Increased prevalence of EPAS1 variant in cattle with high-altitude pulmonary hypertension

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High-altitude pulmonary hypertension (HAPH) has heritable features and is a major cause of death in cattle in the Rocky Mountains, USA. Although multiple genes are likely involved in the genesis of HAPH, to date no major gene variant has been identified. Using whole-exome sequencing, we report the high association of an EPAS1 (HIF2α) double variant in the oxygen degradation domain of EPAS1 in Angus cattle with HAPH, mean pulmonary artery pressure >50 mm Hg in two independent herds. Expression analysis shows upregulation of 26 of 27 HIF2α target genes in EPAS1 carriers with HAPH. Of interest, this variant appears to be prevalent in lowland cattle, in which 41% of a herd of 32 are carriers, but the variant may only have a phenotype when the animal is hypoxemic at altitude. The EPAS1 variant will be a tool to determine the cells and signalling pathways leading to HAPH.
High-altitude pulmonary hypertension (HAPH) in cattle leads to Brisket Disease, which is congestive right heart failure with oedema of the chest muscles and gravity-dependent tissues. Bovine HAPH is heritable in an autosomal dominant mode with incomplete penetrance. The responsible genes are unknown. Brisket disease was first recognized in high-altitude ranching in the early 1900s. Evidence that HAPH in cattle is an autosomal dominant trait was suggested by breeding experiments by Grover and Reeves. A few studies have investigated the genetic basis of HAPH but no genes with a high likely impact have been identified. Epidemiological studies in high-altitude cattle have found influences of age, gender, birth weight and growth rate in addition to heritability. Early death in high-altitude calves is also associated with undiagnosed pulmonary hypertension.

Bos taurus cattle have one of the highest acute and chronic hypoxic pulmonary arterial pressure (PAP) responses of all species. Cattle have a basal sea level mean PAP of approximately 27–29 mm Hg, versus only 14 mm Hg in human. There is no strict definition of pulmonary hypertension in cattle, but an age greater than 12 months and residence at or above 7,000 feet altitude, a mean PAP of 31–41 mm Hg is considered normal, and above 49 mm Hg is considered at high risk for brisket disease. PAPs between 42 and 48 mm Hg are in an uncertain zone. PAP rises with duration at high altitude, the degree of altitude and age of the animal. Some animals develop HAPH at altitudes near 5,000 feet. HAPH affects 5–10% of most high-altitude ranches. Affected cattle may die of right heart failure if not identified and moved to lower altitude. Over 2 million head of cattle reside at high altitude in the United States of America and the annual losses to high-altitude ranches are considered substantial, estimated at multiple millions of dollars a year.

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There is no known measurement at low altitude to predict which cattle will develop HAPH upon ascent. The high rate of development of HAPH in cattle shipped to high altitude suggested us that the variant was common in lowland herds. Human HAPH is a worldwide problem, with over 140 million high-altitude dwellers at risk. We describe a newly identified double variant in the hypoxia-inducible factor (HIF2α) gene, Endothelial PAS domain-containing protein 1 (EPAS1), that is highly associated with HAPH and probably represents a gain of function mutation that is prevalent in low-altitude Angus herds and only expresses disease in the hypoxic state at high altitude.

**Results**

**Phenotype data and initial whole-exome sequencing (WES).** We identified 41 (30 bulls and 11 cows) Angus cattle residing at high altitude from three ranches at altitudes of 4,850, 7,200 and 8,590 feet. All cattle underwent right heart catheterization by an experienced veterinarian (T.N.H.) at rest to measure PAP and draw blood for DNA testing. Twenty cattle had HAPH with PAP > 50 mm Hg and twenty-one cattle had PAP < 39 mm Hg. Our strategy as follows: we initially used comparative WES analysis of DNA from five cattle with extreme HAPH versus five unaffected controls to seek any important variant. To ensure the selected cattle were as genetically diverse as possible, we genotyped ten microsatellite markers on all DNA samples and calculated pairwise ‘distance’ score, calculated as the number of alleles that differed between samples, using SimpleSeq2 (ref. 17). The five cattle with the highest diversity in the high PAP group had mean PAP of 94.2 ± 5.3 and the five cattle with the highest diversity in the low PAP group (unaffected) had mean PAP of 30.8 ± 0.8 (Fig. 1). We generated an average of 10 Gb of sequence per animal as paired-end 100 bp reads by WES with 80-fold coverage. We sequenced over 2000 exons of 2000 genes (Table 1) were found to be homozygous negative in all samples (Supplementary Table 1) with a sequence variation, of which 9 genes (Table 1) were found to be homozygous negative in all unaffected and heterozygous positive for a variant in four of the genes.

**Filtering sequence.** We used an autosomal dominant model for screening (Fig. 2). We used a simple algorithm that showed at least four of the five affected samples carry the same variant and four of the five unaffected samples be homozygous negative and for a candidate variant. This generated a list of 103 genes (Supplementary Table 1) with a sequence variation, of which 9 genes (Table 1) were found to be homozygous negative in all unaffected and heterozygous positive for a variant in four of the genes.
The 13 of the 32 samples had both variants (41%), with every positive sample carrying both variants. To test this hypothesis, we obtained DNA samples from 32 Angus cattle residing in Alabama, USA, to screen for the two EPAS1 variants. In all, 13 of the 32 samples had both variants (41%), with every positive sample carrying both variants.

Replication in second herd and lowland cattle. We tested and replicated the variant data in a second herd, residing at 7,100 feet in Wyoming. These were young cattle, studied about 6 months of age, but they had been born and raised at this altitude. Eleven of the fifteen cattle with PAP ≥ 45 mm Hg but only four of sixteen with PAP ≤ 39 mm Hg carried the variant, χ² -value < 0.007 (Supplementary Table 2). A joint analysis of the original herd with HAPH, which was replicated in a second independent herd. Although the original P-value was not as strong as in typical genome-wide association studies due to the small sample size, the replication finding was statistically highly significant, and the odds ratio suggests a strong effect of the variant in HPAH.

Because of the high rate of development of HAPH in lowland cattle moving to high altitude, we predicted that the variants would be present at a reasonably high frequency in lowland cattle. To test this hypothesis, we obtained DNA samples from 32 Angus cattle residing in Alabama, USA, to screen for the two EPAS1 variants. In all, 13 of the 32 samples had both variants (41%), with every positive sample carrying both variants.

Conservation among species and stability. Since the two variants introduce non-synonymous changes in the HIF2α protein, we investigated existing databases to see if the corresponding residues are conserved among species. These two amino acids are well-conserved across species. Interestingly, yak, which has an identical HIF2α protein sequence to cattle and has adapted to high altitude, is not reported to have either of these changes (http://www.mevolab.net/yak). Sheep are known to have one of the two single-nucleotide polymorphisms found in cattle, at codon 606, but not the second variant. Sheep are much less susceptible to HAPH than cattle. Given that all affected bovine have both the variants, it is possible that both changes need to be present together to alter function of the protein. Another possibility is that the HIF pathway is configured in sheep to tolerate one variant allele.

Table 1 | Nine genes found in 4/5 HAPH and 0/5 unaffected cattle at altitude.

| Gene | Symbol | Function | Cow chromosome | Ref Seq mRNA | Non-synonymous variation | Location (Btau_4.6.1) |
|------|--------|----------|----------------|-------------|--------------------------|-----------------------|
| Budding uninhibited benzimidazoles 1 | BUB1 | Mitotic spindle checkpoint kinase | NM_001102011 | Exon10:c.G1165A:p.V389M | 1571369 |
| Complement component | C4A | Complement factor 4 | NM_001166485 | Exon11:c.A1262G:p.Q421R | 27193512 |
| Feline leukemia virus | FLVCR2 | Calcium transporter | NM_001192143 | Exon9:c.C1239G:p.D413E | 87200257 |
| Leucine-rich repeat-containing protein 17 | LRRC17 | A negative regulator of receptor activator of NF-κB | NM_001078150 | Exon2:c.A95T:p.H32L | 44501073 |
| Pyruvate dehydrogenase phosphatase | PDPR | Pyruvate metabolic electron transport | NM_174781 | Exon3:c.C327G:p.H109Q | 1939296 |
| Regulator of G-protein signalling 18 | RG518 | Regulator of G-protein signalling | NM_001192971 | Exon4:c.C397T:p.R133C | 13749384 |
| Zinc finger MMYM-type protein 6 | ZMYM6 | Regulation morphology and cytoskeleton | NM_001206292 | Exon16:c.G2834C:p.C945S | 111275016 |
| Endothelial PAS domain-protein 1 (hypoxygen-inducible factor 2-α) | EPAS1 | Transcription factor in the induction of genes regulated by oxygen | NM_174725 | Exon12:c.G1816A:p.A606T | 28662654 |
| Rho GTPase activating protein 20 | ARHGAP20 | GTPase activator for the Rho-type GTPases | NM_001206733 | Exon15:c.C3262T:p.P1088S | 20909547 |

EPAS1 (HIF2α)  

Figure 3 | Graphic depiction of the known EPAS1 domains. bHLH, basic Helix-Loop-Helix; PAS, Per-Arnt-Sim domain; ODDD, oxygen-dependent degradation domain; N-TAD, N-terminal transactivation domain; C-TAD, C-terminal transactivation domain. The two variants are in the ODDD domain of the protein.

five of affected. Sanger sequencing of ten additional samples, five affected and five unaffected, narrowed the list to two genes, EPAS1 and pyruvate dehydrogenase phosphatase regulatory subunit (PDPR), in which none of the ten unaffected and eight of the ten affected samples had the variant. EPAS1 contained two variants in close proximity to each other, c.1816G-A and c.1828G-A, both in exon 12. Each sample had either both variants or none, indicating that both were in haplotype. The EPAS1 variants predicted two non-synonymous substitutions, p.A606T and p.G610S, in the oxygen-dependent degradation domain of the HIF2α protein (Fig. 3).

We then designed Taqman assays for the 2 EPAS1 variants and the 1 PDPR variant, and genotyped 10 additional HAPH cattle and 11 additional unaffected animals to fully evaluate the 41 variants. The 4 unaffected cattle that carried the variant had the highest PAPs among all 21 unaffected (36–38 mm Hg), at the upper limit of normal. Taking all 41 cattle, 15 of the 20 samples with PAP > 50 mm Hg were positive for the two EPAS1 variants and 4 of 21 with PAP < 39 mm Hg had both variants, with a P-value of 3.3 × 10⁻⁴.

Armitage trend test for this herd was 1.8 × 10⁻³. Wilcoxon’s rank sum tests on PAP between EPAS1 variant carriers and non-carriers were also significant, P = 4.6 × 10⁻³. PDPR variant did not segregate with high PAP; 10 of the 20 affected samples and 5 of the 21 unaffected had the PDPR variant (P < 0.08). With a likelihood of development of HAPH at 50%, the probability of EPAS1 variants would be 79.7% and the negative predictive value would be 76.4%. The relative risk for HAPH between a carrier and a non-carrier is estimated to be 3.47 (1.55–7.77), and the odds ratio is estimated as 12.75 (2.9–66.4). Trend test P-value was 0.002. We found no variants that segregated with HAPH in a number of known PH genes, including BMP2, ACVRL1, Cav1, VHL and endoglin. There were no other non-synonymous variants in EPAS1 in the cattle.
Mutations in the same oxygen-dependent domain in the human EPAS1 gene show increased stability and decreased degradation associated with increased downstream transcriptional activity, consistent with a gain of function\(^29\). We used computational tools to predict the function of the variants residues in cattle. The analysis showed that each variant was predicted to significantly increase the protein stability of HIF2\(\alpha\). Two stabilizing variants together in cis are predicted to result in an even more stable protein. We note that the two residues were in perfect linkage disequilibrium in our cattle. It is still possible that the variants are in linkage disequilibrium with another variant of effect on the hypoxic response.

Since these variants were predicted to increase HIF2\(\alpha\) stability, we hypothesized that this would result in upregulation of downstream HIF2\(\alpha\) target genes. We thus looked at differential expression between peripheral blood mononuclear cells from cattle with HPAH and HIF2\(\alpha\) variants and unaffected cattle without HIF2\(\alpha\) variants by analysing our previously published gene expression array data\(^6\). We found that in cattle containing the double HIF2\(\alpha\) variant, transcription of HIF target genes was significantly overrepresented (\(P < 0.0001\) by \(\chi^2\) - chi-square test), with 26 of 27 HIF target genes significantly upregulated compared with unaffected cattle (Supplementary Table 3).

Discussion

Using WES and an autosomal dominant model in 20 HAPH and 21 unaffected Angus cattle living at high altitude, we found two variants, in cis, in exon 12 of the EPAS1 gene that encodes a hypoxia inducible factor, HIF2\(\alpha\). The variants were present in 75% of the 20 cattle with mean PAP \(> 50\) mm Hg, in all 5 cattle with extreme HAPH (PAP \(> 94\) mm Hg), and in 19% of 21 unaffected cattle with PAP \(< 39\) mm Hg. We replicated the finding in a second, unrelated herd from Wyoming, in which 11 of 15 cattle with HAPH and only 4 of 16 unaffected were carriers\(^18\). We also found higher mRNA expression in variant carriers with HAPH than in unaffected 26 of 27 genes, known to be targets of HIF2\(\alpha\)\(^6\). This double variant haplotype in EPAS1 is likely a gain-of-function mutation that requires a gene–environment interaction for expression.

Human EPAS1 and HIF1\(\alpha\) are basic helix–loop–helix transcription factors that contain a Per Arnt Sim (PAS) domain and share 48% sequence homology\(^20,21\). During sufficient oxygen availability, the resulting HIF proteins are constantly degraded, but are released for function during cell or tissue hypoxia. Under hypoxic conditions, HIFs are protected by inhibition of oxygen-dependent hydroxylation of specific residues in the oxygen-dependent degradation domain. This prevents interaction with the von Hippel–Lindau ubiquitin ligase complex and proteasomal destruction. HIF2\(\alpha\) is found in all human tissues including lung and lung vasculature. The downstream effects of HIF2\(\alpha\) include regulation of angiogenic factors including VEGF and TGF-\(\alpha\), and cell permeability and stimulation of erythropoietic and glycolytic proteins\(^20–22\).

Rare germline EPAS1 gain-of-function variants can cause familial erythrocytosis and/or pulmonary hypertension\(^32,24\). A von Hippel–Lindau mutation that reduces destruction of HIF2\(\alpha\) is found in Chuvash populations and is associated with modest pulmonary hypertension and erythrocytosis\(^35\). Transgenic mice carrying a G536W variant in EPAS1 developed high right ventricular systolic pressure and medial hypertrophy of pulmonary arteries in addition to erythrocytosis under normoxic conditions\(^26\). Thus, HIF2\(\alpha\) dysregulation is well recognized in association with pulmonary hypertension, frequently in context of erythrocytosis, with or without severe hypoxemia.

In contrast, probable loss-of-function variants in EGLN1 and HIF2\(\alpha\) were found to be enriched in residents of the Tibetan Plateau\(^27–29\). Native Tibetans are recognized to have beneficial adaptation to high altitude, with less polycythemia, less haemoglobin desaturation, preserved ventilatory responses and normal birth weights. The inference from these studies is that loss of function of HIF2\(\alpha\) driven either directly or indirectly by downregulation of modifying proteins may be beneficial in states of chronic hypoxia where chronic HIF protein stabilization may be detrimental.

In summary, we found that two cis variants in EPAS1 (HIF2\(\alpha\)) are highly associated with HAPH in Bos taurus Angus cattle residing at high altitude in the Rocky Mountains, USA. Given what is known about HIF2\(\alpha\) function, our data suggest that EPAS1 is likely a major gene for HPAH in cattle. Other genes are undoubtedly involved in bovine HAPH. Additional studies are needed to clarify its biological role in HAPH in cattle and any role of the variant in human HAPH.

Methods

Collection of samples and measurement of PAP. We (T.N.H.) obtained blood from 27 Angus cattle from four sites, ages 12–16 months, residing at 5,200–7,850 feet, by jugular vein puncture on the same day of right heart catheterization, done to screen for HAPH, and blood from an Alabama herd of cattle residing near sea level. The project was approved by the Vanderbilt Medical Center, Institutional Animal Care Use Committee.

DNA isolation and microsatellite-based genotyping. DNA was isolated from whole blood using QiAmp DNA mini-kit as directed by the manufacturer’s instructions (Qagen) and subsequently quantified using a spectrophotometer.

WES and data preprocessing. We used Agilent SureSelect XT Bovine All Exon kit (Cat. Number 4307480; Life Technologies).

Sanger sequencing and Taqman analysis. All DNA sequencing was performed using BigDye (Life Technologies) according to the manufacturer’s instructions. Taqman assays were obtained from Life Technologies. The assays targeted the variations found on WES and were designed so that the wild-type allele utilized the VIC probe and the minor allele utilized the FAM probe. Analysis was done on 5 ng of genomic DNA using ABI PRISM 7500 instrument (Life Technologies) per the manufacturer’s instructions.

Protein stability analysis. The two EPAS1 variants A606T and G610S were individually submitted to the PolyPhen-2 website (http://genetics.bwh.harvard.edu/pph2/) for analysis.

Gene expression array analysis. Of 171 genes found to be upregulated by hypoxia in the Broad Institute set GSEA Elvidge_Hypoxia_Up\(^4\), 68 have expression values above 7 log base 2 Affymetrix units in either affected or unaffected data sets (thus, within reliable signal values). Of these 68, 26 (38%) are upregulated in the affected group compared with the unaffected (\(P < 0.05\) by uncorrected t-test, \(\alpha = 1.25\); Supplementary Table 2), whereas only one is downregulated (1.5%). Based on the total numbers of up- or downregulated genes expressed above 7 log base 2 Affymetrix units in the overall data set using the same criteria, our expectation values are 7% upregulated and 8% downregulated (or 5 genes upregulated and 5 genes downregulated) based on random variation.
Chi squared is thus calculated as 96.4 with 2 degrees of freedom, for a two-tailed P value of less than 0.0001, Supplementary Table 2.

**Statistical analysis.** Statistical analysis was done using Prism for Mac.

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**Author contributions**

J.H.N. had the idea, initiated the project, oversaw the work and wrote the bulk of the paper. T.N.H. catheterized the cattle, measured PA pressure, drew blood for DNA and consulted on HAPH. J.D.C. assisted in sorting gene candidates from WES. B.W. and Z.K. did the sequencing and Tagman assays. J.A.P.III oversaw the lab, consulted on the ideas, contributed to the writing. C.L. did the biostatistical analysis and assisted in filtering design. R.H. organized the filtering, oversaw the technical work of B.W. and Z.K., contributed to all aspects of the project, did technical analysis and wrote much of the methods section. J.D.W. performed the statistical analysis of the mRNA expression data. K.R.S., R.D.B., M.G.T. and S.R.R. performed the physiologic experiments and blood processing of the validation herd from Wyoming. All authors read, assisted with interpretation and approved the manuscript.

**Additional information**

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

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