1 **Title:** Hypoxia, AMPK activation and uterine artery vasoreactivity

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14 **Running head:** AMPK and uterine artery vasodilation

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Key points summary

- Uterine artery vasodilatation is a key mechanism for increasing utero-placental blood flow and fetal nutrient supply.

- Since the pioneering work of Joseph Barcroft, the natural laboratory of high altitude has been used to study the mechanisms regulating uterine artery blood supply and fetal growth.

- Genes near the metabolic sensor, adenosine monophosphate-activated protein kinase (AMPK) have been implicated in genetic protection from high altitude-associated fetal growth restriction.

- We show that AMPK is present in utero-placental tissues, has vasodilator effects in murine uterine arteries, and that exposure to chronic hypoxia sufficient to decrease fetal growth increases the vasodilator actions of AMPK in opposing phenylephrine-induced vasoconstriction.

- These results point to AMPK as being a key link between maternal vascular responses to pregnancy and fetal growth. Manipulation of AMPK may be a novel mechanism for developing new therapies in pregnancies complicated by chronic hypoxia.

Abstract

Genes near *PRKAA1* (*adenosine monophosphate-activated protein kinase [AMPK] alpha-1*) have been implicated in the greater uterine artery (UtA) blood flow and relative protection from fetal growth restriction seen in altitude-adapted, Andean populations. AMPK activation vasodilates multiple vessels but whether AMPK is present in UtA or placental tissue and influences UtA vasoreactivity during normal or hypoxic pregnancy remains unknown. We studied isolated UtA and placenta from near-term C57BL6/J mice housed in normoxia (n=8) or hypoxia (10% F_iO_2, n=7-9) from day 14-19, and placentas from non-labouring sea level (n=3) or 3100 m (n=3) women. Hypoxia increased AMPK immunostaining in near-term murine UtA and placental tissue. RT-PCR products for AMPK alpha-1 and alpha-2 isoforms and LKB1 (the upstream kinase activating AMPK) were present in murine and human placenta, and hypoxia increased LKB1, AMPK alpha-1 and alpha-2 expression in the high- compared with low-altitude human placentas. Pharmacological AMPK activation by A769662 caused phenylephrine pre-constricted UtA from normoxic or hypoxic pregnant mice to dilate and this dilatation was partially reversed by the NOS inhibitor L-NAME. Hypoxic pregnancy sufficient to restrict fetal growth markedly augmented the UtA vasodilator effect of AMPK activation in opposition to PE constriction as the result of both NO-dependent and -independent mechanisms. We concluded that AMPK is activated during hypoxic pregnancy, and that AMPK activation vasodilates the UtA, especially in hypoxic pregnancy. AMPK activation may be playing an adaptive role by limiting cellular energy depletion and helping to maintain utero-placental blood flow in hypoxic pregnancy.

Keywords: fetal growth restriction, high-altitude adaptation, nitric oxide, utero-placental blood flow, phenylephrine

Introduction

Pioneering studies of Joseph Barcroft and his students drew attention to the value of high altitude as a natural laboratory for understanding the mechanisms by which intrauterine hypoxia reduces fetal growth (Barcroft, 1933; Barron *et al.*, 1964). A key determinant of fetal growth is the pregnancy rise in uterine artery (UtA) blood flow, which is due, in turn, to profound changes in multiple physiological systems (Gant & Worley, 1989). Among the greatest are the structural remodelling and alterations in vasoreactivity of the uterine vasculature that result in approximately 20% of the maternal cardiac output being directed to the utero-placental circulation by term (Osol & Moore, 2014). Isolated vessel studies in experimental animals show that chronic hypoxia is associated with a decreased vasodilator response to flow and to pharmacological agonists in the main UtA and downstream vessels (White *et al.*, 2000; Mateev *et al.*, 2003; Xiao *et al.*, 2010), which may, in turn, contribute to the reduced UtA blood flow and altitude-associated fetal growth restriction seen in high-altitude newcomers (Zamudio *et al.*, 1995; Julian *et al.*, 2008). Multigenerational highland residents (Andeans and Tibetans) are relatively protected from altitude-associated reductions in fetal growth and have a greater pregnancy rise in UtA blood flow compared with altitude newcomers (Moore *et al.*, 1998; Giussani *et al.*, 2001; Moore *et al.*, 2001; Chen *et al.*, 2002; Julian *et al.*, 2007; Julian *et al.*, 2009; Soria *et al.*, 2013). Such protection appears due, in part, to genetic factors given that it is proportional to the degree of highland ancestry and is not the result of the woman's own duration of residence at high altitude (Bennett *et al.*, 2008; Julian *et al.*, 2011; Soria *et al.*, 2013).

Identifying the genes and signalling pathways involved in protecting native highlanders presents a novel means for addressing the mechanisms by which intrauterine hypoxia influences fetal growth. Several gene regions have been acted

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95   upon by natural selection in long-resident populations (Bigham *et al.*, 2009; Beall *et al.*,

96   2010; Bigham *et al.*, 2010; Simonson *et al.*, 2010; Yi *et al.*, 2010; Alkorta-Aranburu *et*

97   *al.*, 2012). In Andeans, prominent among these are single nucleotide polymorphisms

98   (SNPs) near *PRKAA1* (*adenosine monophosphate-activated protein kinase [AMPK]*

99   *alpha-1*) (Bigham *et al.*, 2009; Bigham *et al.*, 2010). Further, the *AMPK alpha-1* variants

100   more common in Andeans are positively associated with infant birth weight as well as

101   with key determinants of fetal growth, namely the pregnancy-associated increase in UtA

102   diameter and the expression patterns of genes in metabolic pathways proposed to play

103   a role in altitude-associated fetal growth restriction (Yung *et al.*, 2012; Bigham *et al.*,

104   2014).

105   AMPK is a ubiquitously expressed enzyme in eukaryotes, that is stimulated by

106   stresses that deplete cellular ATP and thus serves as a metabolic sensor for matching

107   tissue energy demand with supply (Hardie *et al.*, 2012). It is comprised of three subunits

108   (alpha, beta, gamma), each of which has multiple isoforms whose expression levels

109   vary by tissue type (Viollet *et al.*, 2010). The alpha-1 and alpha-2 isoforms are the

110   catalytic subunits and contain the Thr-172 site where AMPK is activated more than 100-

111   fold via phosphorylation by LKB1 and other upstream kinases (Evans *et al.*, 2009;

112   Viollet *et al.*, 2009). In skeletal muscle AMPK activation promotes glucose uptake and

113   mitochondrial biogenesis, and decreases energy demand by inhibiting the mechanistic

114   target of rapamycin (mTOR) and switching on various catabolic enzymes (Hardie,

115   2011). More recently, AMPK activation has been recognized to have vascular effects,

116   acting to stimulate endothelial nitric oxide (NO) production (Wang & Proud, 2006) as

117   well as to regulate smooth muscle function directly (Goirand *et al.*, 2007). Underscoring

118   its importance under conditions of hypoxia, AMPK activation has been implicated in the

119   aetiology of early-onset pre-eclampsia (Yung *et al.*, 2014), cardiorespiratory responses

120   to hypoxia (Evans, 2006), and hypoxic pulmonary vasoconstriction (Evans *et al.*, 2005).

121   It is unknown whether AMPK activation influences UtA vasoreactivity during

122   pregnancy and, if so, whether such effects are altered by exposure to hypoxia. We used

123   a broad-ranging approach to address such questions in which human as well as

124   experimental-animal tissues were studied using multiple methods. Specifically, we

125   hypothesized that AMPK was present in UtA and placental tissue and that its

126   expression was increased during pregnancy by exposure to hypoxia as detected using

127   immunohistochemistry in mice and quantitative RT-PCR in previously-collected human

128   tissues. We further hypothesized that AMPK activation prompted UtA vasodilation

129   and/or altered vasoconstrictor sensitivity to phenylephrine (PE) in vessels isolated from

130   near-term mice, and that such effects were altered by exposure to hypoxia. To test

131   these hypotheses we used the pharmacological AMPK agonist A769662 and compared

132   UtA vasodilator and vasoconstrictor responses in vessels isolated from the normoxic *vs*.

133   hypoxic animals. Finally, we treated vessels with the NOS inhibitor NG-nitro-L-arginine

134   methyl ester (L-NAME) to determine the contribution of NO-dependent and

135   -independent mechanisms to the effects of AMPK activation observed. We considered

136   that such studies would improve our understanding of the mechanisms regulating

137   maternal vascular responses to pregnancy and fetal growth under conditions of chronic

138   hypoxia.

139

140   **Methods**

141   Ethical Approval. All mouse experiments were carried out using procedures

142   consistent with the UK Animals Scientific Procedures Act 1986 and approved by the

143   Local Ethics Review Committee of the University of Cambridge. Placentas were

144   collected from human subjects who provided written informed consent to procedures

145   approved by the University of Colorado Multiple Institutional Review Board (COMIRB,

146   Aurora, CO, USA), the University College Hospital London (London, UK), and the

147   Cambridge Local Research Ethics Committee.

148   <u>Samples and Protocols</u>. Female C57BL6/J mice were placed with males

149   overnight. The presence of a copulatory plug the following morning was taken to

150   indicate day one of pregnancy. Pregnant mice (n=31) were housed in groups of two or

151   three in rooms with 21% O_2, 12 hr light-dark cycles and controlled temperature (21°C)

152   and humidity (60%). They had *ad libitum* access to food (Rat and Mouse No. 3

153   Breeding, Special Diet Services, Witham, UK). At day 14 of pregnancy (term is ~ 21

154   days), animals were randomly assigned to normoxic or hypoxic (10% O_2) treatment

155   groups. Hypoxia was achieved by placing the animals in a chamber containing a PVC

156   isolator (PFI plastics Ltd) and a N_2 generator (N2MID60, Domnick Hunter Ltd, UK) so as

157   to control the percent O_2 within the chamber without changing the CO_2 levels by altering

158   the inflows of air and N_2. O_2 levels were monitored using an O_2 analyser (ICA, UK) and

159   CO_2 levels by a portable CO_2 analyser that was calibrated daily (The Electronic

160   Workshop, Department of Physiology, Development and Neuroscience, University of

161   Cambridge). Normoxic animals were housed in the same room that contained the

162   chambers. Maternal weight, food and water intake were monitored daily. This was

163   achieved in the hypoxic group via a sealed transfer box that could be opened briefly

164   without altering O_2 levels within the main chamber.

165   On day 19 of pregnancy mice were euthanized by cervical dislocation. The

166   uterus was dissected, the numbers of viable fetuses and fetal reabsorptions counted,

167   fetal and placental weights recorded, and fetal biometry taken. Both UtA were removed,

168   with one being used for myography and the other for immunohistochemistry. In

169 approximately half the normoxic and hypoxic animals, one placenta whose weight was

170 closest to the litter mean was prepared for immunohistochemistry.

171 Samples from human placentas were obtained from term, non-labouring women

172 residing either at sea level (n=3) or high altitude (3100 m, n=3) as described previously

173 (Yung *et al.*, 2012).

174 Immunohistochemistry. The murine uterine vessels were placed in ice cold PBS,

175 cleared of adipose tissues and the uterine horn using a dissecting microscope (Leica,

176 Germany), and fixed in 4% paraformaldehyde. The main UtA together with its 1st and

177 2nd order branches was divided into 2-3 longitudinal segments, embedded in a single

178 paraffin block, and sectioned. Whole placentas were washed with PBS, fixed in 4%

179 paraformaldehyde, embedded in paraffin, and sectioned. Paraffin-embedded sections

180 were de-waxed, washed, prepared for antigen retrieval using 0.01 M citric buffer (pH

181 6.0), and incubated overnight using either primary antibody (phospho-AMPKα [Thr172]

182 [40H9] rabbit) from Cell Signalling Technology (Hitchin, UK) diluted in 5% GS/HS in

183 TBS or vehicle alone. The following day, the secondary antibody (anti-rabbit IgG, diluted

184 1:200 in 5% GS/HS) was applied, incubated for 1 hr, developed for staining using DAB

185 and counterstained with hematoxylin. The slides were scanned using a Nanozoomer

186 (Hamamatsu Photonics, Welwyn Garden City, UK), saved as high-resolution files, and

187 images quantified using IPLAb software (v6.0, Scanalytics, Fairfax, VA). DAB-positive

188 areas were selected and segmented in a region of interest based on a selective

189 distribution of saturation and hue values that matched the color of the DAB reactive

190 product and the pattern of staining of the image. Segmented overlays representing the

191 selected pixels of DAB staining were selected in individual UtA and the labyrinthine and

192 junctional zones (separately), quantified and expressed as the percent area of positive

193 DAB staining per area of tissue examined.

194   <u>Quantitative RT-PCR.</u> RNA from mouse (n=3) or human (n=6) placental tissue

195   was extracted using the miRNeasy Mini Kit from Qiagen (Manchester, UK) following the

196   manufacturer's guidelines, and the concentration determined using a Nanodrop 1000

197   spectrophotometer (Thermo Scientific, Hemel Hempstead, UK). cDNA synthesis was

198   carried out using the Transcriptor High Fidelity cDNA synthesis Kit (Roche, UK)

199   following manufacturer instructions. For qPCR analysis, 2.5 µl of cDNA in RNase free

200   water was made up to 25 µl with FastStart Universal SYBR Green Master (ROX, 12.5

201   µl, Roche), Ultra Pure Water (8 µl, SIGMA, UK) and forward and reverse primers for

202   LKB1, AMPK alpha-1 and alpha-2 (Qiagen, UK). The sample was then centrifuged and

203   25 µl added to a MicroAmp^{TM} Fast Optical 96-Well Reaction Plate (Greiner Bio-One,

204   Stonehouse UK), the reaction plate sealed with an optical adhesive cover (Applied

205   Biosystems, Warrington UK) and the plate centrifuged. The reaction was then run on a

206   sequence detection system (Applied Biosystems) using AmpliTaq Fast DNA

207   Polymerase with a 2 min initial step at 50°C followed by a 10 min step at 95°C, a 15 sec

208   step at 95°C (repeated 40 times) followed by a dissociation stage with a 15 sec step at

209   95°C, and followed by a 20 sec at 60°C and a 15 sec step at 95°C. Negative controls

210   included tissue aspirants for which no reverse transcriptase was added, and aspiration

211   of extracellular medium and PCR controls. None of the controls produced any

212   detectable amplicon, ruling out genomic or other contamination.

213   <u>Myography.</u> The murine uterine vasculature was dissected and pinned out in a

214   dish of ice-cold Krebs solution (in mmol/L, all reagents from Sigma UK unless specified

215   otherwise: NaCl 118.5, NaHCO$_3$ 25, KCl 4.75, MgSO$_4$.7H$_2$O 1.2, KH$_2$PO$_4$ 1.2, CaCl$_2$

216   2.5, D-glucose 11.1). Using a bifocal dissecting microscope (Brunel Microscopes Ltd.,

217   UK), first-order 2 mm in length UtA segments were dissected and cleared of any

218   connective or adipose tissue. The vessel segments were then mounted in a four-

219   chamber, small-vessel wire myograph (Multi Wire Myograph System 620M, DMT,

220   Denmark) by threading two wires 40 µm in diameter through the vessel lumen and

221   attaching one to a pressure transducer and the other to a micrometer as previously

222   described (Pulgar *et al.*, 2011; Giussani *et al.*, 2012). The chamber was continually

223   gassed with 5% CO_2 and 95% O_2 to maintain a physiological pH, and gradually heated

224   to 37°C. Throughout the investigation, the Krebs solution was refreshed every 20 min,

225   and the vessels allowed to re-equilibrate for at least 20 min between experiments.

226   UtA were normalised to 0.9 of $L_{13.3\,kPa,}$ and allowed to equilibrate for 20 min.

227   Following normalisation a 'wake up' protocol was performed consisting of exposure of

228   the vessels to a high concentration of potassium (KCl, 125 mmol/L). Vessel viability was

229   verified by a positive constrictor response to phenylephrine (PE, 1×10^{-4} M) and a

230   positive relaxant response to acetylcholine (ACH, 1×10^{-10} M to 1×10^{-4} M). Two vessels

231   from each animal were pre-constricted with a dose of PE determined to produce 70% of

232   the maximal constriction to PE for each vessel. The sub-maximally pre-constricted

233   vessels were then relaxed by addition of the AMPK agonist A769662 (Tocris, USA)

234   dissolved in DMSO in increasing concentrations (1×10^{-6} M to 1×10^{-4} M) at 10-min

235   intervals, and the results from the two vessels per animal averaged. We chose A769662

236   because, unlike 5-amino-1-beta-D-ribofuranosyl-imidazole-4-carboxamide (AICAR) or

237   metformin, it activates AMPK directly, and not other enzymes that are responsive to

238   AMP levels (Goransson *et al.*, 2007). Another advantage is that A769662, unlike

239   AICAR, is not taken up by the adenosine transporter and therefore does not lead to the

240   accumulation of adenosine outside cells or necessitate the use of an adenosine

241   antagonist (Goransson *et al.*, 2007). A third and fourth vessel segment from each

242   animal was also tested using increasing doses of PE (1×10^{-9} M – 1×10^{-4} M), with this

243   then being repeated following 20 min incubation with A769662 (1×10^{-4} M) and finally

244   repeated a third time following incubation with A769662 (1×10^{-4} M) plus the NOS

245   inhibitor L-NAME (1×10^{-5} M). The finding that the third curve was consistently larger

246   than the second provided reassurance that the response of the vessels was not

247   deteriorating over time. Studies were completed in vessels from a total of 17 animals.

248   <u>Data and Statistical Analyses.</u> The contractile response to PE was expressed as

249   a percentage of the maximal constriction produced by KCl (% K_{max}). The curves were

250   fitted with the best-fit line for describing the response to a given agonist. The negative

251   logarithm to base 10 of the dose at which vessels were 50% maximally-constricted to

252   PE (pD_2) was used as an index of vascular sensitivity, and the area under the curve

253   (AUC) was calculated to assess overall reactivity using Prism v6.0 (GraphPad Software,

254   La Jolla CA). Single values were compared between normoxic and hypoxic groups

255   using unpaired t-tests. Differences in immunostaining or mRNA expression between

256   normoxic and hypoxic groups were assessed using one-way ANOVA with Student

257   Neuman Keuls *post hoc* test. The effects of AMPK activation on PE-induced constriction

258   were assessed using two-way ANOVA with Tukey *post hoc* test (Sigma Stat,

259   Buckinghamshire, UK). Data are presented as the mean ± standard error of the mean

260   (S.E.M.). Significance was accepted when the two-tailed p<0.05 and reported as trends

261   when 0.05<p<0.10.

262

263   **Results**

264   <u>Maternal and fetal characteristics.</u> Murine maternal body weights were similar at

265   days 1 and 14, but reduced at day 19 in the hypoxic compared with normoxic groups

266   (Table 1). Litter size was not reduced in the hypoxic group although there was a trend

267   for a greater number of reabsorptions. Fetuses from hypoxic pregnancies weighed less

268   but placental weights were similar to those seen in the normoxic group. Fetuses from

269 the hypoxic compared with the normoxic group had smaller crown-rump lengths,

270 biparietal diameters (BPD) and a greater BPD to body weight ratio, indicating

271 asymmetric growth restriction (Table 1).

272   Placental and birth weights were similar for the non-labouring, sea-level or high-

273 altitude women (Table 1), although birth weight was reduced at high altitude in the

274 larger group of subjects from which these data were derived (Yung *et al.*, 2012)

275   Immunohistochemistry. The uterine vessels from normoxic mice demonstrated

276 staining for phosphorylated (activated) AMPK in arteries of varying size. The hypoxic

277 compared with normoxic animals had greater staining intensity with primary antibody

278 (11.0% *vs.* 22.0% respectively, p<0.0001) whereas the staining intensity did not differ in

279 the vessels from normoxic animals with and without antibody (p=0.12), indicating

280 minimal AMPK expression (Figure 1, left hand panels). Phosphorylated AMPK was also

281 present in all regions of the mouse placenta (Figure 1, right hand panels). Whereas

282 staining intensity did not differ between the JZ and LZ of the placenta from normoxic

283 animals (p=NS), hypoxic exposure markedly increased LZ relative to JZ staining

284 (p<0.0001).

285   Quantitative RT-PCR. Results from quantitative RT-PCR assays showed that

286 mRNA for LKB1, AMPK alpha-1, and AMPK alpha-2 (as a % of β-actin) were present in

287 human and mouse placental tissue. Levels of RT-PCR products for LKB1 and both

288 AMPK catalytic isoforms were greater in the tissues from the high-altitude compared

289 with sea-level human placentas (Figure 2, top panel). In sea-level mouse placentas,

290 AMPK alpha-2 levels were higher in the labyrinthine than junctional zones (Figure 2,

291 bottom panel).

292   Myography. The internal circumference of the isolated UtA at the time of study

293 was similar in the normoxic and hypoxic murine groups (Table 1). There was no

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294   difference in the maximal contraction to KCl or PE, or the contractile sensitivity to PE as

295   measured by the pD_2 (Table 1) or the relaxation response to ACH (10^{-10} M to 10^{-4} M,

296   data not shown).

297   A769662, an AMPK agonist, caused complete, concentration-dependent

298   relaxation in the PE pre-constricted UtA and that relaxation was similar in the normoxic

299   and hypoxic mice (Figure 4 3A). Inhibition of NOS by incubation with L-NAME reduced

300   the relaxation response to A769662 by 30% but did not fully reverse it, indicating that

301   70% of the effect was NO-independent in both the normoxic and hypoxic groups (Figure

302   3B).

303   Incubation with the AMPK agonist A769662 reduced UtA contractile sensitivity to

304   PE in both the normoxic and hypoxic mice as demonstrated by the rightward shifts in

305   the contractile concentration-response curves (Figure 4A-B), with the change due to

306   A769662 being greater in the hypoxic than normoxic groups (Figure 5 4C). Treatment of

307   the vessel with L-NAME increased contractile sensitivity to PE in vessels from normoxic

308   or hypoxic mice (both p<0.05), indicating that a portion of A769662's vasodilator effect

309   was due to increased NO production or activity. However, L-NAME treatment did not

310   fully reverse the effect of A769662, indicating that there was a NO-independent

311   contribution as well. Moreover the NO-independent component was principally

312   responsible for the greater vasodilator effect of AMPK activation seen in the vessels

313   from hypoxic than normoxic animals (Figure 5 4D).

314

315   **Discussion**

316   Our principal findings were that AMPK was present in uterine arteries from near-

317   term mice as well as in mouse and human placental tissue, and that its AMPK

318   expression was increased by hypoxia as judged by immunohistochemistry and mRNA

13

319 expression. Consistent with the possibility that the LKB1-AMPK signalling pathway

320 influenced vascular reactivity, we found that pharmacological activation of AMPK

321 caused PE-preconstricted UtA from pregnant mice to relax due to both NO-dependent

322 and -independent mechanisms. While the vasodilator effect of AMPK activation was

323 similar in UtA isolated from normoxic or hypoxic mice, AMPK activation more markedly

324 opposed PE-induced constriction in the UtA from the hypoxic than the normoxic mice

325 and this was due to NO-dependent and largely NO-independent mechanisms. We

326 therefore concluded that AMPK activation dilates uterine arteries, particularly in

327 response to PE-induced vasoconstriction, and that this latter effect is enhanced by

328 hypoxic pregnancy.

329   AMPK activation in non-pregnant animals has chiefly been studied for its

330 metabolic effects. Drugs such as metformin, which is widely prescribed for lowering

331 glucose levels in individuals with type 2 diabetes, decrease hepatic glucose production

332 by mildly inhibiting the mitochondrial respiratory chain complex I, which in turn activates

333 AMPK (Viollet *et al.*, 2012). In skeletal muscle, AMPK activation promotes glucose

334 uptake and mitochondrial biogenesis, and decreases energy demand by inhibiting the

335 mechanistic target of rapamycin (mTOR) or by switching on the expression of various

336 catabolic enzymes (Hardie, 2011). More recently it has been recognized that AMPK

337 activation also has vascular effects. Vessels express AMPK alpha-1 and alpha-2

338 isoforms; alpha-1 predominates in endothelial cells and both are present in vascular

339 smooth muscle with their relative predominance varying by tissue type (Evans *et al.*,

340 2005; Goirand *et al.*, 2007; Matsumoto *et al.*, 2008). AMPK activation has been shown

341 to improve cardiac function in a rat model of chronic heart failure (Wang *et al.*, 2011); to

342 help initiate hypoxic pulmonary vasoconstriction (Evans *et al.*, 2005); and to augment

343 acetylcholine-induced relaxation in pre-constricted isolated thoracic aorta, mesenteric or

344 resistance-sized cremaster arteries (Goirand *et al.*, 2007; Ford & Rush, 2011).

345 Importantly, the vascular effects of AMPK activation are not dependent on its metabolic

346 effects (Bradley *et al.*, 2010) or the accumulation of adenosine (Evans *et al.*, 2005;

347 Goirand *et al.*, 2007; Bradley *et al.*, 2010) but rather are due to both NO-dependent and

348 NO-independent mechanisms; namely, increased eNOS activity and NO production

349 (Viollet *et al.*, 2010), decreased superoxide and vasoconstrictor prostanoid production

350 (Matsumoto *et al.*, 2008; Li *et al.*, 2010), and direct actions in vascular smooth muscle

351 (Goirand *et al.*, 2007).

352 　　　Our data showing AMPK immunostaining in normoxic mouse uterine vessels and

353 placenta tissue, and mRNA in murine and human placenta for both AMPK alpha

354 isoforms and the upstream kinase responsible for activating AMPK, LKB1, in mouse

355 and human placenta were consistent with prior reports showing that AMPK is expressed

356 in placental tissue (Yung *et al.*, 2012). This is, however, to the best of our knowledge

357 the first report of its presence in murine uterine vessels during pregnancy. The UtA and

358 other uterine vessels undergo profound changes during pregnancy, enlarging their

359 diameters as the result of structural remodelling as well as increased vasodilator

360 responses to flow, acetylcholine and other pharmacological agonists (reviewed in (Osol

361 & Moore, 2014)). Such changes are attributable to effects of oestrogen and other

362 pregnancy hormones serving to increase the production and/or activity of various

363 vasodilators, including NO and large-conductance potassium channels (BK_{CA}), rather

364 than placentation *per se* since the changes begin before placentation is complete, occur

365 even in ectopic pregnancy, and are present albeit to a lesser degree in pseudo-

366 pregnant animals (Burchell, 1967; Rosenfeld *et al.*, 1996; van der Heijden *et al.*, 2005;

367 Collins *et al.*, 2011; Hu *et al.*, 2011). We also showed for the first time that uterine

368 arteries are relaxed by AMPK activation, suggesting that activation of AMPK may be

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369   another mechanism contributing to pregnancy vasodilatation. The vasorelaxant effects

370   of AMPK activation were due, in part, to increased NO production or activity as shown

371   by the effects of NOS inhibition but the major portion of its vasorelaxant effect remained

372   following NOS inhibition, indicating that additional mechanisms are involved. One

373   possibility is that AMPK activation reduced the production of vasoconstrictor

374   prostanoids, similar to what has been reported in mesenteric arteries following

375   metformin treatment in a rat model of type 2 diabetes (Matsumoto *et al.*, 2008). Direct

376   effects on vascular smooth muscle cells may also be involved via actions on the RhoA-

377   Rho associated protein kinase (Rock) pathway (Gayard *et al.*, 2011) or via K^+ channels

378   given that AMPK activation affects multiple K^+ channels (Andersen & Rasmussen,

379   2012), including the BK_{CA} and the ATP-sensitive potassium channel (K_{ATP}), previously

380   implicated in uterine vascular responses to pregnancy and hypoxia (Zhu *et al.*, 2013).

381   Thus further study is required in both non-pregnant and pregnant animals to determine

382   whether pregnancy influences the effects of AMPK activation on UtA vasoreactivity, and

383   the endothelial as well as vascular smooth muscle mechanisms involved.

384   Little is known about the effects of AMPK activation under conditions of hypoxia.

385   Suggesting a beneficial role, Davidge and co-workers have shown that resveratrol,

386   which works in part by activating AMPK (Hardie, 2011; Tennen *et al.*, 2012),

387   administered to the mother under conditions of severe hypoxia dramatically improved

388   fetal survival and increased placental relative to fetal weight but not fetal growth *per se*

389   (Bourque *et al.*, 2012; Banek *et al.*, 2013). Resveratrol treatment also increased UtA

390   blood flow velocity and fetal weight in a catechol-O-methyltransferase knockout mouse

391   model of fetal growth restriction, but vasodilator or vasoconstrictor responses of isolated

392   UtA were unaffected (Poudel *et al.*, 2013). Reseveratrol has also been shown to

393   agument UtA blood flow during pregnancy in nonhuman primates (Roberts *et al.*, 2014).

394   We found that hypoxia in late murine pregnancy sufficient to decrease fetal

395   growth increased the intensity of AMPK immunostaining in the labyrinthine zone, which

396   may be more sensitive to hypoxia given its rich blood supply in contrast to the relatively

397   hypovascularized junctional zone (Dilworth & Sibley, 2013). AMPK alpha-2 mRNA

398   expression was also greater in the labyrinthine than the junctional zone of the mouse

399   placenta, suggesting that it may be the isoform involved in the greater immunostaining

400   but differences in primer efficiency prevent comparison of LKB1, AMPK alpha-1 and

401   alpha-2 expression levels. In humans, the levels of RT-PCR products for both AMPK

402   alpha subunits and LKB1 were greater in the placentas from high- *vs*. low-altitude

403   residents, suggesting higher basal AMPK activity and / or increased capacity to respond

404   to metabolic stress. However, while these same placentas showed evidence of

405   endoplasmic reticulum (ER) stress and mTOR inhibition, the ratio of P-AMPK to total

406   AMPK was not consistently elevated in the small number (n=3) of non-labouring

407   placentas available for study (Yung *et al.*, 2012). In pre-eclamptic women, the ratio of P-

408   AMPK to total AMPK protein levels was inversely correlated with gestational age, being

409   highest in those with the earliest onset of disease, although not higher overall in early-

410   (<34 wk) *vs*. late ($\geq$ 34 wk) onset pre-eclamptic or normotensive women (Yung *et al.*,

411   2014). Thus, further studies in larger numbers of placentas are required to determine if

412   AMPK is activated at high *vs*. low altitude in placental tissue.

413   We were interested in the effect of AMPK activation on UtA vasoreactivity under

414   conditions of hypoxia given our prior studies indicating that the gene region containing

415   *AMPKα-1* has been acted upon by natural selection in Andean residents of high altitude

416   and that the variants more common in Andeans were positively associated with birth

417   weight, UtA diameter and alterations in the expression of genes in the mTOR pathway

418   previously implicated in altitude-associated fetal growth restriction (Bigham *et al.*, 2009;

419   Yung *et al.*, 2012; Bigham *et al.*, 2014). The increased intensity of immunostaining in

420   the mouse uterine arteries suggested that hypoxia increased AMPK abundance.

421   However, since the antibody employed detects both alpha-1 and alpha-2 isoforms, we

422   were not able to determine which isoforms were present in the uterine vessels.

423   Based on previous studies in pulmonary and uterine arteries, we expected that

424   AMPK activation would have less vasodilator effect in the UtA isolated from the hypoxic

425   *vs*. normoxic animals. In pulmonary arteries, AMPK activation under conditions of acute

426   ($\leq$ 60 min) hypoxia prompted vasoconstriction through mobilization of myocyte

427   sarcoplasmic reticulum calcium stores via ryanodine receptors and the release of an

428   endothelium-derived constrictor (Evans, 2006). Inhibitory effects of AMPK activation on

429   Bk_{CA} channels have also been observed, and in a manner that is splice-variant specific

430   (Wyatt *et al.*, 2007; Ross *et al.*, 2011). It is notable, therefore, that Bk_{CA} channels are

431   inhibited in resistance-sized ovine uterine vessels and, in turn, reverse the normal

432   pregnancy reduction in myogenic tone (Hu *et al.*, 2012). Further, we have reported that

433   hypoxia throughout pregnancy reduced the NO-dependent UtA vasodilator response to

434   ACH and flow in isolated guinea pig UtA (White *et al.*, 2000; Mateev *et al.*, 2003).

435   Therefore, our finding that pharmacological AMPK activation prompted similar

436   concentration-dependent vasodilation in preconstricted UtA from normoxic and hypoxic

437   animals was somewhat surprising. Since maximal contraction to PE tended (p=0.07) to

438   be greater in the hypoxic than the normoxic group and in order to evaluate the

439   relationship between AMPK activation and PE constriction more fully, we extended our

440   study to examine the effect of AMPK activation on contractile sensitivity to PE. We

441   found that AMPK activation markedly reduced UtA contractile sensitivity to PE in the

442   UtA from the hypoxic compared with normoxic animals as demonstrated by a rightward

443   shift in the contractile-response curves. The shift was greater in the vessels from the

444 hypoxic compared to normoxic animals, and was partly reversed by treatment with the

445 NOS inhibitor L-NAME, suggesting a role for NO in opposing PE-induced of constriction.

446 While we cannot rule out the possibility that hypoxic exposure increased NO sensitivity,

447 this appeared unlikely given that the relaxation in response to ACH or A769662 was not

448 greater in the UtA from the hypoxic compared to normoxic animals. In addition, even

449 though the NO component was larger in the hypoxic group, the major portion of the

450 greater reduction in contractile sensitivity to PE was NO-independent. We therefore

451 concluded that greater AMPK activation under conditions of hypoxic pregnancy opposes

452 PE-induced vasoconstriction, thus perhaps serving as a compensatory mechanism for

453 maintaining uterine artery blood flow. Further studies are required for determining the

454 mechanisms by which AMPK activation opposes PE-induced vasoconstriction and

455 whether similar effects of AMPK activation are observed when hypoxia is present

456 throughout gestation such as is the case in residents of high altitude.

457   The strengths of our study were its broad-ranging design in which human as well

458 as murine tissues were examined following exposure to normoxia and either shorter- or

459 longer-term hypoxia. Further, multiple methods were used to evaluate the effects of

460 AMPK activation and hypoxia; namely, immunohistochemistry, quantitative RT-PCR and

461 myography. Our results, however, were limited by use of a single AMPK activator. While

462 A769662, unlike other agonists, activates AMPK directly (Goransson *et al.*, 2007), it too

463 has indirect effects by, for example, inhibiting the sodium pump (Benziane *et al.*, 2009).

464 While we used only one agonist, our results were similar to those obtained using

465 metformin, AICAR or exercise to activate AMPK, and thus suggested that the responses

466 observed were the result of AMPK activation and not some other effect of the agonist

467 employed (Goirand *et al.*, 2007; Ford & Rush, 2011; Wang *et al.*, 2011; Kroller-Schon *et*

468 *al.*, 2012) but additional studies using other agonists are required to confirm the

469   mechanisms of A769662 action. Further studies are also needed using western blot or

470   other means to document AMPK activation, and using pair-fed animals to control for the

471   lesser maternal weight gain seen during the period of hypoxic exposure in the present

472   report. The possible effect of duration of hypoxic exposure also requires evaluation

473   since the late-pregnancy onset employed here does not parallel the circumstances of

474   residence at high altitude where hypoxia is present throughout gestation but, on the

475   other hand, it does likely resemble that occurring with late-onset pre-eclampsia.

476   In summary, AMPK is present in maternal uterine vessels as well as in placental

477   tissues, and its abundance increased by short- as well as longer-term hypoxia as

478   indicated by greater immunostaining in uterine vessels from pregnant mice and

479   increased expression of both catalytic AMPK subunits in murine and human placenta.

480   This is, to our knowledge, the first report that pharmacological AMPK activation prompts

481   vasodilatation in UtA via both NO-dependent and -independent mechanisms and that

482   exposure to chronic hypoxia sufficient to restrict fetal growth markedly augmented the

483   ability of AMPK activation to oppose PE-induced vasoconstriction. Together with prior

484   reports that genetic variation near AMPK is related to increased UtA blood flow and

485   maintenance of fetal growth in high-altitude adapted Andeans, we speculate that AMPK

486   activation may be helping to maintain utero-placental blood flow and thereby limit

487   cellular energy depletion. In addition to its role as a metabolic sensor, AMPK may be a

488   key link between maternal metabolic and cardiovascular responses to pregnancy and

489   the regulation of fetal growth.

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Figure Captions

Figure 1. Phosphorylated AMPK (brown staining) is present in uterine arteries (UtA)

and placentas from normoxic mice and increased by hypoxia in the UtA and the

placental labyrinthine (LZ) but not junctional (JZ) or the decidua (DEC) zones. For the

placenta specimens, panels A and B are with and without AMPK antibody, respectively,

at 5x and panels C and D are with AMPK antibody at 20x magnification.

Figure 2. Upper Panel: mRNA for both AMPK alpha subunits and LKB1 are present at

higher levels in human placentas from 3100 m *vs*. sea level. Lower Panel: Murine

placentas also contain both AMPK alpha subunits and LKB1 at sea level, with

expression levels for the alpha-2 subunit being greater in the labyrinthine than junctional

zone. *=$p<0.05$, **=$p<0.01$, ****=$p<0.0001$. Data are mean $\pm$ sem.

Figure 3. Panel A: Uterine arteries pre-constricted with a submaximal dose of PE from

normoxic (n=5) and hypoxic (n=7) pregnant (day 19) mice relax in response to

increasing concentrations of the AMPK agonist, A769662. Panel B: The area under the

curve (AUC), representing nitric oxide (NO)-dependent and -independent components

did not differ in the normoxic and hypoxic groups. Data are mean $\pm$ sem.

Figure 4. Incubation with A769662 (1×10^{-4}M, open circles, dotted lines) decreased

contractile sensitivity to phenylephrine (PE) in UtA from both normoxic (panel A, n=8)

and hypoxic (panel B, n=6-7) pregnant mice compared to values obtained ~~contraction~~

prior to incubation with A769662 (open circles, solid lines). This decrease was

significant in both groups when expressed as the area under the curve (AUC) (panel C,

$p<0.05$, two way ANOVA and *post-hoc* Tukey test). The AUC, representing the change

in sensitivity to PE following incubation with A769662, was significantly greater in the

hypoxic compared to the normoxic animals (panel C, $p<0.05$, unpaired t-test). Treatment

with L-NAME (1×10^{-5} M, black boxes and dashed lines) decreased the contractile

790   response to PE in A769662-treated vessels from normoxic (A) and hypoxic (B) pregnant

791   mice, indicating a significant NO-dependent component (both $p<0.05$), but did not fully

792   restore the contractile response. The contributions of both nitric oxide (NO)-dependent

793   and -independent mechanisms as well as the total relaxation response were greater in

794   the UtA from hypoxic compared with normoxic animals (panel D, $p<0.05$, unpaired t-

795   test). * = $p<0.05$. Data are mean $\pm$ sem.

796

Table 1. Characteristics of the murine and human subjects.

Variable		Normoxic group	Hypoxic group	P value
Mice:				
Sample size		8	7-9	
Maternal body weight, gm	day 1	26.4 ± 1.8	26.2 ± 0.8	0.89
	day 14	31.9 ± 2.2	31.8 ± 0.8	0.93
	day 19	37.4 ± 3.0	32.5 ± 1.1	**0.046**
Litter size, n		6.0 ± 1.1	6.6 ± 0.4	0.59
Reabsorptions, n		0.4 ± 0.2	1.2 ± 0.4	0.06
Fetal weight, mg		1101.6 ± 11.0	816.2 ± 36.3	**<0.0001**
Placental weight, mg		101.4 ± 7.5	95.5 ± 3.8	0.42
Fetal crown-rump length, mm		18.2 ± 0.3	16.3 ± 0.4	**0.002**
Fetal biparietal diameter, mm		5.4 ± 0.2	4.8 ± 0.1	0.03
Fetal biparietal diameter/weight		0.49 ± 0.02	0.58 ± 0.02	**0.006**
UtA internal circumference, μm		847.6 ± 34.1	911.8 ± 39	0.21
KCl maximum contraction, μm		1.08 ± 0.2	0.79 ± 0.1	0.20
PE maximal contraction, μm		124.8 ± 11.8	166.2 ± 17.5	0.07
	PD_2, M	6.8 ± 0.29	7.1 ± 0.3	0.50
PE+A769662 pD_2 without L-NAME, M		5.7 ± 0.17	5.2 ± 0.1	**0.045**
PE+A769662 pD_2 with L-NAME, M		5.9 ± 0.16	5.6 ± 0.2	0.32
Human subjects:				
Sample size		3	3	
Placental weight, gm		670 ± 118	530 ± 35	0.33
Infant birth weight, gm		3813 ± 104	3263 ± 502	0.58
Infant gestational age, wk		39.2 ± 1.4	39.8 ± 1.7	0.78

Values are means ± sem. Bolded p values are <0.05.

Abbreviations: KCl = potassium chloride, L-NAME = N_ω-Nitro-L-arginine methyl ester hydrochloride, PE = phenylephrine, pD_2 = negative logarithm to base 10 of the EC50 or the dose at which 50% of the maximal contraction was achieved.