figure S5f). Total free fatty acids were not available for this metabolomics dataset, thus palmitate was used as a surrogate comparison due to its known strong correlation to total FFAs [18] and independent validation in the clamp cohort in this report (figure S2a).

Discussion

This is the first work to use the hyperglycaemic clamp to investigate β-cell function in individuals with idiopathic PAH. We confirmed a decreased insulin response to hyperglycaemia in PAH, corroborating our findings from an oral glucose tolerance test [9]. The novel contribution of this work is that the reduced insulin response to hyperglycaemia was not attributable to reduced pancreatic β-cell insulin secretion or skeletal muscle insulin sensitivity compared with age-, BMI- and sex-matched controls. Rather, hepatic insulin extraction was significantly elevated in PAH, suggesting hepatic insulin extraction may contribute

FIGURE 2 β -Hydroxybutyrate and hepatic insulin extraction. a) The ketone β -hydroxybutyrate was elevated in pulmonary arterial hypertension (PAH) through 30 min of hyperglycaemia, reaching similar levels as controls by 60 min. [PAH: baseline 0.22 ± 0.02 mM, 180 min 0.12 ± 0.01 mM; control: baseline 0.16 ± 0.01 mM, 180 min 0.12 ± 0.01 mM; two-way repeated measures ANOVA, group $p=0.018$, time $p<0.001$, interaction $p<0.001$]. *: different between groups, Tukey's *post hoc* test $p<0.05$; #: different from baseline within PAH group, Dunnett's multiple comparisons test $p<0.02$. Hepatic insulin extraction was significantly greater in PAH compared with controls during the first phase insulin response [b] 0–10 min; PAH: $84.7 \pm 1.5\%$, control: $77.1 \pm 2.5\%$, $p=0.028$ and through prolonged hyperglycaemia [c] 10–180 min; PAH: $84.4 \pm 1.8\%$, control: $73.5 \pm 2.5\%$, $p=0.033$; significance determined by unpaired Student's *t*-test. Hepatic insulin extraction, molar ratio of plasma insulin/plasma C-peptide expressed as a percentage.

to the poor oral glucose tolerance in observed PAH pathophysiology. Another primary contribution of this work was defining preferential lipid and ketone metabolism associated with PAH clinical measures, along with replicating these findings in a larger cohort using a plasma metabolomics approach. Fatty acid oxidation (acetylcarnitine) and ketogenesis (β OHB) were elevated in PAH, while maintaining a greater propensity to convert available free fatty acids to ketones (defined by the FFA: β OHB ratio) was associated with improved exercise endurance, an important clinical marker in PAH. These data suggest an adaptive shift in nutrient metabolism towards fatty acid and ketone utilisation at the expense of glucose control in PAH.

Our interpretations are based upon the framework that glucose control is primarily regulated by the hormone insulin, governed by three critical control points: pancreatic insulin secretion; hepatic insulin extraction; and skeletal muscle insulin sensitivity. Upon induction of hyperglycaemia, insulin is secreted by the pancreatic β -cells. Prior to reaching arterial circulation, insulin is partially sequestered by hepatic insulin extraction, modifying the circulating insulin concentration. Finally, circulating insulin acts on peripheral tissues to facilitate tissue-specific glucose disposal, skeletal muscle being the largest consumer, whereby the insulin sensitivity of skeletal muscle partially dictates the magnitude of this response. By this manner, these three primary tissues are factors underlying glucose control in PAH: pancreatic insulin secretion, hepatic insulin extraction and skeletal muscle insulin sensitivity. An integrated working model of our findings at these critical control points is illustrated in figure 6, including equivalent pancreatic insulin secretion, elevated hepatic insulin extraction and increased skeletal muscle insulin sensitivity in PAH compared with age-, BMI- and sex-matched controls.

Liver: insulin extraction and ketogenesis

Important to the interpretation of these results, the control group was overweight/obese and sedentary and presented with classic signs of early stages of insulin resistance: elevations in FFAs and insulin secretion

FIGURE 3 Free fatty acids and lipid oxidation. **a)** Total plasma free fatty acids were similar between groups at baseline (pulmonary arterial hypertension [PAH]: $961 \pm 167 \mu\text{M}$; control: $857 \pm 100 \mu\text{M}$) and decreased equally with hyperglycaemia (180 min; PAH: $111 \pm 30 \mu\text{M}$, control: $89 \pm 13 \mu\text{M}$; two-way repeated measures ANOVA, time $p < 0.001$). **b)** Lipidomic profiling of free fatty acids revealed elevated palmitoleate (16:1) in PAH (PAH: $37.3 \pm 4.9 \mu\text{M}$; control: $22.1 \pm 2.9 \mu\text{M}$; $p = 0.023$). **c)** Free-, butyryl- and propionyl-carnitines were similar between groups and remained unchanged at 120 and 180 min of hyperglycaemia. Acetylcarnitine was elevated in PAH. Both groups decreased acetylcarnitine similarly in response to hyperglycaemia. (PAH: baseline $10.2 \pm 0.7 \mu\text{M}$, 120 min $5.3 \pm 0.6 \mu\text{M}$, 180 min $3.9 \pm 0.4 \mu\text{M}$; control baseline $7.7 \pm 0.5 \mu\text{M}$, 120 min $4.0 \pm 0.4 \mu\text{M}$, 180 min $3.0 \pm 0.2 \mu\text{M}$, two-way repeated measures ANOVA, group $p = 0.033$, time $p < 0.001$, interaction $p < 0.001$, Tukey's multiple comparisons tests, within group (Baseline versus 120 min versus 180 min), all $p < 0.05$). *: different between groups $p < 0.05$; #: different from baseline $p < 0.05$; †: Sidak's multiple comparison test, different from baseline, $p < 0.05$.

with reductions in fasting βOHB [19, 20] and hepatic insulin extraction [21, 22]. Pancreatic β -cell function (*i.e.* insulin secretion) was similar between groups, suggesting PAH patients are progressing along the natural history of diabetes. Typically, hepatic extraction is progressively reduced along this continuum, thus, the finding of greater hepatic insulin extraction in PAH was unexpected. To our knowledge, this is the first report suggesting PAH presents with elevated hepatic insulin extraction in response to hyperglycaemia. Given neither pancreatic β -cell insulin secretion nor skeletal muscle insulin sensitivity were indicative of poor glucose control, it remains plausible that the elevation in hepatic insulin extraction plays a role in the poor oral glucose control observed in PAH [23]. Future research should utilise more direct approaches to assess hepatic insulin extraction, such as stable isotopic tracers, invasive portal vein sampling or rigorous modelling calculations in combination with an oral glucose tolerance test [24], to further pursue this line of inquiry in PAH metabolism.

Another important role of the liver in metabolic health is hepatic ketogenesis. Fasted circulating βOHB reflects hepatic ketogenesis [25], thus the elevated βOHB observed in the PAH group is indicative of increased ketogenesis. This agrees with the literature on the metabolism of heart failure [12, 26] where ketones are preferentially utilised by cardiac tissue as an adaptive response to declining heart function [27], as ketones are a more energetically favourable fuel source [28]. Fasting hepatic ketogenesis also positively correlated with hepatic insulin extraction in this population. This relationship between these two hepatic processes is both novel and intriguing because ketogenesis is partly regulated by insulin. Yet, it remains unknown whether the insulin extracted by hepatocytes remains inert prior to degradation, or instead, first induces local insulin signalling within hepatocytes (*e.g.* inhibiting ketogenesis).

FIGURE 4 Correlational analysis with metabolic measures. To gain insight into nutrient metabolism in pulmonary arterial hypertension (PAH), we interrogated relationships between the primary metabolic findings. Insulin sensitivity (M/I) correlated strongly with the (a) hepatic insulin extraction (second phase, $r^2=0.498$, $p=0.010$) and (b) β -hydroxybutyrate (β OHb) ($r^2=0.504$, $p=0.010$) during the clamp. β OHb concentrations correlated with second phase hepatic extraction at (c) baseline ($r^2=0.402$, $p=0.027$) and (d) throughout the clamp ($r^2=0.467$, $p=0.014$). β OHb expectedly correlated with (e) acetyl carnitine ($r^2=0.452$, $p=0.017$) and additionally with (f) palmitoleate ($r^2=0.527$, $p=0.008$). M/I, mg glucose·kg body weight⁻¹·min⁻¹· μ U insulin·mL⁻¹.

An additional regulator of ketogenesis is delivery of FFAs to the liver *via* adipose tissue lipolysis [29], whereby increased FFAs are associated with increased ketones in healthy physiology. To address whether the observed elevation in fasting hepatic ketogenesis was merely a reflection of elevated FFAs, we measured fasting free fatty acids, showing no differences between groups. To address whether the ability to convert prevailing free fatty acids to ketones was related to clinical outcomes in PAH, we calculated a ratio of free fatty acids to ketones (FFA: β OHb) and assessed correlations with primary clinical measures. Independently, FFA or β OHb are associated with poorer clinical measures in this study. In contrast, the FFA: β OHb ratio was negatively associated with clinical severity, suggesting that a greater ability to convert available FFAs to ketones is indicative of better clinical health in PAH. Although ketones have previously been viewed as a mere by-product of metabolism, recent research has implicated ketones as a regulating factor in glucose and lipid metabolism [30], along with possessing independent signalling properties [31]. Taken together, the FFA: β OHb ratio may provide a novel characterisation of metabolism in both PAH and isolated metabolic disease. Further, the physiological relationship between β OHb concentrations and hepatic insulin extraction have, to our knowledge, not been previously reported. This provides incentive to investigate the relationship between hepatic ketogenesis and hepatic insulin extraction in other clinical settings.

TABLE 2 Clinical characteristics of PAH patients (clamp study, n=6)

	Measured value
NYHA class	
II	5
III	1
Echocardiography	
RV dilation present	5
RV dysfunction present	2
RA dilation present	3
RVSP mmHg	71.3±12.8
Pericardial effusion present	3
NT-proBNP pg·mL⁻¹	80.8±24.4
Medications	
Sildenafil	1
Tadalafil	3
Bosentan	1
Macitentan	2
Treprostinil	5
6MWT m	411.7±25.6
Right heart catheter data	
RAP mmHg	8.2±1.3
mPAP mmHg	48.2±4.8
PAWP mmHg	11.5±2.4
Cardiac index L·min ⁻¹ ·m ²	3.08±0.18
PVR Wood units	6.2±0.7

Data are presented as n or mean±SEM. PAH: pulmonary arterial hypertension; NYHA: New York Heart Association functional class; RV: right ventricle; RA: right atrium; RVSP: right ventricular systolic pressure; NT-proBNP: N-terminal pro B-type natriuretic peptide; 6MWT: 6-minute walk test; RAP: right atrial pressure; mPAP: mean pulmonary arterial pressure; PAWP: pulmonary arterial wedge pressure; PVR: pulmonary vascular resistance.

Skeletal muscle: insulin sensitivity

Glucose infused during the clamp is actively transported into skeletal muscle, and in the case of the hyperglycaemic clamp, provides a proxy estimate of skeletal muscle insulin sensitivity (M/I) [15]. This measure of insulin sensitivity is classically reduced in concert with oral glucose tolerance, where individuals with poor oral glucose tolerance are expected to also have poor insulin sensitivity. We and others have previously identified poor oral glucose tolerance in PAH [9, 13], thus it was an unexpected to observe increased skeletal muscle insulin sensitivity in PAH compared with controls. Several explanations for elevated insulin sensitivity in PAH may exist. First, pulmonary arterial hypertension increases basal metabolic rate [9], which was observed in this study when controlling for height, weight, age and sex. We therefore performed an additional analysis to control insulin sensitivity for basal metabolic rate, with unremarkable effects (figure S6a). Another explanation may be an effect of hypoxia in PAH or the overweight/obese control group, which may enhance [32] or impair [33] insulin sensitivity. It is also possible that medications specific to PAH increase insulin sensitivity or blood flow, however, we did not find any relationships with medications and insulin sensitivity within this small cohort.

The FFA:βOHB ratio was associated with improved clinical measures and predicted M/I after controlling for BMI and PAH status. This suggests the hepatic capacity to convert FFAs to ketones may be a novel factor in skeletal muscle insulin sensitivity. Insulin sensitivity was also predicted by hepatic extraction, which is in agreement with recent human literature [34] and instils additional confidence in the validity of these unexpected results. Reductions in ketogenic amino acids in concert with elevated ketones is particularly noteworthy, as fasted-state amino acids primarily originate from muscle tissue and loss of muscle mass is common in advanced PAH and heart failure. This observation begs the question as to whether exogenous provision of ketogenic precursors could protect PAH-induced muscle catabolism and should be pursued further.

Ultimately, larger studies utilising clamp-derived insulin sensitivity are warranted to verify the observation of elevated skeletal muscle insulin sensitivity in PAH compared to age, BMI and sex-matched controls. The addition of a lean, healthy control group, representative of normal skeletal muscle insulin sensitivity, would substantially strengthen the study design. Importantly, identifying the metabolism underlying poor

FIGURE 5 Untargeted plasma metabolomics. a) Volcano plot (excluding metabolites from drug or environmental exposure) representing fold-change of log scaled data, $q < 0.10$. b) Heat map of top 30 differentially expressed metabolites. Both (c) β -hydroxybutyrate (β OHB) and (d) acetylcarnitine were elevated in pulmonary arterial hypertension (PAH) compared with control. Box-and-whiskers plots presented using the Tukey method.

oral glucose tolerance in the context of increased skeletal muscle insulin sensitivity would be an impactful contribution to our understanding of PAH pathophysiology.

FFAs and fat oxidation

Fasting FFAs were similar between groups, but notably, higher than levels typically reported for healthy, non-obese individuals [35, 36]. PAH and controls reduced FFAs similarly during the clamp and, given FFA concentrations are primarily driven by rates of adipose tissue lipolysis, this suggests the adipose tissue of PAH and controls respond similarly to insulin. To gain deeper insight into fatty acid metabolism, we performed short-chain acylcarnitine analysis. Acetylcarnitine, an indicator of complete fatty acid oxidation, was elevated in PAH and was associated with β OHB in both clamp and metabolomics cohorts, supporting the interpretation of preferential lipid and ketone metabolism in PAH. An interesting finding was elevated palmitoleate in PAH, which has been reported to impart beneficial effects on insulin sensitivity and cardiovascular health [37], providing a potential mechanism for the elevated insulin sensitivity in PAH and adding to the growing interest of palmitoleate in metabolic research.

Clinical correlates and implications

Distance walked during a 6-min walk test is an important predictor of mortality and clinical outcomes in PAH, and in the current study was correlated with hepatic insulin extraction, implicating a role for nutrient metabolism in PAH clinical outcomes, not just metabolic health. In both clamp and metabolomic cohorts, FFAs and β OHB were independently associated with poorer clinical measures, while the FFA: β OHB ratio correlated with improved clinical measures. In the context of the literature, these results suggest increased FFAs and β OHB are an early metabolic adaptation in response to the heart failure associated with PAH.

A prioritisation of FFA and β OHB fuel sources requires circulating insulin to be maintained at lower concentrations. In physiological agreement, we observed markedly increased skeletal muscle insulin sensitivity in PAH, permitting equivalent glucose disposal with less than half the insulin concentrations of controls. The factors responsible for maintaining reduced insulin in PAH physiology are unclear, though analysis of C-peptide and insulin relationships implicates elevated hepatic insulin extraction. The

FIGURE 6 Summary of metabolic findings in pulmonary arterial hypertension (results compared with age-, sex- and body mass index-matched controls). The underlying physiology of pulmonary arterial hypertension (PAH) is characterised by elevated blood pressure in the arteries of the lungs, which puts stress on the right ventricle, culminating in progressive heart failure. Poor oral glucose control in PAH has been observed in the literature, but the underlying factors remain to be fully elucidated. Importantly, glucose control is primarily regulated by the hormone insulin, which dictates the disposal of glucose from the blood into peripheral tissues. The impact of insulin-mediated glucose control in response to hyperglycaemia is primarily regulated by three critical control points: pancreatic β -cell insulin secretion, hepatic insulin extraction and skeletal muscle insulin sensitivity. Here, we assessed fasting metabolism in PAH along with the insulin response to hyperglycaemia induced by the gold-standard hyperglycaemic clamp technique. In PAH, fasting metabolism was shifted towards utilisation of ketones and lipids, despite similar insulin levels compared to controls. During experimentally induced hyperglycaemia, we observed reduced circulating insulin in PAH (fig. 1b). We further observed that 1) pancreatic β -cell insulin secretion was equivalent between PAH and controls, 2) hepatic insulin extraction was elevated in PAH and 3) skeletal muscle insulin sensitivity was increased in PAH.

regulation of hepatic extraction is not well understood [38, 39], but several possibilities exist, including liver congestion [40] caused by elevated right atrial pressure, autonomic control of the liver [41], changes in liver blood flow [29] or side effects of PAH medications [42].

It also remains to be resolved if these metabolic observations are regulated in coordinated fashion or merely coincidental findings related to the complex physiology and pharmacotherapy in PAH. However, we speculate that elevated hepatic insulin extraction may be a physiologic means to maintain low insulin levels in the context of elevated peripheral insulin sensitivity and thus support ketone metabolism to supply fuel-efficient ketones to cardiac tissue [27, 28], an adaptation that may be necessary to compensate for declining heart function, but which inadvertently contributes to poor glucose control in individuals with PAH and overweight/obesity.

These data agree with the left heart failure literature, evidencing sustained β -cell function with elevated hepatic insulin extraction [43]. However, the left heart failure literature primarily uses oral glucose testing and typically shows lower fasting insulin levels in populations with more severe heart failure compared with our study population. The novelty in this study is the use of the hyperglycaemic clamp, which by design, creates hyperglycaemia while avoiding the contribution of gut factors (*i.e.* gut peptides) and gastric emptying present with oral glucose procedures. The observed reductions in circulating insulin and elevated hepatic insulin extraction in response to hyperglycaemia support the repurposing of some anti-diabetic agents for consideration in future PAH research. Special interest should be noted for the sodium glucose co-transporter-2 (SGLT2) inhibitors or glucagon-like peptide 1 (GLP1) receptor agonists, as they improve glucose control while having independent cardioprotective effects [44]. Similarly, research is needed to determine the impact of medical nutrition therapy to reduce glucose excursions and support lipid and ketone metabolism on PAH-related outcomes.

Limitations

Due to complexity and patient burden, the hyperglycaemic clamp was limited to a sample size of 12 participants, yet we show strong reproducibility of results with prior work. Furthermore, we validated the primary findings in a separate cohort of 52 participants using unbiased plasma metabolomics. Our interpretations on nutrient metabolism were based on plasma concentrations and thus, we cannot address nutrient flux (production *versus* disposal) or other potentially confounding factors like endogenous glucose production, glucose effectiveness or lipid turnover, which require stable isotope tracer approaches. Hepatic/extrahepatic extraction is difficult to directly quantify without portal vein sampling, thus we utilised a commonly reported surrogate measure (molar ratio of insulin:C-peptide) [45, 46]; additional considerations and limitations on this calculation of hepatic insulin extraction are available elsewhere [47, 48]. M/I assumes a linear response of glucose disposal to increasing insulin concentrations, which has been evidenced within the insulin concentrations observed in this study and remains commonly reported from hyperglycaemic clamps [49, 50]. Hyperinsulinaemic-euglycaemic clamps in combination with oral glucose tolerance testing should be used to verify these results. The clamp cohort was primarily female, and although this is consistent with the predominantly female population of individuals with PAH, we conducted a separate analysis removing the male participants. This analysis revealed unremarkable effects (table S1). Finally, elevated basal metabolic rate is common to PAH pathology and may impact nutrient metabolism, including insulin sensitivity and ketogenesis. We therefore performed an additional analysis to adjust M/I and β OHB for basal metabolic rate, again showing unremarkable effects (figure S6ab). Despite these limitations, this research provides novel insight into the underlying metabolism present in PAH pathophysiology.

Conclusion

This research provides finer detail of the metabolic underpinnings of PAH, and suggests that the poor oral glucose control is impacted by elevated hepatic insulin extraction rather than defects in pancreatic β -cell function or skeletal muscle insulin sensitivity. This is the first work to utilise the highly technical hyperglycaemic clamp to assess β -cell function in PAH, isolating the insulin response to hyperglycaemia *per se*. Finally, these data support the narrative that the shift in nutrient metabolism (preferential lipid and ketone utilisation at the expense of glucose control) is a beneficial adaptation to compensate for the failing right heart. The metabolic implications of this work may help inform future trials investigating therapeutic approaches to improve PAH outcomes, including supporting lipid and ketone metabolism and minimising glucose excursions through dietary, lifestyle or pharmacological strategies.

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