Association Between Serum Concentrations of Hypoxia Inducible Factor Responsive Proteins and Excessive Erythrocytosis in High Altitude Peru

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Abstract

Painschab, Matthew S., Gary E. Malpartida, Victor G. Davila-Roman, Robert H. Gilman, Todd M. Kolb, Fabiola Leon-Velarde, J. Jaime Miranda, and William Checkley. Association between serum concentrations of hypoxia inducible factor responsive proteins and excessive erythrocytosis in high altitude Peru. *High Alt Med Biol* 16:26–33, 2015.—Long-term residence at high altitude is associated with the development of chronic mountain sickness (CMS), which is characterized by excessive erythrocytosis (EE). EE occurs under chronic hypoxia, and a strongly selected mutation in hypoxia-inducible factor 2 α (*HIF2A*) has been found in native Tibetans that correlates with having a normal hemoglobin at high altitude. We sought to evaluate differences in plasma levels of four HIF-responsive proteins in 20 participants with EE (hemoglobin >21 g/dL in men and >19 in women) and in 20 healthy, age- and sex-matched participants without EE living at high altitude in Puno, Peru. We performed ELISA to measure plasma levels of the four HIF-responsive proteins: vascular endothelial growth factor (VEGF), soluble VEGF receptor 1 (sVEGF-R1), endothelin-1, and erythropoietin. As a secondary aim, we evaluated the association between HIF-responsive proteins and echocardiography-estimated pulmonary artery systolic pressure (PASP) in a subset of 26 participants. sVEGF-R1 was higher in participants with vs. without EE (mean 107 pg/mL vs. 90 pg/mL; $p=0.007$). Although plasma concentrations of endothelin-1, VEGF, and erythropoietin were higher in participants with vs. without EE, they did not achieve statistical significance (all $p>0.25$). Both sVEGF-R1 ($p=0.04$) and erythropoietin ($p=0.04$) were positively associated with PASP after adjustment for age, sex, and BMI. HIF-responsive proteins may play a pathophysiological role in altitude-related, chronic diseases but our results did not show consistent changes in all measured HIF-responsive proteins. Larger studies are needed to evaluate for additional genetic and environmental risk factors.

Key Words: chronic mountain sickness; EPO; HIF; pulmonary hypertension; VEGF

Introduction

Over 140 million people worldwide live at altitudes >2500 meters above sea level (León-Velarde et al., 2005). Long-term residence at high altitude is associated with the development of chronic mountain sickness (CMS), which

is characterized by excessive erythrocytosis (EE). Understanding of the pathophysiology and risk factors for CMS, however, is incomplete. Notably, Tibetan highlanders are less likely to develop CMS and average 1–3 g/dL lower hemoglobin concentrations than native Han Chinese and Andean populations living at similar altitudes (Pei et al., 1989;

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León-Velarde et al., 2005; Yi et al., 2007; Simonson et al., 2010). A better understanding of risk factors and biomarkers associated with CMS may thus provide insight into its pathophysiology.

Recent studies have implicated the hypoxia-inducible factor (HIF) pathway as important in the pathogenesis of CMS. Genome-wide association studies and two candidate studies of *HIF2A* (aka *EPAS1*) in Tibetan highlanders have confirmed a genetic variant in the transcription factor HIF-2 α (Simonson et al., 2010; Yi et al., 2010). This mutation and other single nucleotide polymorphisms (SNPs) in the *HIF2A* gene were associated with lower hemoglobin concentrations in Tibetan mountain dwellers (Beall et al., 2010; Simonson et al., 2010; Yi et al., 2010). Mutations in the *EGLN1* gene, which codes for HIF prolyl 4-hydroxylase domain 2 (PHD2), an important negative regulator of HIF proteins, have also been identified in Tibetan mountain dwellers and associated with lower hemoglobin (Lorenzo et al., 2014).

The HIF family of transcription factors consists of two subunits (α and β) that dimerize and which, under normoxia, are hydroxylated by PHD2, then are constitutively ubiquitinated by von Hippel-Lindau (VHL) protein and degraded. However, in the setting of hypoxia, HIF proteins are stabilized and translocated to the nucleus where they regulate gene expression via hypoxia responsive element DNA sequences (Loboda et al., 2012). The hypothesis, then, is that genetic changes in Tibetan highlanders change HIF protein expression or stability, changing downstream transcription of hypoxia responsive elements and blunt the normal erythropoietic response to hypoxia.

A number of HIF-responsive proteins can be measured in plasma and are potentially of interest in altitude-related illnesses. First, erythropoietin is a HIF-responsive protein released from the kidneys in response to cellular hypoxia. Erythropoietin promotes erythropoiesis but also plays an important role in hypoxic vasoconstriction and smooth muscle proliferation (Jelkmann, 2011). Some studies in Andean highlanders have identified a correlation between erythropoietin and CMS, but this finding has been difficult to reproduce consistently (León-Velarde et al., 1991). Vascular endothelial growth factor (VEGF) and its soluble receptor, soluble VEGF receptor 1 (sVEGF-R1), are involved in the regulation of angiogenesis. The relationship between VEGF isoforms, their receptors, and EE is complicated and incompletely understood (Wu et al., 2010; Kajdaniuk et al., 2011; Voelkel and Gomez-Arroyo, 2014); however, a SNP in *VEGFA* has recently been associated with the development of CMS (Espinoza, et al., 2014). Finally, endothelin-1 is a therapeutic target in pulmonary arterial hypertension and has been shown to be elevated in both humans and mice with pulmonary hypertension (Goerre et al., 1995). Data are limited on protein expression of other HIF-responsive proteins in CMS.

In this study, we recruited 20 participants with EE aged ≥ 35 years of age living at high altitude (3825 meters above sea level) in the Peruvian Andes, and 20 age- and sex-matched participants without EE. We hypothesized that if HIF signaling is responsible for development of CMS, then participants with EE would have increased plasma levels of HIF-responsive proteins such as EPO, sVEGF-R1, VEGF, and endothelin-1. Since HIF-responsive proteins also appear to be important in the pulmonary vascular response to hypoxia (Shimoda and Laurie, 2014), we studied the correlations of erythropoietin, sVEGF-R1, VEGF, and endothelin-1 with pul-

monary artery systolic pressure (PASP) and echocardiography-estimated PASP in a subset of participants in whom PASP was measured.

Materials and Methods

Study design

We conducted a nested case-control study in Puno, Peru (3825 meters above sea level) of participants with and without excessive erythrocytosis. Participants were selected from cohort study of adults aged ≥ 35 years (Miranda et al., 2012). The majority of the population under study was predominantly of Aymaran descent with a Quechua minority. Using available laboratory data, we selected a sample of 20 participants with EE, defined as having a hemoglobin > 21 g/dL in men and > 19 g/dL in women (León-Velarde, 2005), and 20 age- and sex-matched participants without EE. Of this group, a subset of 26 also underwent echocardiography. We used a standardized questionnaire to assess sociodemographics and medical history (Miranda et al., 2012). Trained field workers measured blood pressure, weight, height, heart rate, and spirometry (Miranda et al., 2012). Certified phlebotomists collected blood to measure fasting lipids, glucose, insulin, hemoglobin A1c, and high sensitivity C-reactive protein (hs-CRP). All laboratory tests were processed in a centralized testing facility (Miranda et al., 2012). Participants provided verbal informed consent. The study was approved by the Institutional Review Boards of the Bloomberg School of Public Health, Johns Hopkins University (Baltimore, MD, USA), Universidad Peruana Cayetano Heredia (Lima, Peru), and A.B. PRISMA (Lima, Peru).

Assessment of HIF-responsive proteins

Five milliliters of whole blood were collected in an EDTA-coated tube, then centrifuged within 30 min at 1000 *g* for 15 min. Blood samples were drawn early in the morning while fasting. The supernatant was extracted and immediately frozen at -20°C . Sandwich antibody ELISA, designed to measure free plasma protein levels (Quantikine, R&D Systems, Minneapolis, MN) for the four HIF-responsive proteins of interest (erythropoietin, endothelin-1, VEGF, and sVEGF-R1) were performed in duplicate per manufacturer's protocol. Plasma protein levels were calculated based on standard curves determined by four parameter logistic curve-fit from standard samples. Median (range) coefficient of variations were: VEGF 2.2% (0–23%), sVEGF-R1 1.3% (0–10.9%), endothelin-1 1.5% (0–9.1%), and erythropoietin 1.9% (0–5.4%).

Echocardiography

A subgroup of 26 participants had echocardiography performed. This subset did not differ in any baseline characteristics from the full cohort (Supplementary Table S1;

TABLE 1. HYPOXIA INDUCIBLE FACTOR RESPONSIVE PROTEINS AND SELECTED TRANSFORMATIONS

Biomarkers	Selected transformation
sVEGF-R (pg/mL)	$\frac{1}{\sqrt{sVEGF-R1}}$
VEGF (pg/mL)	log(VEGF)
Endothelin-1 (pg/mL)	Endothelin-1
Erythropoietin (pg/L)	log(Erythropoietin)
hs-CRP (mg/mL)	log(hs-CRP)

supplementary material is available online at www.liebertpub.com/ham). Two operators, blinded to participant characteristics, performed a limited echocardiogram with a portable ultrasound system (MicroMaxx, Sonosite, Bothell, WA) as described previously (Caravedo et al., 2014). PASP was estimated using the simplified Bernoulli equation: $PASP = 4v^2 + RAP$, where v is the peak tricuspid regurgitation jet velocity (meters per second) and RAP is the estimated right atrial pressure as per the American Heart Association Consensus guidelines (McQuillan et al., 2001; Lam et al., 2009; Rudski et al., 2010; Caravedo et al., 2013).

Biostatistical methods

The primary objective was to compare plasma levels of four HIF-responsive proteins between participants with and without EE. Several of these proteins were non-normally distributed. Therefore, we used the Box-Cox Power transform to identify the best transformation for each biomarker (Table 1). These transformations were conducted *a priori* to normalize the data. We used the Spearman method to measure correlation among non-transformed variables and the Pearson method to measure correlation among the trans-

TABLE 2. BASELINE CHARACTERISTICS OF PARTICIPANTS GROUPED BY EXCESSIVE ERYTHROCYTOSIS AND HEALTHY CONTROLS WITH NORMAL HEMOGLOBIN

Characteristic	All (n = 40)	Controls (n = 20)	Excessive erythrocytosis (n = 20)	p
<i>Demographics, mean (SD) or %</i>				
Age, years	62 (14)	61 (14)	62 (14)	0.88
Sex, % men	55	55	55	0.75
BMI, kg/m ²	27 (5)	24 (3)	30 (5)	<0.001
Urban residence, %	60	50	70	0.33
Biomass Fuel, % use	42	50	35	0.52
Poverty, %	66	64	67	1
Finished primary school, %	51	47	55	0.88
<i>Hemodynamics, mean (SD)</i>				
HR, bpm	70 (10)	67 (10)	74 (10)	0.03
SBP, mmHg	118 (15)	116 (16)	120 (15)	0.42
DBP, mmHg	74 (9)	72 (9)	77 (10)	0.06
Pulse pressure, mmHg	44 (11)	45 (12)	43 (10)	0.63
Pulmonary artery systolic pressure, mmHg	34 (13)	33 (14)	35 (10)	0.77
<i>Pulmonary function, mean (SD)</i>				
Post-bronchodilator FEV ₁ , L	2.6 (1.0)	2.8 (1.0)	2.4 (1.0)	0.27
Post-bronchodilator FEV ₁ /FVC, %	75.6 (8.3)	75.3 (9.0)	75.8 (7.7)	0.85
Resting oxygen hemoglobin saturation, %	88 (4)	89 (4)	86 (4)	0.04
<i>Past medical history, %</i>				
HTN, %	26	15	35	0.27
DM, %	5	10	0	0.23
Heart disease or stroke, %	12	15	10	1
COPD, %	8	0	15	0.23
Epworth Sleepiness Scale	7 (4)	7 (4)	7 (3)	0.99
<i>Smoking history, %</i>				
Never, %	72	63	80	
Previous, %	26	32	20	
Current, %	2	5	0	0.37
<i>Laboratory values, mean (SD)</i>				
Total cholesterol, mmol/L	5.1 (1.3)	4.7 (1.3)	5.5 (1.4)	0.07
LDL, mmol/L	3.1 (1.1)	2.8 (1.0)	3.4 (1.2)	0.01
HDL, mmol/L	1.1 (0.2)	1.2 (0.3)	1.0 (0.2)	0.11
Triglycerides, mmol/L	1.8 (0.8)	1.4 (0.5)	2.3 (0.8)	<0.001
Hemoglobin A1c, % mmol/mol	6.1 (0.6)	5.9 (0.6)	6.3 (0.5)	0.02
Fasting glucose, mmol/L	5.3 (0.8)	5.0 (0.9)	5.4 (0.7)	0.15
hs-CRP, mg/L	6.2 (22.9)	2.2 (3.0)	10.2 (32.1)	0.07
log(hs-CRP), log(mg/L)	0.8 (1.4)	0.3 (0.5)	1.0 (1.5)	0.06
Hemoglobin, g/L	186 (30)	160 (15)	213 (15)	<0.001
HOMA-IR. (mmol x uU)/L ²	2.0 (1.5)	1.5 (1.2)	2.6 (1.6)	0.03
<i>Medications, %</i>				
Aspirin, %	5	5	5	1
β-Blockers or calcium channel blockers, %	3	5	0	0.49
ACE Inhibitors, ARBs, or diuretics %	3	0	5	1
Statins, %	3	5	0	0.49
Metformin and/or insulin, %	5	10	0	0.23
Bronchodilators, %	3	0	5	1

TABLE 3. DIFFERENCES IN FOUR HYPOXIA-INDUCIBLE FACTOR RESPONSIVE PROTEINS BETWEEN HEALTHY CONTROLS AND PARTICIPANTS WITH EXCESSIVE ERYTHROCYTOSIS

Biomarkers, mean (SD)	All (n=40)	Controls (n=20)	Excessive erythrocytosis (n=20)	P
<i>VEGF</i>				
VEGF, pg/mL	41 (78)	35 (42)	46 (103)	0.81
Transformed VEGF, log pg/mL	1.2 (3.8)	1.1 (3.9)	1.4 (3.7)	
<i>SVEGF-R1</i>				
sVEGF-R1, pg/mL	98 (20)	90 (14)	107 (21)	0.004
Transformed sVEGF-R1, 1/√(pg/mL)	0.0105 (0.0020)	0.0114 (0.0015)	0.00097 (0.0020)	
<i>Endothelin-1</i> , pg/mL	1.4 (0.6)	1.3 (0.5)	1.5 (0.7)	0.24
<i>Erythropoietin</i>				
Erythropoietin, pg/mL	15.6 (13.1)	14 (14.0)	17.2 (12.4)	0.52
Transformed erythropoietin, log(pg/mL)	2.5 (0.8)	2.4 (0.7)	2.5 (0.9)	

formed values. A secondary objective was to determine if these four HIF-responsive proteins were associated with PASP. Finally, we used logistic regression to determine the relationship between each biomarker and EE adjusted for BMI. We also conducted multivariable regression to determine the relationship between each biomarker and PASP adjusted for age, sex and BMI. We conducted statistical analyses in R (www.r-project.org).

Results

Participant characteristics

Mean age was 62 years (SD 14), 55% were male and 60% were urban dwellers (Table 2). Participants with EE and

healthy controls were similar in age and sex. Those with EE had a higher BMI, higher heart rate, higher LDL cholesterol, higher triglycerides, higher HOMA-IR, higher hemoglobin A1c, and a lower oxygen saturation by pulse oximetry than did healthy controls (Table 2). There were no differences by EE status in other measured variables, including PASP. Chronic exposure to biomass fuel smoke was high at 42% in study sample; however, this exposure was equally distributed between groups. A total of 26% were former smokers and 2% were current smokers with no differences between groups.

Correlation among HIF-responsive proteins, hs-CRP and PASP

We present average values of HIF-responsive proteins in Table 3. None of the HIF-related proteins were strongly associated with any other individual protein (Fig. 1), with the exception of sVEGF-R1 and hs-CRP. sVEGF-R1 appeared to be correlated with PASP and this relationship appeared to be J-shaped (Fig. 2).

Association between HIF-responsive proteins and excessive erythrocytosis

In Figure 3, we compared levels of HIF-responsive proteins and hs-CRP by EE status. Plasma levels of sVEGF-R1 were higher in those with EE compared to healthy controls in single variable analysis (Table 3) and in multivariable analysis adjusted for BMI ($p=0.007$). Although plasma

FIG. 1. Correlation matrix for four hypoxia inducible factor responsive proteins, pulmonary artery systolic pressure (PASP) and high-sensitivity C-reactive protein (hs-CRP) in 20 participants with excessive erythrocytosis and 20 sex- and age-matched participants without excessive erythrocytosis. The strength of correlation is represented both by the direction of the ellipse and the color bar. The color bar correlates with strength of association, more blue implying a stronger positive correlation and more red implying a stronger negative correlation. The number inside each circle is the p value for each relationship.

FIG. 2. Association between soluble VEGF receptor 1 (sVEGF-R1) and pulmonary artery systolic pressure (PASP).

FIG. 3. Differences in transformed values of hypoxia inducible factor responsive proteins and high sensitivity C-reactive protein (hs-CRP) for 20 participants with excessive erythrocytosis (EE) and 20 sex- and age-matched healthy controls with normal hemoglobin (no EE).

levels of VEGF, endothelin-1, or erythropoietin were higher in participants with EE than in those without EE, these increases were not significant in either single variable analyses (Table 3) or in multivariable analyses adjusted for BMI (all $p > 0.25$). Both transformed values of sVEGF-R1 and erythropoietin were associated with PASP (Fig. 4), and were also associated with PASP in a linear regression adjusted for age, sex and BMI (all $p \leq 0.04$). We did not find an association between PASP and either VEGF or endothelin-1.

Discussion

Populations living at high altitude demonstrate different profiles of adaptation which result in protection from CMS in some groups, particularly in Tibetan and in some Andean subjects. Mutations in HIF pathway genes, but not changes in

plasma levels of downstream proteins, have been previously described (Simonson et al., 2010; Yi et al., 2010; Lorenzo et al., 2014). Further, HIF-responsive proteins have shown to be important in the pulmonary vascular response to hypoxia (Shimoda and Laurie, 2014). In this study, plasma sVEGF-R1 was positively associated with both EE and PASP, and erythropoietin was associated with PASP. Although we did not find a strong association between serum levels of VEGF, endothelin-1, or erythropoietin and EE, all of these proteins trended towards higher levels in EE.

Phenotypic parallels exist between CMS and the familial causes of erythrocytosis, in which genetic mutations in the HIF pathway cause erythrocytosis under normoxia. Mutations have been found in *HIF2A*, *EGLN1*, and *VHL* genes that promote HIF2 α stability and HIF-dependent gene expression (Percy et al., 2008) and are correlated with erythrocytosis and

FIG. 4. Scatterplot of transformed values of soluble VEGF receptor 1 (sVEGF-R1) and erythropoietin vs. pulmonary artery systolic pressure (PASP).

elevations in pulmonary arterial pressures (Semenza, 2011; Shimoda and Laurie, 2014). In one example, a mutation in the *VHL* gene causes Chuvash polycythemia. A similar study to ours was performed in patients with Chuvash polycythemia, but with findings that were not entirely consistent with our study. In that study, increased plasma levels of VEGF, endothelin-1, and EPO were associated with Chuvash polycythemia compared to healthy controls (Gordeuk et al., 2004; Bushuev et al., 2006). A significantly higher PASP was noted among Chuvash polycythemia patients but higher PASP was associated only with a lower level of VEGF and no correlation was noted with EPO or endothelin-1. This study was limited by a sample size of only 14 in each arm (Gordeuk et al., 2004; Bushuev et al., 2006). Despite similar phenotypes, a different pathophysiological mechanism, a more heterogeneous disease in our population, small sample sizes, or different populations may account for the differences in findings between the two studies.

VEGF and sVEGF-R1 are two HIF-responsive proteins involved in both vasculogenesis and angiogenesis (Gerber et al., 1997). VEGF consists of at least six isoforms of varying lengths. VEGF acts primarily in a paracrine fashion, making plasma measurements an imperfect surrogate (Wu et al., 2010). However, VEGF₁₆₅ and VEGF₁₂₁ are the primary isoforms found in plasma and are the isoforms measured by the assay used in this study (Jelkmann, 2001). Although the interplay between VEGF and its receptors is complicated, sVEGF-R1 acts primarily to inhibit angiogenesis by binding free VEGF and forming dominant negative heterodimers (Wu et al., 2010; Voelkel and Gomez-Arroyo, 2014). In chronic hypoxia, there is mixed experimental evidence for pulmonary hypertension, with some studies showing correlation with protection by VEGF and others with worsening (Tuder and Yun, 2008; Voelkel and Gomez-Arroyo, 2014). In our study, higher levels of sVEGF-R1 were associated with both EE and higher PASP.

Endothelin-1 has been shown to be a key protein in the pathogenesis of pulmonary arterial hypertension at sea level but its role in CMS is incompletely understood. Endothelin-1 antagonists are used for treatment of pulmonary arterial hypertension and have been shown experimentally to at least partially reverse hypoxic pulmonary hypertension (Giaid et al., 1993; Li et al., 1994; Dicarolo et al., 1995; Goerre et al., 1995; Tjen-A-Looi et al., 1996; Runo and Loyd, 2003; Iglarz and Clozel, 2007; Xu and Jing, 2009). Systemic measurements may not reflect local lung measurements, however; animal models of hypoxia have demonstrated a preferential upregulation of endothelin-1 in the pulmonary circulation (Li et al., 1994). Although we expected to find a positive correlation between endothelin-1 and either EE or PASP, no relationships were observed.

Erythropoietin is released primarily from the renal cortex in response to hypoxia and is responsible for stimulation and maturation of red blood cells in the bone marrow. It may also upregulate NO synthetase in the lungs and induce hyperventilation in response to hypoxia in the brain (Bushuev et al., 2006; Soliz et al., 2007). Erythropoietin has also been associated with vascular remodeling and smooth muscle proliferation in the pulmonary vasculature (Karamanian et al., 2014). Higher serum erythropoietin has been correlated with pulmonary arterial hypertension in humans (Karamanian et al., 2014) and erythropoietin administration has been shown to blunt hypoxia-induced pulmonary hypertension in

mice (Samillan et al., 2013). A correlation between higher EPO levels and higher PASP was also seen in this study. As in previous studies (León-Velarde et al., 1991), no difference was seen in plasma erythropoietin levels between those with EE and normal controls.

Our study has a number of potential shortcomings. First, our study design does not allow us to establish causation. Second, pulmonary artery pressures were estimated using a limited echocardiographic study that did not allow for the evaluation for other structural problems (e.g., left heart failure) that may explain elevations in pulmonary artery pressure. Third, the paracrine action of many of the plasma biomarkers complicates their interpretation. Fourth, participants with EE were more likely to have many components of the metabolic syndrome compared to the healthy controls; however, our results were not affected when we adjusted for body mass index. Finally, we did not match participants on co-morbidities; however, the overall prevalence of co-morbidities was low: only 3 participants with EE had COPD and one participant with EE used an ACE inhibitor or angiotensin receptor blocker; and 7 of 20 participants with EE had hypertension.

In summary, HIF-responsive proteins may play a pathophysiological role in altitude-related, chronic diseases but our results did not show consistent changes in all measured HIF-responsive proteins. Larger studies are needed to evaluate for additional genetic and environmental risk factors. Moreover, more comparative studies are needed to better determine the underlying factors that increase risk in Andean highlanders but protect Tibetans.

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WC and MSP conceived the study design and conduct, MSP and ADV performed the echocardiograms; MSP and VGD performed the echocardiogram analysis; GEM performed laboratory analysis; MSP and WC performed the statistical analysis and wrote the first draft of the manuscript. RHG, JJM, TMK, and FLV participated in study design and writing of manuscript. WC takes ultimate responsibility over study design and administration, analysis and writing of the manuscript.

Author Disclosure Statement

The authors have no conflicts of financial interest to disclose.

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