

Antioxidant Defense and Oxidative Damage vary widely among High-Altitude Residents

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Abstract

Objectives: People living at high altitude experience unavoidable low oxygen levels (hypoxia). While acute hypoxia causes an increase in oxidative stress and damage despite higher antioxidant activity, the consequences of chronic hypoxia are poorly understood. The aim of the present study is to assess antioxidant activity and oxidative damage in high-altitude natives and upward migrants.

Methods: Individuals from two indigenous high-altitude populations (Amhara (n=39), Sherpa (n=34)), one multigenerational high-altitude population (Oromo (n=42)), one upward migrant population (Nepali (n=12)), and two low-altitude reference populations (Amhara (n=29), Oromo (n=18)) provided plasma for measurement of superoxide dismutase (SOD) activity as a marker of antioxidant capacity, and urine for measurement of 8-OH-deoxyguanosine (8-OHdG) as a marker of oxidative damage.

Results: High-altitude Amhara and Sherpa had the highest SOD activity, while highland Oromo and Nepalis had the lowest among high-altitude populations. High-altitude Amhara had the lowest DNA damage, Sherpa intermediate levels, and high-altitude Oromo had the highest.

Conclusions: High altitude alone does not associate with high antioxidant defenses; residence length is influential, however. The single-generation upward migrant sample had the lowest defense and nearly the highest DNA damage. The two high-altitude resident samples with millennia of residence had higher defenses than the two with multiple or single generations of residence.

Key Words: 8-OHdG, SOD, high altitude, Nepal, Ethiopia, Sherpa, Oromo, Amhara

Introduction

Acute hypoxia (less than the normal amount of oxygen) causes an increase in oxidative stress and damage. Oxygen serves as the terminal electron acceptor for the mitochondrial electron transport chain that produces CO₂, H₂O, and heat. Relative oxygen scarcity results in a partial reduction to superoxide and hydrogen peroxide. Reactive oxygen species (ROS) can damage intracellular biomolecules, including DNA (Cash et al., 2007; Klimova and Chandel, 2008; Moller et al., 2008; Schumacker, 2011); however they are necessary for initiating transcription of loci underlying adaptive responses such as erythropoietin synthesis (Simon, 2006). Cells defend against ROS by upregulating antioxidants, primarily the powerful superoxide dismutases (SOD) (Comhair and Erzurum, 2010).

Chronic hypoxia is less studied. Reports of healthy highlanders on the Tibetan and Andean Plateaus suggest modest long-term elevation of some, although not all, ROS and antioxidants and thus imply a parallel elevation of oxidative damage. However, their long-term residence suggests the hypothesis of an evolved antioxidant response. Long-term indigenous human populations at altitudes above 2500m include Tibetans and the Sherpas of Nepal, who have lived at altitude for perhaps more than 30,000 years; and the Amhara of Ethiopia, who have lived at altitude for ~5000 years. Shorter-term, multi-generational populations include the Oromo, also in Ethiopia, who have lived at altitude for ~500 years (Beall, 2014). Our understanding of the antioxidant response in high-altitude populations is incomplete due to limitations in samples and

measures selected, and variation in the genetic background of populations, altitudes, and length of exposure (Moller et al., 2008).

We hypothesized that indigenous high-altitude populations have higher levels of SOD to counteract the increased oxidative stress resulting from chronic hypoxia and prevent a rise in DNA damage. To test this, we evaluated plasma SOD activity as a biomarker of defense against ROS (Comhair and Erzurum, 2010) and urine 8-hydroxy-2'-deoxyguanosine (8-OHdG) as a biomarker of oxidative DNA damage (Wu et al., 2004) in two indigenous and one multi-generational samples (Sherpa, Amhara, and Oromo), one upward migrant population (Nepali), and two low-altitude, paired reference populations (Amhara and Oromo).

Methods

Study Samples

Data and samples were collected over the course of several field studies over a ten-year period. The Sherpa (Nepal), Amhara, and Oromo (both Ethiopia) samples have been previously described (Alkorta-Aranburu et al., 2012; Hoit et al., 2011; Jeong et al., 2014). Low-altitude native Nepalis of South and East Asian ancestry had migrated to high altitude and lived in the same locations as the Sherpa sample. Approximately half (N=7) of the Nepalis had been living at high altitude for ~1.5 years, with the remainder between 8 months and 8 years. The Institutional Review Boards at Case Western Reserve University, the Cleveland Clinic, the Addis Ababa University Faculty of Medicine, the Ethiopian Science and Technology Committee, the Nepal Health Research Council, and OXTREC approved the studies. All participants provided written informed consent.

No individuals from high-altitude samples had traveled to altitudes below 2500 m in the previous six months, while no individuals from low-altitude samples had traveled to altitudes above 2500 m in the same timeframe. All study volunteers were healthy (by physician exam or self-report), non-pregnant (by self-report), normotensive, non-anemic, and non-smoking.

To exclude potential confounding by inflammation or infection, we measured C-reactive protein by ELISA (R&D Systems, Minneapolis, MN) and tested red cells from the Ethiopian samples to detect all four human malarial parasites (Hoit et al., 2011). Six

individuals with CRP levels above the upper detection limit of the assay were excluded along with seven individuals with confirmed malaria infection. Blood pressure measurements are the average of three measurements taken while participants rested in a seated position. Pulse and percent oxygen saturation were measured via pulse oximetry (Criticare Model 503 and SpO2; Criticare Systems, Waukesha, WI), and values reported are the average of six readings taken 10 seconds apart.

Sample collection

Venous blood was collected via peripheral venipuncture into heparinized tubes. Hemoglobin concentration was measured immediately in duplicate in whole blood using the cyanmethemoglobin method (Hemocue, Angelholm, Sweden). Blood samples were first centrifuged, and spot urine samples were aliquoted immediately. The aliquots were stored in liquid nitrogen, shipped frozen to Cleveland, and stored at -80°C until analysis.

SOD activity in plasma

SOD activity was measured in plasma as previously described (Nebot et al., 1993). SOD activity measured in a reference Cleveland population was similar to previous reports (24.6 ± 3.7 U/ml, N=10), confirming the accuracy of the assay.

Oxidative damage in urine

Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 2-deoxyguanosine (dG) were measured by liquid chromatography/tandem mass spectrometry as previously described (Teichert et al., 2009), and the ratio of oxidized base (8-OHdG) to non-oxidized base (2-dG) was calculated.

Statistical analysis

Data are reported as mean \pm SEM for normally distributed variables or median with 25th and 75th percentile for non-normally distributed variables. Comparisons across all samples were made by ANOVA or Kruskal–Wallis test, followed by post hoc pairwise comparisons of all groups by Student's t-test or Wilcoxon test as appropriate. All statistical analyses were done in JMP v7.0.1 (SAS, Cary, NC). A p-value <0.05 was considered significant.

Results

Altogether, 42 high-altitude Oromo, 18 low-altitude Oromo, 39 high-altitude Amhara, 29 low-altitude Amhara, 34 Sherpa, and 12 Nepali provided plasma and urine samples. Table 1 describes the samples. Blood pressures were normal, and BMI was in the low-normal range (Table 1). As expected, percent oxygen saturation was lower in high-altitude samples than in low-altitude samples.

[TABLE 1 HERE]

Superoxide Dismutase Activity

Among the high-altitude samples, Amhara and Sherpa had the highest SOD activities, while multi-generational highland Oromo and upward-migrant Nepalis had the lowest among high-altitude residents (Figure 1). At both altitudes, there was a trend towards greater SOD activity in Amhara as compared to Oromo, but neither high-altitude sample differed in SOD activity compared to its low-altitude counterpart.

DNA Oxidation

Among high-altitude samples, urine 8-OHdG/dG levels were lowest in the Amhara ($p < 0.0001$, $p = 0.05$, $p = 0.04$ compared to Oromo, Nepali, Sherpa, respectively). (Figure 1). The Oromo, but not the Amhara, had an altitude-dependent increase in 8-OHdG/dG. Sherpa and Nepali levels did not differ. Oromo and upward-migrant Nepalis had the highest DNA oxidation levels as measured by 8-OHdG/dG levels.

[FIGURE 1 HERE]

Discussion and Conclusion

These findings reject the hypothesis that chronic hypoxia elicits higher antioxidant defenses regardless of ancestry. The lack of a universal pattern of antioxidant defense and oxidative damage is consistent with findings relating to other physiological systems that report different patterns of adaptation described in Tibetans, Amhara, Aymara, and Oromo high altitude populations (Beall, 2014). Instead, we found that the Amhara and Sherpa samples with millennia of residence had higher levels of antioxidant defense than the two high-altitude resident samples with multiple or single generations of residence. The latter samples had the highest DNA damage. This suggests that natural selection acted on Sherpa and Amhara highlanders over the millennia to maintain a healthy balance of defense, damage, and hypoxia signaling.

A limitation of the present study is that only a single antioxidant and measure of oxidative damage were assessed. Other antioxidants and ROS might have roles in the response to hypoxia (Simon, 2006) in addition. Additionally, environmental factors such as increased UV radiation at high altitude (Askew, 2002) and air pollution (e.g. from indoor cook fires (Commodore et al., 2013)) are sources of oxidative stress at high altitude, while a lack of dietary antioxidants might contribute to insufficient defense. Other sources of defense and damage were not quantified in this study.

In conclusion, high altitude alone does not associate with high antioxidant defenses or increased oxidative damage, but rather defense and damage might be related to time at altitude and other environmental factors. A more complete profile of antioxidants, ROS and their signaling function, oxidative damage and environment

should be considered in each population in order to explore the range of possible roles of ROS and antioxidant defenses in high altitude hypoxia.

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Competing interests: The authors declare no competing interests.

Author Contributions: AJJ, MN, RZ, AK, and KSR performed research and collected data. AJJ, RZ, SAAC, SCE, and CMB analyzed data. BB, MN, AG, SCE, and CMB designed the study. AJJ, SCE, and CMB wrote the manuscript.

Fig 1. Measures of plasma SOD activity and urine 8-OH-dG/dG in two low-altitude, paired reference populations (low-altitude Amhara and Oromo), one upward migrant population (Nepali), and three lifelong high-altitude populations (Oromo, Amhara, and Sherpa). (A) Both Sherpa and high-altitude Amhara had higher SOD activity compared to Nepalis ($p=0.03$; $p=0.01$, respectively). Both low- and high-altitude Amhara had a trend towards higher SOD activity than their Oromo counterparts ($p=0.08$; $p=0.06$, respectively), but neither population had an altitude-dependent increase in SOD activity. (B) High-altitude Amhara had the lowest oxidative damage among high-altitude populations ($p<0.0001$; $p=0.05$; $p=0.04$ compared to high-altitude Oromo, Nepalis, and Sherpa, respectively). One high-altitude Oromo individual with an 8-OHdG/dG ratio more than five standard deviations from the mean was excluded with no influence on the results. Overall p-values by Kruskal–Wallis test; pairwise comparisons by Wilcoxon. Black bar: sample median; grey bar: overall median.

References:

- Alkorta-Aranburu G, Beall CM, Witonsky DB, Gebremedhin A, Pritchard JK, Di Rienzo A. 2012. The genetic architecture of adaptations to high altitude in Ethiopia. *PLoS Genet* 8(12):e1003110.
- Askew EW. 2002. Work at high altitude and oxidative stress: antioxidant nutrients. *Toxicology* 180(2):107-119.
- Beall CM. 2014. Adaptation to High Altitude: Phenotypes and Genotypes. *Annu Rev Anthropol* 43:251-272.
- Cash TP, Pan Y, Simon MC. 2007. Reactive oxygen species and cellular oxygen sensing. *Free Radic Biol Med* 43(9):1219-1225.
- Comhair SA, Erzurum SC. 2010. Redox control of asthma: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal* 12(1):93-124.
- Commodore AA, Zhang JJ, Chang Y, Hartinger SM, Lanata CF, Mausezahl D, Gil AI, Hall DB, Aguilar-Villalobos M, Vena JE et al. 2013. Concentrations of urinary 8-hydroxy-2'-deoxyguanosine and 8-isoprostane in women exposed to woodsmoke in a cookstove intervention study in San Marcos, Peru. *Environ Int* 60:112-122.
- Hoit BD, Dalton ND, Gebremedhin A, Janocha A, Zimmerman PA, Zimmerman AM, Strohl KP, Erzurum SC, Beall CM. 2011. Elevated pulmonary artery pressure among Amhara highlanders in Ethiopia. *Am J Hum Biol* 23(2):168-176.
- Jeong C, Alkorta-Aranburu G, Basnyat B, Neupane M, Witonsky DB, Pritchard JK, Beall CM, Di Rienzo A. 2014. Admixture facilitates genetic adaptations to high altitude in Tibet. *Nat Commun* 5:3281.

251 Klimova T, Chandel NS. 2008. Mitochondrial complex III regulates hypoxic activation of
 252 HIF. *Cell Death Differ* 15(4):660-666.

253 Moller P, Risom L, Lundby C, Mikkelsen L, Loft S. 2008. Hypoxia and oxidation levels of
 254 DNA and lipids in humans and animal experimental models. *IUBMB Life*
 255 60(11):707-723.

256 Nebot C, Moutet M, Huet P, Xu JZ, Yadan JC, Chaudiere J. 1993. Spectrophotometric
 257 assay of superoxide dismutase activity based on the activated autoxidation of a
 258 tetracyclic catechol. *Anal Biochem* 214(2):442-451.

259 Schumacker PT. 2011. Lung cell hypoxia: role of mitochondrial reactive oxygen species
 260 signaling in triggering responses. *Proc Am Thorac Soc* 8(6):477-484.

261 Simon MC. 2006. Coming up for air: HIF-1 and mitochondrial oxygen consumption. *Cell*
 262 *Metab* 3(3):150-151.

263 Teichert F, Verschoyle RD, Greaves P, Thorpe JF, Mellon JK, Steward WP, Farmer PB,
 264 Gescher AJ, Singh R. 2009. Determination of 8-oxo-2'-deoxyguanosine and
 265 creatinine in murine and human urine by liquid chromatography/tandem mass
 266 spectrometry: application to chemoprevention studies. *Rapid Commun Mass*
 267 *Spectrom* 23(2):258-266.

268 Wu LL, Chiou CC, Chang PY, Wu JT. 2004. Urinary 8-OHdG: a marker of oxidative
 269 stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. *Clin*
 270 *Chim Acta* 339(1-2):1-9.

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 272

273 **Table 1**

	Amhara* 1200 m	Oromo 1700 m	Nepali 3800 m	Oromo 4000 m	Amhara 3700 m	Sherpa 3800 m	P
N			9/3			18/16	
(Male/Female)	21/8	14/4		25/17	23/16		
Age (years)	33±2	24±1	26±1	27±1	30±1	34±2	0.0002
Systolic BP (mmHg)	120±1	114±2	120±2	119±2	115±2	114±2	0.03
Diastolic BP (mmHg)	79±1	78±1	80±2	78±1	75±1	75±2	0.07
Pulse (BPM)							
Male	78±3	74±3	75±2	73±2 [†]	72±3 [†]	71±3	0.5
Female	78±5	86±3	89±7	90±3 [†]	92±3 [†]	70±2	<0.0001
Height (m):							
Male	1.72±0.02 [†]	1.70±0.02	1.66±0.02 [†]	1.69±0.02 [†]	1.66±0.01 [†]	1.66±0.02 [†]	0.03
Female	1.58±0.01 [†]	1.64±0.03	1.45±0.01 [†]	1.57±0.01 [†]	1.58±0.02 [†]	1.52±0.01 [†]	<0.0001
Weight (kg):							
Male	59.2±1.4 [†]	54.3±1.5	62.3±2.4 [†]	58.4±1.3 [†]	51.2±1.0 [†]	58.8±1.3 [†]	<0.0001
Female	50.6±1.4 [†]	52.8±0.3	49.4±4.3 [†]	53.1±1.5 [†]	47.3±1.3 [†]	54.1±1.4 [†]	0.01
BMI (kg/m ²)							
Male	20.0±0.4	18.7±0.4	22.6±0.7	20.5±0.4	18.5±0.3	21.3±0.4 [†]	<0.0001
Female	20.3±0.7	19.6±0.6	23.5±2.2	21.5±0.5	18.9±0.6	23.3±0.5 [†]	<0.0001
Saturation (%)	97.0±0.3	96.5±0.3	90.9±0.6	86.6±0.6	91.6±0.5	89.5±0.5	<0.0001
Hemoglobin (g/dl):							
Male	15.5±0.2 [†]	16.6±0.3 [†]	17.6±0.5 [†]	18.1±0.3 [†]	16.3±0.2 [†]	16.8±0.3 [†]	<0.0001
Female	13.7±0.4 [†]	14.0±0.5 [†]	14.2±0.8 [†]	16.0±0.4 [†]	15.4±0.3 [†]	15.0±0.2 [†]	0.0005

274 Values are presented as mean±SEM

275 BP, blood pressure; BPM, beats per minute; BMI, body mass index: weight (kg)/(height (m))².

276 *Sample: mean barometric pressure, temperature, relative humidity (%)

277 Low-altitude Amhara: 659 mm Hg, 22°C, 36%

278 Low-altitude Oromo: 635 mm Hg, 15°C, 32%

279 Sherpa/Nepali: 484 mm Hg, 7°C, 67%

280 High-altitude Oromo: 469 mm Hg, 3°C, 40.5%

281 High-altitude Amhara: 498 mm Hg, 6°C, 36%

282 †Significant difference between men and women within a sample

283 P-value across samples by ANOVA

284

285

Figure 1

