

1 **Title page**

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4 **The von Hippel-Lindau Chuvash mutation in mice alters cardiac substrate and**  
5 **high energy phosphate metabolism**

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8 Mary Slingo, Mark Cole, Carolyn Carr, Mary K. Curtis, Michael Dodd, Lucia Giles, Lisa C  
9 Heather, Damian Tyler, Kieran Clarke, Peter A Robbins.

10

11 All affiliations:

12 Department of Physiology, Anatomy and Genetics. University of Oxford,

13 Sherrington Building,

14 Parks Road,

15 Oxford,

16 OX1 3PT

17 UK.

18

19 MS – designed and performed study, performed data analysis, wrote the manuscript.

20 MC – designed and performed study, performed data analysis.

21 CC – performed *in vivo* cine MRI and assisted with analysis.

22 KC – performed gene analysis and assisted with analysis.

23 MD and LG – performed hyperpolarized MRI study and its analysis.

24 LH – assisted with design of study, radiolabelled substrate perfusion, and assisted with  
25 analysis.

26 DT – assisted with cine MRI and hyperpolarized MRI and data analysis.

27 KC and PR – designed study, assisted with data analysis, reviewed the manuscript.

28

29 **Corresponding author for production only:**

30 Dr Mary Slingo

31 +44 7900 953919

32 [maryslingo@doctors.org.uk](mailto:maryslingo@doctors.org.uk)

33

34 **Corresponding author upon publication:**

35 Prof Peter A Robbins

36 Peter.robbins@dpag.ox.ac.uk. Tel: +44 (0)1865 272490.

**37 Abstract**

38

39 Hypoxia-inducible factor (HIF) appears to function as a global master regulator of  
40 cellular and systemic responses to hypoxia. HIF-pathway manipulation is of therapeutic  
41 interest, however global, systemic upregulation of HIF may have as yet unknown effects  
42 on multiple processes. We utilized a mouse model of Chuvash polycythemia (CP), a rare  
43 genetic disorder which modestly increases expression of HIF target genes in normoxia,  
44 to understand what these effects might be within the heart.

45

46 An integrated *in* and *ex vivo* approach was employed. In comparison to wild-type  
47 controls, CP mice had evidence (using *in vivo* MRI) of pulmonary hypertension, right  
48 ventricular hypertrophy, and increased left ventricular ejection fraction. Glycolytic flux  
49 (measured using  $^3\text{H}$  glucose) in the isolated, contracting, perfused CP heart was 1.8-fold  
50 higher. Net lactate efflux was 1.5-fold higher. Furthermore, *in vivo*  $^{13}\text{C}$  magnetic  
51 resonance spectroscopy (MRS) of hyperpolarized  $^{13}\text{C}_1$  pyruvate revealed a 2-fold  
52 increase in real-time flux through lactate dehydrogenase in the CP hearts, and a 1.6-fold  
53 increase through pyruvate dehydrogenase.  $^{31}\text{P}$  MRS of perfused CP hearts under  
54 increased workload (isoproterenol infusion) demonstrated increased depletion of  
55 phosphocreatine relative to ATP. Intriguingly, no changes in cardiac gene expression  
56 were detected.

57

58 In summary, a modest systemic dysregulation of the HIF pathway resulted in clear  
59 alterations in cardiac metabolism and energetics. However, in contrast to studies  
60 generating high HIF levels within the heart, the CP mice showed neither the predicted  
61 changes in gene expression nor any degree of LV impairment. We conclude that the  
62 effects of manipulating HIF on the heart are dose-dependent.

63

64

**65 New and noteworthy**

66

67 This is the first integrative metabolic and functional study of the effects of modest HIF  
68 manipulation within the heart. Of particular note, the combination (and correlation) of  
69 perfused heart metabolic flux measurements with the new technique of real-time *in vivo*  
70 MR spectroscopy using hyperpolarized pyruvate is a novel development.

71

72

73 **Keywords**

74

75 Hypoxia-inducible factor, cardiac metabolism, MRI, hyperpolarized pyruvate, Chuvash

76 polycythemia

**77 Introduction**

78

79 Prolonged hypoxia results in diverse changes within multiple organ systems -  
80 ventilatory acclimatization, increased erythropoiesis, pulmonary vascular remodeling,  
81 and metabolic alterations occur(54). Many of these diverse cellular and systemic  
82 responses to hypoxia are, or are likely to be, coordinated by the hypoxia-inducible factor  
83 (HIF) family of transcription factors. HIF is a heterodimer, comprising an oxygen-  
84 regulated alpha subunit (HIF-1 $\alpha$ , HIF-2 $\alpha$ , or HIF-3 $\alpha$ ) and a ubiquitous beta subunit (HIF-  
85 1 $\beta$ ). The stability of HIF- $\alpha$  subunits is regulated by oxygen-dependent prolyl  
86 hydroxylation (by prolyl-4-hydroxylase domain proteins, PHDs) which enables  
87 recognition by the von Hippel-Lindau (VHL) ubiquitin E3 ligase and subsequent  
88 degradation via the ubiquitin-proteasome pathway(31, 54). Hence, HIF appears to  
89 function as a global master regulator of cellular and systemic responses to hypoxia in all  
90 metazoan species studied to date(36).

91

92 There is considerable interest in understanding further the role of HIF in both individual  
93 organ systems and within integrative physiology, driven in part by the potential benefits  
94 of VHL-HIF pathway manipulation in the treatment of cancer and vascular disease(49).  
95 For example, global, systemic upregulation of HIF may have as yet unknown effects on  
96 multiple cellular and physiological processes. We sought to understand what these  
97 effects might be within the heart.

98

99 Exposure of animals and humans to sustained hypoxia results in a consistent pattern of  
100 myocardial alterations in metabolic gene expression, substrate utilization, energetics,  
101 and function. The dynamic changes in gene expression increase the capacity for glucose  
102 uptake(63) and utilization(1, 62), whilst capacity for fatty acid utilization is  
103 reduced(62). Indeed, myocardial glucose uptake is increased in high-altitude  
104 natives(21) and in rats exposed to hypobaric hypoxia(28). These alterations in substrate  
105 utilization may affect myocardial energetics and function – lower phosphocreatine  
106 (PCr):ATP ratios were observed in Sherpa hearts(20), in lowlanders exposed to 20  
107 hours of normobaric hypoxia (with accompanying diastolic dysfunction)(22), and in  
108 trekkers travelling to Everest base camp at 5300 m (also with impaired diastolic  
109 function)(23).

110

111 It is of note that the cardiac metabolic changes seen in response to hypoxia are similar to  
112 those observed in heart failure(34, 43-45). Indeed, it has been hypothesized that

113 myocardial hypoxia, with consequent activation of the HIF pathway, may play a role in  
114 altering cardiac substrate utilization, energetics and contractile function; these changes  
115 may then cause or exacerbate heart failure. This hypothesis has been explored in  
116 experiments producing very high levels of HIF in the heart, from which it is clear that  
117 major alterations in HIF give rise to metabolic changes and cardiomyopathy(7, 24, 29,  
118 35, 40, 41). However, these experiments do not reveal whether more modest alterations  
119 in HIF, particularly at a systemic level, produce similar results.

120

121 Chuvash polycythemia (CP) is a rare autosomal recessive disorder with the potential to  
122 address this question. CP arises from a single point mutation in *VHL* which diminishes  
123 the binding affinity of the protein for hydroxylated HIF-1 $\alpha$  and HIF-2 $\alpha$ , increasing the  
124 expression of HIF target genes under normoxic conditions(2, 14). Patients with CP  
125 develop polycythemia, pulmonary hypertension, increased ventilatory and pulmonary  
126 vascular sensitivity to hypoxia, and altered skeletal muscle metabolism and  
127 energetics(14, 65), although any effects on cardiac function remain unidentified. Many  
128 aspects of the human disease have been shown to be faithfully recapitulated in the  
129 mouse model(18, 19, 39, 64).

130

131 The CP mouse therefore provides a unique opportunity to study the effects on the heart  
132 of long term, systemic activation of the HIF pathway at more 'physiological' levels,  
133 without the confounding influence of periods of reduced oxygen availability through  
134 hypoxia. The purpose of the present study was to investigate the cardiac metabolic and  
135 functional phenotype of CP, utilizing both *in-* and *ex-vivo* techniques.

136

137 **Methods**

138 *Animals.* CP breeding pairs were donated, and the original mutation was generated on a  
139 C57BL6 background as described previously(18). CP and wild type (homozygous, WT)  
140 mice (from the same breeding colony of mice heterozygous for the *VHL* Chuvash  
141 mutation) were used for all comparisons. All animal procedures were compliant with  
142 both the UK Home Office Animals (Scientific Procedures) Act 1986 and the Local Ethical  
143 Review Procedures (University of Oxford Medical Sciences Division Ethical Review  
144 Committee). Mice were housed within individually ventilated cages, in room air, with  
145 free access to food and water.

146

147 *Hematology and analysis of plasma metabolites.* Following excision of the heart for  
148 perfusion, blood was collected immediately from the chest cavity. Hematocrits, in a  
149 heparinized capillary tube, were measured using a hematocrit centrifuge (Hettich,  
150 Germany); hemoglobin was measured using a HemoCue Hb 201+ (HemoCue, UK). The  
151 remaining blood was centrifuged at 4 °C and the plasma subsequently frozen in liquid  
152 nitrogen. All plasma analysis was performed on an ABX Pentra 400 Clinical Chemistry  
153 analyzer (Horiba ANX S.A.S, UK).

154

155 *Cardiac magnetic resonance imaging (MRI).* Anesthesia was induced using 5%, and  
156 maintained with 1 – 2%, isoflurane in 100% O<sub>2</sub>. Respiratory rate and ECG were  
157 monitored continuously. Cine MRI was performed as described previously(58). Mice  
158 were placed in a purpose-built cradle, which was lowered into a vertical-bore 11.7 T  
159 (500 MHz) system (MagneX, UK) with a 40 mm birdcage coil (Bruker, Germany). A stack  
160 of contiguous, 1 mm thick, true short axis ECG- and respiration-gated cine/FLASH  
161 images were acquired to cover the entire heart. Image data were analyzed using the  
162 ImageJ software (NIH Image, USA). End-systole (ES) and end-diastole (ED) frames for  
163 each slice were identified and the LV endo- and epi-cardial borders outlined in all slices  
164 to give values for ESV (end-systolic volume) and EDV. Stroke volume ( $SV = EDV - ESV$ ),  
165 ejection fraction ( $EF = (SV/EDV) \times 100$ ), and cardiac output ( $CO = SV \times \text{heart rate}$ ) were  
166 calculated. LV mass was calculated as the product of the LV volume and the specific  
167 gravity of myocardium (1.05 g/cm<sup>3</sup>). The mid-ventricular (MV) slice was defined as the  
168 one in which the papillary muscles were most prominent; right ventricle (RV) free wall  
169 thickness was measured in three separate locations in this slice, and subsequently  
170 averaged.

171

172 To quantify septal bowing (the distortion of the LV), a novel method was used. First, the  
173 LV epi-cardial border in early-diastole in the MV slice was outlined, giving an actual  
174 perimeter length ( $P_A$ ) and area ( $A_A$ ). The maximum possible area enclosed by a  
175 perimeter of any given length arises when the figure is a circle. For a perimeter of length  
176  $P_A$ , we denote this area as  $A_C$ . If the LV were perfectly circular, then the ratio of  $A_A$  to  $A_C$   
177 would be 1. As the LV becomes increasingly distorted in shape  $A_A$  falls relative to  $A_C$ , and  
178 hence the ratio will fall below 1. This 'septal bowing ratio' thus enables quantification of  
179 the distortion of the septum using the LV itself as the comparator. We chose to quantify  
180 septal bowing in this way, as it is an approach that is likely to be relatively insensitive to  
181 observer variability. We expect that a relatively linear relationship would exist between  
182 this index and other indices of septal bowing, such as the ratio between the long and  
183 short axes in a short-axis ventricular slice (as introduced by Ryan et al.(56), and which  
184 correlates with pulmonary artery pressure in patients with pulmonary hypertension),  
185 but this has not been tested directly.

186

187 *Gene expression (Real-Time PCR)*. Total RNA was extracted from 20 – 30 mg powdered  
188 (in liquid nitrogen) whole-heart tissue (following Langendorff perfusion) and whole-  
189 lung tissue. Total RNA was extracted using the Rneasy Fibrous Kit (Qiagen, UK),  
190 including a Dnase treatment step, and complementary DNA immediately synthesized  
191 from 1  $\mu$ g RNA using the Applied Biosystems High Capacity cDNA Reverse Transcription  
192 Kit (Life Technologies, UK). Real-Time PCR was performed using an ABI Prism 7000  
193 Sequence Detection System (Applied Biosystems, UK) with TaqMan Universal PCR  
194 Master Mix and TaqMan Gene Expression Assays (choosing manufacturer-  
195 recommended assays: Applied Biosystems, UK). Relative quantification of mRNA  
196 expression levels was determined using the standard curve method and normalized to  
197 beta-actin (heart tissue) or 18s ribosomal RNA (lung tissue).

198

199 *Gene expression (Micro-array)*. Total RNA was extracted from 20 – 30 mg powdered (in  
200 liquid nitrogen) whole-heart tissue using the Rneasy Fibrous Kit (Qiagen, UK), including  
201 a Dnase treatment step. The RNA integrity was assessed on a BioAnalyzer (Agilent  
202 Laboratories, US); all samples had a RNA Integrity Number (RIN)  $\geq$  7. Labelled sense  
203 ssDNA for hybridisation was generated from 200 ng starting RNA with the Ambion WT  
204 expression kit (P/N 4411973) and the Affymetrix GeneChip WT Terminal Labeling and  
205 Controls Kit (P/N 901525) according to the manufacturer's instructions. The  
206 distribution of fragmented sense ssDNA lengths was measured on the BioAnalyser. The  
207 fragmented ssDNA was labeled and hybridised for 17 hours at 45 °C to the Affymetrix

208 GeneChip Human Mouse 1.0 ST Array (Affymetrix). Chips were processed on an  
209 Affymetrix GeneChip Fluidics Station 450 and Scanner 3000. Affymetrix Command  
210 Console was used to generate cel files and Affymetrix Expression Console was used for  
211 the quality control. Arrays were RMA normalised in GeneSpring GX 12 and differentially  
212 expressed genes were identified using Limma with a Benjamini and Hochberg multiple  
213 testing correction of  $\leq 0.05$ . As few genes were detected using this method the data  
214 were re-analysed using PLIER normalisation and a Student's t-test with a p-value cut off  
215 of  $\leq 0.05$  and a fold change difference between wild type and Chuvash of  $\geq 1.3$ .

216

217

218 *Isolated heart perfusion.* Mice were terminally anesthetized with an intraperitoneal  
219 injection of sodium pentobarbitol (500 mg/kg, Merial, UK) and the heart excised and  
220 arrested in ice-cold Krebs-Henseleit buffer; blood was immediately collected from the  
221 chest cavity for subsequent analysis. The ascending aorta was cannulated and the heart  
222 perfused retrograde in Langendorff mode at 37 °C at a constant perfusion pressure of 80  
223 mmHg. A polyethylene balloon, connected to a pressure transducer and used to measure  
224 cardiac function, was inserted into the left ventricle (LV) lumen and its volume adjusted  
225 to produce an end-diastolic pressure (EDP) of 4 – 8 mmHg. Rate pressure product (RPP)  
226 was calculated as the product of LV developed pressure (systolic pressure minus EDP)  
227 and heart rate. Hearts were perfused with 100 ml of a modified Krebs-Henseleit re-  
228 circulating buffer (118 mmol/L NaCl, 3.7 mmol/L KCl, 1.2 mmol/L MgSO<sub>4</sub>, 1.97 mmol/L  
229 CaCl<sub>2</sub>, 0.5 mmol/L Na<sub>2</sub>EDTA, 25 mmol/L NaHCO<sub>3</sub>, 1.2 mmol/L KH<sub>2</sub>PO<sub>4</sub>) containing 11  
230 mmol/L glucose and 0.4 mmol/L palmitate pre-bound to 1.5 % albumin as substrates.  
231 The buffer was continually gassed with a mix of 95% O<sub>2</sub> with 5 % CO<sub>2</sub>.

232

233 *Perfused heart energetics.* Perfused hearts were inserted into an 11.7 T (500 MHz)  
234 vertical bore (123 mm internal diameter) magnet (Magnex Scientific, UK) with a 10 mm  
235 probe (Rapid, Germany) containing concentric <sup>1</sup>H and <sup>31</sup>P sensitive coils. Fully relaxed  
236 <sup>31</sup>P spectra were acquired using pulse-and-collect sequence at a repetition time of 10 s  
237 and a flip angle of 90° (32 averages, total acquisition time of 5 min, steady state.  
238 Approximate doubling of the RPP was then achieved by an infusion of isoproterenol  
239 (concentration received by the heart 2 – 5 nM) and further spectra acquired (minimum  
240 acquisition time 5 min). Spectra were analyzed using the jMRUI software(42) to give  
241 values for phosphocreatine (PCr) and  $\gamma$ ATP abundance, and the ratio of these two.  
242 Spectra for each perfusion condition (standard, or isoproterenol) were averaged to give  
243 final values for PCr:ATP.



244

245 *Measurement of cardiac substrate metabolism.* To determine glycolytic flux in the  
246 perfused heart, 50  $\mu\text{Ci}$  of D[5- $^3\text{H}$ ]-glucose (Amersham, UK) were added to the re-  
247 circulating buffer. Following a 10 min stabilization period, perfusate samples were  
248 collected at 5 min intervals for 35 min.  $^3\text{H}_2\text{O}$  was separated from the D[5- $^3\text{H}$ ]-glucose in  
249 the time samples using Dowex® ion exchange resin (Sigma, UK) and subsequently used  
250 to calculate the glycolytic rate. Due to the position of the  $^3\text{H}$  label, this method gives a  
251 measure of true glycolytic rate since the label is cleaved by enolase in the cytosol.

252

253 Cardiac lactate efflux was determined by measuring lactate concentration  
254 spectrophotometrically in timed perfusate collections using lactate dehydrogenase. This  
255 method gives a measure of net lactate efflux from the heart. Cardiac palmitate oxidation  
256 rates were determined in a separate group of perfused hearts by adding 50  $\mu\text{Ci}$  of [9,10-  
257  $^3\text{H}$ ]-palmitate (Amersham, UK) to the re-circulating buffer and performing a chloroform-  
258 methanol Folch extraction on the time buffer samples to collect the  $^3\text{H}_2\text{O}$ .

259

260 *Measurement of in vivo cardiac metabolism in real-time.* Mice were studied in the early  
261 absorptive (fed) state, between 1 and 11 am. They were anesthetized in isoflurane and  
262  $\text{O}_2$  and intravenous access was gained using a 32 G tail-vein cannula. Mice were then  
263 positioned within a 7 T horizontal-bore MR scanner. Respiratory rate and ECG were  
264 monitored continuously. As described previously(12), 0.15 ml of hyperpolarized  $^{13}\text{C}_1$   
265 pyruvate (0.48 mmol/kg) was injected over 10 s, followed by a 0.05 ml flush of  
266 heparinized saline to clear the delivery line. Sixty individual ECG-gated  $^{13}\text{C}$  MR cardiac  
267 spectra were acquired over 1 min following injection and subsequently analyzed using  
268 the AMARES algorithm in the jMRUI software package (Version 4.0(42)). The peak areas  
269 of  $^{13}\text{C}_1$  pyruvate,  $^{13}\text{C}_1$  lactate,  $^{13}\text{C}_1$  alanine and  $^{13}\text{C}_1$  bicarbonate at each time point were  
270 quantified and used as input data for a kinetic model as described previously(12).

271

272 *Statistical analysis.* Differences between groups were assessed using one-way analysis of  
273 variance using SPSS Statistics 19 (IBM, USA).

274

**275 Results**

276 The purpose of this study was to determine the cardiac metabolic and functional  
277 phenotype of the CP mouse, in comparison with the WT.

278

279 *General hematological and biochemical characteristics of the CP mouse model.* Body mass,  
280 basic hematology and plasma metabolites are summarized in Table 1. Polycythemia in  
281 the CP mice was confirmed by demonstrating a modest increase in both hemoglobin and  
282 hematocrit, consistent with previous reports(18, 19, 64). No differences were seen in  
283 non-fasting plasma metabolites.

284

285 *CP mice have normal cardiac metabolic gene expression.* The whole-heart expression of  
286 key cardiac metabolic genes revealed no differences between WT and CP mice (Figure  
287 1). In order to confirm the methodology, the expression of lung endothelin-1 was  
288 studied and found to be significantly increased in the CP mice, in keeping with previous  
289 findings(19). The full micro-array results are included in the supplemental material.

290

291 *CP mice exhibit features of pulmonary hypertension.* Cardiac function and mass in WT and  
292 CP mice at different ages are shown in Figure 2. A full table of cine MRI results is  
293 included in Table 2.

294

295 RV free wall hypertrophy, a typical feature of pulmonary hypertension, was seen in all  
296 age groups in the CP mice (Figure 2B). These findings were comparable with those  
297 reported in a previous study, in which pulmonary artery pressure and RV free wall  
298 thickness were measured using invasive techniques (19). In addition, marked  
299 interventricular septal bowing, another characteristic feature of pulmonary  
300 hypertension, was seen in all age groups in the CP mice (Figure 2F). The degree of septal  
301 bowing was quantified and is included in Table 2.

302

303 In contrast to the marked changes in the RV, no differences were seen in LV mass or  
304 cardiac output (corrected for body mass; Figures 2A and C). Interestingly, LV ejection  
305 fraction was significantly increased in the younger CP mice, perhaps as a result of the  
306 pulmonary hypertension (Figure 2D).

307

308 *CP hearts exhibit more marked depletion of phosphocreatine under conditions of high*  
309 *workload.* <sup>31</sup>P MR spectroscopy of Langendorff-perfused hearts enabled the  
310 measurement of cardiac energetics under conditions of both normal and high workload.

311 Although cardiac energetics were similar in WT and CP hearts perfused under normal  
312 conditions, an infusion of isoproterenol (causing an approximate doubling in RPP)  
313 resulted in significantly lower PCr:ATP ratios in the CP hearts compared with WT  
314 controls (PCr:ATP  $1.21 \pm 0.08$  in CP versus  $1.56 \pm 0.08$ ,  $p < 0.02$ ) (Figure 3).

315

316 *Glycolytic flux and lactate efflux are increased in the CP heart.* Glycolytic flux in the  
317 perfused heart, measured using  $^3\text{H}$ -glucose, was 1.8-fold higher in the CP heart  
318 compared with WT controls (Figure 4A). Analysis of variance, using genotype (CP or  
319 WT) as the fixed factor and heart mass and RPP as co-variates, demonstrated that the CP  
320 mutation was the only significant determining factor on glycolytic rate ( $p = 0.004$  for  
321 genotype;  $p = 0.095$  for heart weight;  $p = 0.147$  for RPP). Similarly, net lactate efflux was  
322 1.5-fold higher in the CP hearts ( $p = 0.009$  for genotype;  $p = 0.121$  for heart weight;  $p =$   
323  $0.123$  for RPP) (Figure 4B). There was a significant correlation between glycolytic and  
324 lactate efflux rates (Pearson  $R = 0.935$ ,  $p < 0.0001$ ). In contrast to the increased glucose  
325 utilization and lactate production by the CP hearts, a decrease in fatty acid oxidation (as  
326 might be predicted by the Randle Cycle(27)) was undetectable (Figure 4C).

327

328 *In vivo pyruvate metabolism, measured in real-time, is significantly altered in the CP heart.*  
329 The advent of dynamic nuclear polarization (DNP) has enabled the study of cardiac  
330 metabolism in real-time(59). *In vivo*  $^{13}\text{C}$  MR spectroscopy of hyperpolarized  $^{13}\text{C}_1$   
331 pyruvate revealed, in keeping with the perfusion studies, a 2-fold increase in the rate of  
332  $^{13}\text{C}_1$  label incorporation into lactate in the CP hearts ( $p < 0.01$ , Figure 5). Furthermore,  
333 there was a 1.6-fold increase in the rate of label incorporation into bicarbonate ( $p <$   
334  $0.05$ ), indicating elevated flux through pyruvate dehydrogenase (Figure 5).

335

**336 Discussion**

337

338 It is now known that HIF functions as a global master regulator, coordinating diverse  
339 cellular and systemic responses to hypoxia. Studies on patients and mice have  
340 demonstrated that perturbations in the HIF pathway, such as Chuvash polycythemia or  
341 HIF-2 $\alpha$  gain-of-function mutations, result in profound abnormalities in systemic and  
342 cellular processes that are usually under tight control. Marked changes in skeletal  
343 muscle metabolism, ventilation, pulmonary vascular tone, and glucose homeostasis are  
344 all observed in both humans and animals with disorders of the HIF pathway(2, 13-16,  
345 18, 19, 37, 39, 46, 48, 50-53, 57, 64-66, 68, 69). We now demonstrate that chronic,  
346 modest upregulation of HIF due to the Chuvash *VHL* mutation results in altered cardiac  
347 metabolism and energetics in the mouse heart.

348

349 A primordial function of HIF-1 is to find the optimal balance between oxidative and  
350 glycolytic metabolism for a given local oxygen concentration. As such, HIF-1 target  
351 genes, including glucose transporters and glycolytic enzymes(30, 60), lactate  
352 dehydrogenase (LDH)(61) and pyruvate dehydrogenase kinase 1 (PDK-1)(33, 47),  
353 enable increased glucose metabolism. In both hypoxia and hypoxia-mimetic models,  
354 myocardial expression of the master regulator of fatty acid metabolism, peroxisome  
355 proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), is decreased together with its downstream  
356 targets such as PDK-4(1, 55, 62). Alterations in cardiac mitochondrial oxidative  
357 metabolism are also seen(17). Thus, one would predict that exposure to hypoxia, or  
358 chronic activation of the HIF pathway, would result in an increased reliance upon  
359 glycolysis and glucose oxidation, and a shift away from fatty acid beta-oxidation.

360

361 Most studies investigating the role of HIF in cardiac metabolism and function have  
362 employed tactics either to over-express HIF itself, or to impair its degradation by using  
363 altered levels of PHDs or VHL. Mice with cardiomyocyte-specific loss of VHL have  
364 elevated HIF levels in the heart, accompanied by increased expression of glycolytic  
365 genes, cellular lipid accumulation, and progressive heart failure(35). Combined cardiac-  
366 specific loss of both PHD2 and PHD3 results in increased expression of  
367 phosphoglycerate kinase (PGK), decreased expression of PPAR $\alpha$ , myocyte accumulation  
368 of lipid, and severe cardiomyopathy(41). Conditional somatic inactivation of PHDs  
369 produces similar results(40). Inducible cardiac-specific overexpression of an oxygen-  
370 stable form of HIF-1 $\alpha$  results in increased transcript levels of glycolytic genes and  
371 progressively impaired cardiac function(7). Furthermore, long-term constitutive

372 overexpression of HIF-1 $\alpha$  results in increased glucose uptake and the development of  
373 cardiomyopathy(24). Taken together, these studies have shown that very high levels of  
374 HIF in the heart result in a switch in gene expression towards increased glucose  
375 metabolism, with accompanying contractile dysfunction. In contrast, shorter-term  
376 constitutive overexpression of HIF-1 $\alpha$  mRNA in cardiomyocytes did not result in  
377 cardiomyopathy, but this construct only produced a barely detectable increase in HIF in  
378 normoxia. Interestingly, this overexpression was protective during myocardial  
379 infarction, where hypoxia would be expected to be present (32).

380

381 Our study investigated the cardiac effects of the Chuvash *VHL* mutation in mice, which  
382 results in systemic, long-term, modest upregulation of HIF. Our model faithfully  
383 recapitulated findings seen in previous studies on CP patients and mice – raised  
384 hemoglobin and hematocrit, pulmonary hypertension (shown using non-invasive MRI),  
385 and RV hypertrophy (using MRI, rather than histology). However, a surprising feature of  
386 our study was the apparent lack of change in the expression of key metabolic genes  
387 within the heart, despite clear alterations in substrate metabolism and energetics. It is  
388 possible that our whole-heart analysis masked differential changes that could have been  
389 seen in the left, versus the right, ventricle(1, 62). Alternatively, the presence of  
390 pulmonary hypertension and right ventricular hypertrophy may have resulted in  
391 genetic remodeling in addition to that caused by the Chuvash *VHL* mutation. Finally, it is  
392 possible that the LV in the CP mouse is exposed to a chronic increase in local and/or  
393 systemic sympathetic drive(9, 10, 38, 67), which may also alter gene transcription on  
394 the background of that caused by the HIF upregulation itself.

395

396 Previous studies of HIF manipulation within the mouse heart have demonstrated an  
397 increased reliance upon glucose metabolism, with accompanying contractile  
398 dysfunction(7, 24, 35, 40, 41). In the much more modest perturbation of the HIF system  
399 in the current study these two features were not coupled, in keeping with a previous  
400 study demonstrating that short-term HIF mRNA overexpression could be cardio-  
401 protective(32). While there was clear evidence of increased glucose utilization and  
402 altered energetics, LV function was enhanced rather than diminished. It is likely that this  
403 increased ejection fraction is a reflection of the pulmonary hypertension demonstrated  
404 in the CP mice. One potential mechanism is that bowing of the interventricular septum  
405 arises as a consequence of the elevated pulmonary arterial pressure, and this could  
406 result directly in a reduced LV end-systolic volume. Another possible mechanism is that  
407 there is an overall increased sympathetic drive to the heart in order to maintain the

408 cardiac output in the face of an elevated pulmonary vascular resistance. Again, this  
409 would be expected to result in a reduced LV end-systolic volume. It should also be noted  
410 that cell types other than cardiomyocytes may be relevant. For example, endothelial cell-  
411 specific HIF has been shown to influence cardiac glucose uptake, and this would have  
412 effects both *in vivo* and on the isolated perfused-heart preparation (26).

413

414 The balance between myocardial glucose and fatty acid metabolism can be studied using  
415 the combination of radiolabelled substrates and the isolated perfused-heart(6, 8). We  
416 have demonstrated that the hearts from CP mice have elevated glycolytic flux,  
417 accompanied by increased net lactate efflux. This finding is in keeping with previous  
418 genetic studies which have shown that long-term, constitutive over-expression of HIF-  
419  $1\alpha$  in the heart results in increased glucose uptake(24), and that cardiac-specific loss of  
420 HIF- $1\alpha$  causes decreased cardiomyocyte lactate concentrations(25). In our study, the  
421 increase in glycolytic flux was greater than that of PDH flux, meaning that the decrease  
422 in palmitate oxidation did not reach significance. However, following hypoxia, mouse  
423 hearts have been shown to have increased glycolytic flux, increased lactate production  
424 and significantly decreased fatty acid oxidation (11).

425

426 The advent of DNP enables flux through metabolic pathways to be measured in real-  
427 time, *in vivo*. This technique has recently been optimized for use in small animals, such  
428 as mice, allowing genetic models to be studied for the first time(5, 12). The increased  
429 sensitivity (greater than 10, 000-fold(3)) of DNP allows the acquisition of data within  
430 one minute after infusion of a bolus of hyperpolarized  $^{13}\text{C}_1$  pyruvate. This bolus is  
431 required to be relatively large, however during the short time-frame of data acquisition  
432 no perturbation of plasma metabolites (other than pyruvate) or pyruvate  
433 dehydrogenase (PDH) activity occurs(4, 5). Using hyperpolarized  $^{13}\text{C}_1$  pyruvate MR  
434 spectroscopy, we have shown that there is increased rate of label incorporation from  
435 pyruvate into both lactate (via LDH) and bicarbonate (via PDH). By using a combination  
436 of techniques, we have therefore demonstrated that the measurements of metabolic flux  
437 obtained *ex vivo* are in agreement with those made in real-time *in vivo*.

438

439 Metabolic flexibility is required for the heart to continue to perform under conditions of  
440 varying workload. Inflexibility, due to increased reliance upon a substrate such as  
441 glucose (which yields less ATP per molecule, in comparison with fatty acids) may result  
442 in myocardial energy depletion. In agreement with this hypothesis, we have  
443 demonstrated that myocardial energetics, quantified by measurement of PCr and ATP,

444 are reduced in the CP perfused heart under conditions of increased workload. Altered  
445 cardiac energetics are a feature of both heart failure(45) and hypoxia(20, 22, 23); our  
446 study adds to this body of evidence by demonstrating that modest long-term  
447 upregulation of HIF may result in a similar finding.

448

449 In summary, a modest systemic dysregulation of the HIF pathway, caused by the  
450 Chuvash *VHL* mutation, resulted in clear alterations in cardiac metabolism and  
451 energetics. However, in contrast to studies generating high levels of HIF in the heart, the  
452 CP mice showed neither the predicted changes in gene expression nor any degree of LV  
453 impairment. We conclude that the effects of manipulating HIF on the heart are dose-  
454 dependent. In the present study, the pulmonary hypertension associated with CP clearly  
455 had a significant effect on cardiac function. In future studies employing the CP mutation  
456 to study modest HIF dysregulation, targeting this mutation specifically to cardiac  
457 myocytes could obviate the influence of pulmonary hypertension.

458

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461

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466

467 **Disclosures**

468 None

469



470 **Figure Captions**

471

472 **Figure 1. Cardiac and lung gene expression.** Quantitative real-time PCR was used to  
 473 measure expression of key metabolic genes in hearts from WT (n = 9) and CP (n = 7)  
 474 mice, aged 6 – 7 months. To confirm the methodology, expression of endothelin 1 (Edn  
 475 1; dark gray) was also determined in lungs from thirteen WT and eleven CP mice. Values  
 476 are mean  $\pm$  95% confidence interval. VEGF (vascular endothelial growth factor); GLUT  
 477 (glucose transporter); PFKM (phosphofructokinase); PDK (pyruvate dehydrogenase  
 478 kinase); LDHA (lactate dehydrogenase A); PKM (pyruvate kinase muscle); PPAR $\alpha$   
 479 (peroxisome proliferator-activated receptor  $\alpha$ ); Edn 1 (endothelin 1). \*\*\*\* ( $p < 0.001$ ).

480

481

482 **Figure 2. *In vivo* cardiac function.** *In vivo* cine magnetic resonance imaging was used  
 483 to measure cardiac mass and function in aging WT and CP mice (n = 3 – 8 mice per  
 484 group). White columns (WT); black columns (CP). (A) Left ventricle (LV) mass; (B) Right  
 485 ventricle (RV) wall thickness; (C) LV cardiac output, corrected to body mass; (D) LV  
 486 ejection fraction. WT mice had normal cardiac morphology (E. Representative image of  
 487 mouse aged 3 – 6 months). CP mice demonstrated marked interventricular septal  
 488 bowing, particularly in early-diastole (F; arrows. Representative image of mouse aged 3  
 489 – 6 months). \* ( $p < 0.05$ ); \*\* ( $p < 0.02$ ); \*\*\* ( $p < 0.01$ ).

490

491

492 **Figure 3. Perfused-heart energetics.**  $^{31}\text{P}$  magnetic resonance spectroscopy was  
 493 performed on perfused hearts from WT and CP mice (n = 5 in each group), aged 6 – 7  
 494 months. Body mass  $30.1 \pm 2.1$  g for WT, vs  $28.7 \pm 1.2$  for CP (not significant). White  
 495 columns (WT); black columns (CP). Hearts were perfused under normal Langendorff  
 496 conditions using palmitate buffer and spectra were acquired, both without and with an  
 497 infusion of isoproterenol in order to increase the rate pressure product. As expected,  
 498 isoproterenol significantly increased the rate pressure product ( $p < 0.001$ ) and  
 499 decreased the PCr/ATP ratio ( $p < 0.001$ ). No significant difference between WT and CP  
 500 was detected without isoproterenol, but following the increase in RPP the PCr:ATP ratio  
 501 was significantly lower in the CP hearts compared with WT controls. (A) rate pressure  
 502 product; (B) PCr:ATP ratio. \*\* ( $p < 0.02$ ).

503

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505

506 **Figure 4. Perfused heart metabolism.** Glycolytic flux (A) and net lactate efflux (B)  
507 were determined in WT (n = 4) and CP (n = 7) mice, aged 15 – 17 months. Body mass  
508  $33.8 \pm 1.8$  g for WT vs  $29.4 \pm 1.6$  for CP (not significant). Glycolytic and lactate rates  
509 were highly correlated (Pearson R = 0.935,  $p < 0.001$ ). Palmitate oxidation rates (C)  
510 were determined in separate mice (n = 5 in each group), aged 11 – 17 months. Body  
511 mass  $27.4 \pm 1.5$  g for WT vs  $26.2 \pm 2.0$  for CP ( $p = 0.034$ ). \*\*\* ( $p < 0.01$ ).

512

513

514 **Figure 5. *In vivo* real-time cardiac metabolism.** *In vivo* magnetic resonance  
515 spectroscopy of hyperpolarized  $^{13}\text{C}_1$  pyruvate was performed in WT (n = 5) and CP (n =  
516 4) mice, aged 9 – 15 months. Body mass  $32.0 \pm 3.0$  g for WT, vs  $27.0 \pm 3.0$  for CP (not  
517 significant). This technique allowed real-time measurement of the rate of label  
518 incorporation from pyruvate into alanine (A), bicarbonate (B), or lactate (C). \* ( $p <$   
519  $0.05$ ); \*\*\* ( $p < 0.01$ ).

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523 **Tables**  
524

	Wild type (n)	Chuvash (n)	<i>p</i> value
Body mass (male mice, g)	35.9 ± 0.7 (12)	28.4 ± 0.4 (12)	< <b>0.001</b>
Body mass (female mice, g)	25.3 ± 0.3 (4)	23.4 ± 0.5 (8)	<b>0.016</b>
Hematocrit (%)	43 ± 0.6 (13)	51 ± 1.0 (13)	< <b>0.001</b>
Hemoglobin (g L <sup>-1</sup> )	138 ± 3 (16)	158 ± 4 (13)	< <b>0.001</b>
Glucose (mmol/L)	13.4 ± 1.0 (7)	13.4 ± 0.5 (7)	0.96
Hydroxybutyrate (mmol/L)	0.18 ± 0.04 (8)	0.21 ± 0.06 (7)	0.65
Non-esterified fatty acid (mmol/L)	0.15 ± 0.01 (8)	0.13 ± 0.02 (7)	0.33
Triacyl glycerol (mmol/L)	2.5 ± 0.2 (7)	2.2 ± 0.5 (7)	0.56
Cholesterol (mmol/L)	1.7 ± 0.20 (8)	1.6 ± 0.05 (7)	0.44

525 **Table 1. Basic hematological and other parameters.** Hematological values, non-  
526 fasting plasma metabolites, and body masses of mice aged 6 – 7 months are shown.  
527 Results are mean ± SEM; the number of mice used (n) is shown in parentheses.  
528  
529

530

		Wild type (n)	Chuvash (n)	p value
Body mass (grams)	3 months	28 ± 1.8 (4)	24 ± 1.3 (7)	0.093
	6 months	36 ± 1.1 (4)	25 ± 1.2 (9)	<b>&lt; 0.0001</b>
	12 months	37 ± 3.2 (5)	27 ± 0.9 (8)	<b>0.003</b>
LV ejection fraction (%)	3 months	61.5 ± 3.7 (3)	71.5 ± 2.2 (6)	<b>0.043</b>
	6 months	54.0 ± 1.7 (4)	68.4 ± 3.2 (8)	<b>0.014</b>
	12 months	55.5 ± 5.6 (5)	66.5 ± 2.9 (8)	0.078
LV mass (% of body mass)	3 months	0.42 ± 0.01 (3)	0.39 ± 0.02 (6)	0.262
	6 months	0.37 ± 0.04 (5)	0.39 ± 0.01 (7)	0.584
	12 months	0.38 ± 0.04 (5)	0.38 ± 0.02 (8)	0.952
MV RV wall thickness (mm)	3 months	0.35 ± 0.02 (3)	0.47 ± 0.03 (7)	<b>0.021</b>
	6 months	0.34 ± 0.02 (5)	0.45 ± 0.02 (8)	<b>0.001</b>
	12 months	0.35 ± 0.02 (5)	0.51 ± 0.02 (7)	<b>0.001</b>
Septal bowing ratio	3 months	0.91 ± 0.009 (3)	0.83 ± 0.03 (7)	<b>0.038</b>
	6 months	0.92 ± 0.004 (5)	0.80 ± 0.01 (7)	<b>&lt; 0.0001</b>
	12 months	0.93 ± 0.0 (4)	0.83 ± 0.01 (7)	<b>&lt; 0.0001</b>
Heart rate (beats per min)	3 months	420 ± 29 (3)	373 ± 8 (6)	0.069
	6 months	413 ± 10 (5)	398 ± 3 (8)	0.202
	12 months	418 ± 3 (5)	402 ± 7 (8)	0.129
Stroke volume (ml)	3 months	35.1 ± 2.9 (3)	32.2 ± 1.2 (7)	0.282
	6 months	34.5 ± 1.0 (5)	30.8 ± 2.3 (8)	0.24
	12 months	37.1 ± 1.5 (4)	28.1 ± 1.4 (8)	<b>0.003</b>
Cardiac output (ml/min)	3 months	14.8 ± 2.0 (3)	12.5 ± 0.8 (7)	0.226
	6 months	14.3 ± 0.5 (5)	12.2 ± 0.9 (8)	0.116
	12 months	14.6 ± 1.2 (5)	11.3 ± 0.7 (8)	<b>0.025</b>
LV Cardiac index (ml/min/g)	3 months	0.54 ± 0.03 (3)	0.52 ± 0.03 (7)	0.756
	6 months	0.43 ± 0.03 (5)	0.48 ± 0.03 (8)	0.197
	12 months	0.40 ± 0.01 (5)	0.43 ± 0.02 (8)	0.322

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**Table 2. Full cine MRI data.** Results are mean ± SEM; the number of mice used (n) is shown in parentheses. For the 'Septal bowing ratio', the greater the distortion of the LV by the septum, the greater the deviation from the perfect circle ratio of 1.0.

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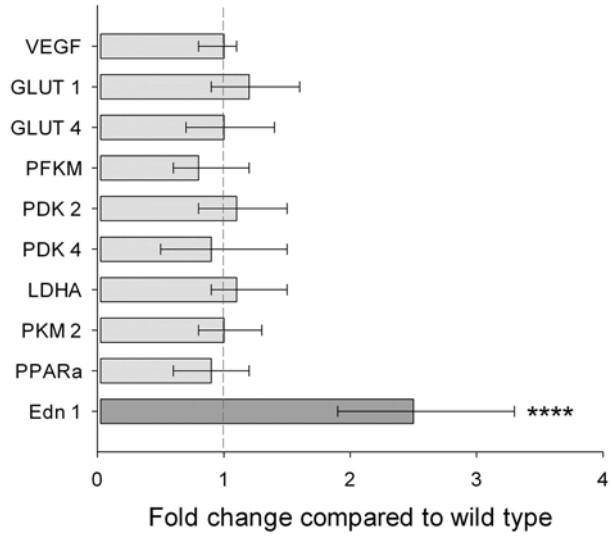
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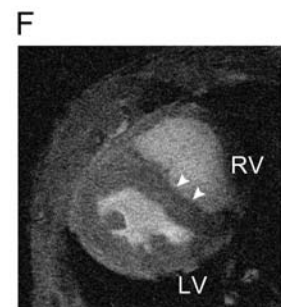
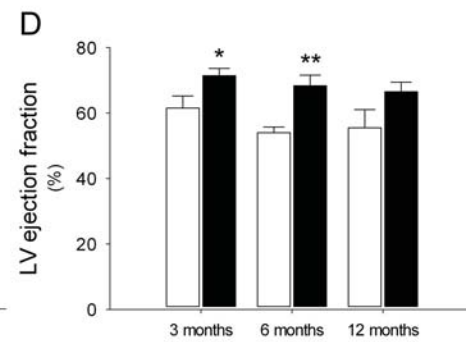
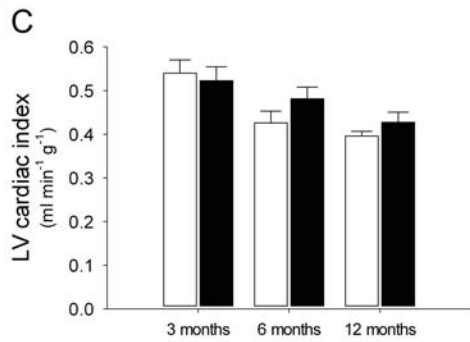
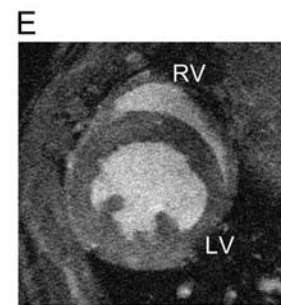
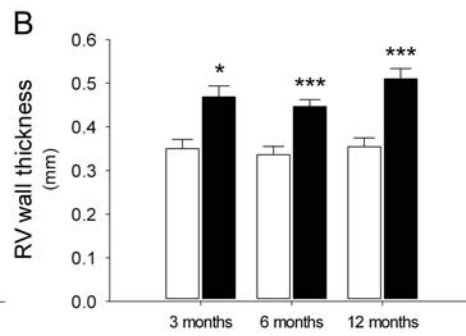
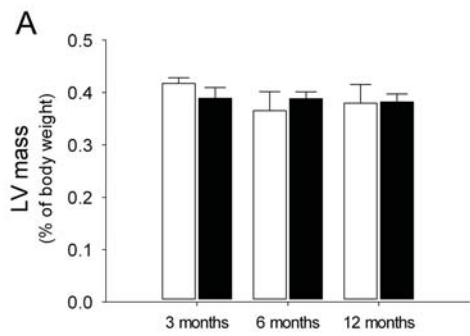
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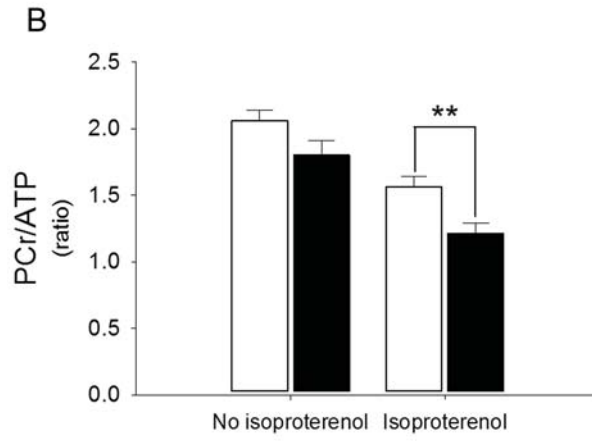
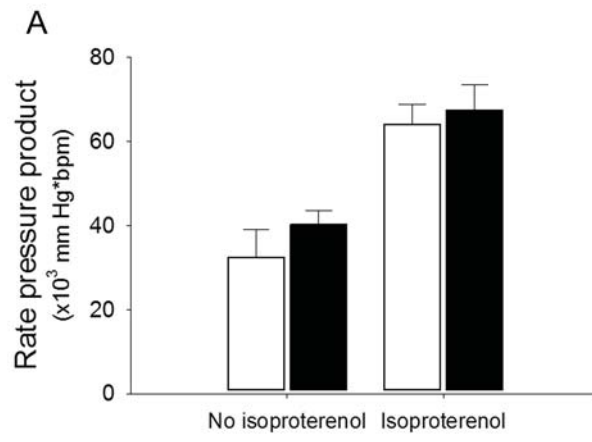


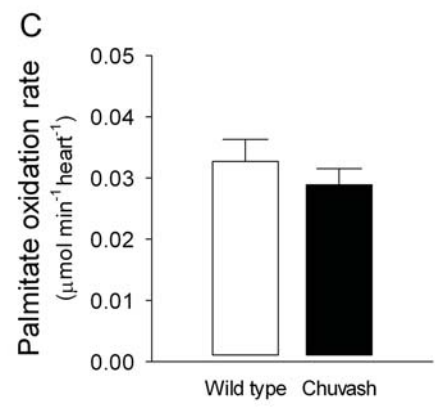
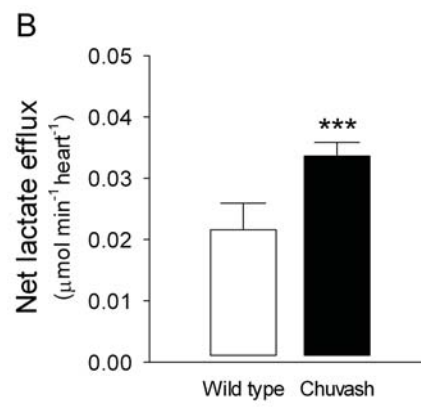
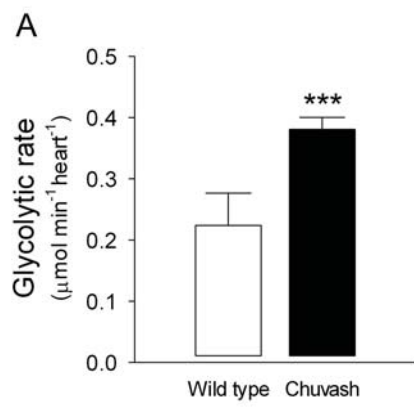
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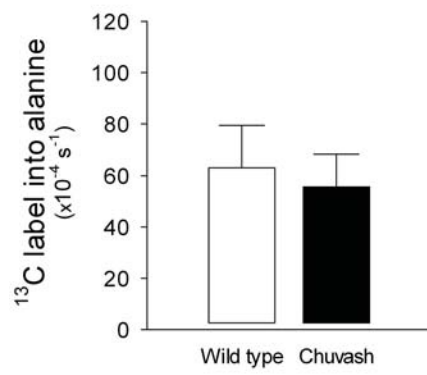




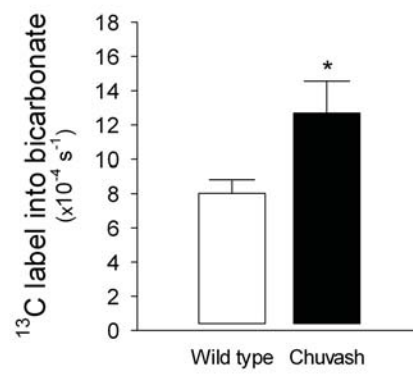




**A**



**B**



**C**

